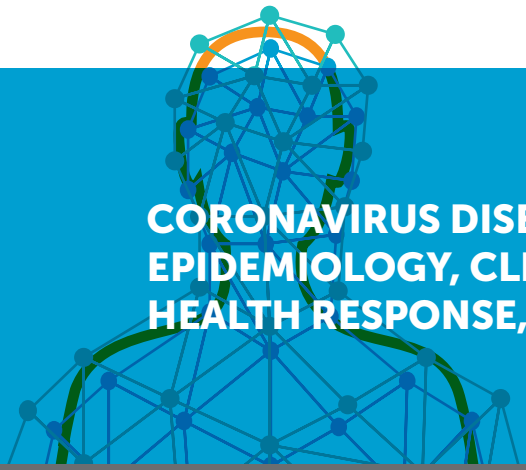

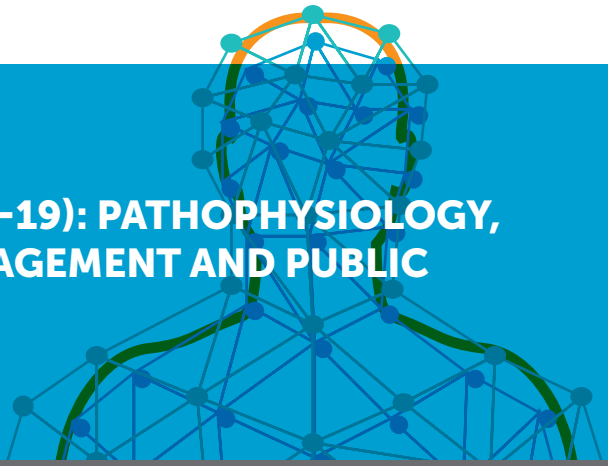

CORONAVIRUS DISEASE (COVID-19): PATHOPHYSIOLOGY, EPIDEMIOLOGY, CLINICAL MANAGEMENT AND PUBLIC HEALTH RESPONSE, VOLUME II

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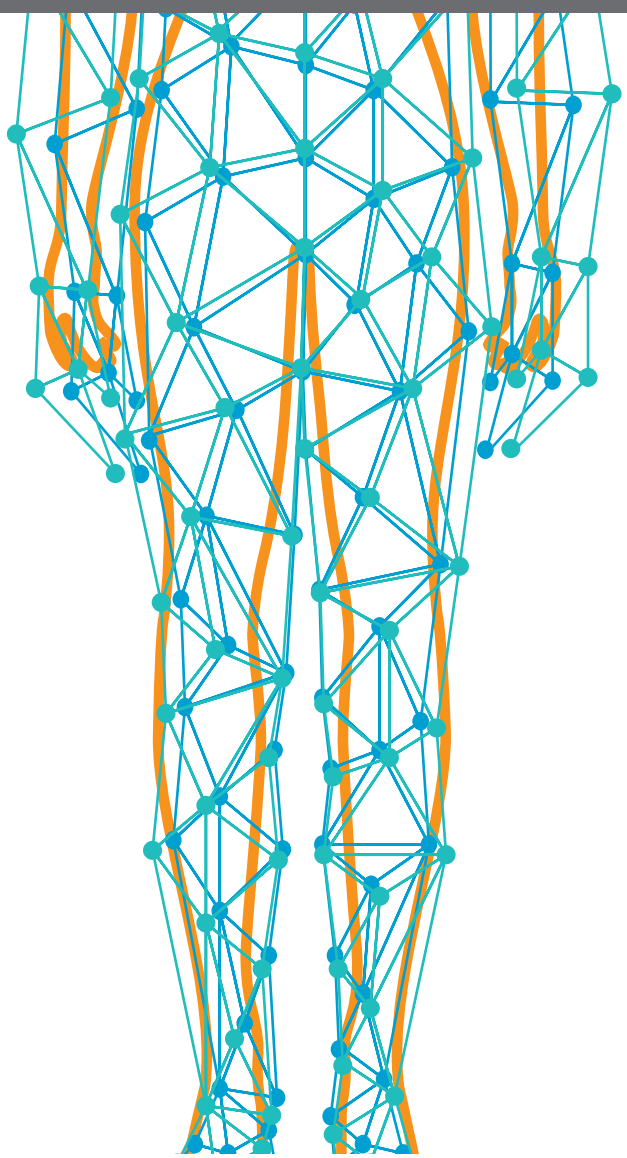

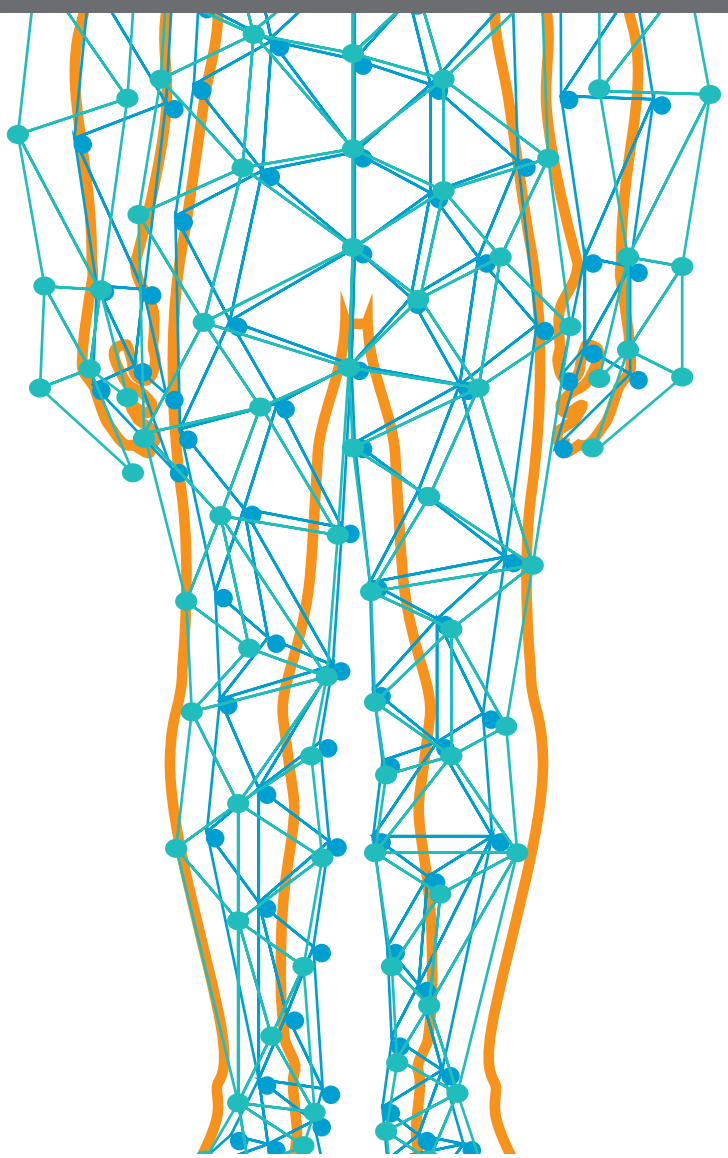

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CORONAVIRUS DISEASE (COVID-19): PATHOPHYSIOLOGY, EPIDEMIOLOGY, CLINICAL MANAGEMENT AND PUBLIC HEALTH RESPONSE, VOLUME II

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Prediction of COVID-19 Waves Using Social Media and Google Search: A Case Study of the US and Canada

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The ongoing COVID-19 pandemic has posed a severe threat to public health worldwide. In this study, we aimed to evaluate several digital data streams as early warning signals of COVID-19 outbreaks in Canada, the US and their provinces and states. Two types of terms including symptoms and preventive measures were used to filter Twitter and Google Trends data. We visualized and correlated the trends for each source of data against confirmed cases for all provinces and states. Subsequently, we attempted to find anomalies in indicator time-series to understand the lag between the warning signals and real-word outbreak waves. For Canada, we were able to detect a maximum of 83% of initial waves 1 week earlier using Google searches on symptoms. We divided states in the US into two categories: category I if they experienced an initial wave and category II if the states have not experienced the initial wave of the outbreak. For the first category, we found that tweets related to symptoms showed the best prediction performance by predicting 100% of first waves about 2–6 days earlier than other data streams. We were able to only detect up to 6% of second waves in category I. On the other hand, 78% of second waves in states of category II were predictable 1–2 weeks in advance. In addition, we discovered that the most important symptoms in providing early warnings are fever and cough in the US. As the COVID-19 pandemic continues to spread around the world, the work presented here is an initial effort for future COVID-19 outbreaks.

Keywords: digital data stream, Twitter, Google Trends, COVID-19, early warning

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1. INTRODUCTION

The COVID-19 pandemic caused by SARS-CoV-2 has been spreading rapidly and continuously posing a significant threat to human lives worldwide. Providing early signals ahead of outbreaks is essential for early public health responses. Prediction systems for other diseases have been built to facilitate management in disease emergencies and making rapid policy decisions (1, 2).

Disease monitoring and surveillance are essential to create situational awareness and initiate timely responses. Since the availability of testing is different from country to country, online platforms can help in monitoring disease occurrences. Web-based platforms can serve as sources where users self-report or search their health-related issues. Social media, in particular Twitter, has been taken into consideration for COVID-19 surveillance purposes.

Several studies attempted to track the volume of health-related online content and associated it with official cases or deaths (3, 4). In a recent work by Mackey et al., English Twitter conversations were collected and used in an unsupervised machine learning approach to assess users' self-reports

of COVID-19 symptoms, testing, and recovery from disease. The results showed that the volume of tweets regarding "symptoms" and "lack of testing" increased at the same time as a surge in the number of confirmed cases in the United States. Similarly, an overlap between COVID-19 cases and discussions on Twitter and Weibo has been shown (5, 6).

In addition to finding a connection between disease cases/deaths and social media posts, Gharavi et al. (7) utilized social media for early reporting of disease cases. A regression analysis was performed for a number of states in the US, which found a connection between the number of tweets related to "cough" and "fever" and officially reported cases with a 5–19 days lag (7).

Search engines have been analyzed to monitor COVID-19 activities too (8, 9). A study utilized multiple digital data sources, including Google Trends to calculate the probability of exponential growth/decay in COVID-19 activities as early signals of the epidemic in Massachusetts, New York, and California states (4). Another study in the United States found a high correlation between search trends and the number of cases with a 7-day lag (10).

In addition to the US, Google search trends were used to predict COVID-19 incidence in Iran (11) and Colombia (12). The study in Iran used Linear regression and long short-term memory (LSTM) models and found that "hand sanitizer," "handwashing," and "antiseptic" were the most effective factors in case predictions.

The present study aimed to examine the potential of online platforms in providing early warnings of first and second waves of COVID-19 outbreaks in the US and Canada for an 8-month period. The main objectives were: (1) to visualize the correlation between digital data sources and COVID-19 official cases; (2) to compare various sources of internet-driven data in terms of their timeliness and precision in providing alert signals of disease waves; and (3) to prioritize COVID-19 symptoms by their values in detecting disease trends.

The first novelty here is utilizing historical and precisely geo-located tweets at provincial/state levels. A growing body of research has been centered around using online content for providing early warning signals of pandemics. The Twitter data used in the existing work of COVID-19 is limited to streaming or standard search APIs that cannot go more than a week back in time (3, 5, 13). Moreover, the above-mentioned studies either had no geographical restrictions on collected tweets (14, 15) or locations have been specified using self-reported information associated with user accounts or tweet contents for a small percentage of tweets (6, 16, 17).

The other novel aspect of the present study lies in comparing the disease predictive value of various data in terms of differences in platforms and keywords. Previous work has explored the correlation between COVID-19 indicator terms of online content and the number of infected individuals (18–20). However, the potential of internet-driven information in providing early warning of COVID-19 outbreaks is still poorly understood.

2. MATERIALS AND METHODS

In this work, we collected Twitter posts and Google search scores related to symptoms and control measures of the COVID-19 outbreak in Canada and the US from January 2020 to September 2020. Subsequently, the weekly time-series of online activities and COVID-19 new cases were employed in anomaly detection and correlation models. Then, we explored and compared the potential of social media and search platforms in providing early warnings of outbreak waves on national and local scales. Furthermore, we compared the ability of COVID-19 symptoms in predicting outbreak waves. An overview of the overall flow of the study is given in **Figure 1**.

2.1. Data Collection

Ground Truth Data: We collected the cumulative number of cases and deaths of COVID-19 in Canada and the United States from Johns Hopkins COVID-19 data repository (21). The data included geographical information, such as province, city, latitude, and longitude. The daily number of new cases was calculated from the initial cumulative numbers. Subsequently, the weekly number of new cases was computed for the US and Canada as well as their states/provinces.

Twitter Data: Twitter Premium Search application programming interface (API) (22) was used to retrieve tweets containing COVID-19 symptoms and preventive measures posted from the specified geographical locations. A list of keywords that were included in or excluded from the Twitter search query is given in **Table 1**.

In total, around 300K tweets were collected from January 2020 to September 2020. This included 202K symptom-related and 95K preventive-related tweets. We determined the province/state that each tweet was posted from using the city names returned by Twitter. The provincial/state number of retrieved tweets associated with Canada and the United States for categories of symptom and precaution keywords is given in **Tables B1, B2**, respectively, in the **Supplementary Material**.

2.1.1. Google Trends Data

The "`Interest_over_time`" scores were acquired from Google Trends (23) given national or local locations and the same keywords we used in Twitter search API. We used provinces/states names to pull the data. The scores indicate the popularity of terms over a specified time range and region. Google Trends scores are based on the absolute search volume for a term, relative to the number of searches received by Google. Scores are quantified as indexes, with 100 showing the maximum search interest and zero showing no interest.

2.2. Visual Trends

The weekly number of tweets and search scores was plotted against the weekly COVID-19 cases on national and provincial/state scales. Given the line plots, one can visually detect the fist/second-half waves of outbreaks for each province/state and compare the online activities with the reported COVID-19 cases. Further, we plotted the distribution

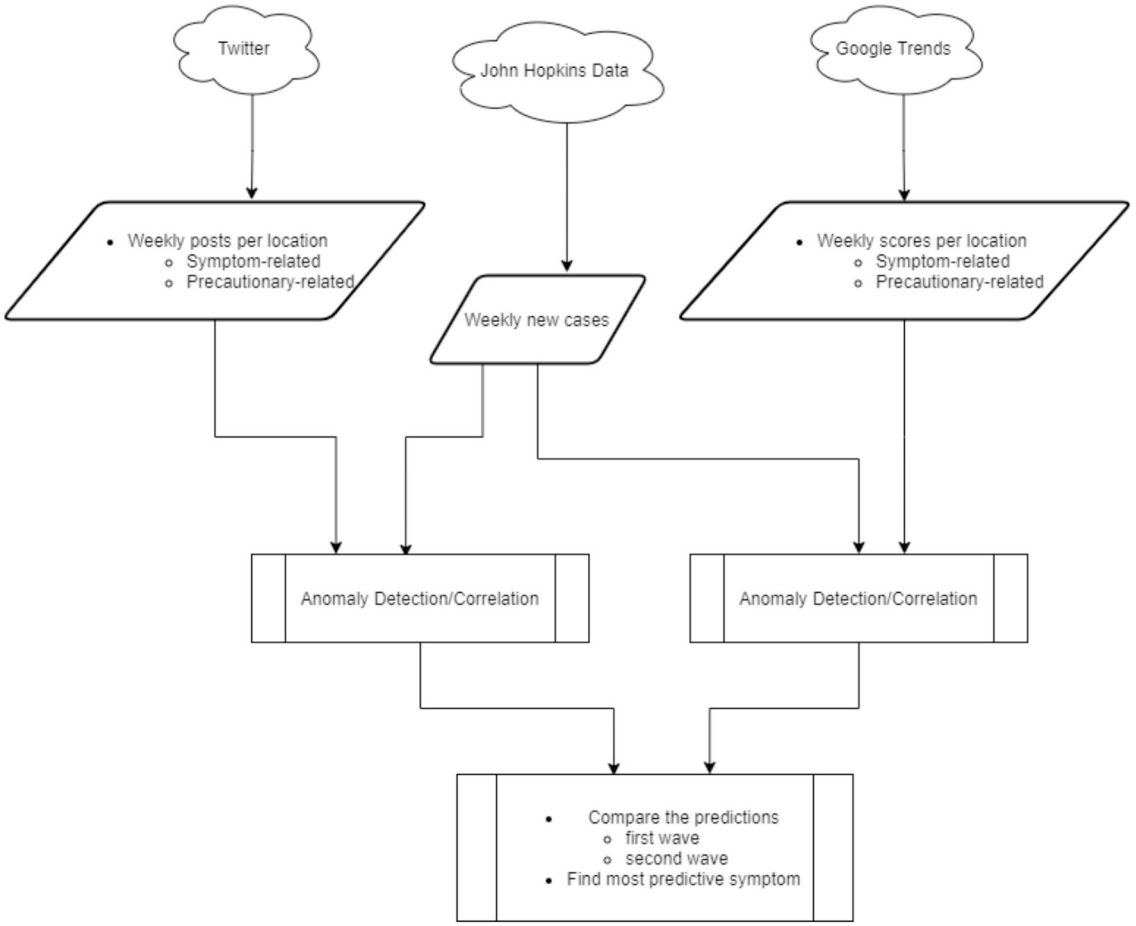

FIGURE 1 | Overall approach.

of tweets across various symptoms, which can help gain insight into how specific terms can be connected to official disease cases.

2.3. Detection of Pandemic Waves

Anomaly detection in time-series is formulated as identifying outliers or unusual data points relative to some standard or usual signals (24). We applied The Seasonal-Hybrid Extreme Studentized Deviate (SH-ESD) (25, 26) algorithm on the weekly time-series of online activities to eventually identify the onset and peak of COVID-19 waves. SH-ESD algorithm was designed in particular for finding anomalies in the cloud infrastructure (26). The algorithm is built based on the Generalized ESD test and includes a statistical test called Grubb's Test and a time-series decomposition method, known as Seasonal-Trend Decomposition based on Loess (STL). Once decomposition extracts the symmetrically distributed residual component of the observed data, Grubb's Test identifies outliers in a sample of residuals (25, 26).

Weekly time-series of cumulated search scores and the number of tweets were calculated on national and local levels for Canada and the US. Subsequently, we employed an R package "AnomalyDetection," which uses SH-ESD method and was released by the Twitter engineering team (27). Finally, we compared the lag time between detected anomalies and the onset and peak times of outbreak waves for all provinces/states. The comparison could help understand the potential of online discussions and searches in providing early warnings of outbreak waves. The onset of a wave was defined as a week when the number of new cases jumped to at least 50, and the peak was defined as the week when the number of new cases reached its maximum in the wave. Finally, we calculated average lags and the percentage of correct detections for symptom and precaution related data for each platform in each nation.

To further evaluate the quality of detections, correlation measures between time series of activities in each province/state and corresponding actual COVID-19 cases were calculated using the Pearson correlation coefficients (r) (28). The coefficient of one ($r = 1$) shows that the two data series are matching and if no correlation exists, the coefficient will be zero ($r = 0$).

2.4. Most Predictive Symptoms

In order to differentiate COVID-19 symptoms for their ability in predicting pandemic trends, we filtered the time-series of tweets by symptoms for each location. Subsequently, the anomaly

TABLE 1 | Twitter query input.

Included symptom keywords	Shortness of breath, cough, fever, sore throat, loss of smell, loss of taste
Included precaution keywords	Face mask, quarantine, wearing mask, wash hand, ovid-19 vaccine, covid-19 vaccine, covid vaccine, corona vaccine, coronavirus vaccine, physical distancing, social distancing
Excluded symptom keywords	Flu, influenza, cold, diabetes, jungle fever, Saturday night fever, fever swamp, baby fever, fever pitch, fever dream, fever 333, dog fever, cat scratch fever, blackouts coastal fever, tattoo fever, Kennel cough, smoke, smoking, allergy, allergies
Excluded precaution keywords	Handle, handling, body wash, hand cream, cold, flu, yogurt, honey, watermelon, cucumber, hair mask

detection analysis was applied to all symptom-specific time-series similar to the previous section. We compared the detected anomalies from time-series of all keywords with the peaks of waves in each province/state and reported the average measures.

3. RESULTS

3.1. Visual Trends

Twitter posts and Google Trends search interests on symptoms and precautionary measures of COVID-19 were plotted weekly against the number of COVID-19 cases. As an example, four curves are given in **Figure 2** showing information from Mid-January till September for Canada. **Figures 2A,C** compare disease cases to the time-series of tweets discussing symptoms and preventive measures, respectively. On the other hand, **Figures 2B,D** present a comparison between disease cases and Google Trends scores for searches on symptoms and preventive measures, respectively. The online activities are plotted with blue color while the official cases are shown in red color. Additional charts related to other locations are included in the **Supplementary Material**.

Visually comparing the ability of platforms in giving early warnings in the beginning of the pandemic, Twitter activities on disease symptoms in the majority of states/provinces showed slightly earlier peaks than Google (e.g., **Figures A44**–**A46**, **A49**–**A51** in the **Supplementary Material**). However, comparing the trends for the second waves, Google searches on symptoms showed more noticeable peaks than Twitter (e.g., **Figures A8**, **A10**, **A12**, **A17** in the **Supplementary Material**).

In general, after peak times, when the number of cases started to decrease, people gradually stopped posting or searching about symptoms. This might be due to knowledge saturation which makes the outbreak monitoring more challenging as time passes. On the other hand, trends for precautions kept steady (e.g., **Figures 2C,D**). The reason behind the steadiness of time-series of precautionary-related data could be due to the impact of news media reporting regulations imposed by governments regardless of the number of cases.

It is worth noting that compared to symptoms, preventive terms were more discussed on Twitter and less searched on Google for all geographical locations. For example, the weekly number of tweets reporting symptoms reached a peak of 500 in Canada while the peak of tweets discussing precautionary measures was 14 times more (**Figure 2**). On the other hand, the maximum cumulative search score of symptom keywords was more than twice the maximum score of precaution keywords. Thus, we could conclude that internet users tend to post on Twitter to discuss control measures and search their symptoms on Google.

Several states of the US, such as Alabama, Tennessee, Utah, and Texas did not experience the first wave of the pandemic (see **Figure 4**). Nevertheless, online discussions and searches about COVID-19 symptoms and control measures soared in March. Having a closer look at a sample of tweets, we noted only 20% of early tweets for the above-mentioned provinces were regarding self-reporting of symptoms. The rest of the tweets were posted from users being anxious about or scared of COVID-19 symptoms. Similarly, internet users likely would search the related terms on Google when they are afraid of pandemic news about other states.

3.2. Detection of COVID-19 Waves

As previously mentioned, some US states had not experienced the first wave of disease. We grouped the US states into two categories: (I) states that had experienced a peak of disease wave before June 2020; (II) states that had experienced a wave peak only after June 2020, which included Alaska, North Carolina, Utah, Alabama, Tennessee, California, Arizona, and Texas. In the latter category, waves actually started before June but reached their peak in the second half of the study period. Examples of the first and second categories are given in **Figures 3**, **4**, respectively.

After applying anomaly detections on the time-series of different sources of internet data for the provinces/states in Canada and the US, we presented the outcomes in **Tables 2**, **3**, respectively. We quantified the average number of weeks that each source of data can provide anomalies before the start and peak of waves. As mentioned in section 2, the onset of a wave was defined as the point when new cases reached at least 50 and the peak as the point when cases got to their maximum. Similarly, we calculated the percentage of waves in provinces/states of these countries that can be detected earlier given a specific source of data (e.g., Twitter or Google Trends). In **Table 3**, we presented separate prediction outcomes for the previously mentioned categories of the US states.

Table 2 shows that except for the precaution-related tweets, the rest of the sources acted the same in the detection time of onsets of waves. The symptom-related tweets showed anomalies 4.3 weeks before the waves peak, which is about 1 week earlier than other sources of data (i.e., 3 weeks). However, the percentage of detection was only 50% which was less compared to the rest of the sources.

Overall, the result presented here demonstrated that Google Trends performed better in terms of the number of early warning weeks and the percentage of correct predictions. Utilizing Google Trends enabled us to identify starts and peaks of waves in Canada in average for about 1 and 3 weeks earlier, respectively. In terms of detection percentages, the symptom-related searches with a

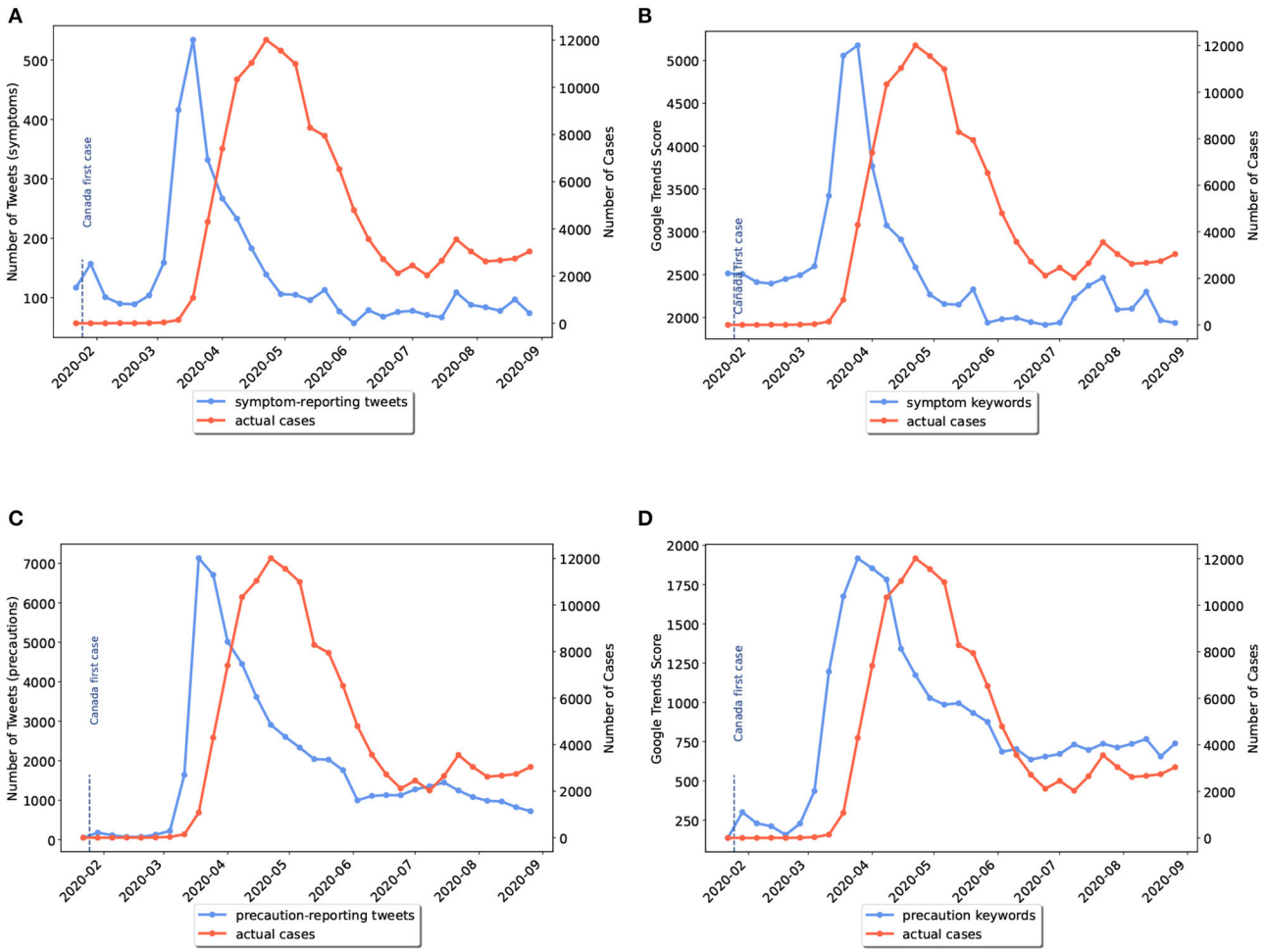

FIGURE 2 | Weekly comparison of online activities and actual number of cases in Canada. **(A)** Symptom-related tweets vs. cases, **(B)** Symptom-related searches vs. cases, **(C)** Precaution-related tweets vs. cases, **(D)** Precaution-related searches vs. cases.

detection percentage of 83% outperformed the precautionary-based searches with a detection percentage of 75%.

Additionally, we observed a strong and statistically significant (p-value < 0.05) correlation between the Twitter/Google activities and the number of cases of the disease in Canada. **Table 4** shows correlations of above 75% with lags of 3–5 weeks for all sources of data.

During the time period covered in the present study, the majority of Canadian provinces had not encountered a major second wave except for British Columbia and Manitoba. The online content generated in these two provinces did not show strong correlations with the actual number of disease cases. Moreover, the analysis used in the present study was not capable of detecting the second waves in Canada.

Similar to Canada, the anomaly detection results for the US is given in **Table 3**. Comparing the ability of Twitter and Google in detecting the start of the first waves, symptom related posts and searches as well as precaution-related searches were capable of detecting 100% of first waves. However, symptom-related tweets could detect the start of first waves about 2–3 days earlier than Google trends and about 6 days earlier than tweets related to precautions. The lag time of symptom-related searches (e.g., 1.54 weeks) matched with the findings of a previous study in China (29). The Baidu searches on symptoms could detect the increase in the number of COVID-19 cases for 6–9 days earlier.

The results revealed that Twitter and Google Trends performed better in detecting the onset of second waves in category II than category I states. Posts and searches identified the start of second waves in 78% of provinces in category II states while the detection percentage for the second wave for the category I states was up to 6%. With regard to time, symptom-related tweets identified the start of second waves in category II about 5 days earlier than other sources.

Overall, higher percentages of detections in early waves than late waves were observed. This could be due to social media

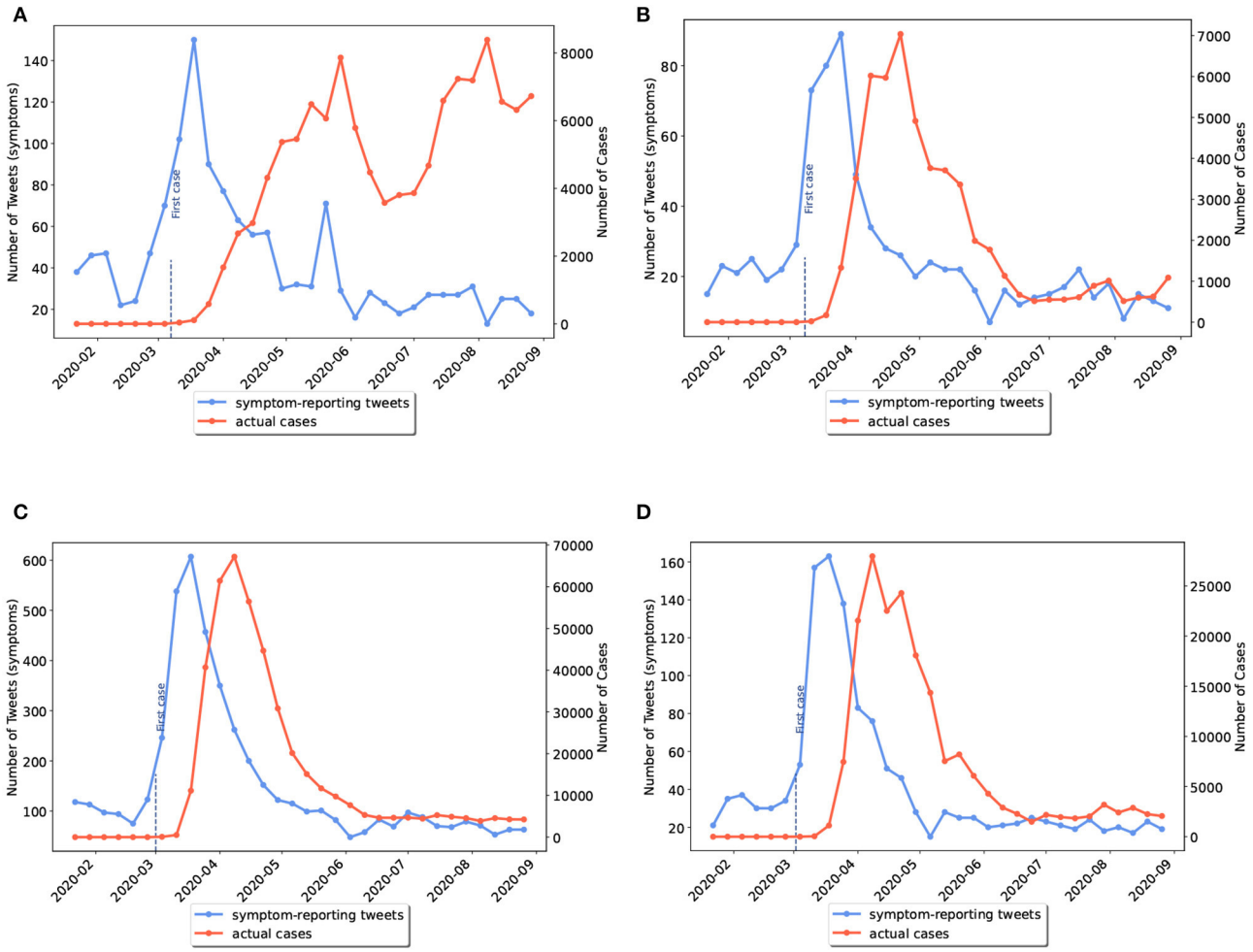

FIGURE 3 | Weekly comparison of symptom-related tweets and the actual number of cases in provinces with first wave of disease (category I). **(A)** Virginia, **(B)** Connecticut, **(C)** New York, **(D)** New Jersey.

users being exhausted and less motivated to post or search on the internet as their level of concern had decreased over time. This is referred to as "pandemic fatigue" in psychology (30).

Furthermore, we observed statistically significant (p-value < 0.05) and strong correlations between online data and disease cases for the US states of category I. A sample of locations is given in **Table 4** and the rest can be found in **Table B3** in the **Supplementary Material**. In general, lags in the second and fourth columns (i.e., symptoms) are higher than the third and fifth columns (i.e., precautions). The same pattern was found in the anomaly detection results in **Table 3**. On the other hand, precaution-related series showed stronger correlations than symptom-related series.

The findings of a study in Taiwan (9) in the early stages of COVID-19 outbreak are consistent with our result (the fifth column) in **Table 4**. Authors found that Google searches on "hand washing" and "face mask" increased 1–3 days prior to the increase in COVID-19 cases. However, our findings in the fourth column (GT symptoms) did not match with the findings in Italy, Spain, UK, USA, Germany, France, Iran, and The Netherlands (8). In comparison with the moderate correlations presented in the fourth column, Walker et al. discovered a strong correlation between the number of searches for "loss of smell"-related information and the number of COVID-19 cases.

3.3. Prediction Values of Symptoms

The progression of tweets related to COVID-19 symptoms during the course of the present study is given in **Figure 5**. Furthermore, a quantitative analysis was performed to find anomalies in symptom-specific time-series of tweets for all US states (see **Table 5**).

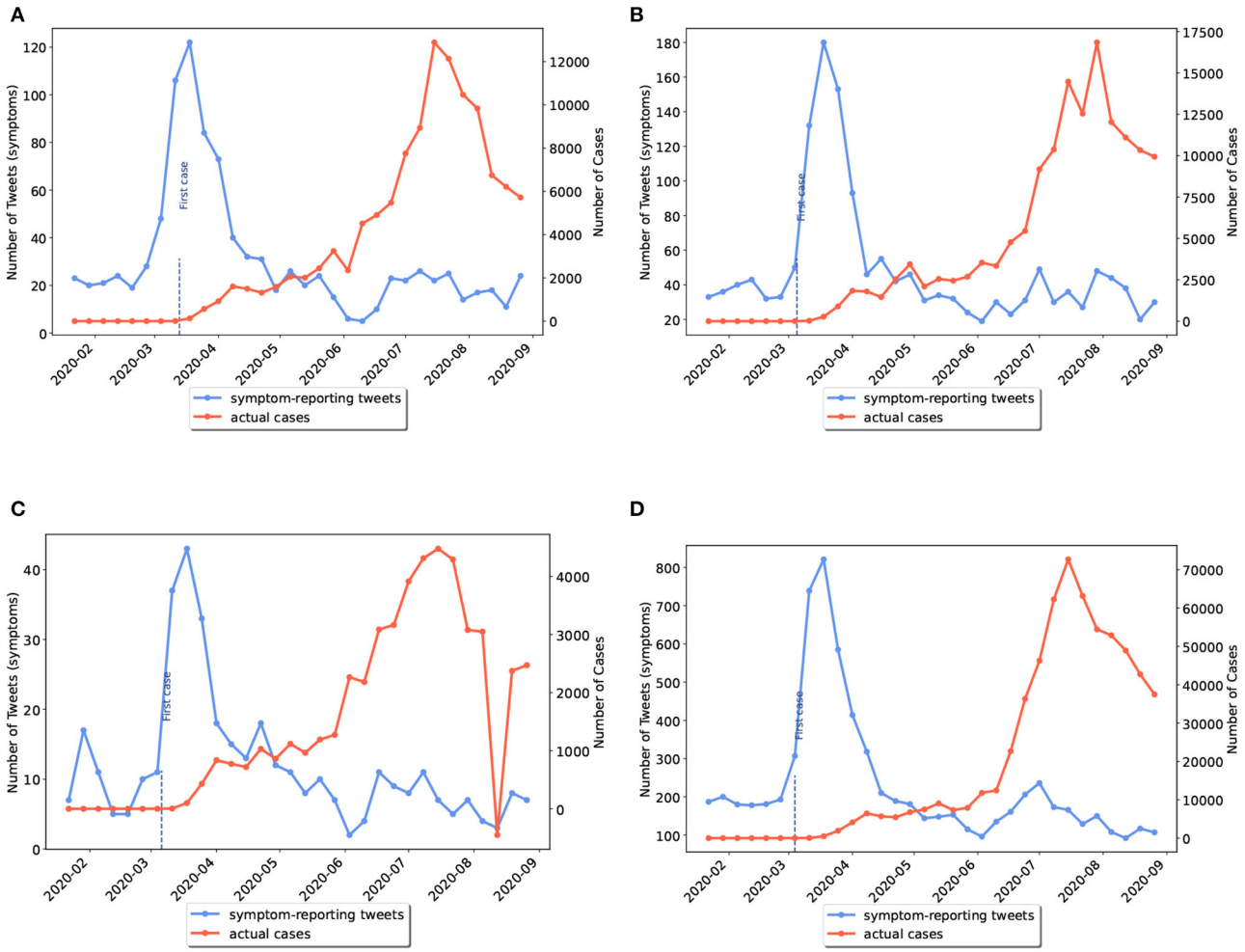

FIGURE 4 | Weekly comparison of symptom-related tweets and the actual number of cases in states with only the second wave of disease (category II). **(A)** Alabama, **(B)** Tennessee, **(C)** Utah, **(D)** Texas.

Manually, we looked at a sample of tweets (1K) for the peak time of symptoms (4 March–25 March) in **Figure 5** and categorized them. We noted that more than 50% of tweets were about self-reporting of symptoms. For example, users reported their symptoms in tweets, such as "I haven't coughed this much in my life. It's a really violent dry cough. My chest hurts." and "My sweet daughter has a high fever for 3 days." The next major category (25%) was the educational tweets, such as "Limit the spread of illnesses like #COVID19: sneeze or cough into a tissue or your elbow, and dispose of used tissues." In the last category (20%) we found comic feeds like "waiting until my roommates asleep to cough."

The volume of tweets related to "sore throat" was high at the beginning of the study period and then decreased. The sample tweets in the first 2 months showed that the majority of discussions were around "sore throat" due to the cold season. After that tweets reporting "tiredness" and "shortness of breath" started to grow. Also, it is visually clear that "cough" and "fever" were better trend indicators of official cases compared with other symptoms.

The quantitative results in **Table 5** are consistent with the visual implications above. We were able to predict first waves of the pandemic in more than half of the US states using tweets regarding "fever" and "cough." Tweets related to all symptoms predicted the peaks of the first wave with an average within the range of 3.3–5.2 weeks earlier than official peaks of cases. Terminologies, such as "tiredness" and "loss of smell" showed the lowest percentage of detections (i.e., up to 20%) among all symptoms.

TABLE 2 | The average prediction value of Canadian provinces (with an early wave).

Source	Start1	Peak1
Twitter		
Symptoms (week lags)	1.19	4.3
Symptoms (detection percentage)	50%	50%
Precautions (week lags)	0.4	2.8
Precautions (detection percentage)	83%	83%
Google Trends		
Symptoms (week lags)	1.2	3.1
Symptoms (detection percentage)	83%	83%
Precautions (week lags)	1.2	3.2
Precautions (detection percentage)	75%	75%

TABLE 3 | The average prediction value of the US states.

Provinces	Source	Start1	Peak1	Start2	Peak2
	Twitter				
Category I	Symptoms (week lags)	**1.83**	5	6	–
	Symptoms (detection percentage)	**100%**	81%	3.2%	0%
	Precautions (week lags)	0.94	4.39	2	3.42
	Precautions (detection percentage)	97%	89%	6%	22%
Category II	Symptoms (week lags)	–	–	1.86	–
	Symptoms (detection percentage)	–	–	**78%**	0%
	Precautions (week lags)	–	–	1.14	2.5
	Precautions (detection percentage)	–	–	**78%**	44%
	Google Trends				
Category I	Symptoms (week lags)	1.54	4.75	7	3.87
	Symptoms (detection percentage)	**100%**	86%	3%	26%
	Precautions (week lags)	1.4	4.75	6	1.75
	Precautions (detection percentage)	**100%**	86%	3%	13%
Category II	Symptoms (week lags)	–	–	1	1.75
	Symptoms (detection percentage)	–	–	**78%**	44%
	Precautions (week lags)	–	–	1.14	4
	Precautions (detection percentage)	–	–	**78%**	11%

The bold values are the best obtained results.

TABLE 4 | Correlation coefficients (r) of weekly online activities and COVID-19 cases.

Location	TW symptoms	TW precautions	GT symptoms	GT precautions
Canada	0.85 (lag = 5)	0.93 (lag = 3)	0.75 (lag = 5)	0.84 (lag = 3)
Massachusetts	0.94 (lag = 5)	0.9 (lag = 3)	0.66 (lag = 5)	0.86 (lag = 4)
Michigan	0.7 (lag = 3)	0.81 (lag = 2)	0.38 (lag = 4)	0.87 (lag = 3)
New Jersey	0.95 (lag = 4)	0.87 (lag = 2)	0.7 (lag = 5)	0.9 (lag = 3)
New York	0.97 (lag = 3)	0.86 (lag = 2)	0.72 (lag = 4)	0.91 (lag = 3)
Vermont	0.9 (lag = 2)	0.83 (lag = 1)	0.63 (lag = 3)	0.88 (lag = 2)

4. DISCUSSION

We aimed to perform a comparative study to understand the potential of Twitter activities and Google searches to be used in early warning systems of COVID-19 pandemic in Canada and the US. Time-series of Twitter posts and Google search scores on several symptoms and precautionary terms were compared with the actual cases qualitatively and quantitatively. Subsequently, we assessed the prediction values of different sources of data in providing early warnings of pandemic waves. Additionally, we made an effort to prioritize symptoms based on their predictive values.

The qualitative results indicated that overall, in the beginning of the pandemic, Twitter posts related to symptoms showed earlier trends compared to Google searches. However, during the second half of the study period (e.g., June–August), Google searches of symptoms could show more noticeable trends. Furthermore, we observed fixed trends of the precautionary time-series after the first waves, which might be due to news media influencing internet users. Visual observations also indicated that internet users tend to discuss preventive measures of COVID-19 on Twitter and search disease symptoms on Google.

Pearson correlation analysis demonstrated an overall strong correlation between official cases and the relevant posts and searches related to Canada. Except for British Columbia and Manitoba, other Canadian provinces have shown correlation coefficients of above 75% with a lag of 3–5 weeks. We did not observe a strong correlation for British Columbia and Manitoba as they did not experience major early waves of disease. Anomaly detections in time-series of Canada revealed that symptom-related Google searches showed the best performance in predicting the onset and peak of first waves about 1 and 3 weeks earlier, respectively.

Although several states in the US did not experience the early waves, the online activities started to grow in March. Similar findings were reported by other studies (31). Increasing activities in social media in the absence of outbreaks is likely due to the panic of the pandemic in other states. We divided states into categories I and II for those with and without early waves, respectively. We observed strong correlations for states in category I. In particular, symptom-related tweets showed the highest correlations. Previous studies have also shown strong (27 days lag) but state different correlations for the US states (32). Additionally, we found that correlation lags for posts and searches of symptoms were higher compared to preventive measures.

The prediction of the first waves in the present study outperforms the detection of second waves. This was aligned with the correlation results being weak for the locations with only the second waves. In other words, the correlations faded as the pandemic proceeded in weeks. This could be due to two following reasons: (1) public began to feel exhausted with the pandemic and were less likely to follow public health practices and (2) COVID-19 related subjects, such as symptoms and remedies became well-known among the public. Thus, the approach presented in the paper is more suitable for the initial wave of an outbreak as it reflects the public's anxiety or curiosity and desire to learn about disease symptoms and control measures. Based on the results presented in this paper,

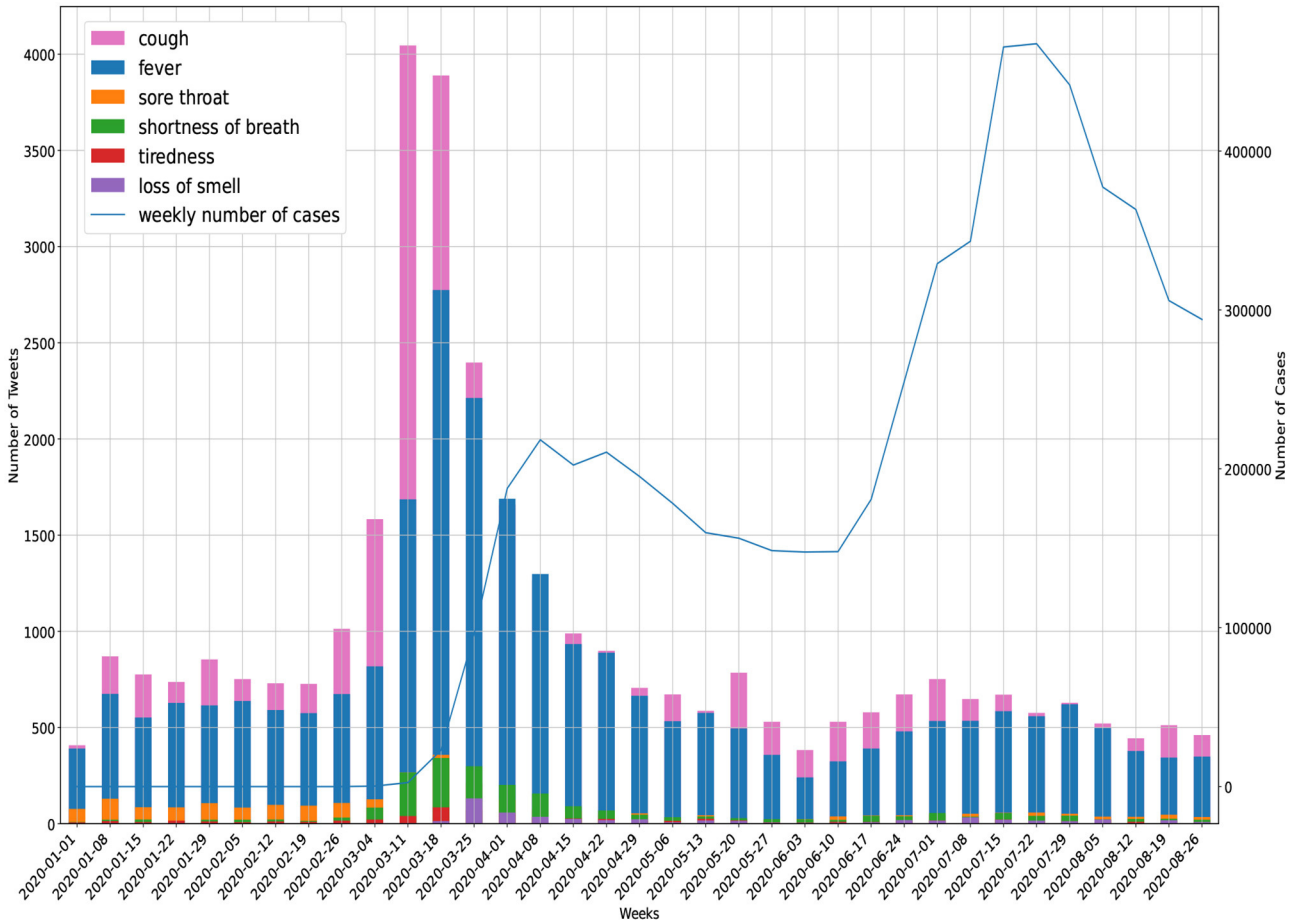

FIGURE 5 | Symptom reporting in Twitter (United States).

it is expected that there will be less engagement through social media in the second and future waves of the outbreak. However, if new symptoms and variants of the virus appear or if public health imposes new control measures in the future, then the proposed approach might be appropriate for the second and future waves.

The analyses on symptom-specific time-series of the US demonstrated that tweets related to "fever" and "cough" had the highest performance in predicting the first waves, which is aligned with the study by Gharavi et al. (7). On the other hand, tweets related to "tiredness" and "loss of smell" could only predict up to 20% of the waves. These results were in contrast with previous studies (8, 33). Walker et al. showed a strong correlation between the frequency of Google search results related to "loss of smell" and the onset of COVID-19 infection in several countries. Similarly, Asseo et al. revealed a correlation between Google searches for "loss of taste" and "loss of smell" symptoms with the number of cases. However, the correlation was found only for a short period of time when people were surprised by new cases and media coverage. A reason behind the differences in findings could be the fact that Walker et al. and Asseo et al. have used Google searches while our study and the study by Gharavi et al. have used Twitter posts.

TABLE 5 | The average prediction values of the US states (detection of early waves).

Source	Peak1 (Twitter)	Peak1 (Google Trends)
Fever (week lags)	4.45	4.03
Fever (detection percentage)	53%	58%
Cough (week lags)	5.2	4.2
Cough (detection percentage)	55%	44%
Tiredness (week lags)	5.2	–
Tiredness (detection percentage)	20%	0%
Shortness of breath (week lags)	4.38	4.27
Shortness of breath (detection percentage)	29%	24%
Loss of smell (week lags)	3.33	–
Loss of smell (detection percentage)	7%	0%
Sore throat (week lags)	4.29	4.24
Sore throat (detection percentage)	38%	55%

The scope of present study is larger in terms of using data with a longer duration and geographical extent compared with a previous studies of the US (4, 7, 10). While we studied all the US states from January 2020 to September 2020, Gharavi et al. performed an analysis for a duration up to April 2020 for the six most affected states of the US. Compared to only "fever" and "cough" terms that were analyzed by Gharavi et al., we employed a wider range of symptoms and the Twitter posts have been filtered during the data collection to avoid irrelevant content.

Despite the strengths of the approach taken in this study and many other existing work, the number of confirmed cases used here might be an underestimate of the actual number of cases due to the lack of testing kits in the beginning of the pandemic (34). Initially, regions had travel-based, symptom-based, or contact-based testing policies that might have not been identified. In the future, it is of interest to indicate whether social media is a better indicator of new cases after regions had open testing for everyone. Moreover, tweets are generated by individuals who are capable of accessing and using social media and search engines. Therefore, it is possible that there may be a bias in favor of certain age groups or individuals belonging to certain socioeconomic groups.

Here, we assumed equal weights for counting tweets. However, engagement metrics, such as re-tweets, replies, follows, favorites and links can be used to assign an important weight to each tweet. Future studies therefore might calculate the weighted sum of tweets in building time-series. Additionally, future work can analyze social media and search signals collectively. Fusion approaches could be used to integrate evidence from several sources, which might lead to more precise predictions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SY: writing–original draft preparation, investigation, methodology, software, data curation, visualization, and validation. RD: supervision, investigation, conceptualization, writing, reviewing, and editing, and validation. SM: writing, reviewing and editing, and investigation. SS: supervision, conceptualization, writing, reviewing, and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.656635/full#supplementary-material

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Efficacy Evaluation of Thymosin Alpha 1 in Non-severe Patients With COVID-19: A Retrospective Cohort Study Based on Propensity Score Matching

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Objective: Thymosin alpha 1 (Thymosin-α1) is a potential treatment for patients with COVID-19. We aimed to determine the effect of Thymosin-α1 in non-severe patients with COVID-19.

Methods: We retrospectively enrolled 1,388 non-severe patients with COVID-19. The primary and secondary clinical outcomes were evaluated with comparisons between patients treated with or without Thymosin-α1 therapy.

Results: Among 1,388 enrolled patients, 232 patients (16.7%) received both Thymosin-α1 therapy and standard therapy (Thymosin-α1 group), and 1,156 patients (83.3%) received standard therapy (control group). After propensity score matching (1:1 ratio), baseline characteristics were well-balanced between the Thymosin-α1 group and control group. The proportion of patients that progressed to severe COVID-19 is 2.17% for the Thymosin-α1 group and 2.71% for the control group ($p = 0.736$). The COVID-19-related mortality is 0.54% for the Thymosin-α1 group and 0 for the control group ($p = 0.317$). Compared with the control group, the Thymosin-α1 group had significantly shorter SARS-CoV-2 RNA shedding duration (13 vs. 16 days, $p = 0.025$) and hospital stay (14 vs. 18 days, $p < 0.001$). No statistically significant difference was found between the Thymosin-α1 group and control group in duration of symptoms (median, 4 vs. 3 days, $p = 0.843$) and antibiotic utilization rate (14.1% vs. 15.2%, $p = 0.768$).

Conclusion: For non-severe patients with COVID-19, Thymosin-α1 can shorten viral RNA shedding duration and hospital stay but did not prevent COVID-19 progression and reduce COVID-19-related mortality rate.

Keywords: severe acute respiratory syndrome coronavirus 2, coronavirus disease 2019, Thymosin alpha 1, Thymosin-α1, efficacy evaluation

INTRODUCTION

Since 2019, the global pandemic of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has influenced almost all countries worldwide. Although considerable efforts have been made to reduce COVID-19 transmission, the overall upward trend of COVID-19 is continuing around the world. As of 31 January 2021, the outbreak of

COVID-19 brings the cumulative numbers to over 102 million reported cases and over 2.2 million deaths globally (1). The disease spectrum of COVID-19 ranges from mild self-limiting disease to severe life-threatening disease, which might progress to acute respiratory distress syndrome, multiple-organ dysfunction syndrome, and death (2, 3).

Immune function dysregulations, including lymphopenia and cytokine storm, were associated with COVID-19 progression (4). Thymosin alpha 1 (Thymosin-α1) is an immune function modifier, which plays an important role in activating and regulating immune cells. Therefore, Thymosin-α1 has been used in diseases with impaired immune function, particularly infections including viral infections (5). In 2003, Thymosin-α1 had been used as an immune enhancer in SARS patients, demonstrating efficacy in controlling the progression of SARS (6). Therefore, Thymosin-α1 has potential as a drug for the treatment of COVID-19 patients.

A recent study showed that Thymosin-α1 reversed T-cell exhaustion and recovered immune reconstitution through promoting thymus output, and then significantly reduced mortality in severe COVID-19 patients (7). Another study also showed that Thymosin-α1 therapy significantly reduced 28-day mortality (HR, 0.11, 95% CI 0.02–0.63, $p = 0.013$) in severe patients with COVID-19 (8). However, the two studies only evaluated the efficiency of Thymosin-α1 on severe patients with COVID-19. To date, there is no available data regarding the efficiency of Thymosin-α1 in non-severe patients with COVID-19. In this study, we aimed to compare clinical outcomes of patients treated with or without Thymosin-α1 therapy in non-severe patients with COVID-19.

METHODS

Participants

A total of 1,511 consecutive confirmed patients with COVID-19 admitted to the Shanghai Public Health Clinical Center from January 20th 2020 to January 31st 2021 were retrospectively analyzed. The Shanghai Public Health Clinical Center is a tertiary teaching hospital, and the only designated hospital for the treatment of adult patients with COVID-19 in Shanghai, China. Exclusion criteria are as follows: (1) Severe cases requiring immediate intensive care unit (ICU) admission at hospital admission ($n = 15$); (2) using corticosteroid therapy before progression to severe cases ($n = 63$); (3) using intravenous immunoglobulin therapy before progression to severe cases ($n = 41$); and (4) using Thymosin-α1 therapy after progression to severe cases ($n = 4$). Finally, 1,388 non-severe patients with COVID-19 at hospital admission were enrolled.

Diagnostic Criteria

The following are the diagnostic criteria: collected nasopharyngeal or throat swab specimens of suspected patients with COVID-19, extracted viral nucleotides in specimens, and detected SARS-CoV-2 RNA by reverse transcription polymerase chain reaction (RT-PCR) assay. Patients with COVID-19 were confirmed according to the positive results of SARS-CoV-2 RNA tests. Severe patients with COVID-19 were diagnosed according to at least one of the following standards (9): (1) respiratory frequency $\geq$ 30 breath/min; (2) resting oxygen saturation $\leq$ 93%; (3) oxygenation index $\leq$ 300 mmHg; (4) mechanical ventilation; and (5) shock or other organ failures.

Details for Standard Therapy

In this study, patients in the Thymosin-α1 group received both Thymosin-α1 therapy and standard therapy, and patients in the control group only received standard therapy. At hospital admission, patients received standard therapy, including oxygen therapy (nasal catheter oxygen inhalation, 3 L/min), antiviral therapy (Traditional Chinese Medicine Decoction, one dose of quaque die; hydroxychloroquine 400 mg quaque die; lopinavir 200 mg/ritonavir 50 mg twice a day; or Arbidol 200 mg three times a day), and allowance of nutrients (three eggs daily, human albumin 10 g quaque die if necessary). During the hospitalization, the oxygen flow rate and drug dosage could be modulated by a joint discussion of at least five experts from the Shanghai Medical Expert Group for the Treatment of COVID-19, based on the change in patients' general conditions, laboratory parameters, and chest CT scans results, and referring to the latest therapy advances in COVID-19.

Details for Administration of Thymosin-α1

The uses of Thymosin-α1 were decided by a joint discussion of at least five experts from the Shanghai Medical Expert Group for the Treatment of COVID-19, based on patients' age, comorbidity, and laboratorial parameters including lymphocyte count, CD8+ T cell count, and CD4+ T cell count. The dose of Thymosin-α1 and date of administration are shown as follows: (1) 1.6 mg, three times a week, for at least 1 week, 82 patients; (2) 1.6 mg, once every 2 days, for at least 6 days, 94 patients; and (3) 1.6 mg, quaque die, for at least 3 days, 56 patients. Thymosin-α1 therapy was initiated within a median of 2 days (IQR, 1–3) of hospital admission.

SARS-CoV-2 RNA Extraction Method and PCR Protocol

SARS-CoV-2 nucleic acids were detected using the automatic magnetic extraction device and accompanying kit (Bio-Germ Medical Technology Co., Ltd, Shanghai, China) and screened with a semi-quantitative RT-PCR kits (Bio-Germ Medical Technology Co., Ltd, Shanghai, China) with amplification targeting the ORF1a/b and N gene. The RT-PCR with 5 μL RNA was used to target the nucleocapsid gene and open reading frame lab gene using a SARS-CoV-2 nucleic acid detection reagent (Bio-Germ Medical Technology Co., Ltd, Shanghai, China). The final reaction mixture concentration was 500 nm for primer and 200 nm for probe, respectively. Conditions for the amplifications were 50°C for 15 min, 95°C for 3 min, followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. The lowest detection concentration is 1×10^3 copies/ml.

Clinical Outcomes and Definitions

In this study, primary clinical outcomes included the rate of patients progressed to severe cases and the COVID-19-related mortality rate. Secondary clinical outcomes included

TABLE 1 | Baseline characteristics of patients.

	All patients	Thymosin-α1 group	Control group	*p*-values
Number of patients	1,388	232	1,156	–
Age (years)	35 (26–47)	38 (28–53)	34 (26–47)	<0.001
Male, *n* (%)	857 (61.7%)	137 (59.1%)	720 (62.3%)	0.355
Comorbidity, *n* (%)	203 (14.6%)	50 (21.6%)	153 (13.2%)	0.001
Vital signs				
Temperature (°C)	37.3 (36.9–37.6)	37.4 (36.8–38.0)	37.2 (37.0–37.6)	0.689
Respiratory rates (/min)	22 (18–24)	22 (18–26)	21 (19–23)	0.571
Heart rates (/min)	75 (68–86)	78 (66–88)	75 (69–85)	0.285
Oxygen saturation (%)	96 (96–99)	96 (95–99)	97 (96–98)	0.369
Laboratory parameters at admission				
WBC count (10^9/L)	6.0 (4.8–7.4)	4.9 (4.0–6.1)	6.2 (5.0–7.5)	<0.001
Lymphocyte (10^9/L)	1.6 (1.2–2.0)	1.1 (0.8–1.4)	1.7 (1.3–2.1)	<0.001
CD4+ T cell (cells/μl)	631 (469–837)	392 (307–551)	671 (523–875)	<0.001
CD8+ T cell (cells/μl)	394 (273–550)	253 (172–388)	417 (299–583)	<0.001
CRP (mg/L)	0.5 (0.5–1.5)	1.5 (0.5–6.5)	0.5 (0.5–0.8)	<0.001
LDH (U/L)	189 (167–218)	229 (205–258)	175 (160–210)	<0.001
D-Dimer (ng/mL)	0.25 (0.18–0.38)	0.32 (0.23–0.50)	0.24 (0.18–0.36)	<0.001
Antiviral therapy				
Chinese medicine	808 (58.2%)	113 (48.7%)	695 (60.1%)	0.001
Hydroxychloroquine	275 (19.8%)	78 (33.6%)	197 (17.0%)	<0.001
Lopinavir/ritonavir	78 (5.6%)	10 (4.3%)	68 (5.9%)	0.343
Arbidol	107 (7.7%)	15 (6.5%)	92 (8.0%)	0.437
Progression to severe cases	12 (0.86%)	4 (1.72%)	8 (0.69%)	0.121

WBC, white blood cell; CRP, C-reactive protein; LDH, lactate dehydrogenase; p-values indicate differences between the Thymosin-α1 group and the control group.

duration of symptoms, SARS-CoV-2 RNA shedding duration, length of hospital stay, and antibiotic utilization rate. In this study, the quantification of the SARS-CoV-2 viral load is not available. Instead, the twice consecutive SARS-CoV-2 RNA negative results with at least 24 h intervals were considered as viral RNA shedding. The SARS-CoV-2 RNA shedding duration was defined as the time from illness onset (symptom onset for symptomatic patients, and first positive SARS-CoV-2 RNA tests for asymptomatic patients) to the occurrence of twice consecutive SARS-CoV-2 RNA negative results with at least 24 h intervals.

Data Collection

Demographic data including age, sex, body mass index, and comorbidity was obtained. Clinical data including epidemiological histories, clinical manifestations, vital signs, laboratory parameters, chest CT scans results, treatments, hospital stays, and primary and secondary clinical outcomes were collected from electronic medical records.

Statistical Analysis

Normally distributed data, non-normal distribution continuous data, and categorical data were presented as mean ± standard deviation, median (interquartile range, IQR), and number (frequency), respectively. The statistical difference was compared using Student's *t*-test for normally distributed data, non-parametric Mann–Whitney test for non-normal distribution continuous data, and Chi-square test for categorical data. Propensity score matching (PSM) is a powerful tool for comparing groups with similar observed characteristics without specifying the relationship between confounders and clinical outcomes (10). The PSM method was used to adjust for differences in the baseline data of patients between the Thymosin-α1 group and control group. Propensity scores were estimated according to the essential covariates that might have affected patient assignment to the Thymosin-α1 group or control group, as well as the clinical outcomes of patients with COVID-19. Univariate and multivariable logistic regression analyses were used to identify the covariates that independently associated with primary clinical outcomes. A 1:1 ratio exposed (Thymosin-α1 group) and unexposed (control group) matched analysis was performed; the caliper was set as 0.25 (11). The statistical analyses were performed using the SPSS software, version 15.0 (SPSS Inc. Chicago, Illinois, USA), the MedCalc software, version 16.1 (MedCalc Software bvba, Ostend, Belgium), and the R software, version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). All significance tests were two-tailed, and $p < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristics of Patients

Baseline characteristics of patients are shown in **Table 1**. The median age was 35 years (IQR, 26–47 years); 857 patients (61.7%) were male, and 203 patients (14.6%) had comorbidity.

TABLE 2 | Variables associated with primary clinical outcomes.

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	***p*-values**	**OR (95% CI)**	***p*-values**
Age (years)	1.138 (1.074–1.206)	<0.001	1.122 (1.033–1.218)	0.009
Male	1.033 (0.246–4.340)	0.965		
Comorbidity	18.015 (3.610–89.892)	<0.001	3.117 (1.415–23.425)	<0.001
Fever (T > 37.3°C)	1.193 (0.266–5.350)	0.817		
Respiratory rates (/min)	1.073 (0.747–1.540)	0.704		
Heart rates (/min)	1.013 (0.978–1.048)	0.475		
Oxygen saturation (%)	0.728 (0.170–3.115)	0.669		
WBC count (10^9/L)	0.930 (0.651–1.328)	0.689		
Lymphocyte (10^9/L)	0.996 (0.992–0.999)	0.023	0.847 (0.811–1.387)	0.194
CD4+ T cell (cells/μl)	0.775 (0.514–0.904)	0.003	0.882 (0.776–0.997)	0.026
CD8+ T cell (cells/μl)	0.996 (0.992–1.001)	0.098		
CRP (mg/L)	1.041 (1.011–1.072)	0.007	1.016 (1.008–1.048)	0.018
LDH (U/L)	1.100 (1.014–1.204)	0.004	1.056 (1.010–1.125)	0.012
D-Dimer (ng/mL)	1.307 (1.055–1.619)	<0.001	1.124 (1.016–1.192)	0.004
Chinese medicine	0.716 (0.178–2.876)	0.638		
Hydroxychloroquine	1.719 (0.409–7.231)	0.460		
Lopinavir/ritonavir	2.417 (0.294–19.897)	0.412		
Arbidol	2.595 (0.315–21.378)	0.375		

WBC, white blood cell; CRP, C-reactive protein; LDH, lactate dehydrogenase. Multivariate analysis was fitted by including factors associated with primary outcomes in univariable analyses ($p < 0.05$).

The median white blood cell (WBC), lymphocyte, CD4+ T cell, CD8+ T cell, C-reactive protein (CRP), lactate dehydrogenase (LDH), and D-dimer were 6.0×10^9/L (IQR, 4.8–7.4), 1.6×10^9/L (IQR, 1.2–2.0), 631 cells/μl (IQR, 469–837), 394 cells/μl (IQR, 273–550), 0.5 mg/L (IQR, 0.5–1.5), 189 U/L (IQR, 167–218), and 0.25 ng/mL (IQR, 0.18–0.38), respectively.

Among 1,388 enrolled patients, 232 patients (16.7%) received both Thymosin-α1 therapy and standard therapy (Thymosin-α1 group), and 1,156 patients (83.3%) only received standard therapy (control group). Compared with patients in the control group, those with higher age (38 vs. 34 years, $p < 0.001$), more common comorbidity (21.6% vs. 13.2%, $p = 0.001$), lower WBC (4.9 vs. 6.2 $\times 10^9$/L, $p < 0.001$), lymphocyte (1.1 vs. 1.7 $\times 10^9$/L, $p < 0.001$), CD4+ T cell (392 vs. 671 cells/μl, $p < 0.001$), and CD8+ T cell (253 vs. 417 cells/μl, $p < 0.001$) were more likely to be treated with Thymosin-α1 (**Table 1**).

Variables Associated With Primary Clinical Outcomes

Variables associated with primary clinical outcomes are shown in **Table 2**. Univariate analysis showed that age, comorbidity, lymphocyte, CD4+ T cell, CRP, LDH, and D-dimer were associated with primary clinical outcomes ($p < 0.05$). Multivariable analysis identified age (OR = 1.122, 95% CI, 1.033–1.218, $p = 0.009$), comorbidity (OR = 3.117, 95% CI, 1.415–23.425, $p < 0.001$), CD4+ T cell (OR = 0.882, 95% CI, 0.776–0.997, $p = 0.026$), CRP (OR = 1.016, 95% CI, 1.008–1.048, $p = 0.018$), LDH (OR = 1.056, 95% CI, 1.010–1.125, $p = 0.012$), and D-dimer (OR = 1.124, 95% CI, 1.016–1.192, $p = 0.004$) as the variables independently associated with primary clinical outcomes.

Characteristics of Patients After PSM

As statistically significant differences existed in the baseline characteristics between the Thymosin-α1 group and control group, we selected patients by the PSM method according to the 1:1 ratio. The factors that independently associated with primary clinical outcomes (age, comorbidity, CD4+ T cell, CRP, LDH, and D-dimer) were matched between the Thymosin-α1 group and control group. After PSM, the baseline characteristics of patients were well-balanced between the Thymosin-α1 group and control group ($p > 0.05$) (**Table 3**).

Evaluation of Efficacy for Thymosin-α1

The evaluation of efficacy for Thymosin-α1 in propensity-matched groups is shown in **Table 4**. The proportion of patients progressed to severe COVID-19 was 2.17% for the Thymosin-α1 group, and 2.71% for the control group ($p = 0.736$). The COVID-19-related mortality was 0.54% for the Thymosin-α1 group and 0 for the control group ($p = 0.317$). Compared with the control group, the Thymosin-α1 group had significantly shorter SARS-CoV-2 RNA shedding duration (13 vs. 16 days, $p = 0.025$) and hospital stay (14 vs. 18 days, $p < 0.001$). No statistically significant difference was found between the Thymosin-α1 group and control group in duration of symptoms (median, 4 vs. 3 days, $p = 0.843$) and antibiotic utilization rate (14.1% vs. 15.2%, $p = 0.768$). In this study, there were no allergic reaction and drug eruption in both Thymosin-α1 group and control group.

TABLE 3 | Baseline characteristics of patients after propensity score matching.

	Thymosin-α1 group	Control group	*p*-values
Number of patients	184	184	—
Age (years)	37 (28–52)	37 (29–44)	0.193
Male, *n* (%)	105 (57.1%)	109 (59.2%)	0.673
Comorbidity, *n* (%)	37 (20.1%)	35 (19.0%)	0.793
Vital signs			
Temperature (°C)	37.3 (36.5–37.8)	37.3 (36.7–37.6)	0.655
Respiratory rates (/min)	21 (18–25)	22 (19–24)	0.469
Heart rates (/min)	76 (65–86)	74 (68–84)	0.841
Oxygen saturation (%)	96 (95–99)	96 (95–99)	0.696
Laboratory parameters at admission			
WBC (10^9/L)	5.1 (4.0–6.1)	5.3 (4.3–6.1)	0.566
Lymphocyte (10^9/L)	1.1 (0.8–1.5)	1.2 (0.8–1.5)	0.378
CD4+ T cell (cells/μl)	383 (312–568)	378 (308–559)	0.459
CD8+ T cell (cells/μl)	256 (175–390)	254 (180–386)	0.707
CRP (mg/L)	1.5 (0.5–5.7)	1.5 (0.5–5.1)	0.364
LDH (U/L)	218 (198–242)	214 (186–238)	0.620
D-Dimer (ng/mL)	0.31 (0.23–0.48)	0.30 (0.21–0.42)	0.134
Antiviral therapy			
Chinese medicine	101 (54.9%)	112 (60.9%)	0.246
Hydroxychloroquine	65 (35.3%)	58 (31.5%)	0.439
Lopinavir/ritonavir	8 (4.3%)	10 (5.4%)	0.629
Arbidol	12 (6.5%)	14 (7.6%)	0.684

WBC, white blood cell count; CRP, C-reactive protein; LDH, lactate dehydrogenase; p-values indicate differences between the Thymosin-α1 group and the control group.

TABLE 4 | Evaluation of efficacy for Thymosin-α1 in propensity-matched groups.

	Thymosin-α1 group	Control group	*p*-values
Number of patients	184	184	—
Primary outcomes			
Developed to severe cases	4 (2.17%)	5 (2.71%)	0.736
Died	1 (0.54%)	0	0.317
Secondary outcomes			
Duration of symptom (days)	4 (2–6)	3 (2–5)	0.843
Viral RNA shedding duration (days)	13 (10–19)	16 (11–20)	0.025
Hospital stays (days)	14 (11–21)	18 (13–23)	<0.001
Antibiotics therapy, *n* (%)	26 (14.1%)	28 (15.2%)	0.768
Probable adverse effects			
Allergic reaction	0	0	–
Drug eruption	0	0	–
Liver injury	40 (21.7%)	36 (19.7%)	0.607

Liver injury is defined as ALT > 40 IU/L during the hospitalization.

No significant difference was found between Thymosin-α1 group and control group in liver injury (21.7% vs. 19.7%, $p = 0.607$).

Cox Analysis for Comparison of Time Variables Between Groups

Cox regression analysis showed that Thymosin-α1 therapy is associated with a shorter SARS-CoV-2 RNA shedding duration (HR 1.29; 95% CI 1.05–1.59: $p = 0.015$) (**Figure 1A**) and hospital stay (HR 1.37; 95% CI 1.11–1.68: $p = 0.003$) (**Figure 1B**), compared with the control group.

DISCUSSION

Although it is important to explore the potential benefits that Thymosin-α1 can bring in patients with COVID-19, so far, clinical studies on the efficiency of Thymosin-α1 are still limited. In this study that evaluated the efficacy of Thymosin-α1 in non-severe patients with COVID-19, we found that Thymosin-α1 treatment did not alter disease progression and mortality rate, but it significantly reduced SARS-CoV-2 RNA shedding duration and hospital stay. In this study, we compared non-severe patients with Thymosin-α1 therapy to those with standard therapy, rather than a specific drug, since there is as yet no effective drug for non-severe COVID-19 patients.

The duration of SARS-CoV-2 RNA shedding is often considered in determining an appropriate period of isolation as it is often used as a marker of infectivity. Therefore, the importance of shortened duration of SARS-CoV-2 shedding is the public health implications for reducing COVID-19 transmission. It can hardly be denied that the medical resources, especially the number of hospital beds, are insufficient after the outbreak of the COVID-19 epidemic in many countries and areas. Shortening hospital stay is helpful for relieving the pressure on medical resource including the number of hospital beds. Therefore, based

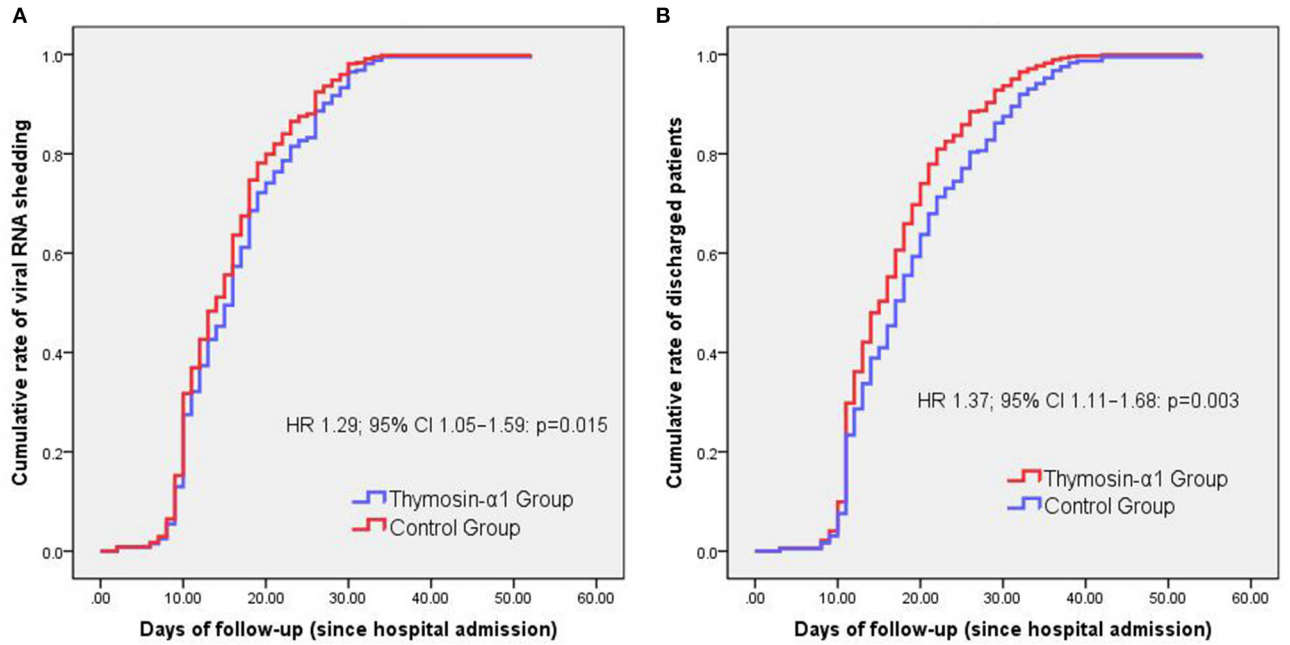

FIGURE 1 | Cox analysis for comparison of time variables between groups. The only one patient who died was excluded when we compared the cumulative rates of discharged patients over days of follow-up between groups. Thymosin-α1 therapy is associated with a shorter SARS-CoV-2 RNA shedding duration (HR 1.29; 95% CI 1.05–1.59: $p = 0.015$) **(A)** and hospital stay (HR 1.37; 95% CI 1.11–1.68: $p = 0.003$) **(B)**, compared with the control group.

on the results that Thymosin-α1 significantly reduced hospital stay and duration of SARS-CoV-2 RNA shedding, we suggested that Thymosin-α1 could be used as a drug for the treatment of non-severe COVID-19 patients.

Thymosin-α1 can boost immune response via activation of T cell proliferation, differentiation, and maturation that is beneficial for virus clearance (12). Therefore, Thymosin-α1 treatment can support patients with low T cell count since it can help boost immunity. The study by Liu et al. recommended COVID-19 patients whose CD8+ T cell count or CD4+ T cell count lower than 400 or 650/μL, respectively, applies Thymosin-α1 injection to improve their immune function (7). Based on our experience and the results of previous studies, we suggested Thymosin-α1 therapy to patients with old age, comorbidity, and reduced lymphocyte, CD8+ T cell, and CD4+ T cell. In this study, patients in the Thymosin-α1 group had higher age, more common comorbidity, lower lymphocyte, CD4+ T cell, and CD8+ T cell count than patients in the control group.

A study by Dominari et al. showed that Thymosin-α1 significantly promoted the proliferation of activated T cells, and this led to a critical prevention of lymphopenia in elderly COVID-19 patients with comorbidity (13). Yu et al. enrolled 25 severely and critically ill patients with COVID-19 and found that patients in the Thymosin-α1 treatment group had a higher number of lymphocytes than patients without Thymosin-α1 treatment (14). Previous studies on severe cases also suggested that treatment with Thymosin-α1 can markedly decrease 28-day mortality and attenuate acute lung injury in critical type COVID-19 patients (7, 8). As a complement to previous studies, we assessed the effect of using Thymosin-α1 as a supportive treatment for non-severe COVID-19 patients. The findings in this study showed that among non-severe COVID-19 patients, Thymosin-α1 therapy significantly reduced hospital stay and the duration of SARS-CoV-2 RNA shedding. In addition, the safety profile of Thymosin-α1 is good and it is virtually devoid of toxicity. Therefore, we suggested that, besides the fact that it should be used on severe cases, Thymosin-α1 could also be used on non-severe COVID-19 patients.

So far, only the nucleotide analog prodrug remdesivir is approved by the US FDA for the treatment of seriously ill patients with COVID-19 (15), although the WHO recommends corticosteroids for the treatment of patients with severe or critical COVID-19 (16). In addition, convalescent plasma is available for use in patients with severe or life-threatening COVID-19 through Emergency Use Authorization (17). However, to date, no effective drugs have been identified to treat non-severe patients with COVID-19. A recent study reported among non-severe patients with COVID-19, treatment with bamlanivimab and etesevimab was associated with a statistically significant reduction in SARS-CoV-2 viral load, compared with placebo (18). In this study, we found that Thymosin-α1 therapy significantly reduced SARS-CoV-2 RNA shedding duration and hospital stay. Compared with bamlanivimab and etesevimab, Thymosin-α1 is more clinically accessible, more inexpensive, and much safer.

In this study, exclusion criteria did not include patients receiving antiviral and antibacterial drugs. We did not exclude patients receiving antibacterial drugs, because secondary clinical outcomes included antibacterial drug utilization rate. We did

not exclude patients receiving antiviral drugs, because most of the patients (1,263 patients, 91.0%) in this study received antiviral drugs, including Traditional Chinese Medicine (808 patients, 58.2%), hydroxychloroquine (275 patients, 19.8%), lopinavir/ritonavir (78 patients, 5.6%), and Arbidol (107, 7.7%). In order to eliminate the effects of antiviral drugs on the clinical outcomes, the PSM method was used to adjust for differences in the use of antiviral drugs. After PSM, the use of antiviral drugs including Chinese Medicine (54.9% vs. 60.9%, $p = 0.246$), hydroxychloroquine (35.3% vs. 31.5%, $p = 0.439$), lopinavir/ritonavir (4.3% vs. 5.4%, $p = 0.629$), and Arbidol (6.5% vs. 7.6%, $p = 0.684$) is well-balanced between the Thymosin-α1 group and control group ($p > 0.05$) (**Table 3**). In this study, multivariable analysis identified underlying disease as one of the variables independently associated with primary clinical outcomes. Although we did not classify underlying disease as an exclusion criterion, the PSM method was used to adjust for differences in the underlying disease.

This study had several limitations. First, although this study showed that Thymosin-α1 has some benefits to non-severe COVID-19 patients, it should be interpreted with caution because of the inherent nature of the retrospective study. More clinical trials are needed to determine the effect of Thymosin-α1 on non-severe patients with COVID-19. Second, in this study, the patient population who progressed to severe COVID-19 or death was small, which made detecting statistically significant differences between groups more difficult for the primary clinical outcomes. Third, in this retrospective study, the SARS-CoV-2 viral load is not available, so we did not know whether Thymosin-α1 treatment can reduce virus titers. Fourth, genetic factors and the presence of some significant SNP in the host are notable factors in the course of COVID-19. Further studies will be needed to confirm the relationship between host genetics and the effect of Thymosin-α1. Fifth, although it is an interesting research point, the difference in viral detection among nasopharyngeal vs. throat swabs in terms of positivity rates and Ct values is unavailable in this retrospective study. However, nasopharyngeal and throat swab specimens from COVID-19 patients have been compared in previous study (19). Vlek reported that combined throat swabs yield a similar sensitivity to detect SARS-CoV-2 as nasopharyngeal swabs and are a good alternative sampling method, despite a lower Ct value for the nasopharyngeal samples (19).

In conclusion, among non-severe patients with COVID-19, Thymosin-α1 treatment did not alter disease progression and mortality rate, but it significantly reduced SARS-CoV-2 RNA shedding duration and hospital stay. No statistically significant difference in duration of symptoms and antibiotic utilization rate were observed between the Thymosin-α1 group and control group. Prospective randomized controlled clinical trials are needed to further assess the clinical benefit of Thymosin-α1 in non-severe patients with COVID-19.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Clinical Research Ethics Committee of the Shanghai Public Health Clinical Center. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

QL: study concept and design and drafting of the manuscript. CH, LF, WX, WL, and XX: data collection. CH, LF, WX, and QL: analysis and interpretation of data. LC: critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Public Knowledge and Practices Regarding Coronavirus Disease 2019: A Cross-Sectional Survey From Pakistan

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Objectives: Effective mitigation of coronavirus diseases (COVID-19) pandemic requires true adoption of precautionary measures by the masses, that primarily depends upon their knowledge and practices behaviors. The current study aimed to assess the knowledge; practices of Pakistani residents regarding COVID-19 and factors associated with adequate knowledge and positive practices.

Material and Methods: A cross-sectional online survey was conducted from 15-April 2020 to 20 May 2020 among 689 Pakistanis by using a validated self-administered questionnaire (Cronbach's alpha 0.77). The questionnaire included questions on the assessment of demographics, the source of information, knowledge, and practice of COVID-19 on google forms and shared links with the WhatsApp groups, Facebook pages and other online platforms. Regression analysis was applied to find potential predictors of knowledge and practices.

Results: Of 689 participants, 431 (62.6%) were male, 64.3% ($n = 443$) were aged <30 years, and\328 (47.6%) of participants were married. 48.19% ($n = 332$) had adequate knowledge; 81% ($n = 555$) had positive practices regarding COVID-19 and majority (66.62%, $n = 459$) seek knowledge from social media. Knowledge was significantly higher (OR > 1.00, $p < 0.05$) among educated and higher income participants. Positive practices were significantly (OR > 1.00, $p < 0.05$) related to the older age (≥50 years), higher education, higher income and good knowledge regarding COVID-19.

Conclusion: The study concluded that Pakistani residents had average knowledge and good practices toward COVID-19 yet there are gaps in specific aspects of knowledge, and practice that should be focused in future awareness and educational campaigns. The study recommends the ministry of health authorities to promote all precautionary and preventive measures of COVID-19-consisting of a better-organized approach to all strata of society: less privileged people, older ones and less educated people, in order to have equilibrium of knowledge about COVID-19; hence effective implementation of precautionary measures.

Keywords: awareness, public, COVID-19, pakistan, knowledge, practices

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INTRODUCTION

On 11 March 2020, the World Health Organization (WHO) declared the outbreak of coronavirus disease (COVID-19) to be a pandemic, with disease spread in 114 countries and more than 4,000 deaths (1). On 31 May 2020, there were 6,175,290 cases of COVID-19, with 371,228 confirmed deaths in 213 countries and territories worldwide (2). Pakistan, a lower middle-income country (LMIC), ranked 5th among the most populous countries in the world to be considered the new COVID-19 hotspot with a fragmented health care system (3). As far as COVID-19 is concerned, Pakistan has a uniquely challenging situation due to its vulnerable geographical location, as it shares borders with China and Iran due to poor screening capacity, leading to delayed implementation of preventive measures (4). At the beginning of January 2020, the WHO sent technical assistance to Pakistan, at which time Pakistan did not have a COVID-19 case or even a COVID-19 test capability. WHO and Pakistan's Ministry of Health have begun to investigate possible cases and to distribute disease risk information brochures to passengers arriving initially at airports (5). Pakistan's first case of COVID-19 was reported on 26 February in Karachi, having a travel history of Iran (5). Within 6 weeks, Pakistan developed a test capacity of up to 30,000 tests per day, imposed lockdowns across the country, suspending public transportation systems, and restricting air and sea travel (5). Due to the lockdown and limited follow-up to standard operating procedures (SOPs), the country's healthcare systems have been burdened by rapidly increasing cases (6). Pakistani Prime Minister Imran Khan has taken the initiative and launched the "Smart lock-down" campaign to limit unnecessary movements in cities (6). Pakistan has also set up a National Command Operations Center (NCOC), which combines military and government to produce and distribute cheap masks locally and developed the guidelines to curb COVID-19 (6). However, as of 31 May 2020, Pakistan has 68,270 laboratory-confirmed cases and 1,483 COVID-19 associated deaths. Sindh (n = 27,360) has the highest number of cases followed by Punjab (n = 25,056), Khyber Pakhtunkhwa (KP) (n = 9,540), Baluchistan (n = 4,193), Islamabad (n = 2,418), Gilgit Baltistan (678), and Azad Jammu Kashmir (n = 251) (7). Punjab (n = 475) being the most populous province has the highest number of deaths followed by Sindh (n = 465), KP (n = 453), Baluchistan (n = 46), Islamabad (n = 27), Gilgit Baltistan (10), and Azad Jammu Kashmir (n = 7) (7).

People may not have access to regular and reliable sources of disease etiology information in the context of LMICs, leaving them ill-equipped to minimize the risk of infection in emerging outbreaks (8–10). Concerns have arisen about the possibility of obstructing public health communication due to misinformation (11–13). As WHO Director-General Dr. Tedros Adhanom Ghebreyesus said, "we're not just fighting an epidemic; we're fighting an infodemic" (14). Excessive or understated pandemic estimates may either give the public a fuel panic or a falsified sense of security (15). Confusion of basic information about how the virus can be reduced and how it can be exposed puts people at risk of infection (16). Data suggest that knowledge, awareness, perception, and attitude of the general public regarding disease play an essential role in the control and management of disease as observed in epidemics of SARS and MERS (14). Poor knowledge of the general public regarding preventive measures as avoiding crowded areas, wearing masks, properly washing hands, and maintaining social distance also pose a significant gap in control of the spread of COVID 19 (17). High number of people with a lack of symptoms could be a possible way of the virus spread (18). Deeming public awareness to be crucial in preventing the spread of COVID−19, which otherwise lacks effective treatment and preventive measures, vast public awareness campaigns are critical in the fight against it (19).

The Pakistani National Institute of Health (NIH) has played a vital role in designing and circulating protocols regarding COVID-19 transmission and prevention, as well as launching public awareness campaigns (7). However, final success depends upon the adherence of people to guidelines and preventive measures that are strongly linked to their understanding and awareness toward disease (20). Survey highlighting awareness level is useful to get information regarding public health education, response and recovery efforts, and social mobilization (21). Data from the study is pivotal for policy development and public health implementation to respond to the outbreak shortly and consistently quickly. In context of the explanation above, the current study aimed to evaluate the current level of awareness regarding transmission, symptoms, and preventive measures of COVID-19 among the general population in Pakistan. Additionally, this study will provide a snapshot of the extent of precautionary measures practiced by the Pakistani population.

METHODS

Study Design

A cross-sectional survey-based study was conducted during the months of April and May 2020, days of strict lockdown to implement social distancing to avoid the spread of the pandemic. The investigators opted for an online data collection method because it was not possible to carry out a population-based survey in this critical/censorious situation.

Sampling, Study Population and Data Collection Method

The sample size calculated by Raosoft was 583, assuming a response rate of 50%, confidence interval (CI) 95%, Z as 1.96, and margin of error d as 4%. Considering, an additional 20% (n = 116) for any error in questionnaire filling, a final sample size of 699 will be required. The survey was started on 15 April 2020, and response acceptance was closed on 20-May 2020, when required sample size was achieved. Pakistani people 16 years of age or older who voluntarily agreed to fill out the form/questionnaire were selected. Participants were given no monetary benefits to participate in the study due to lack of funds.

The questionnaire was designed on google forms and the generated link was shared with the WhatsApp groups. Link was also shared personally with the contact list of investigators.

Measure

A survey instrument was designed based on substantial literature analysis (22–24), material related to emerging respiratory

diseases including COVID-19 by WHO (25) and guidelines issued by national institute of health (NIH), Islamabad Pakistan (7). After the preliminary draft questionnaire was drawn up, it was validated in two stages. In the first place, the study tool was discussed with pharmacy and medical researchers and professionals to give their expert opinion on its simplicity, relativity and relevance. Secondly, a pilot study was conducted by selecting a small sample ($n = 60$) to make the questionnaire simpler and more comprehensive. The questionnaire was amended based on the suggestions made by the participants and its consistency with the published literature. After a thorough discussion, the authors finalized the questionnaire and then distributed it to the participants for their response. The coefficient of reliability was calculated using SPSS v.20 and Cronbach's alpha value. It was found to be 0.77. The data from the pilot study were not included in the final analysis.

TABLE 1 | Demographic characteristics of study population ($N = 689$).

Variables	Characteristics	Number of participants (n)	Percentage (%)
Gender			
	Female	258	37.4
	Male	431	62.6
Age			
	< 30 years	443	64.3
	31–39 Years	148	21.5
	40–49 years	69	10.0
	More than 50 years	29	4.2
Marital status			
	Single	361	52.4
	Married	328	47.6
Monthly Income			
	0–24,999	367	53.3
	25, 000–49,999	165	23.9
	≥50,000,	157	22.8
Employment status			
	Employed	160	23.2
	Unemployed	529	76.8
Residence			
	Rural	160	23.2
	Urban	529	76.8
Education status			
	None	71	10.3
	≤10 years	39	5.7
	11–12	131	19.0
	13 or more	448	65.0

The questionnaire included questions on the assessment of demographics, the source of information, knowledge, and practice of COVID-19. The demographic characteristics included gender, age, marital status, monthly income, residence, employment status and education. One item was regarding the source of information about COVID-19. Awareness section comprised of 20 items; regarding etiology (2-items), symptoms (7-items), risk group (1-item), transmission (6-items), treatment (2-items) and precautions/preventions (2-items). Each question was responded as Yes, No, and I don't know. The correct answer was marked as 1 while wrong answer was marked as 0. Total score ranges from 0 to 14, and a cut off level of ≤15 was set for poor knowledge and ≥16 (More than 75%) for good knowledge.

The practice section included 6 items related to the use of face mask, and implementation of other precautionary measures were included in the practice section. Each item was responded as yes (1-point), No (0-point), and sometimes (0-point). Practice items total score ranged as 0-6, where 5–6 score was considered as good practice, and a score of 1–4 indicated poor practice of preventive measures for COVID-19.

Ethics

The study was performed following the declaration of Helsinki. Due to lockdown, Universities were closed, hence study protocol

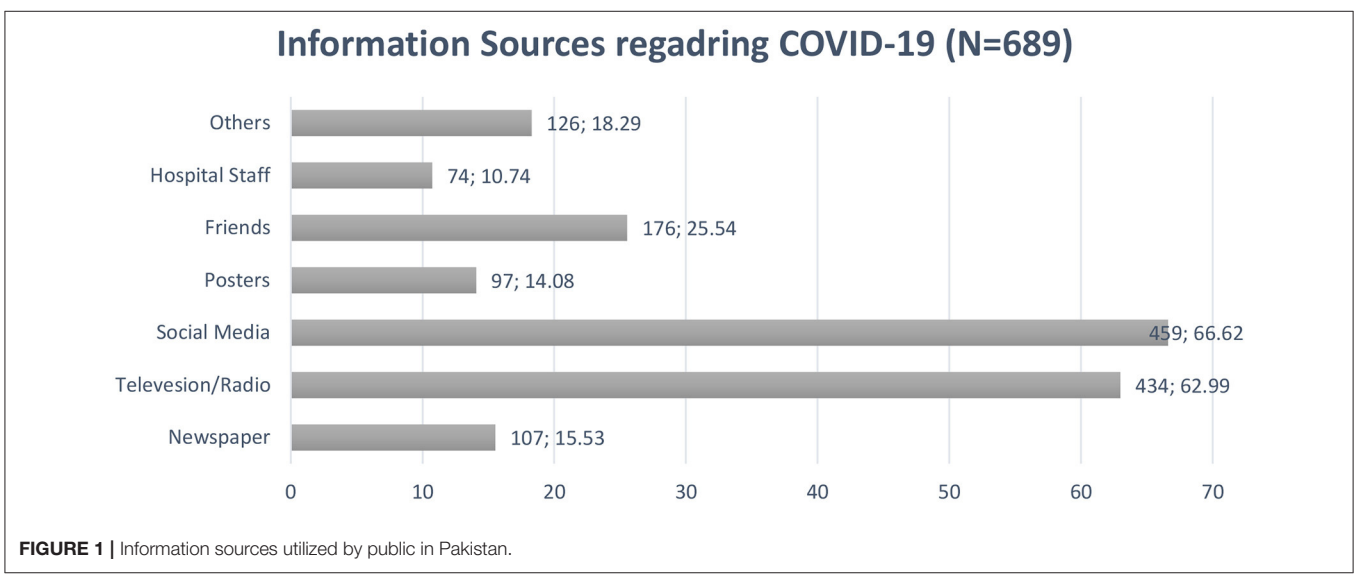

FIGURE 1 | Information sources utilized by public in Pakistan.

was approved from the "Tehsil headquarter hospital samundri" hospital board (767/THQ/HR). The study questionnaire contained a consent portion that stated purpose, nature of the survey, study objectives, volunteer participation, declaration of confidentiality, and anonymity.

Statistical Analysis

Data were entered in Microsoft Excel and later imported into SPSS V.21 for statistical analysis. Numerical variables were measured as mean and standard deviations, while categorical variables were expressed as frequencies and percentages. Inferential statistics were applied depending upon the nature of data and variables. Chi-square tests were used to find differences in knowledge groups (good vs. poor) and practice (good vs. poor) by demographic characteristics. Binary logistic regression models have been used to identify possible determinants of good knowledge and practice, both unadjusted and adjusted (adjusted for age, gender and other demographic variables). Results were expressed as crude odds ratio (COR), and adjusted odds ratio (AOR) accompanied by 95% confidence interval (CI). A p-value of < 0.05 will be considered significant in all tests.

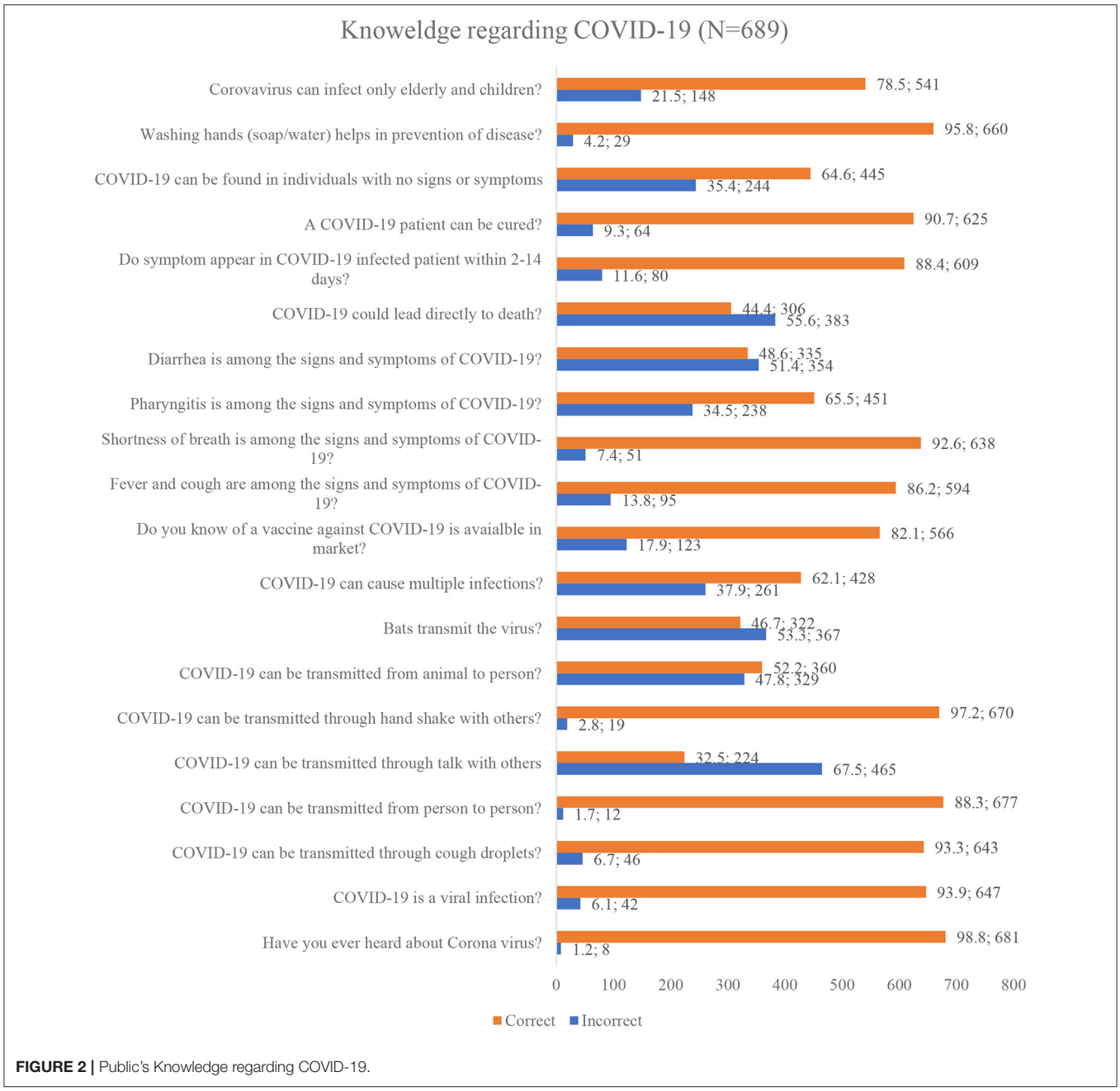

FIGURE 2 | Public's Knowledge regarding COVID-19.

RESULTS

Out of the total 699 responses collected, 10 questionnaires were excluded due to missing information, and 689 responses were analyzed in the final analysis. Most participants were male (62.6%, $n = 431$), 64.3% ($n = 443$) were aged < 30 years, and less than half (47.6%, $n = 328$) of participants were married. More than half (53.3%, $n = 367$) of respondent had monthly income of (Pakistani rupee) PKR 0 - 24,999, 76.8% ($n = 529$) were unemployed, and 65% ($n = 448$) participants had education of 13 years or more (**Table 1**).

Figure 1 provides a summary of the information sources utilized by respondents. Majority of participants used social media (66.62%, $n = 459$) as a source to seek information regarding COVID-19 followed by television/radio (62.99%, $n = 434$) and friends (25.54%, $n = 176$).

Knowledge Regarding COVID-19

Figure 2 summarizes the responses of participants for knowledge items of the questionnaire. Mixed responses were obtained regarding 20 items. The majority of the 689 participants had heard about the disease, and 93.9 % ($n = 647$) know that virus is the causative agent of COVID-19. Response to questions regarding transmission of disease indicated 93.3% ($n = 643$) participants correctly identified that virus can transmit trough droplets, 97.2% ($n = 670$) subjects were well aware that infection can be transmitted by shaking hands, and 98.3% ($n = 677$) respondents had correct knowledge regarding the transmission of the virus from person to person. On the other hand, when the question asked regarding fatality of disease, 55.6% ($n = 383$) subjects respond that COVID-19 directly leads to death, 35.4% ($n = 244$) respondent did not know that COVID-19 can be found in a person with no signs and symptoms, and 21.5% ($n = 148$) reported that virus could affect only elderly and children.

Practices Regarding COVID-19

Figure 3 summarizes the practices of the general population regarding COVID-19. All of the 689 participants respond to all 6 items regarding COVID-19. Most respondents had good practice regarding each item with the highest practice showed toward washing of hands with soap or cleaning with sanitizers (94%, $n = 648$), and a similar proportion (93.8%, $n = 646$) of participants revealed that they avoid going in a crowded place. A lower percentage of good practice was observed among the general population to wear a face mask (79.8%, $n = 550$).

Difference in Knowledge and Practice Status by Demographics

More than half of participants had poor knowledge (51.81%, $n = 357$), while 332 (48.19%) individuals had adequate

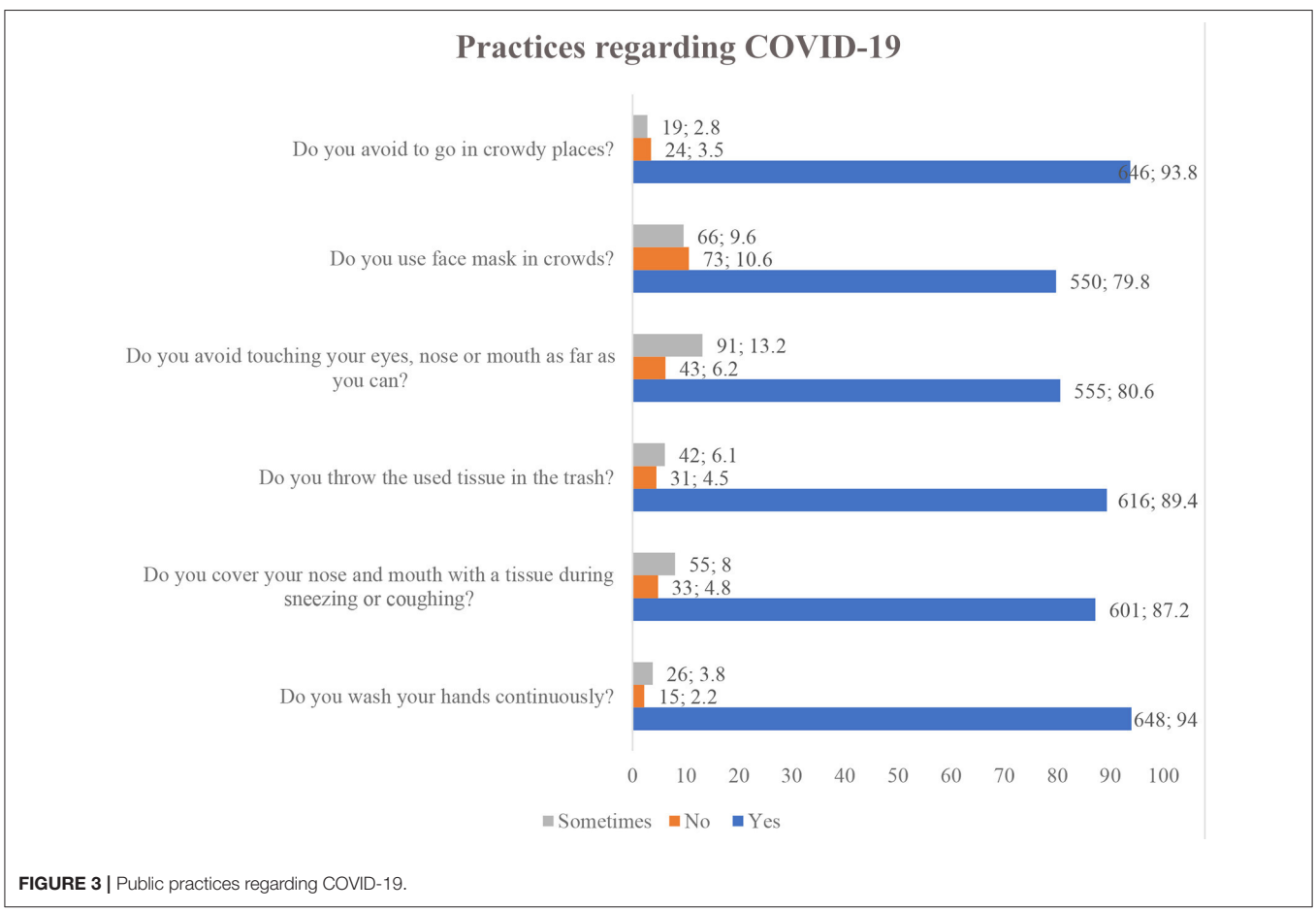

FIGURE 3 | Public practices regarding COVID-19.

knowledge regarding COVID-19. Chi-square tests were applied to find differences in knowledge status by sample characteristics. Knowledge status was significantly differed by monthly income as participants with higher income have adequate knowledge compared to lower-income counterparts ($\chi^2 = 25.85, p < 0.001$). The findings showed that knowledge status differed significantly from marital status ($\chi^2 = 4.606, p = 0.032$), to employment status ($\chi^2 = 4.968, p = 0.026$). Similarly, most participants with higher education had adequate knowledge compared to less-educated counterparts ($\chi^2 = 24.07, p < 0.001$) while the status of practice did not differ significantly in terms of gender, age, and residence ($p > 0.05$) (**Table 2**).

Findings indicated that 81% ($n = 555$) participants had good practice in following precautionary measures regarding COVID-19. Chi-square analysis revealed that participant's practices regarding COVID-19 were significantly differed by monthly-income ($\chi^2 = 8.979$, $p = 0.011$), residence ($\chi^2 = 6.154$, $p = 0.013$), and education status ($\chi^2 = 16.716$, $p = 0.001$). While the status of the practice did not differ significantly in terms of gender, age, marital status, and employment status ($p > 0.05$) (**Table 2**).

Binary Logistic Regression Analysis for Factors Associated With Good Knowledge and Practice

Adjusted and un-adjusted regression analysis was applied to find possible predictors of good knowledge and practice among the general population in Pakistan. The regression model adjusted for all independent variables showed that participants with higher monthly income (≥50,000) had higher odds (AOR: 2.133, 95% CI: 1.394–3.867, $p < 0.001$) of adequate knowledge compared to the reference category. Similarly, participants who have an education of 13 years or more had higher odds (AOR: 1.501, 95% CI: 0.858–2.627, $p = 0.041$) of good knowledge compared to less-educated counterparts (**Table 3**).

Finding showed that age group of 50 years or more (AOR: 1.087, 95% CI: 0.428–1.207, $p = 0.020$), monthly income of PKR 25, 000 - 49,999 (AOR: 1.875, 95% CI: 1.055–3.331, $p = 0.032$), education of 11–12 years (AOR: 1.18, 95% CI: 0.567–2.489, $p = 0.049$), education of 13 years or more (AOR: 1.250, 95% CI: 0.647–2.415, $p = 0.031$), and good knowledge (vs. Poor knowledge: AOR: 1.80, 95% CI:

TABLE 2 | Difference in knowledge and practice status by demographics.

Characteristics	Categories	Knowledge		χ^2 (P)	Practice		χ2 (P)
		Poor knowledge	Good knowledge		Poor practice	Good practice	
		n (%)	*n* (%)		*n* (%)	*n* (%)	
Total		357 (51.81)	332 (48.19)		134 (19)	555 (81)	
Gender				0.273 (0.601)			0.690 (0.406)
	Female	137 (53.1)	121 (46.9)		46 (17.8)	212 (82.2)	
	Male	220 (51.0)	211 (49.0)		88 (20.4)	343 (79.6)	
Age				5.020 (0.170)			7.011 (0.071)
	<30 years	217 (49.0)	226 (51.0)		83 (18.7)	360 (81.3)	
	31–39 Years	88 (59.5)	60 (40.5)		20 (19.6)	119 (80.4)	
	40–49 years	36 (52.2)	33 (47.8)		11 (15.9)	58 (84.1)	
	More than 50 years	16 (55.2)	13 (44.8)		11 (37.9)	18 (62.1)	
Marital status				4.606 (0.032)			0.534 (0.465)
	Single	173 (47.9)	188 (52.1)		74 (20.5)	287 (79.5)	
	Married	184 (56.1)	144 (43.9)		60 (18.3)	268 (81.7)	
Monthly Income				25.85 (<0.001)			8.979 (0.011)
	0–24,999	223 (60.8)	144 (39.2)		85 (23.2)	282 (76.8)	
	25, 000–49,999	65 (39.4)	100 (60.6)		20 (12.1)	145 (87.9)	
	≥50,000,	69 (43.9)	88 (56.1)		29 (18.5)	128 (81.5)	
Employment status				4.968 (0.026)			2.554 (0.110)
	Employed	215 (48.6)	227 (51.4)		78 (17.6)	364 (82.4)	
	Unemployed	142 (57.5)	105 (42.5)		56 (22.7)	191 (77.3)	
Residence				0.784 (0.376)			6.154 (0.013)
	Rural	78 (48.8)	82 (51.2)		42 (26.3)	118 (73.8)	
	Urban	279 (52.7)	250 (47.3)		92 (17.4)	437 (82.6)	
Education status				24.007 (<0.001)			16.716 (0.001)
	None	44 (62.0)	27 (38.0)		16 (22.5)	55 (77.5)	
	≤10 years	32 (82.1)	7 (17.9)		17 (43.6)	22 (56.4)	
	11–12	74 (56.5)	57 (43.5)		25 (19.1)	106 (80.9)	
	13 or more	207 (46.2)	241 (53.8)		76 (17.0)	372 (83.0)	

TABLE 3 | Binary logistic regression analysis for factors associated with good knowledge.

Characteristics		Factors associated with good knowledge					
		COR	95% CI	P	AOR	95% CI	P
Gender							
	Female	Reference	–	–	Reference	–	–
	Male	1.086	0.797–1.479	0.601	1.153	0.819–1.624	0.415
Age							
	<30 years	Reference	–	–	Reference	–	–
	31–39 Years	0.655	0.449– 0.955	0.028	0.642	0.393–1.050	0.078
	40–49 years	0.880	0. 530– 1.462	0.622	0.831	0.441–1.567	0.567
	More than 50 years	0.965	0.367– 1.660	0.519	0.859	0.358–2.062	0.859
Marital status							
	Single	Reference	–	–	Reference	–	–
	Married	0.720	0.533–0.972	0.032	0.742	0.487–1.131	0.742
Monthly Income							
	0–24,999	Reference	–	–	Reference	–	–
	25, 000–49,999	2.382	1.636–3.470	<0.001	2.420	1.576–3.717	<0.001
	≥50,000,	2.975	1.753–3.884	<0.001	2.133	1.394–3.867	<0.001
Employment status							
	Employed	Reference	–	–	Reference	–	–
	Unemployed	0.700	0.512–0.958	0.026	0.819	0.566–1.186	0.291
Residence							
	Rural	Reference	–	–	Reference	–	–
	Urban	0.852	0.598–1.214	0.376	0.704	0.480–1.035	0.704
Education status							
	None	Reference	–	–	Reference	–	–
	≤10 years	0.356	0.138–0.920	0.033	0.348	0.130–0.931	0.035
	11–12	1.255	0.695–2.286	0.451	1.086	0.580–2.035	0.797
	≥13	1.897	1.135–3.172	0.015	1.501	0.858–2.627	0.041

1.201–2.700, $p = 0.004$) were the substantial determinants of good practice regarding COVID-19 among general population in Pakistan (**Table 4**).

DISCUSSION

In view of the rapid spread of COVID-19 and the increase in the number of cases in Pakistan, it is necessary to have a clear picture of the state of public awareness and their practices in the context of the precautionary measures. In addition, Pakistan is a populous country and is facing enormous pressure on non-communicable diseases (26). Both factors increase the country's vulnerability to this deadly infection and results in higher mortality and morbidity. Moreover, Pakistan's history of dealing with epidemics required a high level of preparedness by government as well as masses (27). Global efforts have been made to reduce the transmission of this contagious infection. These efforts include political efforts by the governments, together with personal attitudes and behaviors, which depend on the awareness of the public about the disease.

Findings revealed that almost half of the population had good knowledge, and 80% had a precautionary approach. News channels such as the internet and social media platforms had become commonly used information sources compared to traditional channels such as newspapers etc. Social media was the primary source of information to be used by the public (66.62%) in Pakistan to obtain information on COVID-19. This could be explained by the fact that 64.3% of study participants were under 30 years of age and had University level education. This stratum is the main user of the Internet in Pakistan, according to a recent survey by the Pakistan Telecommunications Authority (PTA), 63% of the 76 million people who have access to the Internet are under 30 years of age (28).

This finding has implications that although social media platforms could be an easily accessible source of information, there is a potential risk of misinformation (13, 29). As with this pandemic of COVID-19, there is also a pandemic of misinformation on the Internet that leads to negative reactions from the public (13). Mainly, false information regarding the potential benefits of certain drugs such as hydroxychloroquine stimulated the irrational use of this drug by masses, and this results in a shortage of these medicines and becomes unavailable to patients who need (13).

Results show that 48.19% ($n = 332$) of the population had adequate knowledge of the nature, transmission, risk groups and

TABLE 4 | Binary logistic regression analysis for factors associated with good practice.

Characteristics		Factors associated with good practice					
		COR	95% CI	P	AOR	95% CI	P
Gender							
	Female	Reference	–	–	Reference	–	–
	Male	0.846	0.569–1.256	0.406	0.810	0.528–1.1242	0.334
Age							
	<30 years	Reference	–	–	Reference	–	–
	31–39 Years	0.946	0.591–1.515	0.818	0.740	0.396–1.381	0.344
	40–49 years	1.121	0.611–2.417	0.578	0.920	0.394–2.145	0.847
	More than 50 years	1.377	1.072–1.829	**0.015**	1.087	0.428–1.207	**0.020**
Marital status							
	Single	Reference	–	–	Reference	–	–
	Married	1.152	0.788–1.682	0.465	1.445	0.834–2.503	0.189
Monthly Income							
	0–24,999	Reference	–	–	Reference	–	–
	25, 000–49,999	2.185	1.291–3.700	0.004	1.875	1.055–3.331	**0.032**
	≥50,000,	2.330	1.831–3.130	0.234	1.962	1.251–2.120	0.379
Employment status							
	Employed	Reference	–	–	Reference	–	–
	Unemployed	0.731	0.497–1.074	**0.111**	0.891	0.573–1.386	0.609
Residence							
	Rural	Reference	–	–	Reference	–	–
	Urban	1.691	1.113–2.568	0.014	1.363	0.876–2.120	0.170
Education status							
	None	Reference	–	–	Reference	–	–
	≤10 years	0.376	0.162–0.875	0.561	0.415	0.173–0.995	0.649
	11–12	1.233	0.608–2.501	**0.023**	1.188	0.567–2.489	**0.049**
	13 or more	1.424	0.775–2.618	**0.015**	1.250	0.647–2.415	**0.031**
Knowledge							
	Poor	Reference	–	–	Reference	–	–
	Good	1.878	1.271–2.774	**0.002**	1.801	1.201– 2.700	**0.004**

Bold values shown significant factors.

precautionary measures of COVID-19. This rate of adequate knowledge is lower as reported in a Pakistani study (64.8%) (30), a Malaysian study (80.5%) (31) and a Chinese study (90%) (23) while higher than a Ethiopian study (36.7%) (12). However, this is in agreement with the findings of Abdelhafiz et al., who reported Egyptians had average (16.39 ± 2.63, range: 7–22) knowledge regarding COVID-19 (22). A possible reason for less knowledge reported in this study could be explained by the fact that most respondents attained COVID-19 related information from social media. Owing to unauthenticated and the use of social media to get information can explain the existence of myths and misinformation among the public (32).

Of note that more than half (55.6%, $n = 383$) of the study population incorrectly reported that COVID-19 directly leads to death. While wolf et al. reported that only 14.2% of US adults think that COVID-19 may cause death (24). Possible speculation is that factors such as the outbreak itself and consequential lockdown result in severe psychological impact as khan et al. reported 87.73% of the studied population feared the current situation, which leads to fatigue, anxiety, and depression (33). Additionally, misinformation surging on the internet and related economic pressure also put immense pressure and creates negative feelings about the situation (34).

The adjusted regression model revealed that higher education and monthly income are substantial predictors of good knowledge ($P < 0.05$). These results are in line with an Egyptian study which also stated that public awareness was important in terms of its socioeconomic status and level of education ($P < 0.002$) (22). A Chinese study concluded that higher education (middle school or lower vs. Master β: 1.346, $P < 0.001$) played a significant role in increasing good knowledge (23). While an American study didn't found any difference in respondents' knowledge regarding symptoms by poverty level (68.5% vs. 73.1%, $P > 0.05$) (24), this finding is of particular importance for the Government and the authorities concerned to focus on the less privileged stratum of society to ensure the effective implementation of precautionary measures.

Findings indicated that 80% of participants had positive practices in following precautionary measures. This is in line

with the results of Zhong et al., and Alahdal et al., who also reported that the majority (>90%) and (81%) of participants were following precautionary measures (23, 35). Possible speculation of a higher rate of good practices depsite of only 50% population had good knowledge could be that of campaigns lauched by Government describing causes, symptoms, and route but these awareness campaigns primarily focused on highlighting precautionary measures such as wearing a facemask, social distancing, and hand hygiene practices.

Note that 20.2% of the participants did not wear a face mask when they left their homes. This poroprtion is much higher than the chineese study, which reported that only 2% of the population studied did not wear a face mask (23). While Pakistani study reported that 14.2% of the people surveyed did not wear a face mask. Despite the vigorous broadcast of precautionary measures, this risk of taking action could be attributed to the younger age of the participants. Additionally, this might be because face mask shortage in different parts of the country due to high demand as well as price hiking also affects the affordability of the less income stratum. The government had taken several measures to ensure the availability and price control of all personal protective equipment (PPEs).

The adjusted multivariable logistic regression model demonstrated that older age, higher education, and knowledge regarding COVID-19 are the factors that substantially related to the positive attitude among the public in Pakistan. Zhong et al. found that knowledge was significantly associated with positive practices as Chinese individuals with higher education regarding COVID-19 were less likely to visit crowded places (OR:0.90, $p = 0.001$) and not wearing a face mask (OR:0.78, $p < 0.001$) (23). While a Pakistani study also concluded that younger age (vs. 30 years; OR = 3.08, $p < 0.001$) and lower education (matriculation vs. Master degree, OR = 6.829, $p < 0.001$) were the characteristics potentially associated with poor practices regarding COVID-19 (30).

To control the pandemic, there is a need for continuous monitoring of the implementation of preventive measures, the review of existing interventions and the updating of such responses. This study helps to inform the current state of awareness and practices of preventive measures and adds to the findings of a previously conducted study in Pakistan (30).

Regarding the policy implications, the findings will reconsider the involvement of the community as a key approach to the fight against any outbreak, including COVID 19. Generally, the current survey data most likely showed that the government's recommendation for the desired preventive action is positive for public health education.

LIMITATIONS

There are several implicit limitations to the study. First, as this is an online survey design, the response depends primarily on honesty and partly on the ability to recall and may, therefore, be subject to bias recall. However, due to a policy of lockdown and social distancing, the survey hand filling was not possible. Second, the sample size is not large enough, and most of the respondents were from one province (Punjab), which limits the generalizability of the population in the whole Pakistan. An additional limitation of the study is that most of the respondents belong to a population age group least affected by the pandemic (i.e., younger adults), while there is a lower representation of the age group most affected (i.e., older adults).

CONCLUSION

This quick online survey shows that around half of Pakistani residents had keen awareness, and 81% had positive practices following precautionary steps. The study is also capable of highlighting gaps in specific aspects of knowledge and practice that should be addressed in future awareness and education campaigns. Findings have also shown that fewer credible sources are used by the general population, which should be discussed immediately because it eventually affects information and is demonstrated in attitudes and practices. The study suggests that the Ministry of Health supports both the corrective and therapeutic steps of COVID-19, consisting of a better-organized approach to all strata of society, i.e., the less privileged, the elderly and the less educated, to provide a balance of knowledge of COVID-19 and hence successful implementation of the precautionary measures.

IMPACTS

- Pakistani residents had average knowledge and good practices of following precautionary measures for COVID-19.
- Findings have shown that education and economic status are potential predictors of good knowledge and positive practices.
- The study findings require future interventions to be targeted at all strata of society: the less privileged, the elderly and the less educated, to balance knowledge toward COVID-19.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by from the Samundari hospital board (767/THQ/HR). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

The manuscript idea, concept, writing, and layout was done by MS, AA, and SN. IN, AG, and MM provided critical help in writing, statistical and layout designing. IN, AA, and

ZA provided critical input regarding data analysis at every step of the manuscript writing process. MS, SK, and ZA proofread the manuscript and provided input in formulating the draft. All authors contributed to the article and approved the submitted version.

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REVIEW
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Review of the Microbiological Diagnostic Approaches of COVID-19

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On March 12, the World Health Organization declared a pandemic following the exponential increase of SARS-CoV-2 cases. The rapid spread of the virus is due to both its high infectivity and the free circulation of unrecognized infectious cases. Thus, diagnostic testing is a key element to prevent further dissemination of the virus. Urged by WHO's call, laboratories worldwide have been working on nucleic acid tests protocols and immunoassays that became available, albeit poorly validated, within a comparatively short time. Since then, external studies evaluating these diagnostic tests have been published. The present study is a review of the COVID-19 diagnostic approaches, discussing both direct and indirect microbiological diagnoses. A compendium of the literature on commercial assays kits available to date is provided together with the conclusions drawn as well as RT-PCR protocols published by the WHO. Briefly, diagnostic accuracy varies according to time elapsed since symptom onset and evolves together with understanding of the COVID-19 disease. Taking into account all these variables will allow determining the most adequate diagnostic test to use and how to optimize diagnostic testing for COVID-19.

Keywords: diagnostic testing, SARS-CoV-2, sensitivity, cross-reactivity, optimizing diagnostics

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INTRODUCTION

In December 2019, Chinese authorities reported an outbreak of cases of pneumonia of unknown etiology in Wuhan, China, of unknown cause. Characterization of the disease in a cluster of reported cases of pneumonia was associated with the spread of a novel coronavirus named SARS-CoV-2 (1). The rapid increase of Coronavirus Disease 2019 (COVID-19) cases, already being reported outside the Asian continent and evidence of human-to-human transmission, led to the declaration of a pandemic by the World Health Organization (WHO) on March 12th, 2020 (2, 3). Soon after the isolation of this new type of coronavirus (CoV) from bronchoalveolar lavage fluid, its viral genome sequence was released on the open access website virological.org (GISAID) (4, 5) to begin the development of diagnostic kits. Since then, a race to develop and distribute reliable diagnostic assays has been encouraged by World Health Organization (6).

SARS-CoV-2 is the seventh CoV known to infect humans and the third causing a severe acute respiratory syndrome, after SARS-CoV in 2002 and MERS-CoV in 2012 [the characteristics of the three CoV outbreaks are summarized in **Table 1** (7, 8)]. Like SARS-CoV, the novel CoV belongs to the Betacoronavirus genus, subgenus *Sarbecovirures* (14).

To date, marking 12 months after the emergence of the pandemic, there have been more confirmed SARS-CoV-2 cases around the globe than MERS and SARS. The reasons for such a rapid spread include a high infectivity [studies assessing efficient SARS-CoV-2 cell entry mechanisms have uncovered that the novel CoV has a higher binding affinity to the human angiotensin-converting enzyme 2 (hACE2) than SARS-CoV (15, 16)], high transmissibility of the virus (17, 18), a longer incubation period, an efficient immune evasion (15) and a delayed response from government and institutions (19).

The measures adopted prior to physicians' advice to stay at home were not sufficiently effective to curb the virus, thereby explaining the high number of undiagnosed infectious cases that went unrecognized (18). Both the long incubation period and the large number of mild infections with limited or absent symptomatology contribute to a deficient early diagnosis. Moreover, it seems that patients can be highly contagious during the pre-syndromic period, which, in addition to the lack of adequate and sensitive diagnostic tests, has made case identification and isolation difficult (20, 21).

Diagnostic tests must be specific enough to discriminate SARS-CoV-2 from other CoV with which it shares a high degree of homology (22). Thus, sensitive and specific diagnostic testing is crucial to prevent further spread of the virus.

The present review aims to describe the current approaches to SARS-CoV-2 diagnosis including the different types of tests available and the current limitations and successful findings achieved during the previous 7 months of testing. Based on the current evidence and what has been learned to date about the infectivity and physiopathology of the virus, as well as the kinetics profile of the specific antibodies to SARS-CoV-2, we provide suggestions on how to optimize diagnostic testing for COVID-19 and the usefulness of antibody detection.

METHODOLOGY

Urged by the call from the WHO to develop reliable diagnostic tools, laboratories worldwide have developed several commercial assays that have been granted an Emergency Use Authorization (EUA) by the Food and Drug Administration (23). Among all the assay tests released, it is important to distinguish direct from indirect assays (24). Direct tests detect the virus replication (active infection), and include real time reverse-transcriptase polymerase chain reaction (rRT-PCR) and antigen-based rapid diagnostic tests (Ag-RDTs). On the other hand, indirect tests search for host antibody response, detecting both active and past infections. These tests include Antibody-based Rapid Diagnostic Tests, usually by lateral flow (Ab-RDTs), and antibody-based diagnostic tests (Ab-DTs) using either enzyme-linked immunosorbent assays (ELISAs) or chemiluminescence enzyme immunoassays (CLIAs).

Since the beginning of the pandemic, 7 different in-house developed molecular assays protocols (RT-PCR) have been posted on the WHO website (25). These protocols have been elaborated by different investigation centers: Hospital Charité (Berlin, Germany) (26, 27), Hong-Kong University-Faculty of Medicine (HKU Med) (Hong-Kong, China) (28), Center for Disease Control of China (China CDC) (29), Institut Pasteur (Paris, France) (30), Center for Disease Control of USA (US CDC) (Atlanta, USA) (31), National Institute of Infectious Diseases (Tokyo, Japan) (32), and the National Institute of Health (Bangkok, Thailand) to guide laboratories involved in testing SARS-CoV-2 worldwide (33). The WHO has shown no preference for any of these assays and none has been endorsed or validated by the organization. Here we compare these different RT-PCR assays and summarize the finding in **Table 2**.

With regard to immunoassays and Ag-RDTs, website of the Foundation for Innovative New Diagnostics (FIND) lists all the SARS-CoV-2 tests commercially currently available or under development (37, 38). Diagnostic kits are submitted by the manufacturer itself or taken from publicly published information. High-speed production and the urgent need for diagnostic tests has resulted in the launching of poorly validated assays in the market (39). The present review only includes commercial kits fulfilling the inclusion criterion of diagnostic kits supported by published literature, tested independently from

TABLE 1 | Characteristics of the three coronavirus outbreaks [information extracted from Wang et al. (7) from the study by Chen et al. study (8)].

	SARS-CoV-2	MERS-CoV	SARS-CoV
Outbreak date	December, 2019	June, 2012	November, 2002
Location of first detection	Wuhan, China	Jeddah, Saudi Arabia	Guangdong, China
Target receptor	ACE2	CD26	ACE2
Confirmed cases	119,791,453[a]	2,494	8,096
Confirmed deads	2,652,966[a]	858	744
Case fatality rate	3%	37%	10%
Ro	1.4–3.5[b]	<1	0.4–2.9[d]
Incubation period (days)	Range from 2 to 14[c]	5	2–7

[a] *Confirmed cases and deads updated on 16 March 2021 (9).*

[b] *On January 23, the WHO estimated R0 to be between 1.4 and 2.5 (10). However, other preliminary studies such as that conducted by the Imperial College of London estimates R0 to be even higher at up to 3.5 (11).*

[c] *SARS-CoV-2 Incubation period information was taken from the Centers for Disease Control and Prevention (CDC) webpage (12).*

[d] *The SARS-CoV R0 value was taken from the "Consensus document on the epidemiology of severe acute respiratory syndrome (SARS)" published in 2003 by the WHO. From the initial phase of the epidemic, excluding superspreading events, R0 was estimated to be 2.9. Once control measures were implemented, the R0 value was reduced to 0.4 (April, 2003) (13).*

TABLE 2 | Comparison of the different RT-PCR assays protocols published by the World Health Organization.

Institute (Country)	Target gene	Throughout the text referred to as	Oligonucleotide	Sequence	Amplicon size (bp)	Polymerase	Thermocycler used in the reference publication	Volume of RNA extract
Charité (Germany) (26, 27)	E[a]	E assay (Charité)	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	113	SuperScript™ III Platinum® One-Step Quantitative RT-PCR System	Light Cycler® 480II (Roche) or Applied Biosystems ViiA ™ 7 (ThermoFisher)	5 µl
			E_Sarbeco_R	ATATTGCAGCAGTACGCACACA				
			E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ				
	RdRp[b]	RdRp assay (Charité)	RdRp_SARSr-F	GTGA**R**ATGGTCATGTGTGGCGG	100			
			RdRp_SARSr-R	CARATGTTAAA**S**ACACTATTAGCATA				
			RdRp_SARSr-P1[c]	FAM-CCAGGTGG**W**AC**R**TCATC**M**GGTGATGC-BBQ				
			RdRp_SARSr-P2[d]	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ				
	N	N assay (Charité)	N_Sarbeco_F	CACATTGGCACCCGCAATC	128			
			N_Sarbeco_R	GAGGAACGAGAAGAGGCTTG				
			N_Sarbeco_P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ				
HKU Med (China) (28, 34)	N[a]	N assay (HKU Med)	HKU-N-F	TAATCAGACAAGGAACTGATTA	110	TaqMan Fast Virus Master mix	Applied Biosystems ViiA™ 7 (ThermoFisher)	4 µl
			HKU-N-R	CGAAGGTGTGACTTCCATG				
			HKU-N-P	FAM-GCAAATTGTGCAATTTGCGG-TAMRA				
	ORF1b (nsp14)[e]	ORF1 assay (HKU Med)	HKU-ORF1-F	TGGGG**Y**TTTACRGGTAACCT	132			
			HKU-ORF1-R	AAC**R**CGCTTAACAAAGCACTC				
			HKU-ORF1-P	FAM-TAGTTGTGATGC**W**ATCATGACTAG-TAMRA				
China CDC (China) (29)	N	N assay (China CDC)	CCDC-N-F	GGGGAACTTCTCCTGCTAGAAT	99	Unspecified	Unspecified	Unspecified
			CCDC-N-R	CAGACATTTTGCTCTCAAGCTG				
			CCDC-N-P	FAM-TTGCTGCTGCTTGACAGATT-TAMRA				
	ORF1ab (nsp10)	ORF1 assay (China CDC)	CCDC-ORF1-F	CCCTGTGGGTTTTACACTTAA	119			
			CCDC-ORF1-R	ACGATTGTGCATCAGCTGA				
			CCDC-ORF1-P	FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1				
Institut Pasteur (France) (30)	RdRp IP2	RdRp-IP2 assay (Pasteur)	nCoV_IP2-12669Fw	ATGAGCTTAGTCCTGTTG	108	SuperScript™ III Platinum® One-Step Quantitative RT-PCR System	Light Cycler® 480 (Roche)	5 µl

(Continued)

TABLE 2 | Continued

Institute (Country)	Target gene	Throughout the text referred to as	Oligonucleotide	Sequence	Amplicon size (bp)	Polymerase	Thermocycler used in the reference publication	Volume of RNA extract
			nCoV_IP2-12759Rv	CTCCCTTTGTTGTGTTGT				
			nCoV_IP2-12696bProbe(+)	HEX-AGATGTCTTGTGCTGCCGGTA-BHQ1				
	RdRp IP4	RdRp-IP4 assay	nCoV_IP4-14059Fw	GGTAACTGGTATGATTTCG	107			
		(Pasteur)	nCoV_IP4-14146Rv	CTGGTCAAGGTTAATATAGG				
			nCoV_IP4-14084 Probe(+)	FAM-TCATACAAACCACGCCAGG-BHQ1				
	E[e]	E assay (Charité)	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	113			
			E_Sarbeco_R	ATATTGCAGCAGTACGCACACA				
			E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ				
US CDC (USA) (31)	N	N1 assay (US CDC)	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	72	TaqPath™ 1-Step RT-qPCR Master Mix, CG (Thermo Fisher)	Applied Biosystems™ 7500 Fast (ThermoFisher)	5 µl
			2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG				
			2019-nCoV_N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1				
	N	N2 assay (US CDC)	2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA	67			
			2019-nCoV_N2-R	GCGCGACATTCCGAAGAA				
			2019-nCoV_N2-P	FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1				
	N[f]	N3 assay (US CDC)	2019-nCoV_N3-F	GGGAGCCTTGAATACACCAAAA	72			
			2019-nCoV_N3-R	TGTAGCACGATTGCAGCATTG				
			2019-nCoV_N3-P	FAM-AYCACATTGGCACCCGCAATCCTG-BHQ1				
	Human Rnase P	HRnaseP assay (US CDC)	RP-F	AGATTTGGACCTGCGAGCG	Unspecified			
			RP-R	GAGCGGCTGTCTCCACAAGT				
			RP-P	FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ1				
National Institute of Infectious Diseases (Japan)[g] (32)	N	N assay (N.I.Infectious Diseases)	NIID_2019-COV_N_F2	AAATTTTGGGGACCAGGAAC	Unspecified	Unspecified	LightCycler96 system (Roche)	5 µl
			NIID_2019-COV_N_R2	TGGCAGCTGTGTAGGTCAAC				

(Continued)

TABLE 2 | Continued

Institute (Country)	Target gene	Throughout the text referred to as	Oligonucleotide	Sequence	Amplicon size (bp)	Polymerase	Thermocycler used in the reference publication	Volume of RNA extract
			NIID_2019-COV_N_P2	FAM-ATGTCGCGCATTGGCATGGA-BHQ				
National Institute of Health (Thailand) (33)	N	N assay	WH-NIC N-F	CGTTTGGTGGACCCTCAGAT	Unspecified	Invitrogen superscript™ III Platinum One-Step Quantitative	Unspecified	5 µl
		(N.I.Health)	WH-NIC N-R	CCCCACTGCGTTCTCCATT				
			WH-NIC N-P	FAM-CAACTGGCAGTAACCA- BQH1				

FAM, 6-carboxyfluorescein; HEX, hexachlorofluorescein; TAMRA, tetramethylrhodamine; BBQ, blackberry quencher; BQH1, black hole quencher.

Table adapted from Etievant et al. study (35) and Vogels et al. study (36).

Red Bold capital letters indicate degenerated nucleotides (W is A/T; R is G/A; M is A/C; S is G/C; Y is C/T).

[a] *Target used as a first screening tool.*

[b] *Target used for confirmation and further discrimination between SARS-CoV and SARS-CoV-2.*

[c] *Probe specific for SARS-CoV-2 detection.*

[d] *Probe specific for SARS-CoV-2, SARS-CoV and bat-SARS-related CoVs detection.*

[e] *Target used for confirmation.*

[f] *On March 15th, the N3 primer and probe was removed from the Diagnostic panel.*

[g] *Apart from developing the RT-PCR protocol, the National Institute of Infectious diseases (Japan) besides developing the RT-PCR protocol also developed a nested RT-PCR protocol, which is not presented in here.*

the manufacturer, and providing both sensitivity and specificity values. Sensitivity was assessed comparing test performance against a gold standard technique. The final selection was updated on 13th May, 2020 and is presented as supporting material in this review (**Supplementary Tables 1–4**).

DIRECT DIAGNOSTIC TESTING

Molecular Assays Protocols Published by the WHO and Developed by Referral Laboratories

To date, rRT-PCR is considered the gold standard technique for the diagnosis of SARS-CoV-2 infection since the symptomatology is non-specific and inconclusive, and other biological markers are non-exclusive of SARS-CoV-2 (40, 41). As SARS-CoV-2 is a positive-stranded RNA virus, reverse transcription into cDNA is needed prior to amplification. Genomic characterization of this novel CoV revealed conserved Betacoronavirus genome arrangement comprised from 5′ to 3′: the open reading frame (ORF) 1a/b [encoding for non-structural proteins (nsp)] and genes encoding structural proteins such as: the spike (S), the envelope (E), the membrane (M), and the nucleocapsid (N). Non-structural proteins are involved in transcription and replication, including the RNA-dependent RNA polymerase (RdRp) also named Nsp12. Additionally, the SARS-CoV-2 genome encodes for some accessory ORF proteins: ORF3a, ORF6, ORF7a, ORF7b, and ORF8 [(22, 42); see **Figure 1**].

The rRT-PCR protocol of Corman et al. (Charité) is the first to have been published and is one of the protocols most commonly implemented by laboratories worldwide (26, 27, 43). When virus isolates or samples from infected patients were not yet available, the Corman approach was based on both the close genetic relatedness to SARS-CoV and the use of synthetic nucleic acid technology. Based on the social media announcement of a SARS-related CoV and on the possibility of an increased sequence variability, an assay targeting the gene encoding the E protein was developed as a first screening tool (with wide sensitivity for detecting even phylogenetic outliers) followed by a confirmatory *rdRp* gene assay for further discrimination of SARS-CoV-2 RNA from SARS-CoV RNA (**Table 2**). Upon release of the novel CoV genome sequence, SARS-related virus sequences downloaded from GenBank were aligned with the SARS-CoV-2 sequence to confirm selected primer matching. The assay showed no cross-reactivity when tested with all endemic human CoV [NL63 (HCoV)229E, etc.] Shortly after the publication of the work of Corman, the European Virus Archive Global (EVAg) made available SARS-CoV positive controls and a panel of cell-culture RNA from different CoV available to check the specificity of the newly developed assays (44).

The same strategy was followed by Chu et al. (28) and HKU Med (34) (**Table 2**), who developed two-target rRT-PCR—primers against the ORF1b and N—sequence regions. The primers were intentionally made to be reactive to multiple viruses from the clade *Sarbecoviruses,* since there was still not enough information of the virus genetic diversity. When

aligned with the SARS-CoV-2 genome sequences, which were gradually being posted on GISAID, the primers were confirmed to match perfectly. Sequence variations among genome targets were translated into degenerate nucleotides on the primers. With the exception of the novel SARS-CoV-2 and SARS CoV, no other *Sarbecoviruses* have ever been detected in humans (14). This affirmation, and the fact that the last reported human SARS case dates back to 2004, supports positive reported cases being attributed to SARS-CoV-2 (45). Amplification of the gene encoding for the N protein was found to be more sensitive than the ORF1b gene assay, suggesting that the first assay could be used as a screening assay using the latter as a confirmatory test.

Scientists from the Institute Pasteur, chose targeting two RdRp targets (IP2 and IP4) using the *E* gene assay from the protocol of Charité, which had just been published, as a confirmatory assay (30). The US CDC opted for the use of three primer-probe sets targeting three *N* gene encoding regions. The innovative strategy in this case was to use an additional primer set targeting the human RNase P gene. Failure to detect the RNase P gene would indicate poor biological sampling suggesting an invalid test result (31). Little is known about the other three protocols: the Chinese CDC protocol targets both the ORF1ab and N genes, while the Thailand protocol only targets the latter. The Japanese protocol uses pan-coronaviral primers that have worked in the past, and at the same time target multiple Spike proteins and Nucleocapsid regions, using both nested and rRT-PCR [(32); **Table 2**]. Overall, nucleic acid amplification tests targets so far include the N, the E, the S proteins, and the RNA-dependent RNA polymerase encoding genes (46).

Sensitivity and Specificity Assessment of Primer-Probe Sets Published by the WHO

During the months that followed, WHO protocols were widely implemented in laboratories worldwide. Since then, some studies have assessed and compared the sensitivity and specificity of the different RT-PCR, reporting their limitations and assisting laboratories in their choice of protocol.

Although all primer-probe sets perform well when tested and can be used to detect SARS-CoV-2, there are some differences in regard to sensitivity: the RdRp assay (Charité) was found to have the lowest sensitivity [it only partially detected SARS-CoV-2 RNA for all $10–10^2$ viral RNA copies/μL concentrations while other assays were able to detect the RNA (Ct values <40)] (36). Etievant et al. also reported a worse sensitivity with the RdRp assay (Charité) compared to other assays when testing the lowest dilutions (35).

Vogels et al. reported that the E (Charité), the ORF1 (HKU Med), the N (HKU Med), the N (China CDC), the N1 (US CDC), and the N3 assays (US-CDC) were the most sensitive primer-probe sets, being able to detect SARS-CoV-2 at 1 and 10 viral RNA/μL (36). The study by Etievant et al. agrees with the primer-probes listed and also adds the RdRp-IP2 assay (Pasteur) and the RdRp-IP4 assays (Pasteur) (of note, the study by Vogels did not assess the Institute Pasteur's assays). The study by Etievant goes one step further, and while accepting that almost all the test are reliable for detection when used in clinical samples (excluding the RdRp and N assays by Charité and the US CDC N2 assay), they found that the N (China CDC), N1 (US CDC), N3 (US CDC), RdRp-IP2 (Pasteur), and the RdRp-IP4 assays (Pasteur) were the most sensitive, highlighting their low limit of detection, Ct values, and performance when testing different RNA concentrations obtained by serial dilutions (35).

The main difference between the Vogels and Etievant studies is the HKU primer-probes assessment. While Vogels states that the ORF1 assay (HKU Med) is one of the most sensitive assays, the Etievant study places it below the N1 (US CDC) and the N assays (China CDC). The discrepancy in sensitivity in the case of the ORF1 assay (HKU Med) may be because Vogels standardized PCR conditions for primer-probes comparisons and did not reproduce the protocols characteristics, unlike Etievant, who did follow different PCR conditions. While it's not clear whether this influences the sensitivity, Vogels reproduced a more realistic scenario since not all laboratories will be able to work within protocols conditions. Another possible explanation could be the use of different RNA extraction kits: Etievant extracted nucleic acid using the EMAG platform (Biomerieux, France) while Vogels used the MagMax Viral/Pathogen nucleic acid isolation kit (Thermofisher). Concerning specificity, background amplification was not observed in any of the nasopharyngeal swabs collected prior to the COVID-19 tested with primer-probe sets (36).

Although all the protocols have been considered reliable for achieving an accurate diagnosis, some irregularities have been noted. Etievant reported that the E (Charité) and the N2 assays (US CDC) were positive for all the dilutions tested including negative samples and controls. When analyzing amplicon size, both unspecific amplification and contamination were noted. It has been previously reported (47) that some other laboratories have received Corman et al. (26) *E* gene and *RdRp* gene tests cross-contaminated with synthetic controls.

Mismatches in Primer Binding Regions May Result in a Decreased SARS-CoV-2 Detection

With regard to the RdRp assay (Charité), low performance (35, 36), and suspected nucleotide substitutions on primer annealing genome regions have been reported. Vogels had calculated the accumulated genetic diversity up to 22 March 2020 using the 992 SARS-CoV-2 genome sequences available at GISAID (36). The mutagenic capability of RNA virus depends on RdRp fidelity among other factors as this enzyme is implicated in both replication and proofreading activities. Several mutations that may compromise its activity have already been found on the RdRp encoding region, being one of the possible causes of the sudden increase in the number of mutations outside the Asian continent with the spread of the pandemic (48). Characterization and tracing of mutations can be valuable for designing and reviewing diagnostic assays. Vogels detected nucleotide mismatches in 12 primer-probe sets (belonging to the 7 different molecular assays protocols posted by the WHO) that have occurred in at least two of the 992 SARS-CoV-2 genomes [primer-probe mismatches referred to are listed in Figure 6 of

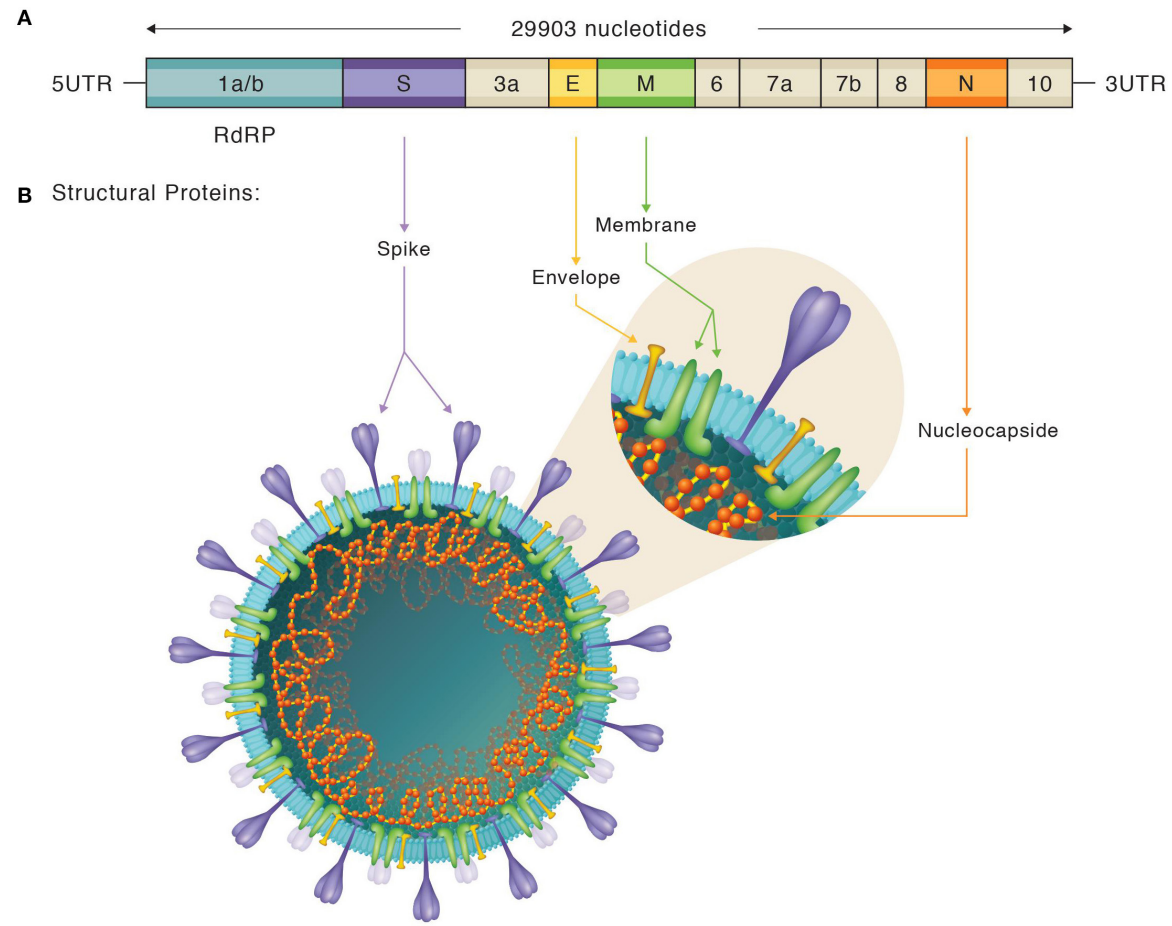


FIGURE 1 | Depiction of SARS-CoV-2 genome and structure. **(A)** Organization of the SARS-CoV-2 genome. Both structural and non-structural proteins are represented. Figure adapted from both the Khailany et al. and Chan et al. studies (22, 42). **(B)** Illustration of SARS-CoV-2 structure, locating, and labeling all the structural proteins. Spike proteins subdomains are also shown.

(36)]. The most noted mismatch is the CT substitution (on genome position 15.519) present in 990 of the 992 SARS-CoV-2 genomes (36). The primer *RdRp_SARSr* (Charité) contains a degenerate nucleotide (S) on the corresponding nucleotide (12th position of the primer) that is intended to pair with G or C (nucleotides found at this position on SARS-CoV and bat-SARS-related CoV genomes). The degenerated nucleotide was purposely added to help the primer anneal to SARS-CoV and bat-SARS-related CoV genomes (26). This substitution compromises primer annealing to the SARS-CoV-2 genome, thus explaining the poor sensitivity reported. Another variant detected was TC substitution on 39 genomes (at genome position 28.688) compromising *2019-nCoV_N3* (US CDC) forward primer annealing (**Table 2**). This was detected and the primer was removed from the diagnostic panel (31). The three nucleotide substitutions (GGG→AAC) at genome positions 28.881–28.883 in 12.7% of the SARS-CoV-2 genomes, comprising the *CCDC-N* (US China) primer target sequence, do not seem as critical since their placement upstream on the primer does not seem to compromise their capability to anneal and amplify (36, 49).

With the posting of the SARS-CoV-2 sequences from the beginning of the pandemics, GISAID has updated the information regarding the variability of the primer target sequences. The last update in March 2020 reported that the N (from China CDC and HKU Med) and the N3 assays (US CDC) had the highest rate of mutations in the 3′ end of the primer (defined as the last 5 nucleotides of the primer sequence). These mutations could partially compromise sensitivity (50).

Avoiding False Negatives When Performing the RT-PCR Test

Even though RT-PCR is considered the gold standard technique to diagnose COVID-19, recurrent notifications of false negatives have cast doubts on this methodology. Although most false negatives can be associated with poor sample collection usually of nasopharyngeal or oropharyngeal swabs, false negatives can also be attributed to the technique itself, aside from personnel skill, and to a lack of knowledge of the characteristics of the virus (virus shedding route and viral load kinetics), which is needed to address where and when to detect the virus (51).

Up to now, SARS-CoV-2 detection has consisted in first targeting a wide range of members of the *Sarbecoviruses* family, and second, in using specific probes for the further discrimination of SARS-CoV-2. This was done on purpose since at the time the first molecular assays were developed, little was known about the genetic diversity of the virus. Targeting a broad range of specimens implies designing primers that recognize a range of variability in detriment of specificity, thereby resulting in a higher number of false negatives. New approaches have attempted to identify SARS-CoV-2 directly, by establishing globally conserved targets, such as the COVID-19-nsp2 or the COVID-19-RdRp/Hel assays (52, 53).

Concerning the shedding pattern, SARS-CoV-2 viral load kinetics differs from that of SARS-CoV, resembling the influenza virus and also peaking soon after symptom onset (54, 55). Transmission of SARS-CoV occurs several days after symptom onset: the peak viral load is reached 7–10 days after illness onset (5×10^5 copies per swab) (56) and by day 15, viral levels are lower than on admission (57). Symptoms appeared after 2–7 days of incubation: therefore, isolation measures are very effective, since by the time symptoms appear and diagnosis has been determined, the subjects are still not at the peak of infectiousness. With SARS-CoV-2, transmission occurs earlier in the course of infection, when symptoms are either absent or mild (55). The highest viral load is reached 5 days before illness onset ($7{,}11 \times 10^8$ copies per throat swab), the same time that it takes for symptomatology to appear. By the time patients are admitted to hospital and testing is performed, the shedding peak in the upper respiratory tract has already been reached, and possible contagions have already occurred (56). The viral load starts decreasing from the 5th day after illness onset. After this point the chances of detecting viral load are progressively smaller—and the possibility of a false negative is higher. In this case, if the patient was diagnosed in an advanced state of the illness, immunological assays would likely be a better option than repeatedly performing nucleic acid tests.

Finally, successful virus isolation from throat swabs, as well as identification of viral subgenomic messenger RNAs provided proof of virus replication in the upper respiratory tract (56). Another study described nasal swabs as the optimal type of sample for SARS-CoV-2 detection, followed by throat swabs, which are more problematic in mild cases or samples collected beyond 15 days after symptom onset (58). Evidence of multiple SARS-CoV-2 shedding routes and body site specific virus replication depending on the severity have been published (58, 59).

An Improved Version of the Accurate Molecular Testing

Both the low turnaround time and the impossibility of processing a large number of samples at the same time are the major drawbacks of molecular testing approaches. For over a decade, the Cepheid (Sunnyvale, USA) has been working on the innovation of the molecular diagnosis, developing an automated molecular testing platform named GeneXpert System, which allows point-of-care testing (60). Following the announcement of the novel SARS-CoV-2, Cepheid launched a SARS-CoV-2 molecular diagnostic test (the Xpert Xpress SARS-CoV-2 cartridge) that was granted EUA on March 21st. This technology works as follows: the sample is added into the cartridge and the latter is loaded into the GeneXpert System which automatedly runs samples and generates results within 30 and 40 min depending on whether the result is positive or negative. A study assessing the Xpert Xpress SARS-CoV-2 assay conducted in the Netherlands has recently been published, reporting equal performance compared to in-house RT-PCR (61). The WHO has stated that the assay is "well-suited to complement a wider testing strategy." In addition, one of the major advantages is that the GeneXpert System is already available and distributed in some countries, as it is used as a diagnostic assay for tuberculosis and to test drug-resistant specimens (62). Another example is the Qiagen product (Venlo, the Netherlands) named QIAstat-Dx Respiratory SARS-CoV-2 panel, that received EUA on March 31st. This panel also uses a cartridge that detects and differentiates among 22 respiratory targets, including the SARS-CoV-2, by targeting both the *ORF1ab* and the *E* genes (63). DiaSorin Molecular LLC has also developed and manufactured the Simplexa TM COVID-19 direct kit that is run on the thermocycle LIAISON® MDX. The sample, which undergoes no extraction step, is loaded into an amplification disc (into which 8 different samples can be run at the same time). The assay targets both the ORF1ab and the S encoding regions (64, 65). The DiaSorin Simplexa product has demonstrated good performance, being slightly less sensitive than the Cepheid Xpert Xpress SARS-CoV-2 assay (66). Lastly, the Roche and the Hologic platforms named Cobas 6800 and Panther® system, respectively, have been widely implemented in microbiological laboratories. These automated systems also allow integrated extraction, amplification and detection of specimens. They offer a higher throughput, and a shorter hands-on time, thereby being less demanding (67, 68).

INDIRECT DIAGNOSTIC TESTING

Basis of Indirect Testing and Rapid Diagnostic Tests

Indirect testing relies on the presence of host antibodies. SARS-CoV-2 antibody response has proven to be similar to that seen against many other acute viral infections (41, 69). IgM is the first immunoglobulin (Ig) to develop after antigen exposure, being an indicator of the early phase of infection, while IgG only appears at a later phase. IgM response is characterized as being more active during the first days after the onset of infection and then declining, while IgG levels increase and remain high for a much longer period of time (40). Despite showing a high activity, IgM is known to have a lower affinity compared to IgG (70). At the time of writing, several studies have shed light on host antibody response, as the value of diagnostic testing depends heavily on the understanding of response. The COVID-19 serological assays currently available target either IgM or

TABLE 3 | Commercial tests performance grouped by antibody detected and antigen used to.

Type of test	Antibody detected	Antigen used for detection	Sensitivity	Specificity	Company	References
ELISA	Total antibodies (Ab)	Receptor Binding domain	93.10%	99.10%	Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. (China)	(41)
			93.00%	100.00%		(71)
			97.50%	100.00%		(72)
	IgM	Nucleocapsid protein	77.30%	100%	Zhuhai Livzon Diagnostics Inc. (China)	(73)
			68.20%	100.00%	Zhuhai Lizhu Reagent Co., Ltd. (China)	(69)
			46.10%	82.00%	Guangzhou Darui Biotechnology Co., Ltd. (China)	(74)
		Receptor Binding domain	82.70%	98.60%	Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. (China)	(41)
			77.10%	100.00%	Beijing Hotgen Biotech Co., Ltd. (China)	(69)
			92.50%	100.00%	Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. (China)	(72)
	IgG	Nucleocapsid protein	83.30%	95.00%	Zhuhai Livzon Diagnostics Inc. (China)	(73)
			64.70%	99%	Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. (China)	(41)
			70.10%	100%	Zhuhai Lizhu Reagent Co., Ltd. (China)	(69)
			23.00%	100%	Guangzhou Darui Biotechnology Co., Ltd. (China)	(74)
			88.80%	100%	Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. (China)	(72)
		Receptor Binding domain	74.30%	100%	Beijing Hotgen Biotech Co., Ltd. (china)	(69)
		Spike protein subdomain 1	67%	96%	Euroimmun Medizinische Labordiagnostika (Germany)	(71)
		Nucleocapsid protein and pike protein subdomain 2	88%	97%	Mologic Ltd. (UK)	(39)
	IgA	Spike protein subdomain 1	93%	93%	Euroimmun Medizinische Labordiagnostika (Germany)	(71)
LFIA	Total antibodies (Ab)	Receptor Binding Domain	97.5%	95.2%	Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. (China)	(72)
	IgM	Receptor Binding domain	88.80%	98.10%	Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. (China)	(72)
		Unspecified	43.20%	98%	Artron Laboratories Inc. (Canada)	(11)
			57.10%	100%	Zhuhai Livzon Diagnostics Inc. (China)	(73)
			55.80%	–		(75)
	IgG	Nucleocapsid protein	86.30%	99.50%	Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. (China)	(72)
		Unspecified	14.40%	100%	Artron Laboratories Inc. (Canada)	(11)
			81.30%	100%	Zhuhai Livzon Diagnostics Inc. (China)	(73)
			54.70%	–		(75)
	IgM-IgG	Receptor Binding domain	30%	89%	Jiangsu Medomics Medical Technologies (China)	(99)
			88.66%	90.63%		(17)
		Unspecified	82.40%	100%	Zhuhai Livzon Diagnostics Inc. (China)	(76)
			90%	100%	Dynamiker Biotechnology (China)	(71)
			90%	100%	CTK Biotech (USA)	
			93%	100%	AutoBio Diagnostics (China)	
			83%	100%	Artron Laboratories Inc. (Canada)	
			18.40%	91.70%	Vivachek Biotech (China)	(21)
			88.90%	100%	Hangzhou Alltest Biotech Co., Ltd. (China)	(77)
CLIA	Total antibodies (Ab)	Receptor Binding domain	96.30%	99.30%	Xiamen InnoDx Biotech Co., Ltd. (China)	(72)
	IgM	Nucleocapsid protein and spike protein	48.10%	100%	Shenzhen YHLO Biotech Co., Ltd. (China)	(78)
			100%	97.33%		(59)
		Receptor Binding domain	86.30%	99.30%	Xiamen InnoDx Biotech Co., Ltd. (China)	(72)
	IgG	Nucleocapsid protein and spike protein	88.90%	90.90%	Shenzhen YHLO Biotech Co., Ltd. (China)	(78)
			100%	99.56%		(59)

IgG, or both, or IgA or total antibodies and are shown in **Table 3**.

The development of rapid diagnostic tests (RDTs) began a short time after the outbreak of SARS-CoV-2, pursuing point-of-care (POC) diagnostic goals, which provide results "*at the time and site of an encounter.*" RDTs have proven to be effective for detecting other pathogens in the past (79). The Ab-RDTs included in the present review are based on the lateral flow immunoassay (LFIA) technology which, in turn, is based on the capillary migration principle. Briefly, sample targets flow along a membrane and bind to their matching antibody at the test line, providing a visual result (40, 80). In addition, fluorescent detection has also been developed. Compared to molecular assays, RDTs are less expensive, easier to perform, faster, do not need qualified personnel, and sample collection carries a lower risk of exposure. On the other hand, they have shown a considerably lower sensitivity and specificity (11, 39, 73).

Besides Ab-RDTs, ELISAs, a well-established type of immunoassay technique, and CLIAs have also been developed and are included in the present review (**Supplementary Tables 1**, **3**). ELISAs and CLIAs provide quantitative data while Ab-RDTs only give qualitative results.

Assessment Based on External Evaluation of Serological Assays

The heterogeneity of the testing conditions among the studies included such as the different number of days since symptom onset during sample collection and the different number of patients in which testing was performed, prevents the pooling of data to perform statistical analyses. In addition, some of the articles here reported data that have not yet been peer-reviewed, due to the recent onset of the pandemic. Therefore, this systematic search aims to be a compendium of the currently available literature on the commercial kits to test SARS-CoV-2 listed on the FIND website. Conclusions drawn by the different studies are collected and compared, with advice on which features provide better results. At present, FIND is evaluating some of the commercial immunoassays listed under the manufacturer's request, using a standardized independent protocol (81); with the objective of providing impartial data to guide laboratories in their choice of immunoassay. Until the FIND report is available, this review intends to provide recommendations based on the data available.

The final selection of articles included in the present review comprised 18 articles testing 7 different commercial ELISAs, 2 different commercial CLIAs, 9 different commercial Ab-RDTs based on LFIA technology, and 1 Ag-RDT (see **Supplementary Tables 1–4**). Some commercial assays are tested in more than one article, thus allowing comparisons of performance.

The tests differ, among other aspects, in the laboratory technique, the antigens used for antibody detection, and the type of antibody targeted. In addition, negative COVID-19 specimens used to test kit specificity across studies have a variable origin. Some studies assessed specificity testing in samples collected from healthy individuals prior to the SARS-CoV-2 outbreak, while others used samples from subjects with a negative COVID-19 result. It is important to note that almost none of the Ab-RDTs manufacturers provide information on which antigen was used for antibody detection. Finally, all the studies, with the exception of one, agree with the use of RT-PCR as the gold standard method for comparing test sensitivity.

The specificity of all the commercial immunoassays was generally very satisfactory, while the sensitivity was far from adequate. The tests showing the best performance, according to sensitivity values, were ELISAs, followed by CLIAs and finally Ab-RDTs (**Table 3**), although even the best commercial assays missed a number of false negatives. A test showing high performance according to specificity, results in an accurate positive predictive value (PPV) when applied to a high prevalence scenario. However, as infection incidences decline, the PPV decreases as well, resulting in an equal number of true and false positives (24). According to the current kits, which have a less than perfect specificity, and with the upcoming scenario of a low prevalence endemic infection, many more infectious cases will be missed (82).

Most of the studies evaluated sensitivity and specificity separately for IgM and IgG, with IgG mainly performing better than IgM. In scenarios in which this was not the case, the results are attributed to the difference in time of sample collection from symptom onset. A possible explanation would be that IgM detection covers a narrower phase of the infection time course than IgG. IgM appears earlier but also fades first, while IgG persists (40). Another theory could be the lower specificity attributed to IgM (70, 74). Some studies have reported an additional sensitivity value either testing total antibodies or considering a positive result if either of the two Igs was positive [(41, 69, 71, 75, 77); Xiang et al., 2020b]. Likewise, a higher sensitivity value was obtained when taking into account the two immunoglobulins together. The search for either of the two Igs covers a broader phase of the infection, increasing test sensitivity; Nonetheless, testing IgM and IgG separately is a better option than targeting total antibodies, as Igs titers provide valuable information of the course of the disease. Apart from IgG and IgM, only one study searched for IgA. The Euroimmun IgA ELISA showed both a low sensitivity and specificity, being more prone to cross-react with negative sera [**Supplementary Table 1**; (71)].

Finally, the ELISA immunoassay studies reporting the best performance used a double sandwich assay instead of a capture or an indirect ELISA (41, 71).

Whether test performance is affected when tested in milder COVID-19 cases remains unknown (71). Most of the studies included in this review tested commercial kits in severe COVID-19 cases that attended hospital in whom RT-PCR was performed.

Overall, Ab-RDTs are far from reliable in terms of sensitivity and specificity. Regardless of how attractive point-of-care diagnosis is, at present it cannot compensate for its poor performance. However, future improvements in these aspects, will make Ab-RDTs a promising solution for large-scale screening.

Being Immunogenic and Avoiding Cross-Reactivity: Two Features Pursued by Candidate Antigens

Serological assays rely on the recombinant antigen with which they are coated. The antigen chosen must not only be immunogenic to ensure a high sensitivity but must also comprise specific epitopes to avoid cross-reactivity. In SARS-CoV, the N and the S proteins were found to be the dominant immunogenic antigens (83). This previous knowledge and the certainty that the novel CoV shares a high degree of similarity with SARS-CoV (82% of nucleotide identity) and presents the same structural proteins including S and N (22), makes the use of these two antigens promising in protein-based serological assays for detecting antibodies against SARS-CoV-2.

The N protein, is one of the major structural viral proteins and is involved in the transcription and replication of the genetic information of the virus and further encapsulation and packaging of the virions (69). It is a small, non-glycosylated protein, that is easy to clone and purify (**Figure 1**). During the SARS-CoV outbreak, N-protein based serological tests reported a high sensitivity paired with a low specificity, with a high rate of false-positive results (84). The same tests showed cross-reactivity among different known human CoVs (85) and autoantibodies in autoimmune diseases (86).

The other major immunogenic candidate is the S protein, a transmembrane glycosylated protein forming homotrimers that mediate CoV entry into the host cells (87). The S protein comprises two subdomains: S1, involved in specifically binding to the ACE2 receptor, and S2, responsible for membrane fusion. In turn, the S1 subdomain is made up of an N-terminal ($S1^A$) domain and a receptor binding domain (RBD) [(14, 88); **Figure 1**]. The S protein is much longer than the N protein, and thus, it is difficult to obtain in full-length, and presents glycosylation sites. Denaturalized or non-glycosylated forms might modulate antibody recognition, leading to false-negative results (84).

In the years following the SARS-CoV outbreak, there was controversial literature as to whether the majority of neutralizing antibodies were directed against the N or S protein. Buchholz et al. reported that neutralizing antibodies against S proteins conferred protection and ultimately prevented host cell infection (89, 90). On the contrary, when studying the antibody response of SARS-infected individuals, Leung et al. observed that the N was the most frequently target, followed by the S (91).

The SARS-CoV-2 pandemic has revived the same doubts concerning which antigen protein is the most adequate for detecting antibodies. To date, the kits available include the use of the RBD, and the N, or the S protein (**Table 3**).

Most Promising Antigen Used for SARS-CoV-2 Detection

The Alphacoronavirus, another CoV genus, is known for being responsible for a number of seasonal common cold cases every year. Consequently, a large proportion of the population possesses antibodies against one of the four human endemic CoVs (92). Phylogenetic closeness and conserved immunogenic proteins might result in cross-reactivity and false-positive results when testing for SARS-CoV-2, as seen previously with SARS-CoV.

Okba et al. tested several in-house and commercial assays that used different recombinant antigens showing that the S1 subdomain is more appropriate for SARS-CoV-2 detection than the S2 (or, by extension, the full-length S protein), as the latter is more conserved in CoV (88) (percentage amino acid identity of coronavirus conserved proteins to the novel coronavirus proteins can be found on Okba et al. work). N protein-based serological tests also proved to be sensitive, even though the N antigen appears to be more conserved than the S protein. All the assays tested showed no cross-reactivity among other CoV except for SARS-CoV sera, possibly due to the highest degree of similarity Okba et al. (88). The authors do not consider this an issue since the last case of SARS reported dates back to 2005 (45) and SARS-CoV specific antibodies are no longer detectable in serum of SARS-infected subjects that had been tracked for 6 years (93).

A study comparing commercial test performance between a rN-based ELISA (Zhuhai Lizhu Reagent Co., Ltd.) and a rS(RBD)-based ELISA (Beijing Hotgen Biotech Co., Ltd.) was conducted by Liu et al. (69) (**Supplementary Table 1**). The results showed that the rS-based ELISA was more sensitive for detecting IgM antibodies, with the S antigen being more immunogenic. Early response against the S protein compared to the N antigen is given as a possible explanation. However, previous literature on SARS-CoV disagree with this, and suggest that antibodies against the S protein are developed later in the infection (94).

Amanat et al. developed two in-house ELISA versions coated with the S protein antigen: the first with the full-length protein, the second with only the RBD. The full-length version showed stronger reactivity possibly due to the larger number of epitopes that encodes the larger version of the antigen (95). Stronger reactivity associated with a larger antigen fragment has also been described by Lassaunière et al. (**Supplementary Table 1**), who compared two commercial ELISA kits: the RBD-based ELISA from Beijing Wantai Biological Pharmacy Enterprise Co., Ltd and the S1-based ELISA from Euroimmun Medizinische Labordiagnostika (71). The latter showed cross-reactivity to serum containing HKU1 and adenovirus antibodies, suggesting than epitopes outside the RBDs are prone to inducing cross-reactivity.

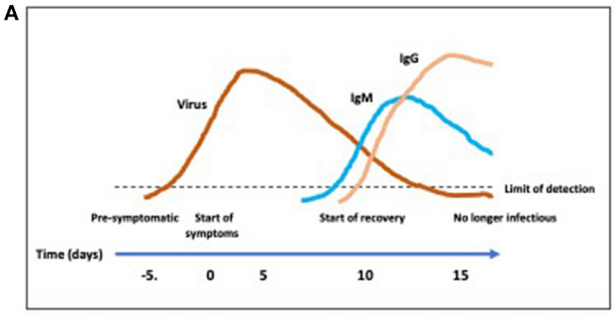
RBD has demonstrated to be especially variable, varying more than the S2 subdomain or even than the N protein, being the major differentiator between the SARS-CoV-2 and the remaining CoVs, and is thus, becoming a promising antigen (14, 22, 88). While some studies have reported encouraging results with the use of SARS-CoV S-directed polyclonal antibodies to inhibit SARS-CoV-2 entry into host cells (which seems to be a contradiction), the explanation lies in the fact that successful antibodies do not target the ACE2 binding site within the SARS-CoV-2 RBD, but rather the S2 subunit (16, 87, 96). Even if the RBD has proven to be specific enough to avoid cross-reactivity, further studies are needed to ascertain whether it is immunogenic enough in comparison with the N-protein. Using two different

antigens to check for antibodies might be a solution to avoid false negatives.

Sensitivity Performance Varies Depending on Time Since Symptom Onset

The heterogeneity of the sensitivities reported across immunoassays is too high to be attributed only to the type of antibody detected or the antigen used in the assay. It should be noted that the number of days since symptom onset at which samples were collected to test commercial immunoassays vary across studies (**Supplementary Tables 1–4**). Indeed, in some cases, the authors decided to stratify samples according to time elapsed since illness onset, thus reporting different sensitivity values, and as expected, the performance was better with each passing day, as expected (69, 71). The increasing sensitivity depending on time determines the underlying growth profile of specific antibodies to SARS-CoV-2. The values in the table show that after day 8 from disease onset, antibody sensitivity exceeds that of RNA testing (41), suggesting that the decision of the type of diagnosis test should be based on time elapsed since illness onset.

Considering that RT-PCR sensitivity is a dynamic value across time, it raises the question as to whether to use this technique as the gold standard method to compare test sensitivity. If RNA testing is performed in the second week after illness onset or later, the sensitivity falls, missing false-negatives, and thereby providing misleading immunoassays sensitivity values.



B

IgM	IgG	Interpretation	Assay type preference
-	-	Presymptomatic or non-contact phase	Direct testing: Ag-RDT and RT-PCR
+/-	+/-	Initial phase (aprox. 3-7 days)	
+	+/-	2nd Phase (8-14 days)	Indirect testing: ELISA, CLIA and Ab-RDT
++	++	3rd Phase > 15 dies (Immunized)	
+ o -*	++	Past infection (immunized)	

FIGURE 2 | The course of infection according to antibody lecture. **(A)** Antigen shedding and antibody kinetics profile along the course of infection. Antigen levels persist despite the appearance of antibodies. Thus, active infection can be diagnosed either by either antigen or antibody detection. **(B)** Interpretation of the presence of antibodies. *IgM+ can appear as in some cases IgM can last overtime.

OPTIMIZING DIAGNOSTIC TESTING

It is essential to understand the difference between a diagnostic test and a test designed to study immunization status. The goal of the latter, which will not be discussed below, is the search for past infections, while the former searches for active infections (24). Diagnosis aims to detect subjects carrying active infections for further isolation and preventing the spread of the virus among contacts, together with providing early treatment. In order to achieve this goal, the appropriate tests should look at both antibodies and antigens.

Likewise, it is important to define what is meant by a screening test. Due to their easy-to-use and low turnaround condition, screening tests can cover a wide range of the population, which explains why they are so appealing for tracking infectious diseases and can be used to either diagnose unrecognized SARS-CoV-2 infectious cases or to determine seroprevalence levels among the population (24). If used as a diagnostic test, screening tests normally require further confirmation due to the low sensitivity and specificity they present (97).

Optimizing diagnostics entails improving the choice of the diagnostic test based on the time since illness onset and understanding of COVID-19 disease, which in turn determine whether to look for antibodies or antigens (98). Furthermore, when performing diagnostic testing it is necessary to learn how to read and interpret the results [(99); **Figure 2**].

Viral antigens and genome are specific markers of the virus that precede both symptomatology and immunoglobulin response (98). In the pursuit of an early diagnosis, direct testing becomes the first option. Unlike the many Ab-RDTs available, there is only one Ag-RDT listed on the FIND website (manufactured by the Bioeasy Biotechnology Company) that is backed by two independent evaluations (98, 100).

This data should not be misinterpreted, as they exist other Ag-RDT authorized by other sources besides the one just mentioned. As stated at the beginning, the present review only includes commercial kits fulfilling the inclusion criterion of diagnostic kits supported by published literature, tested independently from the manufacturer, and providing both sensitivity and specificity values. Following such a strict criterion resulted in the inclusion of only one Ag-RTDs following FIND data search. However, since July 2020, the Food and Drug Administration has conceded 10 Emergency Use Authorizations for SARS-CoV-2 Ag-RDT. Approved Ag-RDT, listed in the Food and Drug Administration webpage, have been developed by manufacturers such as Quidel Corporation, Becton Dickinson and Company (BD), LumiraDx UK Ltd., among others (101). Besides, another six Ag-RDT have been approved by Japan's Pharmaceutical and Medical Devices Agency (102). Recently, several publications (103–105) have reported the sensitivity and specificity of Panbio COVID-19 Ag test with overall sensitivities that went from 73.3 to 91.7%, whereas when restricting the Ct to <32 the sensitivity went up to between 86 and 98%.

Currently, the World Health Organization (106) recommends using Ag-RDTs to respond to suspected outbreaks of COVID-19 in remote settings, institutions and semi-closed communities; to monitor trends in disease incidence in communities or when there is a widespread community transmission. In addition, they considered to test asymptomatic contacts of cases even if the Ag-RDT is not specifically authorized for this use. This last point is controversial and more studies should be carried out in order to support this statement.

If it were not for the poor accuracy reported, Ag-RDTs would have been a suitable option for large-scale detection of infectious cases before the appearance of symptoms, since despite being highly sensitive, RT-PCR molecular tests have a slower turnaround time, and are therefore less suitable for population screening. Consequently, RT-PCR molecular tests have been the standard technique to diagnose and screen contacts among reported infectious cases.

Active infections can also be diagnosed by detecting antibodies against SARS-CoV-2. A negative nucleic acid test result does not necessarily exclude the possibility of being infectious. Likewise, a positive immunoassay test result does not necessarily translate into antigen clearance. It has been demonstrated that RNA levels persist despite the appearance of antibodies (41). This raises many concerns related to discharge criteria (51, 107). A diagnosis based on antibody detection, however, is constrained by the time-dependent appearance of Igs (71). Antibodies against SARS-CoV-2 are detected from the middle stage of the course of infection (73). More specifically, IgM overtakes the detection cut-off value at day 9 after onset, and peaks at day 18. On the other hand, IgG is produced at some point between day 9 and 12 after onset, showing a rapid surge by day 15, and continuing to rise more steadily until day 39 (108). Another study reported that seroconversion times for total antibodies, IgM and IgG are 11, 12, and 14 days, respectively (41). Comparison of seroconversion rates between non-critical and critical cases showed no significant differences (41). Since little is known about the asymptomatic and autoimmune antibody kinetics profile, no advice is given on how to optimize the diagnosis of these groups of subjects based on the immune response.

Immunoassays are considered as a complement to RNA testing, especially after the second week after symptoms onset [**Table 4**; (41, 51, 109)]. They have proven to be helpful when nucleic acid tests continue to be negative in suspected patients, possibly because too many days have passed since infection and lower antigen levels mislead results (41, 77). In addition, simultaneous detection of both IgM and IgG can reveal valuable information about the time course of the infection, thus giving useful leads for treatment. In conclusion, combining RNA testing with antibody detection significantly improves diagnosing sensitivity, which is the ultimate goal.

THE IMPORTANCE OF THE DETECTION OF BOTH ANTIBODIES

Besides being a complement to RNA testing in the diagnosis of COVID-19, antibody detection using immunoassay tests has many other applications. In the present situation, immunoassays are being used within the context of epidemiological studies to determine which are the seroprevalence levels among the population (41). Furthermore, SARS-CoV-2 based immunoassays are helpful to guide the identification of possible human donors for collecting convalescent serum, which is considered a possible promising treatment (95). Finally, immunoassays may play a crucial role during vaccine trials and in recognizing possible animal hosts for SARS-CoV-2 (41, 88).

The studies conducted so far have revealed a significant correlation between antibody titers and that the clinical severity of the disease remains beyond the second week after illness onset—the higher the antibody titers, the worse the prognosis (41). Moreover, it has been reported that antibody detection rates are lower in younger subjects (5, 74). However, even though the cause is not yet known, and further research is needed, what is clear is that antibody measurement can be a marker of disease severity and may be helpful in treatment decision making.

Moreover, Wu et al. measured SARS-CoV-2 specific neutralizing antibodies (NAbs) among recovered patients and observed that about 30% of subjects developed very low NAbs levels, suggesting the presence of alternatives pathways besides NAbs production against the virus (5).

Despite the recent concern of the WHO in regard to the lack of data demonstrating immunization, and whether immunization protects against SARS-CoV-2 reinfection, a study conducted in China demonstrated that reinfection did not occur in Rhesus macaques after recovering from SARS-CoV-2 infection (110). Finally, due to the recent onset of the pandemic, data concerning how long antibodies last is not yet known (41).

DISCUSSION

This review was aimed at analyzing the current COVID-19 diagnostic approaches available, with the added difficulties of the recent onset of the pandemic and the huge amount of incoming information. The constant development of new commercial diagnostic tests will subject any conclusion drawn here to obligatorily be revised. In the meantime, even though it is too soon to derive definitive results, the present work intends to be a helpful guide in terms of optimizing diagnosis.

The high degree of homology shared with other human CoVs and the high number of mild COVID-19 cases demonstrate the need for both sensitive and specific diagnostic tests. RT-PCR is currently the most accurate test. Two automatized platforms can currently be used: (1) Integrated platforms which provide a result in 1 h or 1 h and a half, although they cannot process many samples at once, and (2) Integrated platforms which process more than 90 samples at once but the turnaround time is of around 3.5–4 h. Meanwhile, immunoassays, for which time-dependent accuracy is their major inconvenience, are considered as a complement to nucleic acid tests, especially after 14 days since illness onset. Moreover, these tests have many other applications

TABLE 4 | Comparison of current SARS-CoV-2 current diagnosis approaches reviewed.

Type of Assay		Target type		Time needed	Site where testing takes place	Advantage		Limitation		Suggested use	
		Looks for	Targeting at								
Direct testing	rRT-PCR	Virus replication (active infection)	Virus genome	3–4 h	Laboratory	Highly sensitive and specific		High turnaround condition*, requires skilfull personnel and its expensive		COVID-19 diagnosis and screening of contacts among infectious cases	
	Ag-based RDT		Viral antigens	15 min	POC diagnosis	Easy-to-use, low turnaround condition, cheaper		Low sensitivity and specificity. Needs further diagnosis confirmation.		A Large-scale screening diagnostic test	
Indirect testing	ELISAs	Host antibody response (active and past infection)	Antibodies[a]	1–3 h	Laboratory	Sample collection supposes a lower exposure risk	Highly sensitive and specific	Time-dependent on the host antibody development	High turnaround condition, requires skilfull personnel and its expensive	Identifying possible human donors for collection of convalescent serum, during vaccine trials and recognizing possible animal hosts for SARS-CoV-2	A complement to RNA testing, especially since the 2nd week after symptoms onset. Immunoglobulins detection reveal information about the time course of the infection.
	CLIAs		Antibodies[b]	1–3 h	Laboratory						
	Ab-based RDT		Antibodies[c]	15 min	POC diagnosis		Easy-to-use, low turnaround condition, cheaper		Low sensitivity and specificity		Screening seroprevalence levels among the population

[a] *Either IgM or IgG, or both, or IgA, or total antibodies.*
[b] *Either IgM or IgG, or both, or total antibodies.*
[c] *Either IgM or IgG, or both.*
**The turn-around time of rapid tests lasts about an hour.*

besides diagnosis, as noted along the text. Finally, the primary goal of RDTs is to obtain point-of-care diagnosis, but they lack sensitivity and specificity and need further diagnostic confirmation.

There is a need for sharing findings as well as providing transparent results when testing different diagnostic kits. As mentioned previously, at the time of writing FIND is conducting a generalized evaluation of the commercial kits available, using a standardized independent protocol, in order to provide practical advice based on robust evidence-based results.

AUTHOR CONTRIBUTIONS

AM-V, CB-D, and JV contributed to the design of the text and writing. AM-V design the tables. JV design figures. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.592500/full#supplementary-material

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Disparities of SARS-CoV-2 Nucleoprotein-Specific IgG in Healthcare Workers in East London, UK

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Introduction: SARS-CoV-2 antibody detection serves as an important diagnostic marker for past SARS-CoV-2 infection and is essential to determine the spread of COVID-19, monitor potential COVID-19 long-term effects, and to evaluate possible protection from reinfection. A study was conducted across three hospital sites in a large central London NHS Trust in the UK, to evaluate the prevalence and duration of SARS-CoV-2 IgG antibody positivity in healthcare workers.

Methods: A matrix equivalence study consisting of 228 participants was undertaken to evaluate the Abbott Panbio™ COVID-19 IgG/IgM rapid test device. Subsequently, 2001 evaluable healthcare workers (HCW), representing a diverse population, were enrolled in a HCW study between June and August 2020. A plasma sample from each HCW was evaluated using the Abbott Panbio™ COVID-19 IgG/IgM rapid test device, with confirmation of IgG-positive results by the Abbott Architect™ SARS-CoV-2 IgG assay. 545 participants, of whom 399 were antibody positive at enrolment, were followed up at 3 months.

Results: The Panbio™ COVID-19 IgG/IgM rapid test device demonstrated a high concordance with laboratory tests. SARS-CoV-2 antibodies were detected in 506 participants (25.3%) at enrolment, with a higher prevalence in COVID-19 frontline (28.3%) than non-frontline (19.9%) staff. At follow-up, 274/399 antibody positive participants (68.7%) retained antibodies; 4/146 participants negative at enrolment (2.7%) had seroconverted. Non-white ethnicity, older age, hypertension and COVID-19 symptoms were independent predictors of higher antibody levels (OR 1.881, 2.422–3.034, 2.128, and 1.869 respectively), based on Architect™ index quartiles; participants in the first three categories also showed a greater antibody persistence at 3 months.

Conclusion: The SARS-CoV-2 anti-nucleocapsid IgG positivity rate among healthcare staff was high, declining by 31.3% during the 3-month follow-up interval. Interestingly,

the IgG-positive participants with certain risk factors for severe COVID-19 illness (older age, Black or Asian Ethnicity hypertension) demonstrated greater persistence over time when compared to the IgG-positive participants without these risk factors.

Keywords: sero-surveillance, healthcare workers, point-of-care, antibody detection, SARS-CoV-2

INTRODUCTION

Since March 2020, the United Kingdom has enforced three separate restriction policies for its population to limit social interaction and movement in the hope of mitigating the impact of the Coronavirus Disease-19 (COVID-19) pandemic caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2).

As nations globally experienced immense pressure on their healthcare systems, the psychological and economic impacts of the pandemic have been equally challenging. This has resulted in an unprecedented worldwide effort for vaccine development alongside the establishment of robust and rapid diagnostic tests, especially as non-specific early clinical manifestations require accurate diagnosis, ensuring appropriate clinical management, surveillance, and effective control strategies (1, 2).

Serological tests are being developed and evaluated to detect humoral immune responses, specifically immunoglobulins (Ig)G, IgM and total Ig to SARS-CoV-2 (3), to be widely employed across communities irrespective of the presence or absence of symptoms, thus complementing diagnosis outside of the window of positivity for polymerase chain reaction (PCR)-based SARS-CoV-2 test (the gold standard) (4). There are currently two types of antibody tests available: (i) quantitative laboratory tests with antibodies titrated by enzyme-linked immunosorbent assay (ELISA) or Chemiluminescent Microparticle Immunoassay (CMIA), (ii) point-of-care (POC) tests, mainly based on lateral flow chromatographic immunoassays (4, 5), designed primarily to provide easy and relatively inexpensive access to diagnostics.

Lateral flow POC tests for the rapid detection of antibodies can effectively complement PCR diagnosis and antigenic tests for SARS-CoV-2 infection, as IgM and IgG seroconversion occur within 10–12 days and 12–14 days, respectively, after the onset of symptoms (6–9). IgM levels begin to decline by week 5 and almost disappear after week 7, whereas IgG levels persist beyond week 7 (10) reflecting IgG as a more robust indicator of prior exposure (11, 12). Further investigations are required to understand the dynamics of the early humoral immune response to realise the full potential of serological testing for SARS CoV-2.

In this study, we first validate the CE-marked Abbott Panbio™ COVID-19 IgG/IgM Rapid Test Device (Panbio™ test). This *in vitro* diagnostic rapid test (immunochromatographic assay) for the qualitative detection of IgG and IgM antibodies to SARS-CoV-2 nucleocapsid (N) protein, is intended for use in a POC setting and has previously been validated mainly for use with serum and plasma (13). Here we further assess the Panbio™ test for its use with fingerstick capillary and venous whole blood in addition to serum and plasma, which form the matrix equivalence arm (ME) of the study. We then focus on determining the seroprevalence and duration of COVID-IgG and IgM antibodies in healthcare workers (HCWs). Previous studies of COVID-19 patients from across the world (14–17), have shown that HCWs had a 10% greater risk of infection due to the nature of their work and viral exposure to the virus from the hospital setting (18). Our aim was to assess the prevalence of a past immune response to the SARS-CoV-2 virus among HCWs, as measured by detecting seroconversion of SARS-CoV-2-specific IgG and IgM antibodies using the Panbio™ test with confirmation using the Architect™ SARS-CoV-2 IgG test, and to evaluate the persistence of SARS-CoV-2 antibodies at a 3-month follow-up visit. Monitoring HCWs may facilitate early detection of healthcare-associated outbreaks which would allow implementation of management strategies assisting containment (19).

MATERIALS AND METHODS

Population Recruitment

Matrix Equivalence Study

Two hundred twenty-eight adults (>18yrs) were recruited over a four-week period from mid-May 2020 after an open invitation was sent locally to the general public living within the Barts Health NHS Trust area, in East London, UK. Individuals known to have had a previous COVID-19 illness (including PCR-confirmed COVID-19) as well as those who were not thought to have been previously exposed to SARS-COV-2, were offered the opportunity to participate. All participants provided informed consent according to the local ethics committee approval (Approved 22/04/2020, South Central - Berkshire Research Ethics Committee ref: 20/SC/0191, ISRCTN60400862) (20).

Healthcare Worker Study

Two thousand and fourteen members from the local staff population were recruited during months June—August 2020 from three hospital sites within the Barts Health NHS Trust, in East London, UK. Concordantly with the ME study, individuals with either known or unknown previous exposure to SARS-COV-2 were offered the opportunity to participate, and all participants provided informed consent according to the local ethics committee approval (Approved 29/05/2020, London - Camden & Kings Cross Research Ethics Committee, ref 20/HRA/2675, ISRCTN15634328) (21).

Study Design

After obtaining written informed consent, study staff verified that each participant met study inclusion and none of the exclusion criteria. The study ISRCTN registrations gives further details of these (20, 21). Demographic information, a brief medical history relating to COVID-19, prior testing results and risk factors,

including occupational risk where appropriate, were collected from each participant. Blood samples were then collected.

The ME study aimed to enroll a 1:1 ratio of SAR-Cov-2 positive and negative participants until 103 evaluable positives subjects were enrolled. A total of 228 participants were recruited.

For the HCW study, from the 2014 participants a subset of 706 were invited to re-attend after 3 months. This was based on a preliminary 90% power analysis using the Fisher's exact method (**Supplementary Table 5**), which required an estimated 2% antibody positive participants and 8% antibody negative participants at enrolment returning. A steering committee decided this was appropriate ensuring all returning participants were representative of the entire enrolment cohort. Therefore, all 476 positive participants at enrolment and 230 negative participants at enrolment, randomly selected to match the study site and occupational status using a random selection algorithm, were invited. Five hundred and forty-five participants (399 IgG antibody positive participants and 146 antibody negative participants at enrolment) returned for follow up testing, **Figures 1A,B**.

Sample Collection

For the ME study, venepuncture was performed on each participant utilising the site's standard blood collection method. EDTA plasma vacutainers (6 ml in total) and one 6 ml serum vacutainer were collected. Additionally, one fingerstick capillary specimen was collected from each participant. In the HCW study, each participant was required to donate only 6 ml total blood in an EDTA plasma vacutainer at each visit. To generate serum and plasma, venous blood samples were centrifuged at room temperature at 3,000 g for 15 min, aliquoted and frozen on the day of collection.

Panbio™ COVID-19 IgG/IgM Rapid Test Device

The Panbio™ COVID-19 IgG/IgM Rapid Test Device (Fingerstick Whole Blood/Venous Whole Blood/Serum/Plasma) (Panbio™; Abbott Rapid Diagnostics Jena GmbH, Jena, Germany) assay detects IgG against the SARS-CoV-2 nucleocapsid (N) protein as well as SARS-CoV-2 IgM antibodies provided as a separate result. The clinical performance of the test device is described in the **Supplementary Section**. Testing was conducted according to the manufacturer's instructions for use. Briefly, samples of 20 μl (fingerstick and venous whole blood) or 10 μl (serum and plasma) were applied to the specimen well of the test device, followed by two drops (∼60 μl) of buffer and a timer was started. Each ME study sample was interpreted at 10 min and again at 20 min by the same study staff member. For the HCW study, each plasma sample was interpreted at 15 min. All staff interpreting Panbio™ tests were blinded to the participants' previous exposure to SARS-CoV-2.

Reference Testing

Architect™ SARS-CoV-2 IgG Test

Frozen aliquots of plasma or serum were used to conduct study reference testing, in accordance with local laboratory standard operating procedures. The primary reference test performed on the Abbott Architect i2000 chemiluminescent microparticle immunoassay (Architect) was for SARS-CoV-2 IgG (Abbott Diagnostics, IL, USA; Architect) which detects IgG against the SARS-CoV-2 nucleocapsid (N) protein. The clinical performance of the immunoassay is described in the **Supplementary Section**. Antibody levels ≥1.4 (manufacturer's arbitrary units; Architect Index: ratio between the sample to the internal calibrator absorbance; S/C or S/CO) were considered positive (22). The Architect Index result was used as a semi-quantitative measure of antibody positivity (3).

All 228 samples from the ME study were tested on the Architect™. Whilst in the HCW study, based on the Panbio™ high sensitivity (99.1%, 95% CI:95.3, 100.0) only the samples that gave a positive enrolment Panbio™ test reading were analysed on the Architect™. At the 3-month HCW study follow-up, all samples that were newly Panbio™ test positive as well as samples that were Panbio™ test positive at enrolment (irrespective if they gave Panbio™ negative results after 3-months) were further tested on the Architect™.

Roche Elecsys® Anti-SARS-CoV-2 Test

Discrepant samples with a positive Panbio™ test device reading and a negative Architect™ test reading were further analysed on the Roche Cobas e801 analyzer using the Elecsys® anti-SARS-CoV-2 assay (Elecsys®; Roche Diagnostics International Ltd, Rotkreuz, Switzerland; Roche Elecsys®) according to the manufacturer's instructions. The assay detects IgG against the SARS-CoV-2 nucleocapsid (N) antigen, as well as SARS-CoV-2 IgM and IgA antibodies which are provided as a combined result (3, 9).

EDI™ Novel Coronavirus COVID-19 ELISA Kits

The Panbio™ COVID-19 IgG/IgM test device results for the ME study were also evaluated against the EDI™ Novel Coronavirus COVID-19 ELISA kits (Epitope Diagnostics, Inc., San Diego, USA); these consisted of separate kits designed to identify human IgG and IgM reacting to multiple epitopes of SARS-CoV-2 full length nucleocapsid (N) protein. The EDI™ Novel Coronavirus COVID-19 IgM ELISA test was the only available SARS-CoV-2 IgM reference test in the study; further discrepant IgM result resolutions were not conducted.

Matrix Equivalence

A matrix equivalence analysis for the SARS-CoV-2 IgG result was conducted for the Panbio™ test using fingerstick whole blood samples, venous whole blood samples and serum samples in comparison with the Panbio™ test using venous plasma samples from the same participant.

Interpretation of the Results—Composite Reference Method

As part of the discrepant result resolution, the Panbio™ test performance for IgG was evaluated against a composite reference result, that is, Architect™ test and the Elecsys® test. The composite reference result was considered positive if either the Architect™ or Elecsys® reference test result was positive. For the

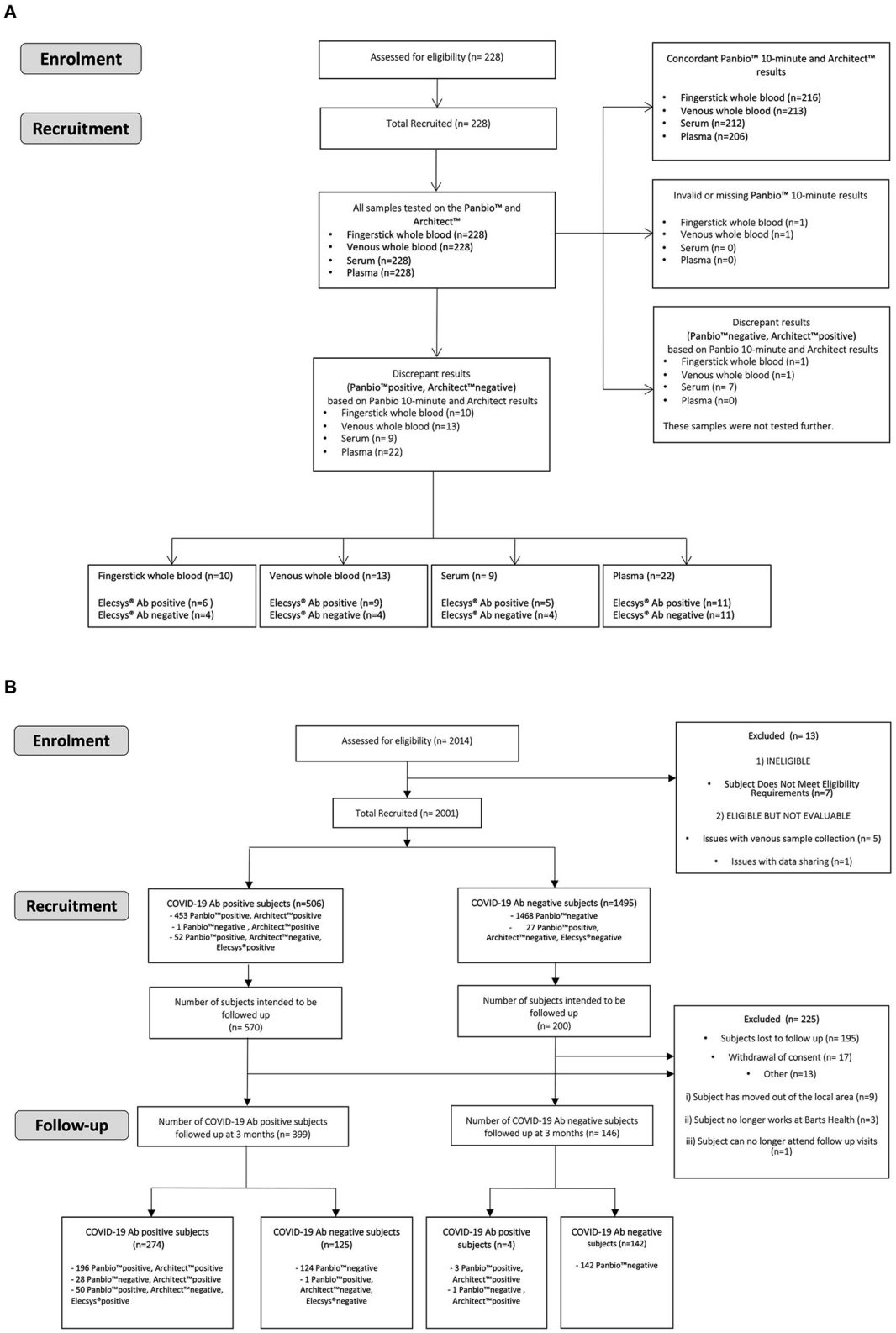

FIGURE 1 | Consort diagram reporting participant flow of: **(A)** ME study and **(B)** HCW study.

TABLE 1 | Final SARS-CoV-2 (Covid-19) IgG antibody result status after discrepant result resolution.

Panbio™ COVID-19 IgG/IgM test device	Architect™ SARS-CoV-2 IgG test	Roche Elecsys® anti-SARS-CoV-2 test	Final IgG result
Negative	-	-	Negative
Negative	Negative	-	Negative
Negative	Positive	-	Positive
Positive	Positive	-	Positive
Positive	Negative	Positive	Positive
Positive	Negative	Negative	Negative

Dash indicates further testing was not required.

HCW study, participants were considered SARS-CoV-2 antibody positive vs. negative as described in **Table 1**.

Data Analysis

The software PASS v13 (Pass Software, Rijswijk, The Netherlands) was used for sample size calculation and the software SAS v9.4 (SAS, Cary, North Carolina, USA) and GraphPad prism version 9.0 (GraphPad Software LLC, California, USA) was used for statistical analyses. The study data was anonymised at source and the data analysis was performed partially by the study sponsor and the authors. For analysis of IgG levels at enrolment and persistence over three months, an Architect Index $\geq$1.4 was considered positive and 4 categorical levels of Architect Index were derived as quartiles of the baseline positive population. Ordinal logistic regression was conducted to characterize the relationship between baseline demographics, medical history, and COVID-19 symptoms and the four categories of Architect Index. The ordinal logistic regression assumes that the relationship between Architect Index and the subject's IgG concentration is monotonic, but not necessarily linear. To compare IgG prevalence amongst groups, one-way ANOVA or Student T test were used.

RESULTS

Study Population for the ME Study

A total of 228 participants comprised of 103 males and 125 females ranging in age from 20 to 69 years were enrolled in the ME study. The participant ethnicities were White (124; 54.4%), Asian (78; 34.2%), and Black (17; 7.5%). One participant was mixed race (0.4%) and 8 participants (3.5%) did not disclose ethnicity information. One hundred twenty-three participants (53.9%) reported past COVID-19 symptoms, whereas 105 (46.1%) did not. The most common symptoms were fatigue (90; 39.5%), fever (89; 39.0%) and muscle ache (84; 36.8%). Fifteen COVID-19 IgG antibody-positive participants (6.6% of the study population) had experienced an asymptomatic SARS-CoV-2 infection. Forty-one participants (18.0%) had been hospitalized for COVID-19 illnesses. Eleven participants (4.8%) had been admitted to Intensive Care, and 3 participants (1.3%) had required invasive ventilation. Eighty-nine participants (39.0%) had a past PCR-confirmed diagnosis of COVID-19. For 87/89, the date of the PCR result was available; the positive PCR results had been obtained 13–80 days prior to study enrolment ($n = 87$, mean 51 $\pm$ 14.2 days). In total, 115 of 228 participants had a positive reference test for antibodies against SARS-CoV-2 based on Architect™ or Elecsys® testing, resulting in a total SARS-CoV-2 antibody prevalence of 50.4%.

Evaluation of the Abbott Panbio™ COVID-19 IgG/IgM Rapid Test Device

The positive percent agreement (PPA) and negative percent agreement (NPA) of the Panbio™ COVID-19 IgG test was assessed with the Architect™ SARS-CoV-2 IgG assay as the primary reference method (**Table 2A**). The discrepant results (Panbio™ positive / Architect™ negative samples) were resolved by the Roche Elecsys® SARS-CoV-2 assay (**Table 2B**), where a composite reference result consisted of the Abbott Architect™ SARS-CoV-2 IgG test and Roche Elecsys® anti-SARS-CoV-2 test and was considered positive if either the Architect™ or the Elecsys® test was positive. For samples without an Elecsys® result, the Architect™ result was the composite reference result.

The IgG results demonstrated a high PPA of the Panbio™ COVID-19 IgG/IgM test in comparison with the Architect™ SARS-CoV-2 IgG test, when used with fingerstick and venous whole blood and with plasma; the NPA was lower. With serum, the Panbio™ COVID-19 IgG/IgM test PPA was lower than with other sample types. The NPA at 10 min using the composite reference result increased for all sample types. The Architect™ assay detects SARS-CoV-2 IgG antibodies only, whereas the Elecsys® result consists of a composite SARS-COV-2 IgG, IgM and IgA result. However, the participants with a positive Elecsys® reference result and a negative Architect™ reference result all had a negative EDI™ Novel Coronavirus COVID-19 IgM ELISA test result, indicating a lack of IgM influence on the final reference test result. The PPA was decreased for whole blood and serum but remained >93.9%. Several of the false negative Panbio™ COVID-19 IgG/IgM test results were obtained for participants whose COVID-19 infection had been asymptomatic. There were no significant differences between the 10- and the 20-min readings (data not shown). In the study, all participants with a positive EDI™ Novel Coronavirus COVID-19 IgM ELISA test result also had a positive SARS-C0V-2 IgG reference result on the Architect™ assay.

Additionally, a matrix equivalence analysis was conducted for the Panbio™ COVID-19 IgG/IgM test using fingerstick whole blood samples, venous whole blood samples and serum

TABLE 2 | Panbio™ IgG Positive percent agreement (PPA) and Negative percent agreement (NPA) using a 10-minute read time with (A) the Abbott Architect™ SARS-CoV-2 IgG test as the primary reference method, (B) a composite reference method of Architect™ and Elecsys® based on discrepant result resolution of Panbio™ positive/Architect™ negative results.

	Total	True positive	False positive	True negative	False negative	PPA (95% CI) (%)	NPA (95% CI) (%)
(A)							
Fingerstick whole blood	227*	102	10	114	1	99.0 (94.7, 100.0)	91.9 (85.7, 96.1)
Venous whole blood	227**	102	13	111	1	99.0 (94.7, 100.0)	89.5 (82.7, 94.3)
Serum	228	96	9	116	7	93.2 (86.5, 97.2)	92.8 (86.8, 96.7)
Plasma	228	103	22	103	0	100.0 (96.5, 100.0)	82.4 (74.6, 88.6)
(B)							
Fingerstick whole blood	227*	108	4	108	7	93.9 (87.9, 97.5)	96.4 (91.1, 99.0)
Venous whole blood	227**	111	4	108	4	96.5 (91.3, 99.0)	96.4 (91.1, 99.0)
Serum	228	101	4	109	14	87.8 (80.4, 93.2)	96.5 (91.2, 99.0)
Plasma	228	114	11	102	1	99.1 (95.3, 100.0)	90.3 (83.2, 95.0)

*One subject had no result on fingerstick capillary whole blood testing at 10 min. **One subject had an invalid test result using venous blood at 10 min. Exact Clopper-Pearson method used to calculate 95% CI = 95% confidence interval.*

samples in comparison with the Panbio™ COVID-19 IgG/IgM test using venous plasma samples (**Supplementary Table 1**). The IgG test reached 95% negative agreement, but did not reach 95% positive agreement, for a Panbio™ COVID-19 IgG/IgM test fingerstick whole blood, venous whole blood or serum test result when compared with a Panbio™ plasma test result obtained from the same participant. The Panbio™ COVID-19 IgG/IgM test device results for the ME study were also evaluated against the EDI™ Novel Coronavirus COVID-19 ELISA kits, providing an accuracy of ≥84% for IgG and ≥73% for IgM (**Supplementary Tables 2**, **3**). To show the sensitivity reference frame **Supplementary Table 4** shows an agreement between the performance of Panbio™, Architect™ and Elecsys® for the ME study PCR positives.

The results obtained from the ME study provided the rationale to use plasma-based samples for the Panbio™ to conduct the Health-Care Worker study.

Study Population for the Health-Care Worker Study

Of the 2014 healthcare workers at Barts Health NHS Trust (London, UK) enrolled into the HCW study (**Table 3**), between June-August 2020, a total of 2001 were evaluable. They comprised of 551 (27.5%) males, 1,449 (72.4%) females and 1 (0.05%) undisclosed gender at enrolment, with an age range of 18- to 77-years. The participants included 1292 (64.6%) frontline HCWs who had direct contact with patients within the emergency department (ED), intensive care unit (ICU) and COVID-19 wards (frontline ever), as well as 709 (35.4%) non-frontline staff who included all other clinical and non-clinical staff (frontline never). The most represented ethnic groups identified within the cohort between enrolment and the 3-month period were White (ranging 46.6–48.3%), Asian (18.7–23.6%), and Black (17.4–18.2%). There were 61 participants who self-identified as mixed race (3.0%) at enrolment, 182 declared their ethnicity as Other (9%), whilst 4 participants (0.2%) preferred not to disclose information regarding ethnicity. Further detailed breakdown of ethnic groups, their corresponding age, gender and occupational categories are listed in **Supplementary Tables 6**, **7A,B**.

At the 3-month follow up (between September-November 2020), 545 subjects were evaluable consisting of 153 (28.1%) males and 392 (71.9% females) between the ages of 18–77yrs. Subject descriptor distribution at follow up was similar to that at enrolment.

Past COVID-19 symptoms were reported by 977 (48.8%) participants, whereas 1022 (51.1%) reported no symptoms and this information was not available for 2 (0.1%) participants. The most common symptoms (**Supplementary Table 9**) were fatigue (28.2%), headache (26.6%), fever (26.3%), aches and pains (25.4%), and cough (25%). Other commonly reported symptoms were loss of taste (22.5%), sore throat (18.8%), shortness of breath (13%) and runny nose (12.4%). Less common symptoms, reported by <10% of the study population, were diarrhoea, skin rash, conjunctivitis and loss of speech. 8.3% of participants declared to have experienced other symptoms (not specified).

SARS-CoV-2 Specific Antibody Prevalence at Enrolment and at 3-Month Follow-Up

From the 2001 participants at enrolment, 532 were Panbio™ IgG positive. Four hundred fifty three of these participants were confirmed SARS-CoV-2 positive by Architect™, and an additional 52 participants were confirmed positive by the Elecsys® test. Twenty seven participants with a positive Panbio™ result had a negative antibody result based on the laboratory testing; these 27 participants were classified as antibody negative. Forty one Panbio™ IgG-negative samples (positive at enrolment) were analysed by Architect™; one had a positive Architect™ result. In total, 506 (25.3%) participants were determined to be antibody positive at enrolment.

At the 3-month follow-up, 545 total eligible participants returned, of whom 399 had a confirmed positive SARS-CoV-2 antibody result at enrolment and 146 were antibody negative at enrolment. At the 3-month follow-up, 278 participants in

TABLE 3 | Subject disposition.

	Enrolment		3-month follow-up	
	Total (*n*)	**% Total population**	**Total (*n*)**	**% Total population**
Enrolled Subjects:	2014	100	575	100
Enrolled subject status				
Evaluable	2001	99.35	545	94.8
Unevaluable	13	0.65	30	5.2
Reasons for unevaluable				
Withdrawal	8	0.39	30	5.2
Unable to obtain sample	5	0.25	0	0.0
Gender distribution				
Female	1449	72.4	392	72.0
Male	551	27.5	153	28.0
Undisclosed	1	0.1	0	0.0
Total	2001	100	545	100
Age range 18–32	628	31.4	120	22.0
33–47	740	37.0	195	35.8
48–62	557	27.8	205	37.6
63–77	76	3.8	25	4.6
Total	2001	100	545	100
Work status				
Frontline	1292	64.6	397	72.8
Non-frontline	709	35.4	148	27.2
Total	2001	100	545	100
Ethnic group				
Asian/Asian British	472	23.59	102	18.7
Black, African, Caribbean/Black British	349	17.44	99	18.2
White	933	46.63	263	48.3
Mixed/Multiple ethnic groups	61	3.05	17	3.1
Other	182	9.05	63	11.6
Unknown	4	0.25	1	0.2
Total	2001	100	545	100

Total subjects during initial enrolment and subsequent 3-month follow up with: gender, occupational status, age range and ethnicity distribution; n and % of total population.

total had a final SARS-CoV-2 antibody positive result; of these 278 participants, 29 had a Panbio™ negative result and 50 had an Architect™ negative result. At the follow-up, 274/399 participants (68.7%) had retained their antibody-positivity from enrolment and 125/399 (31.3%) of those who were antibody-positive at enrolment had a negative antibody result at follow-up. 4/146 participants (2.7%) whose antibody result was negative at enrolment had seroconverted. No subject developed disease requiring hospitalisation.

Considering the total HCW cohort at enrolment (**Table 4A**) the highest IgG prevalence was reported for male HCWs aged between 48–62 yrs (39.6%) and 63–77 yrs (35.0%). Similarly, in females the age group with the highest IgG positivity was 48–62 yrs (32.0%) followed by 63–77 yrs (30.4%). The prevalence was higher for males than females for all age groups. To note, these age groups were arbitrarily chosen for the cohort by dividing the age range into four equal categories.

Among the self-assigned ethnic groups, those of Asian and Black ethnicity within the HCW study showed the highest prevalence of COVID-19 antibody positivity; the lowest antibody prevalence was observed in the White ethnic cohort (**Table 4B** and **Supplementary Table 7B**).

When categorising the participants according to their professional roles (**Table 4B**) IgG prevalence was higher amongst frontline workers (28.3%) compared to non-frontline workers (19.9%).

SARS-CoV-2 Specific Antibody Levels at Enrolment

Confirmed IgG positive results using the Architect Index were grouped into four separate levels:1.4–2.65, 2.66–4.16, 4.17–5.79, and ≥5.79, based on the quartiles of their distribution. Looking at the association between age groups and the categorical level of the Architect Index, participants in the age groups 48–62 yrs, and 63–77yrs were more likely to have a higher Architect Index compared to participants in the group 18–32 yrs (OR = 2.422 and 3.034 respectively via ordinal logistic regression) (**Table 5** and **Supplementary Tables 8A–C**). No significant differences were observed between the different genders and occupational status, however, a significant difference was observed between the different ethnic groups. Those identifying as Asian, Black, Mixed or of Other ethinicity were more likely to exhibit a higher Architect Index compared to participants of White ethnicity (OR = 1.881, 1.451, 1.166, and 1.418, respectively), where Asian ethnicity was statistically significant.

Past PCR Diagnosis, Symptoms, and Co-morbidities

Fifty four participants (2.7%) had a past PCR-confirmed diagnosis of COVID-19. 18.6% (94) of COVID-19 IgG antibody-positive participants had experienced asymptomatic SARS-CoV-2 infection at enrolment whilst 411 participants (81.2%) experienced associated symptoms. Information regarding symptoms was missing from 1 participant (0.2%).

Participants' medical history was also analysed, taking into account past hospitalisations and comorbidities (**Supplementary Table 9**). The association of co-morbidities and categorical level of the Architect Index (**Table 6**) illustrates that participants with hypertension were more likely to have a higher Architect Index value, compared to normotensive participants (OR = 2.128 via ordinal logistic regression). Individuals with obesity or diabetes also showed higher Architect Index values, however, this did not reach statistical significance. An ordinal logistic regression model showed no significant interactions between age, ethnicity, hypertension and COVID-19 symptoms (**Table 7D**).

The relationship between self-reported COVID-19 symptoms and the categorical level of the Architect Index was analysed

TABLE 4 | Evaluation of % IgG prevalence in relation to: (A) age and gender, (B) ethnicity and occupational role.

		Enrolment					
	Age range	*N* (% of study population)	IgG prevalence (%)		Age range	N (% of study population)	IgG prevalence (%)
(A)							
Female	18–32	461 (23.0)	17.4	Male	18–32	167 (8.3)	26.3
	33–47	535 (26.7)	22.1		33–47	205 (10.2)	24.4
	48–62	397 (19.8)	32.0		48–62	159 (7.9)	39.6
	63–77	56 (2.8)	30.4		63–77	20 (1.0)	35
	Total	1449	23.6		Total	551	29.8

	Enrolment	
Ethnic group	**N (% study population)**	**IgG prevalence (%)**
(B)		
Asian/Asian British	472 (23.6)	25
Black, African, Caribbean/Black British	349 (17.4)	33
White	933 (46.6)	21
Mixed/Multiple ethnic groups	61 (3.0)	23
Unknown	4 (0.2)	25
Other	182 (9.0)	35
COVID-19 occupational exposure		
Frontline worker	1292(64.6)	28.3
Non-frontline worker	709 (35.4)	19.9

Results are based upon all three assays Panbio™, Architect™ and Elecsys® as described in study design.

TABLE 5 | Relationships between Architect Index levels catergorised within age, gender, ethnicity and occupational roles.

Odds ratio estimates			
Effect	**Point estimate**	**95% Wald confidence limits**	
Age:			
Age group 33–47 vs. 18–32	1.538	0.970	2.439
Age group 48–62 vs. 18–32	2.422	1.536	3.820
Age group 63–77 vs. 18–32	3.034	1.331	6.914
Gender			
Male vs. female	0.945	0.658	1.355
Ethnicity			
Asian vs. white	1.881	1.209	2.927
Black vs. white	1.451	0.930	2.262
Mixed vs. white	1.166	0.432	3.142
Other vs. white	1.418	0.829	2.424
Occupational status			
Frontline vs. non-frontline	1.209	0.834	1.753

TABLE 6 | The association of co-morbidities and categorical level of the Architect Index.

Odds ratio estimates			
Effect	**Point estimate**	**95% Wald confidence limits**	
Diabetes—yes vs. no	0.974	0.491	1.932
Hypertension—yes vs. no	2.128	1.323	3.424
Respiratory illness—yes vs. no	1.093	0.586	2.038
Obesity—yes vs. no	1.748	0.584	5.227
Coronary illness—yes vs. no	1.010	0.303	3.373

by ordinal logistic regression. Participants reporting at least one of the possible COVID-19 defining symptoms by Public Health England (PHE) (new continuous cough, temperature $\geq$37.8°C, anosmia or ageusia) were more likely (OR = 1.869) to have higher Architect readings than those who did not report COVID-19 specific symptoms (**Table 7D**). From the 977 participants with self-reported symptoms, 542 (55.5%) were confirmed antibody negative.

An association between the days of duration of COVID-19 symptoms and the Architect Index was evaluated by using Ordinal Logistic Regression. Symptom duration of 11–15 days had greater probability (OR = 2.024) of a higher Architect Index when compared to symptom duration of 7 days or less. Symptom duration of 16 days or more had a higher Architect Index (OR = 2.29) when compared to symptom duration of 7 days or less (**Supplementary Table 10**).

Difference in SARS CoV-2 Antibody Status and Levels Over a 3-Month Interval

During the 3-month study time course, 274/399 (68.7%) of the follow-up participants remained IgG positive; 3 (0.7%)

had a higher Architect Index than at enrolment. Two of these participants reported at least one PHE possible COVID-19 defining symptom during the follow-up time-course. One hundred and twenty five (31.3%) participants converted from IgG positive to IgG negative whilst 4/146 antibody negative participants seroconverted to IgG positive (2.7%). The remaining 142 (97.3%) did not develop antibodies (**Table 7A**). When considering the overall antibody dynamics of SARS-CoV-2 IgG positive participants, a decline in the Architect Index readings was observed within the 3-month study period (**Figure 2**).

To establish any relationships between the 125 participants whose IgG declined to undetectable levels after a 3-month interval, the subjects were analysed according to their ethnic groups or categorised according to work status, age, or medical history (**Table 7B** and **Figure 3**).

The persistence of SARS-CoV-2 IgG at 3-months was assessed by classifying subjects as persistently positive if Architect Index remained ≥1.4 and negative if Architect Index dropped below 1.4 (**Table 8**). As expected, participants with the lowest category of positive Architect Index (1.40 to 2.65) at enrolment had the lowest probability of remaining positive at 3 months (11.4%) whilst participants with higher levels of Architect Indices at enrolment (>4.16) had the highest probabilities of remaining positive at 3 months (70.2–78.6%) (**Table 8**).

DISCUSSION

Since March 2020, UK hospitals have screened healthcare workers given their high exposure to SARS-CoV-2, as previously highlighted (23, 24). Nosocomial transmission has been an important amplifier in epidemics of both SARS and Middle East respiratory syndrome (25). Therefore, the rationale for hospital trusts has been to maintain the health and welfare of staff, to enable rapid identification and isolation of infected healthcare workers resulting in the protection of vulnerable patients (26) and the wider community. Over time it has become evident that the spectrum of COVID-19 symptoms is broad, ranging from asymptomatic cases, pauci-symptomatic (subclinical), pre-symptomatic (go on to develop symptoms later), or post-infection (viral RNA still detectable from a previous infection) (27).

Many HCW's remain asymptomatic (17–20%) (28–30) and modelling data indicates screening could reduce transmission by 16–23% (31), underscoring the need for widespread screening programmes of this population.

Whilst recent studies of HCWs report on the longitudinal evaluation of SARS-CoV-2 antibodies (32–35), we utilise a hospital setting to screen the performance of the Panbio™ COVID-19 IgG/IgM rapid test. Different from molecular testing, detection of a humoral immune response to the virus is an indirect marker of infection and provides a long-lasting measure of SARS-CoV-2 infection (36). As complementary diagnostic tests, they can confirm infection in symptomatic patients with high clinical suspicion who present late after illness onset, when the sensitivity of nucleic acid detection is lower. Indeed, negative antibody results of PCR positive individuals is not sufficient to exclude infection (false positivity of the molecular test) as antibody levels may be undetectable if the serologic test is performed too early (6–9) or if subjects are immunocompromised. Alternatively, absence of (or discordant) antibody responses may be due to effective clearance of infection mediated by T cells (37–39).

Robust serological tests have also demonstrated added value in epidemiological investigations for contact tracing, linking clusters of cases retrospectively (40), and determining the prevalence of the infection in high-risk categories such as HCWs or care home residents and staff (41–43).

We report a high concordance between the rapid Panbio™ COVID-19 IgG/IgM rapid test and the laboratory-based Architect™ and the Elecsys® tests indicating this may be a useful POC test for coronavirus antibody detection. However, the value of the IgM test was limited. The highest positive percent agreement (PPA) for the Panbio™ test was obtained using plasma samples (PPA 99.1%, CI:95.3, 100.0). Therefore, we analysed plasma from HCWs using the Panbio™ assay for qualitative analysis and confirmed positive results with the semi-quantitative Architect™ SARS-CoV-2 IgG assay.

We report the serologic status of 2001 hospital staff members at recruitment between June-August 2020, and 545/2001 who were longitudinally sampled at a 3-month interval from their first sampling time-point (September-November 2020). Our heterogeneous cohort reflects the unique ethnic diversity of Barts NHS Trust staff in East London, UK. From our study cohort of 2001 HCWs, we observed 506 SARS-CoV-2 (anti-N) IgG positive participants (25.3%) at enrolment. Fifty four participants (2.69% of the total cohort) had a PCR confirmed infection with a variable range of Architect Index values.

Several published studies (22, 42–44) show a varying association between age and IgG prevalence; we report higher IgG prevalence in older age groups of 48–62 yrs and 63–77 yrs, irrespective of gender. We also observed IgG prevalence was higher among males compared to females, which correlates with initial findings that linked gender-bias as a risk factor (45). Gender has been shown not to play a role in infection rates (32), but our findings could suggest gender equitable solutions are required for management of COVID-19 prevention.

Asian, Black and Other ethnicities reported higher IgG prevelance compared to White participants in our cohort. However, only the Asian ethnic group was statistically significant. This is a particularly important observation as Black and Asian ethnicities are at a higher risk of severe disease (45–48). It should be noted that our study did not account for mediators such as socioeconomic inequalities that may link ethnicity to antibody response.

Although reporting IgG concentrations would have been ideal, there was no opportunity to do this at the time the study was conducted. While the Architect Index is intended for the qualitative detection of IgG, the reported units of Architect Index are related to IgG concentration by a monotonic calibration curve. It is a limitation of the study that the calibration curve was not determined at the time of the measurements. However,

TABLE 7 | Persistence and decline of SAR-CoV-2 antibody status over the 3-month study interval.

Enrolment IgG status	*N*	3-month follow up IgG status	*N* (% of enrolment)
Followed-up participants (*N* = 545)			
(A)			
IgG positive	399	IgG positive	274 (68.7%)
		IgG negative	125 (31.3%)
IgG negative	146	IgG positive	4 (2.7%)
		IgG negative	142 (97.3%)

	Confirmed IGG positive				
	Enrolment (*N* = 399)	**Month 3 (*N* = 274)**	**% Persistence**	***P*-value of persistence**	**% Decline**
(B)					
Age					
18–32	85	47	55.3	<0.0001	44.7
33–47	139	84	60.4		39.6
48–62	156	125	80.1		19.9
63–77	19	18	94.7		5.3
Gender					
Male	121	77	63.6	0.1525	36.4
Female	278	197	70.9		29.1
Occupational role					
Frontline	291	198	68	0.6558	32
Non-Frontline	108	76	70.4		29.6
Ethnicity					
Asian	81	57	70.4	0.0055	29.6
Black	82	67	81.7		18.3
White	171	102	59.6		40.4
Mixed	13	11	84.6		15.4
Other	51	36	70.6		29.4
Missing	1	1	100		-

	Confirmed IGG positive				
	Enrolment (*N* = 399)	**Month 3 (*N* = 274)**	**% Persistence**	***P*-value of persistence**	**% Decline**
(C)					
Medical History					
Cardiovascular conditions					
No	392	267	68.1	0.0714	21.9
Yes	7	7	100		0
Diabetes					
No	376	257	68.4	0.5767	31.4
Yes	23	17	73.9		26.1
Hypertension					
No	346	225	65.0	< 0.0001	35
Yes	53	49	92.5		7.5
Obesity					
No	391	266	68.0	0.0536	32
Yes	8	8	100		0
Respiratory disorders					
No	364	253	69.5	0.2469	30.5
Yes	35	21	60.0		40

(Continued)

TABLE 7 | Continued

Effect	Point estimate	95% Wald confidence limits	
(D)			
Odds ratio estimates			
Age group: 33–47 vs. 18–32	1.519	0.958	2.409
Age group: 48–62 vs. 18–32	2.209	1.378	3.539
Age group: 63–77 vs. 18–32	2.903	1.246	6.765
Ethnic group: Asian vs. White	1.851	1.190	2.882
Ethnic group: Black vs. White	1.547	0.984	2.432
Ethnic group: Mixed vs. White	1.138	0.424	3.054
Ethnic group: Other vs. White	1.431	0.825	2.483
Hypertension: Yes vs. No	1.494	0.895	2.494
Covid Symptoms: Yes vs. No	1.869	1.260	2.771

(A) Changes in the total follow-up population, (B) changes for age (multiple comparisons: 33–47 vs. 18–32 p = 0.4489; 48–62 vs. 18–32 p < 0.0001; 63–77 vs. 18–32 p = 0.0013; 48–62 vs. 33–47 p = 0.0002, 63–77 vs. 33–47 p = 0.0034), gender, occupational roles, ethnicities (multiple comparisons: Black vs. white p = 0.0005), (C) medical history. P-values < 0.05 were considered significant and (D) Odd ratio estimates from the ordinal logistical regression for age, ethnicity, hypertension and COVID-19 symptoms.

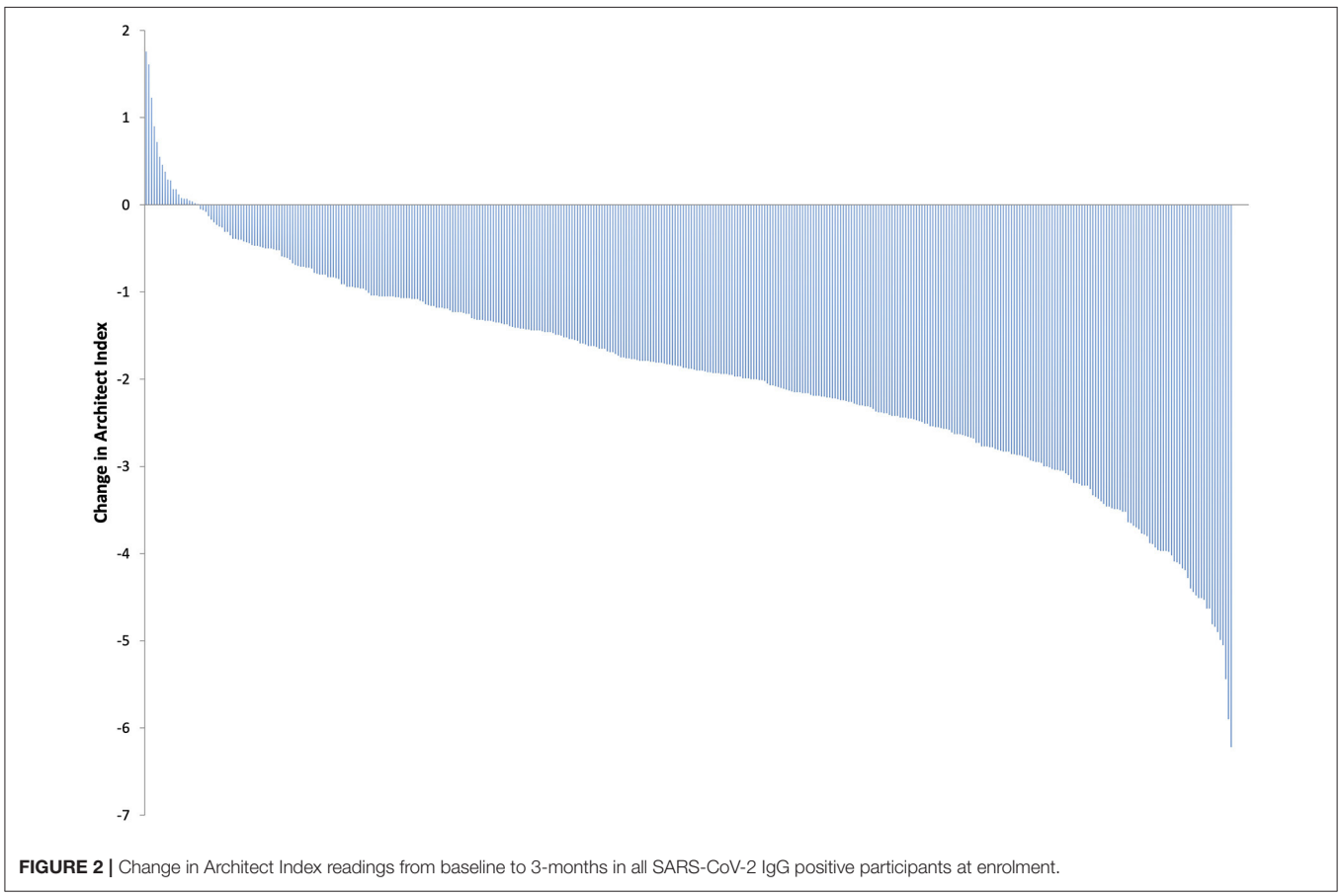

FIGURE 2 | Change in Architect Index readings from baseline to 3-months in all SARS-CoV-2 IgG positive participants at enrolment.

by segmenting the population into quartiles of the Architect Index the relationship between IgG concentration and baseline characteristics can be explored. Complementing previous studies earlier in the course of the pandemic, which identified certain co-morbidities as risk factors for more severe manifestations of COVID-19 (45, 49–51), we report that hypertension is associated with higher Architect Indices (OR = 2.128). In parallel to the higher IgG readings at enrolment within the Black and Asian ethnic groups as well as the older participants and those with hypertension, at the 3-month follow-up these groups showed a smaller decrease in antibody positivity compared to the reference groups (White, 18–32 yrs and normotensive,

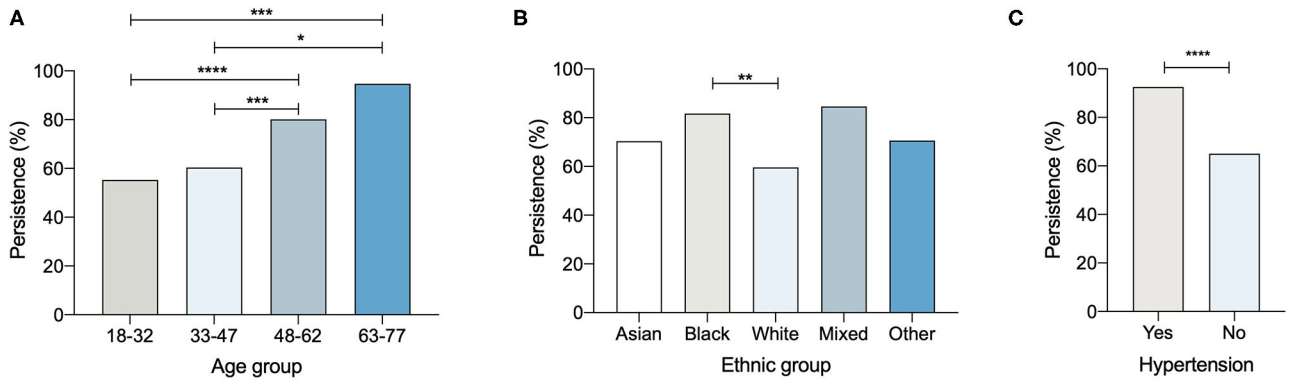

FIGURE 3 | Percentage persistence of COVID-19 antibody positivity and **(A)** age, **(B)** ethnic group **(C)** hypertension (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; ****$p < 0.001$).

TABLE 8 | Persistence of IgG positivity after 3-months according to enrolment SARS-CoV-2 IgG levels.

Architect index range	IgG positives at enrolment (*n*)	IgG status after 3 months			
		Positive (*n*)	Negative (*n*)	Non-follow up at 3 months (*n*)	Positivity rate
1.4–2.65	114	13	80	21	11.4%
2.65–4.16	114	43	52	19	37.7%
4.16–5.79	114	80	11	23	70.2%
>5.79	112	88	2	22	78.6%

n, number of participants.

respectively). Additionally, IgG prevalence at enrolment was higher among frontline workers (28.3%) compared to non-frontline workers (19.9%) with a similar decline rate observed after 3 months.

We observed that participants who declared COVID-19 defining symptoms (as specified by the PHE) had higher Architect Index readings than those with other symptoms (e.g., headache, runny nose, diarrhoea) or who were asymptomatic (OR = 1.869). Additionally, we observed that participants with a symptom duration of more than 11 days were twice as likely to have elevated Architect Indices. This is consistent with findings which suggest the presence and duration of symptoms along with disease severity were more likely to influence the development of an adequate and persistent serum immune response (51).

Among the 545 participants included in the 3-month follow-up analysis, 3 out of the 399 IgG positive subjects (0.7%) at enrolment were observed to have higher Architect Index values at follow-up, which could be explained by reinfection or the timing of the first sample, possibly taken whilst immunity was building. To date, there is limited evidence of reinfection by SARS-CoV-2, although it is generally assumed that reinfections by coronaviruses occur (52–54). In our study, a reinfection could be an explanation for 2 of the 3 subjects, as they reported COVID-19 defining symptoms a week prior to follow-up testing. The third participant only reported symptoms before enrolment in March 2020. Notably, only 4 out of the 146 antibody negative participants at enrolment (2.7%) became positive after 3 months. Similar to the much larger SIREN study (55), which recruited nationally, our local cohort data suggests that those that had a known previous infection did not become re-infected during the study period. However, we were not able to determine an association between previous infections and lower risks of new infections due to the lack of PCR information. Additionally, the low rates of seroconversion (2.7%), indicative of new infections during the September-November period (although not confirmed by PCR), may have resulted from improved workplace containment practices.

The most striking finding of this study is that 31.3% of the IgG positive participants at enrolment were found to have a negative result after 3 months, furthermore, most participants (94%) experienced a decline in their IgG antibody readings, including those with high initial Architect Indices. Our study confirms data reported by the Centre for Disease Control and Prevention, where 94% of HCWs experienced a fall in IgG levels within 60 days, with 28% sero-reverting (56).

In our study we measured SARS-CoV-2 anti-N IgG using the Abbott Architect^{TM}, which has been reported to correlate with neutralizing antibody titres (57, 58). However, we recognise that this does not provide an entire picture of the anti-SARS-CoV-2 humoral immunity in the analysed participants. Anti-N and anti-S IgG have been shown to present different kinetics; anti-N IgG, detected using different automated assays,

appears earlier in infection but disappears faster than anti-S IgG (59).

SARS-CoV-2 anti-N IgG and anti-SARS-CoV-2 neutralising antibodies have shown a similar decline over time (60) hence we consider it plausible that our findings can be extrapolated to describe antibody production in general. Further studies regarding total anti-SARS-CoV-2 IgG kinetics are necessary to address this knowledge gap.

The humoral immune response against human endemic coronaviruses is known to wane over time (allowing potential reinfections after 6–12 months) (61), while specific antibodies against SARS-CoV-1 have been detected for up to 17 years post infection (62, 63). Recent studies have shown different proportions of sero-reversion in SARS-CoV-2 infected individuals, determined by anti-Spike and anti-Nucleocapsid assays (34, 35). The magnitude of the neutralising antibody (anti-Spike1 nAb) has been shown to be dependent on the severity of infection resulting in individuals with mild disease having modest nAb titers having an undetectable neutralising response 50 days after the onset of symptoms (64). Whereas other studies looking at mild-moderate disease have shown detectable levels for up to 5 months (65).

In this study, we report on the Panbio^{TM} as a point of care test using the Architect assay as a semi-quantitative confirmatory assay, thereby defining a potential role of the Panbio^{TM} in epidemiological sero-surveillance or the assistance in management of COVID-19 in the future. HCW subjects with pre-defined risk factors for serious COVID-19 illness demonstrated greater prevalence, higher levels and greater persistence of SARS-CoV-2 IgG antibody than those deemed low risk. It is accepted that we will require an arsenal of tools at our disposal to diagnose early, manage and contain community outbreaks. In line with this, we envisage that the Panbio^{TM} will be incorporated as part of a battery of tests to provide diagnostic information and facilitate interventions, as it will allow the distinction between antibody production induced by natural infection and vaccination, the latter inducing anti-S, but not anti-N antibody production. Future studies should focus on better understanding antibody prevalence and persistence especially in high-risk populations aided with POC testing methods in the COVID-19 diagnostic algorithm.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by South Central-Berkshire Research Ethics Committee ref: 20/SC/0191, ISRCTN60400862. London-Camden & Kings Cross Research Ethics Committee, ref 20/HRA/2675, ISRCTN15634328. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GF and PK: study concept and design and study supervision. NC, CU, and UG: acquisition of data. KD, NC, CU, and VS: data analysis. KD, NC, CU, VS, and UG: statistical analysis. KD, DL, RB, MN, ST, MH, TC-M, and UG: admin/technical and material support. PK: ethics. NC, CU, and VS: drafting of manuscript. UG, KD, GF, and PK: critical review of manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.642723/full#supplementary-material

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MINI REVIEW
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Geographical Variations in Host Predisposition to COVID-19 Related Anosmia, Ageusia, and Neurological Syndromes

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The novel coronavirus disease (COVID-19), has become the most critical global health challenge in recent history. With SARS-CoV-2 infection, there was an unexpectedly high and specific prevalence of olfactory and taste disorders (OTDs). These high rates of hyposmia and hypogeusia, initially reported as up to 89% in European case series, led to the global inclusion of loss of taste and/or smell as a distinctive feature of COVID-19. However, there is emerging evidence that there are striking differences in the rates of OTDs in East Asian countries where the disease first emerged, as compared to Western countries (15.8 vs. 60.9%, p-value < 0.01). This may be driven by either variations in SARS-CoV-2 subtypes presenting to different global populations or genotypic differences in hosts which alter the predisposition of these different populations to the neuroinvasiveness of SARS-CoV-2. We also found that rates of OTDs were significantly higher in objective testing for OTDs as compared to subjective testing (73.6 vs. 60.8%, p-value $= 0.03$), which is the methodology employed by most studies. Concurrently, it has also become evident that racial minorities across geographically disparate world populations suffer from disproportionately higher rates of COVID-19 infection and mortality. In this mini review, we aim to delineate and explore the varying rates of olfactory and taste disorders amongst COVID-19 patients, by focusing on their underlying geographical, testing, ethnic and socioeconomic differences. We examine the current literature for evidence of differences in the olfactory and gustatory manifestations of COVID-19 and discuss current pathophysiological hypotheses for such differences.

Keywords: anosmia, ageusia, olfactory and gustatory dysfunctions, COVID-19, geographical variations, socio-economic variations, ethnic variations

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INTRODUCTION

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the resultant coronavirus disease 2019 (COVID-19) is the largest pandemic in recent history. As of 24th January 2021, there have been 96, 877, 399 confirmed cases and 2, 098, 879 confirmed deaths in 224 countries and territories, according to the World Health Organization (WHO). The first cases of COVID-19 were described in Wuhan, China, in late 2019 (1), with initial presenting complaints related to acute respiratory illnesses (ARI) (2–4). However, as the pandemic developed, relatively

minor symptoms such as anosmia and ageusia were discovered to be disproportionately important to the presentation and understanding of COVID-19 pathophysiology.

Olfactory and taste disorders (OTDs) were first described in February 2020 by Mao et al. (5) in their retrospective case series describing neurological manifestations amongst COVID-19 patients in Wuhan, China. Out of 314 patients, they reported 5.1% hyposmia and 5.6% hypogeusia (5). As the pandemic spread to Europe, media and anecdotal accounts from medical practitioners supported such reports of OTDs (6). In early April, Lechien et al. (7) published a multicentre cross-sectional study based in several European countries, with 417 patients, of which 85.6 and 88.8% were found to have olfactory dysfunction and gustatory dysfunction, respectively. Shortly after this, multiple otolaryngology chapters released statements recommending that OTDs be considered as symptoms of COVID-19 (8–10). This was followed by the Centers for Disease Control and Prevention (CDC), United States of America (USA), and the Ministry of Health, Singapore adding "loss of smell or taste" to the list of symptoms of COVID-19 in mid-April. The World Health Organization and the Department of Health and Social Care, United Kingdom (UK), officially added "loss of taste or smell" to their respective list of symptoms of COVID-19 in early May.

Anosmia, ageusia and the entire spectrum of OTDs are of importance to our understanding of COVID-19 because they provide an opportunity to learn more about the neurotropic effects of the SARS-CoV-2 virus and allow us to study the potential long-term neurological effects that SARS-CoV-2 infection can lead to, even in patients with mild COVID-19 infections. It is interesting to note, that despite the initial surge of COVID-19 cases in Asia, the literature highlighting OTDs was primarily based on patients in Europe and the USA.

In this mini review we explore the different possible reasons behind these geographical differences in OTD rates, such as the initial stress on Asian healthcare systems, different viral genotypes and differing pathogenic susceptibility of different populations. We also examine variations seen in OTD rates in studies utilizing subjective testing as compared to objective testing. We describe the differences seen between different ethnic groups and explore if genetic determinants can account for the disproportionate affliction of minority races, and other factors such as comorbidity burden and socio-economic status. We also highlight developing trends such as the gender differences in anosmia and ageusia as well as the use of real-time trackers.

METHODOLOGY

We performed searches for studies examining olfactory and gustatory dysfunction amongst COVID-19 patients in databases such as PubMed, Google Scholar and Web of Science. In view of the time-sensitive nature of the COVID-19 pandemic, preprint databases such as Medrxiv and Biorxiv were also utilized to capture latest developments. Search terms utilized included "Anosmia in COVID-19," "Ageusia in COVID-19," "Olfactory disorders in COVID-19," "Gustatory disorders in COVID-19" and other related search terms. Original studies, commentaries and review articles were considered during the literature review. Studies with original data on OTDs were included for comparison and analysis, with the original reported rates of OTDs reflected without any secondary analysis. Pooled averages were calculated for comparison between different geographical regions. Statistical analysis was carried out by SPSS version 20.0 (SPSS, IBM Corporation, IL, USA), and Pearsons Chi-square tests were performed, with $p < 0.05$ regarded as statistically significant.

HYPOTHESIZED PATHOPHYSIOLOGICAL PROCESSES FOR THE DEVELOPMENT OF ANOSMIA AND AGEUSIA

SARS-CoV-2 is closely related to severe acute respiratory syndrome coronavirus (SARS-CoV) and the Middle East respiratory syndrome coronavirus (MERS-CoV) – which have each caused their own epidemics associated with extrapulmonary manifestations and high mortality rates (11, 12). The functional receptor allowing for SARS-CoV-2 entry into host cells is human angiotensin-converting enzyme 2 (ACE2) (13), and this viral entry is facilitated by transmembrane protease serine 2 (TMPRSS2), similar to SARS-CoV (14, 15). ACE2 is found in the human airway epithelia, lung parenchyma, vascular endothelia, kidney cells and small intestine cells (16, 17).

SARS-CoV-2 is postulated to be able to infect the CNS in a similar manner to SARS-CoV, via a hematogenous and trans-neuronal route, with cell entry mediated by ACE2 receptors (18). SARS-CoV-2 in the bloodstream may interact with ACE2 expressed in the capillary endothelium of cerebral vessels, and allow viral access to the brain, after which the virus can interact with ACE2 receptors expressed in neurons (18). Viral interaction with the olfactory bulb and cortex may lead to neuronal damage and resultant hyposmia or anosmia (18–21). The trans-neuronal spread of the virus has also been hypothesized to damage the peripheral neurons directly (18, 22, 23). However, olfactory neurons do not express significant levels of ACE2 and TMPRSS2 (24–27) and neuronal damage to the olfactory bulb and cortex cannot account for case reports of rapid and transient anosmia (7), in view of such damage requiring significant time for recovery (27).

Another proposed mechanism for anosmia is damage to non-neuronal structures that support olfactory function, such as olfactory epithelium sustentacular cells, microvillar cells, Bowman's gland cells, horizontal basal cells and olfactory bulb pericytes (25). These olfactory epithelium sustentacular cells have abundant expression of ACE2 and TMPRSS2 (24, 25, 28, 29). Local infection of these non-neuronal structures is proposed to cause significant inflammatory responses affecting olfactory sensory neurons or olfactory bulb neurons, and may even result in neuronal death (25). Reports of transient anosmia, with rapid recovery, may then be explained by the faster regeneration rate of sustentacular cells, as compared to olfactory neurons (20, 27).

Regarding ageusia, ACE2 receptors are known to diffusely express on the mucous membranes of the oral cavity, with a high concentration on the tongue (30). It is thought that ACE2

modulates taste perception, and that SARS-CoV-2 binding to the receptor may lead to taste dysfunction by damaging the gustatory cells, even though the exact mechanism is unclear (31, 32). One proposed mechanism is the binding of SARS-CoV-2 to sialic acid receptors, an ability it shares with MERS-CoV (33). This binding of SARS-CoV-2 to sialic acid receptors may result in the acceleration of degradation of gustatory particles, resulting in blunting of the patient's taste (31). Another possibility is that ageusia happens concomitantly with anosmia due to the close functional correlations between the olfactory and gustatory chemosensory systems (34).

Emerging evidence on neuroimaging characteristics of anosmic patients may also assist to elucidate the definite pathophysiology of COVID-19 associated OTDs. Magnetic Resonance Imaging of COVID-19 patients with OTDs have shown olfactory bulb injury (19) and changes (35–37), suggesting the viral invasion of these nerve structures with resultant sensorineural dysfunction. The persistence of OTDs is also an area of interest, with studies suggesting a persistence of symptoms in up to 24% of COVID-19 patients (38, 39), and interesting trends such as younger patients and female patients having a higher tendency for such persistence (40). It may be only possible with time to elucidate the exact pathophysiological elements leading to OTDs in the context of SARS-CoV-2 infection, and histological biopsies of COVID-19 patients are likely to greatly aid this effort (27).

GEOGRAPHICAL VARIATIONS

Anosmia, ageusia and OTDs amongst COVID-19 patients were first recognized to be common in Europe, several months after the first few COVID-19 epicenters in Asia. Asian studies were consistently publishing lower percentages of patients presenting with anosmia and ageusia compared to those being reported in Europe and the USA, with one study reporting the prevalence of chemosensory dysfunction in Caucasians to be three times higher than that in Asians (27, 41–44). **Table 1** illustrates the difference in pooled average prevalence of olfactory disorders, taste disorders and combined olfactory and/or taste disorders between Eastern and Western populations, with a map graphically representing the higher prevalence of OTDs in Western countries. The pooled average for the Western population was close to 4 times that of the Eastern populations (15.8 vs. 60.9%, p-value < 0.01) as seen in **Table 1**. Three main reasons have been postulated in the literature with regards to this difference between Western populations and Eastern populations.

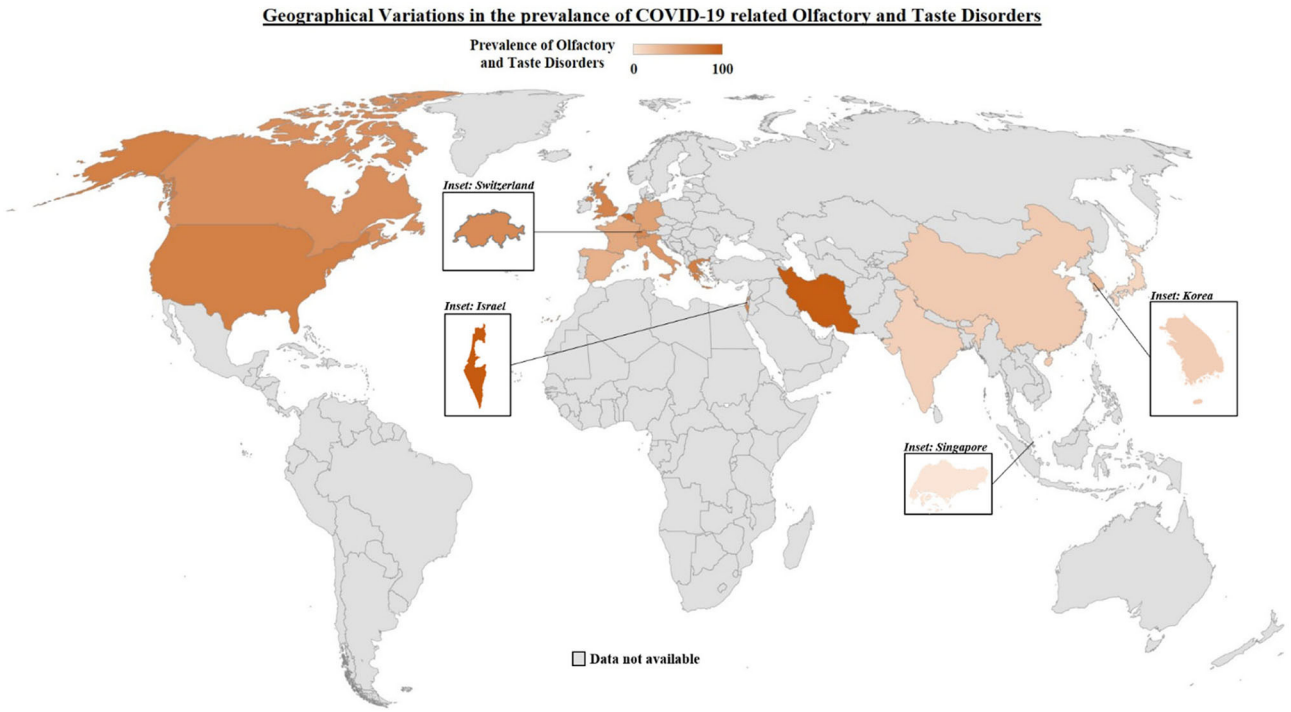
Firstly, there was the shock element of the initial outbreak. In the initial stages of the outbreak, when it was first recognized in Asia, patients who were critically ill would have been prioritized and hospitalized. Indeed, the literature from the early days of the pandemic highlighted concerns regarding mortality and need for intensive care therapy (76, 77), suggesting that the patients presenting to the healthcare institutions were indeed more unwell. It has been suggested that minor symptoms such as anosmia and ageusia may have been overlooked in preliminary cohorts in the pandemic, both by medical professionals, as well as patients themselves (7, 43). This could have led to an under-reporting of actual anosmia and ageusia rates in Asian countries in the initial stages of the outbreak. However, over time, this has become a less viable explanation, in view of studies from other Asian countries also showing significantly lower rates of anosmia and ageusia as compared to Western nations (40, 45).

The second possible reason is that of differing viral genotypes in Asia as compared to Europe and the USA. A phylogenetic analysis of 160 SARS-CoV-2 genomes by Forster et al. (78) found 3 central variants of the virus: Types A, B and C. Types A and C were found to be more prevalent amongst Europeans and Americans, compared to Type B which was more prevalent amongst Asians (78). Types A and C are speculated to have high pathogenicity for the nasal cavity, hence resulting in the higher prevalence of olfactory and taste disorders in Western populations (43, 78). Mutations in the receptor binding domain (RBD) of the virus spike protein (subunit S1) may also result in differing viral tropism and infectivity (79). Mutations in the RBD have been shown to affect its binding to the ACE2 receptor (80, 81), and these mutations can impact the pathogenicity of the virus (82). Indeed, early studies probing interactions between ACE2 coding variants and SARS-CoV-2 virus have pointed to certain populations having a higher predisposition for SARS-CoV-2 binding (83). The emergence of new variants such as the UK variant (84) and South African variant (85) in late 2020 and early 2021 lend further credence to the presence of differing viral genotypes in distinct geographical territories. These variants may have differing rates of infectivity of the olfactory epithelium which may influence the prevalence of OTDs (27).

Finally, differing pathogenic susceptibility, in the form of genetic variations of host proteins and receptors such as ACE2 and TMPRSS2, may have led to the difference in anosmia and ageusia rates between different populations. Variations in ACE2 expression in different populations have been reported (86, 87), with one study finding increased ACE2 expression in tissues in East Asian populations (88). Variations in TMPRSS2 protein frequency have also been observed with European populations having much higher levels of pulmonary expression as compared to East Asian populations (20). Genetic differences in ACE2 variants, characterized by post-translational modifications such as glycosylation, may also contribute to the varying susceptibility of different populations to anosmia (27, 89). Such genetic differences resulting in differing OTD rates were corroborated by a Singaporean study, which collected nationality and ethnicity data, and found that Caucasians were 3.05 times more likely to have OTDs as compared to Chinese, South East Asian and West Asian races (51). Further research is required to delineate the link between ACE2/TMPRSS2 expression and susceptibility to olfactory and taste disorders.

The high susceptibility to OTDs amongst Western populations as compared to East Asian populations, raises the specter of whether these same Western populations are facing a higher burden of SARS-CoV-2 related peripheral and central nervous system disorders. The same reasons of possibly different viral genotypes and differing pathogenic susceptibility can also be used to explain any corresponding spike in both PNS

TABLE 1 | Geographical and testing variations.



World Map of the prevalence rates of Olfactory and Taste Disorders globally.
(The intensity of color suggests a higher prevalence of OTDs.)

S/N	Author	Country	Sample Size	Olfactory and taste assessment method	Olfactory disorders only (%)	Taste disorders only (%)	Any olfactory and/or taste disorders (%)
Asian (ex-Middle East) studies							
1	Mao et al. (5)	China	214	Subjective	5.1	5.6	NA
2	Lee et al. (40)	Korea	3191	Subjective	4.2	3.1	15.3
3	Wee et al. (45)	Singapore	154	Subjective	NA	NA	22.7
4	Chua et al. (46)	Singapore	31	Subjective	22.6	NA	NA
5	Qiu et al. (47)	China	239	Subjective	20.0	3.0	32.0
6	Kim et al. (48)	Korea	172	Subjective	39.5	33.7	NA
7	Komagamine et al. (49)	Japan	628	Subjective	10.0	9.1	NA
8	Mishra et al. (50)	India	74	Subjective	14.8	NA	NA
9	Tham et al. (51)	Singapore	1065	Subjective	11.8	4.6	12.6
Pooled averages					**8.3**	**5.1**	**15.8**
Middle Eastern and Western studies							
10	Hopkins et al. (6)	England	382	Subjective	60.0	88.9	NA
11	Giacomelli et al. (52)	Italy	59	Subjective	5.1	10.2	33.9
12	Yan et al. (53)	USA	128	Subjective	68.0	71.0	NA
13	Levinson et al. (54)	Israel	45	Subjective	35.7	33.3	69.0
14	Menni et al. (55)	England	579	Subjective	NA	NA	59.0
15	Spinato et al. (56)	Italy	283	Subjective	NA	NA	64.4
16	Klopfenstein et al. (57)	France	114	Subjective	47.0	40.3	Nil
17	Beltran et al. (58)	Spain	79	Subjective	45.2	45.2	39.2
18	Menni et al. (59)	England	7178	Subjective	NA	NA	65.0
19	Zens et al. (60)	Germany	65	Subjective	47.6	NA	NA
20	Patel et al. (61)	England	141	Subjective	56.7	63.1	NA

(Continued)

TABLE 1 | Continued

S/N	Author	Country	Sample Size	Olfactory and taste assessment method	Olfactory disorders only (%)	Taste disorders only (%)	Any olfactory and/or taste disorders (%)
21	Luers et al. (62)	Germany	72	Subjective	74.0	69.0	68.0
22	Bertlich et al. (63)	Germany	47	Subjective	31.9	19.1	NA
23	Haehner et al. (64)	Germany	69	Subjective	31.8	NA	NA
24	Borobia et al. (65)	Spain	2226	Subjective	12.8	NA	NA
25	Tudrej et al. (66)	France	816	Subjective	19.1	23.0	29.7
26	Qiu et al. (47)	Germany	39	Subjective	18.0	3.0	69.0
27	Qiu et al. (47)	France	116	Subjective	6.0	NA	49.0
28	Gelardi et al. (67)	Italy	72	Subjective	11.0	25.0	47.0
29	Speth et al. (68)	Switzerland	103	Subjective	61.2	65.0	NA
30	Carignan et al. (69)	Canada	134	Subjective	51.5	63.4	64.9
31	Abalo-Lojo et al. (32)	Spain	131	Subjective	NA	NA	55.0
32	Lee et al. (70)	Canada	56	Subjective	42.9	57.1	NA
Pooled averages (Subjective only)					**26.1**	**46.9**	**60.8**
33	Kaye et al. (71)	USA	237	Objective	73.0	NA	NA
34	Moein et al. (72)	Iran	60	Objective	98.0	NA	NA
35	Hornuss et al. (73)	Germany	45	Objective	40.0	NA	NA
36	Lechien et al. (7)	Europe	417	Objective	85.6	88.8	NA
37	Vaira et al. (74)	Italy	72	Objective	14.4	12.5	73.6
38	Tsivgoulis et al. (75)	Greece	22	Objective	72.0	NA	NA
Pooled averages (Objective only)					**74.2**	**77.5**	**73.6**
p-values (Subjective vs. objective)					*<0.01*	*<0.01*	*0.03*
Pooled averages					**33.4**	**52.3**	**60.9**
p-values (Asian vs. Western)					*<0.01*	*<0.01*	*<0.01*

and CNS manifestations in Western populations as compared to Eastern populations. We should note that directly comparing prevalence of neurological symptoms between studies has proven to be difficult, largely due to the heterogenous nature of recorded neurological symptoms such as headache, giddiness and altered mental state – especially as they may be manifestations of systemic disease as well (90, 91). Nevertheless, comparing CNS syndromes, such as encephalitis, and PNS syndromes, such as mono or polyneuropathies, reveals no evidence of increased rates of such syndromes amongst Western populations compared to Eastern populations thus far (92, 93).

TESTING VARIATIONS

The majority of the literature concerning COVID-19 and OTDs has been based on patient self-reporting (94). This may inevitably lead to inconsistences (52, 94), such as recall bias on the part of the patient, or confirmation bias on the part of the medical professional. Objective forms of testing have been proposed and utilized in some studies, such as the University of Pennsylvania Smell Identification Test (UPSIT), Questionnaire of Olfactory Disorders–Negative Statements (95, 96), COVID-19 Anosmia Reporting Tool (71), Sniffin' sticks test, and Korean version of Sniffin' sticks test (KVSS) (97). Broadly, studies utilizing objective testing for anosmia and ageusia have found a higher prevalence of olfactory and gustatory disturbances amongst COVID-19 patients (72, 98). **Table 1** highlights the differences in OTD rates between objective and subjective testing, seen in the differing prevalence rates, in favor of objective testing (60.8 vs. 73.6%, p-value = 0.03). We can hypothesize that the reasons behind under-reporting of anosmia or ageusia may be due to difficulties in perceiving a reduction in sense of smell or taste (99) as well as difficulties in finding and receiving an appropriate level of care (100), which may be linked to socio-economic issues, further elaborated on below. It has to be appreciated however, that self-reporting of symptoms may often be the only feasible and practical way of data collection, especially with pandemic precautions and restrictions (44).

ETHNIC, COMORBIDITY AND SOCIO-ECONOMIC VARIATIONS

COVID-19 has disproportionately affected racial minorities across the world, with infection rates and mortality rates two to three times higher in these minorities than their proportion in the population (101–106). Ethnic, socio-economic and comorbidity variations all have a role in accounting for this higher affliction rate amongst racial minorities (105).

Variations in OTDs, due to COVID-19, between different ethnicities residing in the same region, have yet to be described fully in the literature. We know from pre-COVID studies that anosmia is more prevalent amongst African-Americans as compared to Caucasians in the USA (107). Dong et al.

TABLE 2 | Gender Variations in COVID-19 related olfactory and taste disorders (OTDs) and COVID-19 trackers.

S/N	Author	Year	Summary/Interpretation
Gender Variations in COVID-19 related OTDs			
1	Lechien et al. (7)	2020	In a study of 417 mild-to-moderate COVID-19 patients, females were found to be significantly more affected by olfactory and taste dysfunctions then males. This was attributed to gender-related differences in inflammatory reaction processes.
2	Hopkins et al. (6)	2020	Online survey of 382 patients reporting self-diagnosed new onset of olfactory and taste dysfunction, of which 74.6% were female. However in view of this being a voluntary online survey, it may simply reflect gender differences in completing such voluntary online questionnaires rather than gender-related differences in prevalence of olfactory and taste dysfunction.
3	Tham et al. (51)	2020	Out of 1065 patients with laboratory-confirmed COVID-19, the female gender was found to be significantly associated with olfactory and taste disorders on multivariate analysis. This was again attributed to gender-related differences in the inflammatory reaction process.
4	Giacomelli et al. (52)	2020	Cross-sectional survey of 59 COVID-19 positive patients of which females were found to have a higher prevalence of olfactory and taste disorders as compared to males (52.6% vs. 25%).
5	Foster et al. (120)	2020	Amongst 949 COVID-19 positive patients, anosmia was significantly associated with younger age, higher BMI as well as female sex. The proportion of females amongst patients with anosmia was significantly higher than that of patients without anosmia (64.7% vs. 52.8%). Anosmia was found to be an independent positive prognostic factor of a less severe COVID-19 infection.
7	Talavera et al. (121)	2020	Amongst 576 COVID-19 positive hospitalized patients, anosmia was present in 25.3%. Patients with anosmia were more frequently female, had less comorbidities such as hypertension and diabetes, and were less likely to be smokers. Hospitalized COVID-19 patients with anosmia had a lower adjusted mortality rate and a less severe course of the disease.
Proposed mechanisms for gender differences in COVID-19 related OTDs			
8	Lefevre et al. (122)	2019	Higher levels of inflammatory cytokines were recorded in males as compared to both women and patients with Klinefelter syndrome following whole blood stimulation, even after adjusting for sex steroid levels. This suggests that males may have a more severe disease process as compared to females.
9	Hewegama et al. (123)	2009	In a comparison study of T-cell gene expression between males and females, females were found to have a higher expression of inflammatory and cytotoxic effector molecules under conditions of repeated stimulation. The authors hypothesized that this may contribute to the development and severity of autoimmune diseases in women.
10	Jaillon et al. (124)	2019	Study examining variation in innate immunity, measured by the level of tumor necrosis factor (TNF) in lipopolysaccharide (LPS)-stimulated whole-blood culture found that females have a nearly 30% lower innate immune response.
11	Marriot et al. (125)	2006	Review article outlining differences in innate immune responses between males and females, particularly that viral infections are more severe and require hospitalization more in males than females, corresponding with higher levels of TNF-α in males than females. Females were also found to mount more effective adaptive immune responses to viral pathogens. These favorable differences in innate immune responses are a consequence of higher estrogen levels, which augment immune responses after infection and have been shown to increase resistance to infections.
12	Bwire et al. (126)	2020	Amongst COVID-19 patients, males have been found to have a higher mortality and morbidity. Biological factors such as genetics and immunology play an important role, but the impact of gender behavior cannot be discounted. Males were found to have a higher burden of pre-existing conditions such as diabetes, hypertension and obesity. Males were also found to have higher rates of smoking and alcohol consumption as well as having a tendency to be less likely to comply with preventive measures such as hand washing, stay home orders and donning of face masks. These may all have contributed to the higher morbidity and mortality amongst males compared to females.
13	Kopel et al. (127)	2020	Review article about gender variations in COVID-19 infection. Females are less likely to produce extreme immune responses as compared to males due to X-chromosome and sex hormone modulated innate and adaptive immunity differences. This study also explores gender differences in ACE2 receptor highlighting that ACE2 expression is higher in males than females, but also high in pregnant female patients. This suggests that pregnant female patients may be more susceptible to COVID-19 infection that non-pregnant female patients.
COVID-19 Trackers			
14	**COVID-19 Symptom Study** *Menni et al. (59) Drew et al. (128)*	2020	The COVID-19 Symptom Study (previously known as COVID-19 symptom tracker) is a smartphone-based application that was launched in the United Kingdom and United States on March 2020. The application captures self-reported information including age, health risk factors and location. It has registered millions of participants and studies with this dataset found that the proportion of participants who reported olfactory and taste disorders was higher in those with a positive COVID-19 test result compared to those with a negative test result.
15	**COVID-19 Symptom Tracker** *Zens et al. (60)*	2020	The COVID-19 Symptom Tracker is a smartphone-based application, which was designed in Germany and was launched in Germany on April 2020. It captures self-reported demographic and medical history as well as prompting users to report symptoms of COVID-19 on a daily basis. This application registered 11,829 participants who completed the symptom questionnaire at least once, and found that loss of smell was one of the top 5 strongest predictors for COVID-19 infection.

(Continued)

TABLE 2 | Continued

S/N	Author	Year	Summary/Interpretation
16	**Google Trends analysis** *Walker et al. (129) Cherry et al. (130)*	2020	Analysis of internet search engine interest (Google Trends) for terms relating to olfactory and taste disorders and then correlating them with region specific COVID-19 data. An analysis of such trends found that there was a strong correlation between daily search volumes related to anosmia/ageusia and increases in daily COVID-19 cases and deaths in the same geographical region. Tracking of such search interest can assist public health planning on a regional and/or national level.
17	**COVIDCast** *Flaxman et al. (131)*	2020	COVIDCast is the largest public repository of geographically detailed, real time indicators of COVID-19 activity in the United States of America run by the Delphi lab at Carnegie Mellon University. It gathers data from Facebook via national daily surveys as well as de-identified medical insurance claims. It has garnered more than 15 million responses since starting in April 2020 and has approximately 55,000 participants daily. It does not collect symptom information related to olfactory and taste disorders.
18	**Coronaisrael survey** *Rossman et al. (132)*	2020	Real-time nationwide survey of coronavirus symptoms via an online survey (https://coronaisrael.org/) which is filled out anonymously, collecting primarily geographical data. The survey attained a cumulative number of close to 75,000 responses within 10 days. The newer version of this questionnaire included loss of smell and taste as symptoms of COVID-19 infection. This tracker is a member of the coronavirus census collective.
19	**HowWeFeel** *Segal et al. (133)*	2020	HowWeFeel is a symptom tracker mobile application which administers a 30-second survey on the participants well-being to collect epidemiological data. This data is anonymous and gathers health and demographic data to educate the researchers about infection trends in the community. This tracker is member of the coronavirus census collective.
20	**The Sex, Gender and COVID-19 project** (134)	2020	Live tracking of COVID-19 statistics globally, with a specific focus on sex and gender. As of 24 January 2021, for every 10 Intensive Care Unit (ICU) admissions amongst females, there are approximately 19 ICU admissions amongst males. This tracker does not collect data with regards to olfactory and taste disorders.
21	**CoEpi (Community Epidemiology In Action)** (135)	2020	CoEpi is an open-source mobile application that uses Bluetooth proximity data to anonymously track and alert users who have been in close proximity to symptomatic users. The application captures symptoms related to COVID-19 and other transmissible illnesses. This tracker has yet to publish data which it has collected.
22	**Beat COVID-19 Now** (136)	2020	Beat COVID-19 Now is a symptom tracker mobile application and webpage developed by the Swinburne University of Technology in Australia and captures self-reported COVID-19 symptom information from users worldwide. This tracker has yet to publish data which it has collected.

described the prevalence of anosmia amongst African-Americans as 22.3%, as compared to 10.4% amongst Caucasians, but were unable to account for this stark racial disparity (107). As such, it would not be surprising if anosmia rates in African-American COVID-19 patients were higher than in other ethnicities. A possible explanation may be in the differences in ACE2 expression. A reduced molecular expression of ACE2 in African-descent populations has been described (108), which should theoretically lead to a lower incidence of COVID-19 in these populations, contrary to reality. Vinciguerra et al. proposed that whilst this reduced expression of ACE2 can lead to lower susceptibility to SARS-CoV-2 infection, once infected, the clinical manifestations may be worse, due to progression of inflammatory and thrombotic processes as a result of such reduced ACE2 expression (109). TMPRSS2 may also play a part in the ethnic variations in anosmia. Ethnic differences in TMPRSS2 gene-related activity in prostate tissue have been associated with a higher incidence of prostate cancer in African-American men, as compared to Caucasian men, in the USA (110). This ethnic difference was found to be similar for nasal gene expression of TMPRSS2. In a study of 305 unique nasal epithelial samples, African-Americans were found to have statistically significantly higher TMPRSS2 expression as compared to Asian, Latino, mixed race and Caucasian individuals (111). TMPRSS2 is known to be essential in SARS-CoV-2 cell entry (15), suggesting a possible reason behind the higher burden of COVID-19 infection amongst African-Americans in the USA, possibly holding true for anosmia as well.

Comorbidity burden has been positively correlated with the severity of COVID-19 and mortality (112). This is of particular interest when analysing the impact of COVID-19 on minority races, as comorbidity burdens in ethnic minorities have been found to be higher (101, 105, 113, 114). Several comorbidities such as cardiovascular disease, diabetes, chronic kidney disease and chronic obstructive pulmonary disease have been reported in higher percentages amongst COVID-19 mortality statistics (115). The impact of comorbidities are further highlighted when considering that mortality rates amongst African-American and Caucasian patients in the USA are not significantly different when comorbidities are corrected for (116). However, anosmia tends to affect individuals with fewer comorbidities, except for asthma, which was found to be of a high proportion in patients presenting with anosmia (7, 57). This could possibly be due to anosmia being the only symptom in mild and moderate COVID-19 infections, which tend to occur more often in patients with no or low comorbidity burdens (7). This implies that COVID-19 patients with only isolated OTDs may have a milder disease process.

Possibly the most important piece in explaining the higher proportion of racial minorities being infected with COVID-19 is the socio-economic aspect of the disease. Poverty has been associated with a higher risk of intensive care unit admissions in the USA (117), and a large study in Brazil

found that patients from lower socio-economic regions had a higher mortality rate (102). Patients in lower socio-economic regions also have more comorbidities, suggesting that structural health disparities and poor access to healthcare result in poorly controlled chronic diseases (102, 103). People from lower socio-economic classes were also unable to comply with pandemic measures such as social distancing or working from home, due to their crowded living conditions or the blue-collar occupations that many hold (101, 105, 115). In Scotland, COVID-19 patients living in areas with the greatest socio-economic deprivation had a higher frequency of critical care admission and a higher adjusted 30-day mortality, with healthcare facilities in areas with higher socio-economic deprivation also operating at higher occupancy rates (118). The relationship between OTDs and socio-economic status alludes to the differing access to healthcare between different socio-economic groups. A pre-COVID-19 study in South Korea found that high-income population groups had a 1.4 times higher incidence of anosmia as compared to low-income population groups (119). The authors attributed this to the accessibility of medical care to patients with different income levels, and concluded that anosmia can be frequently underestimated by the elderly and low-income due to their economic situation, which hinders them from seeking medical care (119). We can hypothesize that the incidence of OTDs in lower socio-economic groups may be higher in the COVID-19 outbreak, but may not be reflected in the data due to socio-economic factors that hinder their access to healthcare. Future studies on the prevalence of OTDs in different socio-economic groups affected by COVID-19 will help to corroborate this hypothesis.

In addition to the inequalities described above, there may be emerging evidence that gender distinguishes both susceptibility to COVID-19 and associated complications such as anosmia and ageusia (**Table 2**); further study is required to explain such differences. The ability for public health and research groups to mobilize the efforts of its "citizen scientist" community during this pandemic has also been key to illustrating emerging or unusual trends, such as OTDs, in the form of trackers (**Table 2**). Despite the limitations of these trackers, they provide both helpful and near real-time updates of disease prevalence as well as gauge societal attitudes toward such group efforts in global health.

CONCLUSION

Anosmia and ageusia have become well-recognized symptoms of this current pandemic. Much has changed since the original case reports about olfactory and taste disorders, but there are still many questions that remain unanswered regarding how biological and societal factors influence the impact of SARS-CoV-2. In this mini review, we categorize and collate current available literature in order to describe the differences in OTDs seen in different geographical regions as well as amongst different ethnicities and socio-economic conditions. We believe our study to be the first mini review to compare and contrast the variously reported global variations in OTDs. Concurrently, we have provided an up-to-date report on the disproportionate influence of ethnic, comorbidity and socio-economic factors toward such variations. Understanding such inequalities may highlight areas of consideration for allocation of resources and focused attention. Further research is also required to elucidate the exact pathophysiological mechanisms underpinning the phenomena of anosmia and ageusia in COVID-19 and account for other variations, such as the importance of gender toward the clinical phenotype of disease.

AUTHOR CONTRIBUTIONS

AK, CL, and NK: conceived of the direction and scope of the review and performed the literature reviews and compilation of references. AK and SL: wrote the manuscript with supervision and direction from CL and NK. All authors contributed to the article and approved the submitted version.

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Seroprevalence of SARS-CoV-2 Assessed by Four Chemiluminescence Immunoassays and One Immunocromatography Test for SARS-Cov-2

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The onset of the new SARS-CoV-2 coronavirus encouraged the development of new serologic tests that could be additional and complementary to real-time RT-PCR-based assays. In such a context, the study of performances of available tests is urgently needed, as their use has just been initiated for seroprevalence assessment. The aim of this study was to compare four chemiluminescence immunoassays and one immunochromatography test for SARS-Cov-2 antibodies for the evaluation of the degree of diffusion of SARS-CoV-2 infection in Salerno Province (Campania Region, Italy). A total of 3,185 specimens from citizens were tested for anti-SARS-CoV-2 antibodies as part of a screening program. Four automated immunoassays (Abbott and Liaison SARS-CoV-2 CLIA IgG and Roche and Siemens SARS-CoV-2 CLIA IgM/IgG/IgA assays) and one lateral flow immunoassay (LFIA Technogenetics IgG–IgM COVID-19) were used. Seroprevalence in the entire cohort was 2.41, 2.10, 1.82, and 1.85% according to the Liaison IgG, Abbott IgG, Siemens, and Roche total Ig tests, respectively. When we explored the agreement among the rapid tests and the serologic assays, we reported good agreement for Abbott, Siemens, and Roche (Cohen's Kappa coefficient 0.69, 0.67, and 0.67, respectively), whereas we found moderate agreement for Liaison (Cohen's kappa coefficient 0.58). Our study showed that Abbott and Liaison SARS-CoV-2 CLIA IgG, Roche and Siemens SARS-CoV-2 CLIA IgM/IgG/IgA assays, and LFIA Technogenetics IgG-IgM COVID-19 have good agreement in seroprevalence

assessment. In addition, our findings indicate that the prevalence of IgG and total Ig antibodies against SARS-CoV-2 at the time of the study was as low as around 3%, likely explaining the amplitude of the current second wave.

Keywords: SARS-CoV-2, serological test, seroprevalence, immunoassays, rapid tests

INTRODUCTION

In December 2019, an outbreak of an unexplained pneumonia was reported in the city of Wuhan, Hubei province, China. A novel coronavirus was identified as the etiological agent (named severe acute respiratory syndrome coronavirus 2—SARS-CoV-2), the associated disease defined as COVID-19 (COrona VIrus Disease, 19 stands for the year the virus was first detected).

The exponential growth of affected individuals led the WHO to declare a global pandemic; since then, the virus has greatly impacted, infecting over 80 million worldwide with more than 1.5 million deaths.

SARS-CoV-2 belongs to the coronavirus family; these are enveloped, single-stranded, positive-sense RNA viruses. Seven coronaviruses infect humans; those are classified in two genera: Alpha and Beta.

NL63 and 229E are alphacoronaviruses distantly related to SARS-CoV-2 and cause cold-like illnesses.

SARS-CoV-2 belongs to the Betacoronavirus genus, Sarbecovirus subgenus, which includes SARS-CoV responsible for the 2002/2003 outbreak and sharing 80% homology with SARS-Cov2 and MERS-CoV, responsible for the 2012 and 2015 outbreaks, respectively, and HKU1 and OC43, associated with mild upper respiratory illness, belong to other Betacoronavirus subgenera (Merbecovirus and Embecovirus, respectively) and are less related to SARS-CoV-2 (1–3).

Human coronaviruses bind different receptors. SARS-CoV-2 primarily infects pneumocytes, by binding angiotensin-converting enzyme 2 (ACE2) receptors using the transmembrane Spike (S) protein. The S protein present on the surface of the virion is one of four structural proteins (spike, nucleocapsid, membrane, and envelope) found in all coronaviruses and is responsible for both the binding to the host receptor and the fusion of the virion with the cell membrane (3). The S protein is composed by three homotrimers, each consisting of three identical polypeptide chains; each chain contains two subunits, S1 and S2. Subunit S1 makes up the majority of the S protein surface area and includes the receptor-binding domain (RBD) allowing SARS-CoV-2 to bind to the ACE2 receptor. The RBD shares only 73% similarity with SARS-CoV, and 21–25% similarity to other human coronavirus S1 subunits (4); the genetic differences in RBD dictate the viral receptor specificity.

The S2 subunit tethers the S protein to the virion membrane and includes the machinery required for virus–cell fusion (5, 6). S2 is more conserved than S1 (90% similarity with SARS-CoV-2, and 35–43% similarity with the other coronavirus S2s). Due to its location on the surface of the virus and its physiologic importance, the immunogenicity of coronavirus S protein was predicted. The serum of SARS-CoV-convalescent patients showed high titers of antibodies against the S protein (7), and in neutralization assays anti-S antibodies have shown to protect cells from SARS-CoV infection (7, 8).

Coronavirus-infected patients also exhibit antibodies with a high reactivity against the structural nucleocapsid protein (N protein) (9); this protein is very abundant, although only within the virion. Anti-N antibodies are believed to not protect cells from infection (10), since they are highly prevalent in the post-infection phase, being likely generated after digestion of viral proteins by macrophages and other antigen-presenting cells to B cells (9). Nevertheless, diagnostic assays for anti-N antibodies are easier to produce and can be useful to detect previous infection.

To address the pandemic, reliable diagnostic assays are required. Real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) tests are the main diagnostic approaches and, so far, the most reliable. Real-time PCR testing requires experienced personnel and well-equipped laboratories, making mass testing of populations difficult.

To detect viral RNA, nasopharyngeal or oropharyngeal swabs are used. The limit of detection (LOD) for the molecular test can vary between 50 and 1,000 viral copies/mL (Laboratory Corporation of America Accelerated Emergency Use Authorization (EUA) Summary COVID-19 PT-PCR Test; available online at https://www.fda.gov/media/136151/download, accessed March 30, 2020). The clinical sensitivity of SARS-CoV-2 PCR tests is not well-defined, with a positive PCR test being the standard for diagnosis in most studies. Despite the high sensitivity and specificity, false-negative results at real-time PCR are an important issue. These can be due both to mutations in primers targeting regions and to the natural disease course of COVID-19. Timing of specimen collection is crucial to clinical sensitivity: early in the course of infection, both in clinical disease and in asymptomatic infections, low Ct readouts are obtained (<20), indicative of high viral loads (ranging from 10×10^4 copies to $>10^6$ copies/mL). Conversely, in the late phase of the infection, the viral load rapidly drops, with high Ct readouts (>32), frequently yielding non-conclusive, indeterminate results. Negative results can be obtained using assays without an adequate LOD or when little RNA is collected, making difficult the diagnosis and posing problems in contact tracing.

Finally, real-time PCR assays are not useful in identifying patients with a previously recovered SARS-CoV2 infection. Therefore, despite the high diagnostic potency, the limitations of the real-time assays make necessary the use of serological tests. The association of real-time PCR assay and serology testing improves the diagnosis of COVID-19.

Due to the drop in viral RNA, serological assays may allow to detect patients in the late stages of the infection. Serological

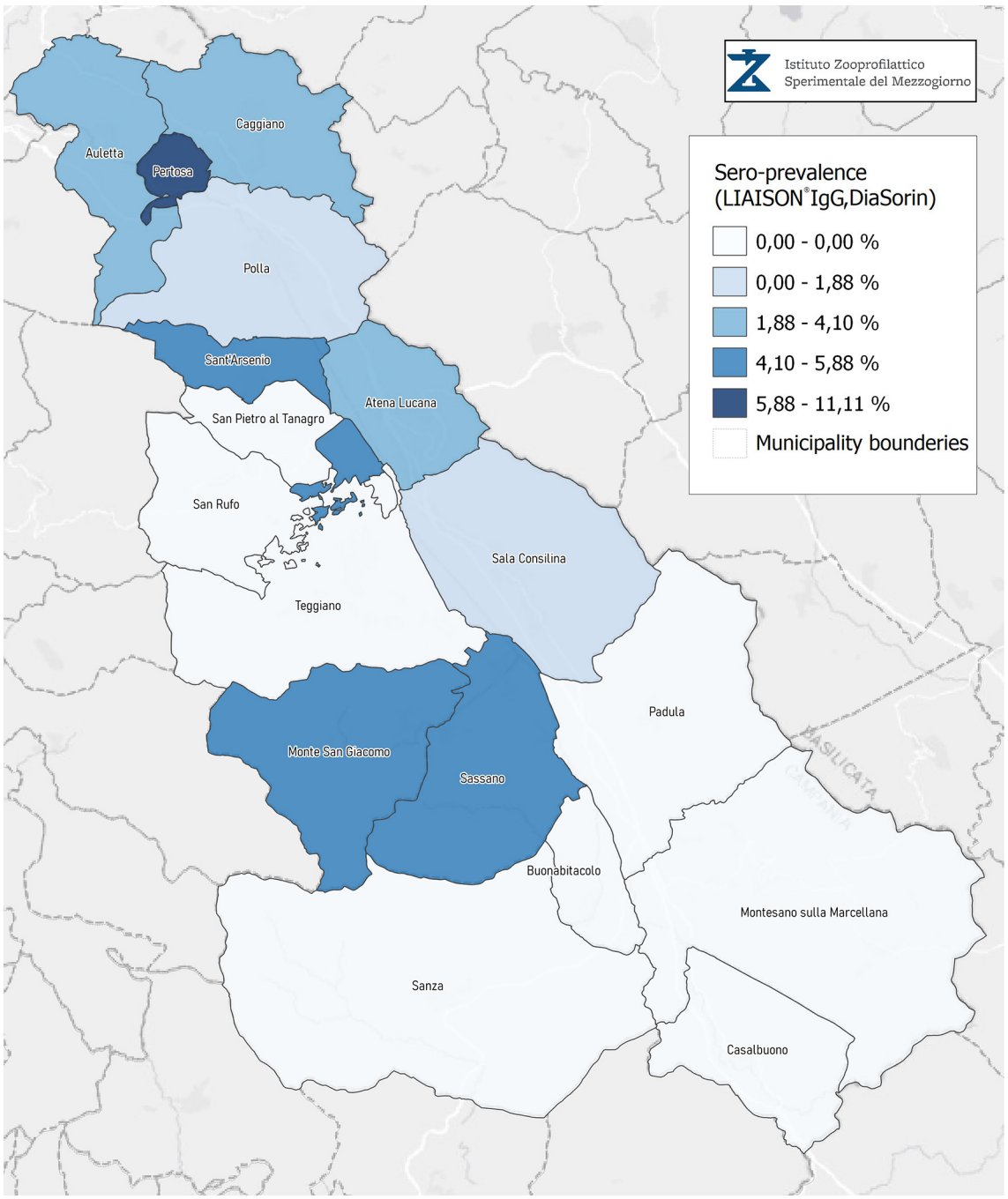

FIGURE 1 | Seroprevalence assessed in Diano Valley municipalities by Liaison IgG Diasorin.

assays could be helpful in identifying patients who have recovered from SARS-CoV2 infection, avoiding in case of contacts more expansive and time-consuming molecular tests. Serological assays can allow to deploy workers with a previous infection in high-risk settings (COVID 19 wards, ICU, etc.). Moreover, serology testing is of great importance in the seroprevalence studies, to identify donors for passive immunization or serum-transfer therapies and for the selection of the vaccine candidates.

The correlation between antibodies and the protection from reinfection is still controversial, although few cases of reinfection have been reported; serology testing of SARS-CoV2 will address this issue.

SARS-CoV-2 serological tests are already commercially available. Initially, serological tests for the detection of IgM and IgG were developed. It was believed that IgM antibodies were produced earlier than IgG; however, later studies showed

TABLE 1 | Absolute number and prevalence of COVID-19 infections as measured by the different serological tests grouped by age, sex, and area.

Demographic information ($N = 3,083$)	Positive cases (prevalence)					Agreement	
	Liaison IgG	Abbott IgG	Technogenetics IgG	Siemens total Ig	Roche total Ig	Agreement (%)	Fleiss' kappa
Age							
<18	5 (3.60)	4 (3.15)	3 (2.26)	5 (3.60)	5 (3.60)	98.53	0.80
18–65	60 (2.48)	52 (2.18)	49 (2.09)	44 (1.82)	43 (1.78)	99.16	0.61
>65	12 (1.94)	11 (1.82)	14 (2.32)	9 (1.45)	11 (1.82)	98.89	0.71
Sex							
Female	39 (2.47)	35 (2.27)	34 (2.23)	33 (2.09)	30 (1.90)	98.50	0.67
Male	38 (2.38)	32 (2.03)	32 (2.03)	25 (1.56)	29 (1.81)	98.45	0.60
Area							
Atena Lucana	12 (3.27)	11 (3.15)	7 (1.91)	7 (1.91)	9 (2.45)	98.39	0.65
Auletta	7 (2.46)	4 (1.44)	7 (2.46)	4 (1.44)	5 (1.76)	98.88	0.79
Caggiano	22 (4.13)	20 (3.79)	9 (1.78)	18 (3.38)	17 (3.19)	97.58	0.64
Polla	10 (1.61)	6 (0.98)	6 (0.98)	4 (0.64)	3 (0.48)	98.79	0.37
Sala Consilina	22 (1.88)	23 (2.00)	33 (2.90)	25 (2.14)	25 (2.14)	98.71	0.70
Total	76 (2.48)	71 (2.34)	66 (2.14)	58 (1.89)	58 (1.89)	98.42	0.64

In addition, raw overall agreement and Fleiss kappa agreement indices are reported.

that IgM and IgG antibodies are detectable with the same timing or with a short time difference (1–2 days) (11–13). More recently, assays detecting total antibodies have been developed. The detection rates of the serologic tests range from 11% in the early phase of the infection to 100% 14 days post-infection. Targets of these assays are the antibodies against the Spike protein, the S1 receptor-binding domain, and N-protein.

Due to the fast spreading of the Sars-Cov2 infection, a great number of serological assays have been developed and different methodologies have been exploited. Most are immunochromatographic assays using the lateral flow format (rapid assays), are easy to perform, do not require instruments, and use capillary blood. The relevant advantage is to obtain a diagnosis without sending samples to centralized laboratories. However, a low diagnostic performance of rapid assays has been reported, for instance in samples with a low antibody concentration, as in early phases of seroconversion, it may yield false-negative results. False-positive results, likely due to cross reactions, were frequently reported (14, 15).

Chemiluminescent tests are considered the most sensitive by methodology and provide results with great accuracy and precision. These tests are highly automated and, in some cases, allow a semiquantitative evaluation (16).

The availability of different serological assays detecting total anti-N or anti-S antibodies or the different antibody classes (IgG or IgM), the different technologies used, and poor knowledge about Sars-CoV2 infection make necessary to evaluate the diagnostic performances of the different assays commercially available, in order to improve diagnostic efficacy and seroprevalence assessment.

In the present seroprevalence study, we evaluated the performance of one lateral flow assay (Technogenetics), two chemiluminescent assays testing for total SARS-CoV-2 antibodies against N protein (Roche) or against S1 (Siemens), and two chemiluminescent assays testing IgG antibodies against N protein (Abbott) and against S protein (DiaSorin).

Moreover, we compared the seroconversion timing by analyzing the sera of confirmed SARS CoV2 patients using three different chemiluminescent immunoassays (CLIA).

PATIENTS AND METHODS

Patients

A total of 3,185 citizens of the Campania Region were tested for anti-SARS-CoV-2 as part of a screening program. More than 90% of the tested individuals were domiciled in municipalities of the Diano Valley of the Salerno Province, with 1,168, 622, 536, 369, and 285 individuals, respectively, domiciled in Sala Consilina, Polla, Caggiano, Atena Lucana, and Auletta, respectively. The median age (interquartile range) of the entire cohort was 51 years (37–61), with 1,580 females (49.6%).

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the University of Naples "Federico II" (Project Identification Code 140/20/ESCOVID19). The patients/participants provided their written informed consent to participate in this study.

Serum samples were collected, refrigerated, and transported to the laboratory for testing. All samples were tested using the different analyzers.

Methods

The Elecsys Anti-SARS-CoV-2 is an electrochemiluminescence immunoassay (ECLIA) detecting total antibodies including IgG using a recombinant protein representing the nucleocapsid antigen (N antigen). Results are reported as a cutoff index (COI) and interpreted as negative (COI $<$ 1.0) or positive (COI $\geq$ 1.0). Positive and negative controls were prepared using pooled patient samples according to manufacturer instructions. Controls

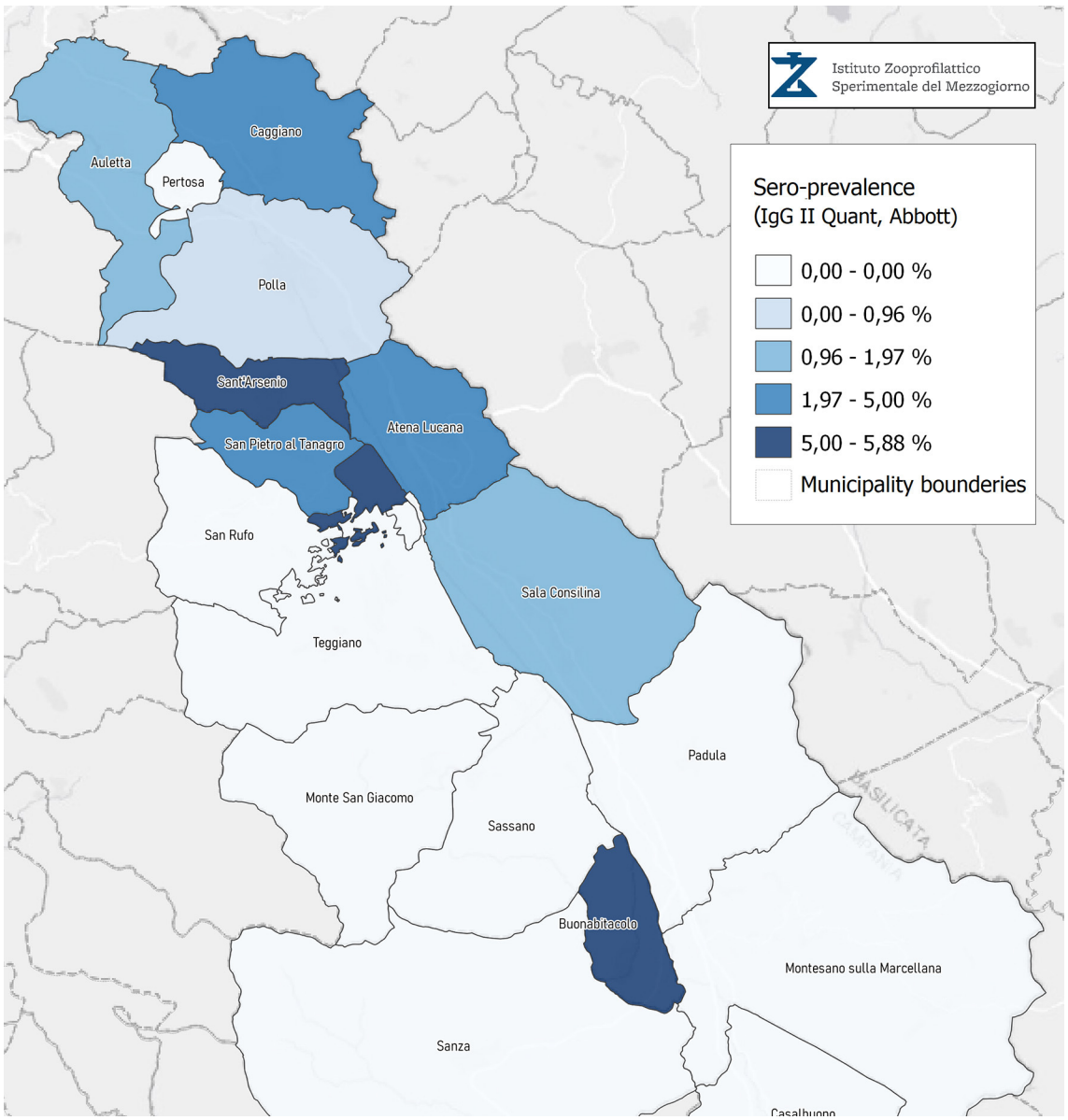

FIGURE 2 | Seroprevalence assessed in Diano Valley municipalities by IgG II Quant Abbott.

and patient samples were analyzed on a Cobas e411 instrument (Roche) according to manufacturer instructions.

The ADVIA Centaur COV2T assay is a one-step antigen sandwich immunoassay using acridinium ester chemiluminescent technology, in which antigens are bridged by antibodies present in the patient sample. The solid phase contains a preformed complex of streptavidin-coated microparticles and biotinylated SARS-CoV-2 recombinant S antigens. Results are determined according to the Index Value. Samples were considered reactive: $\geq$1.0 Index or non-reactive: <1.0 Index. Samples were analyzed using the ADVIA Centaur XPT instrument.

The Abbott SARS-CoV-2 IgG test assay uses nucleocapsid protein for antibody detection. The assays were performed on an Abbott Architect i1000 analyzer following the manufacture instructions. Samples with a signal-to-cutoff (S/CO) ratio $\geq$1.4 were considered positive.

LIAISON SARS-CoV-2 S1/S2 IgG (DiaSorin) is an indirect chemiluminescent immunoassay for the quantitative detection of IgG antibodies against S1/S2 proteins [cutoff of 12 arbitrary units (AU)/mL, classifying gray zone results of 12–15 AU/mL as positive].

The subjects were also analyzed with COVID-19 IgM/IgG Rapid Test Technogenetics, an immunochromatographic test for

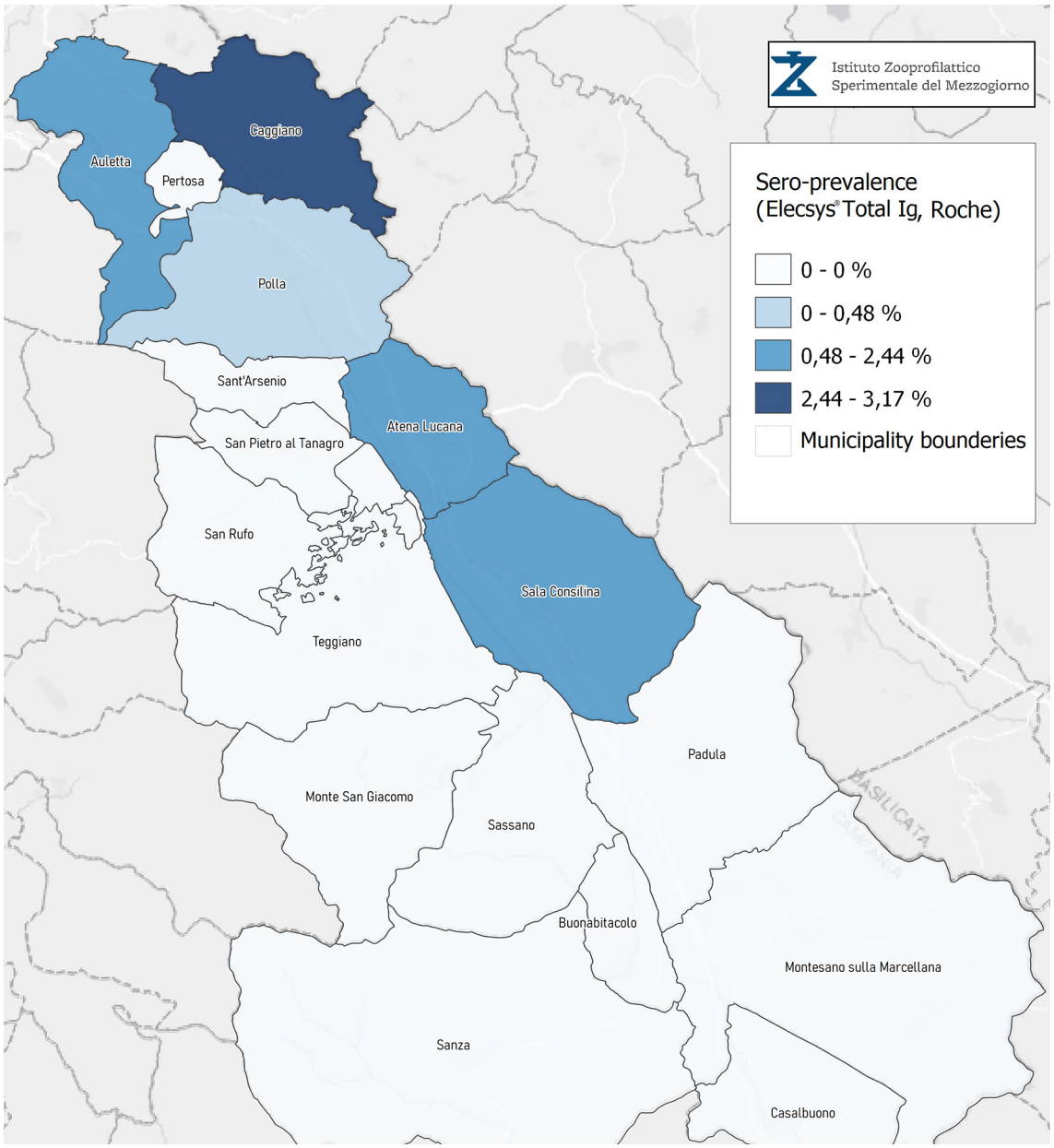

FIGURE 3 | Seroprevalence assessed in Diano Valley municipalities by Elecsys Total Ig Roche.

the qualitative determination of IgM and IgG class antibodies against COVID-19 in human serum, plasma, and whole blood. A specificity of 99.4% and a sensitivity of 100% at day 16 after infection is reported by the manufacturer.

Statistical Analysis

Descriptive data of COVID-19 positivity were expressed as absolute number and prevalence. The prevalence measured by each of the serological tests was estimated by computing the ratio between the positive cases and the total number of tested subjects belonging to each considered category. Agreement among the Liaison Igg, Abbott IGG, Technogenetics IGG, Siemens, and Roche tests was measured as overall raw agreement and Fleiss' kappa coefficient. Agreement between the COVID-19 rapid IGG test and each IGG serological test (Liaison, Abbott, and Technogenetics) and between the rapid test and the Roche and Siemens tests was measured as absolute count, percentage of overall raw agreement (on positive and negative cases), and Cohen's kappa coefficient. Fleiss' kappa and Cohen's kappa coefficients can be interpreted as follows: <0.20, poor agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, good agreement; and >0.81, very good agreement (17). All statistical analyses were performed using the R statistical environment, version 4.0.2 (18).

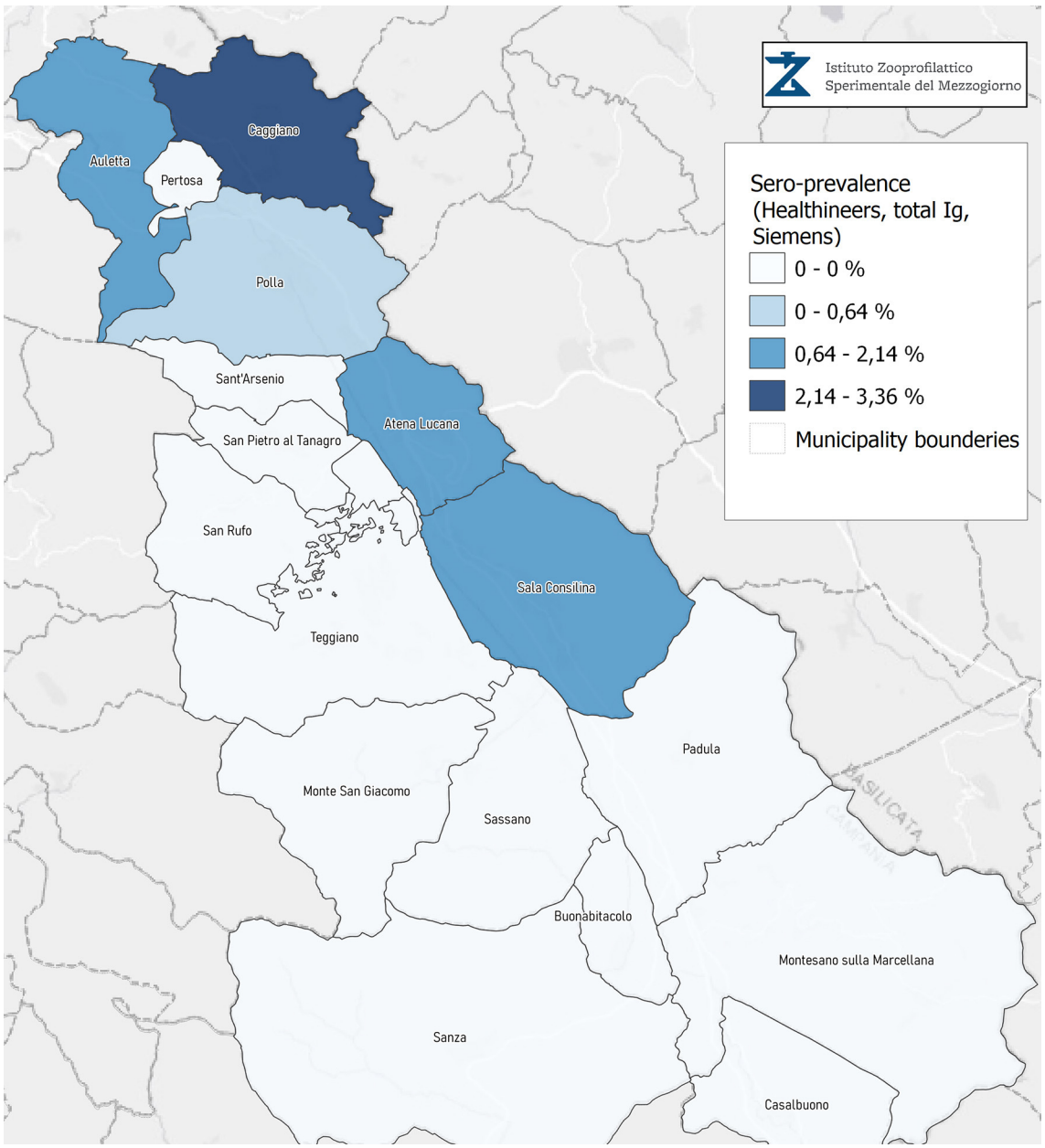

FIGURE 4 | Seroprevalence assessed in Diano Valley municipalities by Healthineers, total Ig Siemens.

RESULTS

A total of 3,185 citizens dwelling in multiple municipalities of the Campania Region were tested for anti-SARS-CoV-2, as part of an institutional screening program promoted (IZSM). More than 90% of the tested individuals were domiciled in municipalities of the Diano Valley of the Salerno Province; the geographical distribution is shown in **Figure 1** and **Table 1**. The median age (interquartile range) of the entire cohort was 51 years (37–61), with 1,580 females (49.6%).

Seroprevalence in the entire cohort was 2.41, 2.10, 1.82, and 1.85% according to the Liaison IgG (**Figure 1**), Abbott IgG (**Figure 2**), Roche (**Figure 3**), and Siemens (**Figure 4**) total Antibodies tests (see **Table 1**).

Seroprevalence appeared slightly higher when assessed using Liaison and Abbott IgG tests as compared to Siemens and Roche tests. Seroprevalence appeared to be higher in younger citizens, independently on the test used. Finally, the highest seroprevalence was found in municipalities of Caggiano and Atena Lucana, independently on the test used. A total of 3,185 citizens were tested for SARS-CoV-2 antibodies using anti-IgG and anti-IgM rapid tests on capillary blood. A total of 63 (2%) and 14 (0.4%) individuals tested positive on anti-IgG and IgM rapid antibody detection tests. When we explored the agreement

TABLE 2 | Comparison of rapid IgG assay and anti-S IgG Abbott and DiaSorin assays.

	Rapid IgG–	Rapid IgG+	Agreement (%)	Cohen's kappa
Liaison–	3,055	21	97.45	0.58
Liaison+	36	41		
Abbott–	3,031	17	98.84	0.69
Abbott+	22	45		
Technogenetics–	2,978	33	97.47	0.42
Technogenetics+	39	27		

Agreement between the rapid test and the Liaison, Abbott, and Technogenetics IgG tests. The results are expressed as absolute count, raw overall agreement, and Cohen's kappa coefficient.

TABLE 3 | Agreement between the rapid test and the Roche and Siemens tests.

	Rapid–	Rapid +	Agreement (%)	Cohen's kappa
Roche–	3,081	26	98.33	0.67
Roche+	16	43		
Siemens–	3,082	26	98.36	0.67
Siemens+	15	43		

The results are expressed as absolute count, raw overall agreement, and Cohen's kappa coefficient.

among the rapid tests and the serologic assays (**Tables 2**, **3**), we reported good agreement for Abbott, Siemens, and Roche (Cohen's kappa coefficient 0.69, 0.67, and 0.67, respectively), whereas we found a moderate agreement for Liaison (Cohen's kappa coefficient 0.58).

DISCUSSION

Increased mass testing and contact tracing together with physical distancing and restriction of movement were efficacious in decreasing transmission rates of SARS-CoV-2 (19). Unfortunately, this kind of measures has unfavorable societal and economic impacts potentially resulting in significant recession; therefore, alternative strategies to control the pandemic are required.

An approach to maintaining epidemiological vigilance and allowing a fast response to the rise of viral infections is to identify and quantitate people with immunity against SARS-CoV-2 in the whole population. This approach could discriminate immune people as health-care workers allowing to reopen activities and borders and follow the development of the herd immunity. Different methods for serological tests are currently available (20).

To strengthen surveillance systems, it is therefore important to evaluate serological assays that can be used in large-scale studies. In this sero-epidemiological study for SARS-CoV-2, we evaluated different serological tests in a large study population of Diano Valley (Campania Region).

Our findings indicate that the prevalence of IgG and total Ig antibodies against SARS-CoV-2 at the time of the study was as low as around 3%, likely explaining the amplitude of the current second wave.

Since the study was designed to obtain data on the Diano Valley area, we were able to reveal differences among the different urban settlements. Caggiano and Atena Lucana showed the highest prevalence around 4%, whereas in Polla the lowest prevalence was observed, confirming a difference in the spread of viral infection among these settlements. Further studies are required to explain these differences. A limitation of our study is the lack of a comparison with epidemiological data in emergency time. However, to our knowledge, this study is one of the largest population-based SARS-CoV-2 seroprevalence studies in Southern Italy with more than 3,000 participants.

The use of two IgG antibody tests, two total Ig antibody tests directed against N or S antigens, and a rapid test allows us to specify a range of seroprevalence between 0.48 and 4.13%.

These estimates clearly indicate a lower magnitude of seroprevalence in Southern compared to Northern Italy (21, 22), partially explaining the extent of the second wave in the Campania region.

As reported for other coronaviruses (23, 24), prevalence was higher in younger citizens both when using the point-of-care test and when using the CLIA. The lower prevalence in young people might be explained on the base of a more efficient immunological response (25, 26). A lower nasal gene expression of the angiotensin-converting enzyme 2 receptor in younger might also explain this lower seroprevalence (27).

Our results also highlighted the performances of different commercial assays to assess the rate of infection in a target population. At variance with other studies, we observed a good agreement for Abbott, Siemens, and Roche automated immunometric assays and Technogenetics rapid commercial assays. However, previous studies compared rapid assays and CLIAs in symptomatic SARS-Cov-2 patients. In these patients, anti-SARS-Cov-2 antibodies are observed 5 days or more from the appearance of the symptoms. In our screening study, we assessed the seroprevalence of a population without any section, thereby detecting previous and resolved SARS-Cov-2 infections. Our data demonstrate that the Technogenetics rapid assays can be useful for epidemiological studies, whereas the assessment of the diagnostic performance of this assay requires further studies.

Interestingly, the seroprevalence appeared slightly higher using IgG anti-S (Liaison assay) and anti-N (Abbott assay) with respect to anti-total Antibodies Siemens (anti-S) and Roche (anti-N). This effect is likely due to a less efficient detection of IgG in total assays compared to IgG-specific assays, as previously reported. A recently published meta-analysis demonstrated that IgG tests had better sensitivity when the samples were taken a week after the onset of symptoms (28). Accordingly, IgM antibodies showed lower specificity than IgG (28).

Several factors could affect the ability of antibody tests to identify infected people, including quality of the sample, low antibody levels, and timing of the test (29). Kinetic studies (30, 31) showed that IgM reaches a peak between days 5 and 12 and then drops, whereas IgG reaches a peak after day 20 or so as IgM antibodies disappear.

Evidence indicates that total antibody tests seem to be more sensitive than single-antibody testing (28). Furthermore, S-based tests were reported as more specific due to poor cross-reactivity with low conserved regions of spike proteins of other coronaviruses (SARS-CoV) (32). In addition, it has been demonstrated that tests detecting antibodies anti-S antigen are more sensitive with respect to test detecting anti-N antibodies, probably since the immune response against S antigen seems earlier with respect to the response to N antigen (28).

The sensitivity and specificity of antibody tests are relevant issues for both diagnosis and epidemiological surveillance. False-positive results may allow to consider immune people who have never been infected and may alter prevalence estimates, mortality rate, and herd immunity assessment.

False-negative findings may prevent to contain viral spread.

Meta-regression analysis showed that CLIAs showed comparable sensitivity (∼90%) but slightly decreased specificity (95–98%) with respect to ELISA tests (higher than 99% and sensitivity ∼93%). The lateral flow immunoassay test showed specificity as high as that of the ELISA test (∼99%) and a lower sensitivity (∼80%).

Accordingly, our results suggested that despite the suboptimal sensitivity, antibody tests could integrate nucleic acid testing both in the diagnosis of SARS-Cov-2 infection and in the assessment of seroprevalence in the entire population (13). When designing seroprevalence studies, attention should be paid to the sensitivity and specificity of the antibody's tests. In the diagnostic assessment, a combined strategy as retesting a negative result with a different method to ameliorate specificity could be advantageous.

In addition, some practical aspects should be considered: for wide screening, completely automated CLIA methods could be advantageous, although rapid tests as immunochromatographic cards should be useful (when centralized laboratories are not available).

Further studies on a large population are needed to compare serological tests and nucleic-acid testing to better define which is the best approach for diagnosis and which for seroprevalence assessment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the University of Naples Federico II (Project Identification Code 140/20/ESCOVID19). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PC, DT, and GPo: conception and design, analysis, interpretation of data, critical revision of the manuscript for important intellectual content, and supervision. AG, BP, CB, DD, MCC, LV, GL, AnC, AP, GB, VF, LA, MT, MC, PR, TS, AF, OC, AlC, GPe, and MD'A: acquisition of data. DT and GPo: drafting of the manuscript. DP: statistical analysis. PC and GPo: administrative, technical, or material support. All authors contributed to the article and approved the submitted version.

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Diagnostic Delay of Pulmonary Embolism in COVID-19 Patients

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Pulmonary embolism (PE) is a frequent, life-threatening COVID-19 complication, whose diagnosis can be challenging because of its non-specific symptoms. There are no studies assessing the impact of diagnostic delay on COVID-19 related PE. The aim of our exploratory study was to assess the diagnostic delay of PE in COVID-19 patients, and to identify potential associations between patient- or physician-related variables and the delay. This is a single-center observational retrospective study that included 29 consecutive COVID-19 patients admitted to the San Matteo Hospital Foundation between February and May 2020, with a diagnosis of PE, and a control population of 23 non-COVID-19 patients admitted at our hospital during the same time lapse in 2019. We calculated the patient-related delay (i.e., the time between the onset of the symptoms and the first medical examination), and the physician-related delay (i.e., the time between the first medical examination and the diagnosis of PE). The overall diagnostic delay significantly correlated with the physician-related delay ($p < 0.0001$), with the tendency to a worse outcome in long physician-related diagnostic delay ($p = 0.04$). The delay was related to the presence of fever, respiratory symptoms and high levels of lactate dehydrogenase. It is important to rule out PE as soon as possible, in order to start the right therapy, to improve patient's outcome and to shorten the hospitalization.

Keywords: pulmonary embolism, SARS-CoV-2, diagnostic delay, thrombosis, misdiagnosis

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus (SARS-CoV-2), a novel coronavirus first detected in Wuhan City, Hubei Province of China in December 2019 (1). The Italian outbreak began in February 2020 and involved mainly Northern Italy, with Lombardy being on the front line (2). The COVID-19 pandemic progressively engulfed Europe and then most part of the world, with a massive impact on public health, politics and economics. As of October 20th, COVID-19 almost reached 40,300,000 cases with more than 1,115,000 deaths worldwide (3). Our academic tertiary referral hospital played a pivotal role in managing the emergency (4).

The clinical spectrum of this infection ranges from asymptomatic forms to multi-organ failure. According to a recent study that observed 5,700 COVID-19 patients hospitalized in the New York City area, the most common symptoms at admission were fever and tachypnea with dyspnea. Mortality rates ranged between 1.98 and 26.6% (in the 18-to-65 and older-than-65 age groups, respectively) and were significantly higher among patients who received mechanical ventilation (5).

SARS-CoV-2 infection can lead to severe complications such as acute respiratory distress syndrome (ARDS), acute renal failure, acute cardiac injury, and septic shock (6). Venous thromboembolism (VTE), that includes deep vein thrombosis (DVT) and pulmonary embolism (PE), is another potentially life-threatening complication reported in COVID-19 patients. An association between VTE and SARS-CoV-2 infection was first described by Zhai et al., who identified thrombotic events in 2.9% of a cohort of COVID-19 patients (7). In previous Asian series, thromboembolic events have been reported in roughly one fourth of COVID-19 patients admitted to the intensive care unit, and these findings correlated with a poor prognosis. Since then, dozens of papers on VTE incidence in COVID-19 patients have been published so far.

Generally, the diagnosis of PE can be challenging, mainly due to non-specific signs and symptoms, and diagnostic delay is common. Previous studies described an average time between symptom onset and PE diagnosis that varied from 4.8 to almost 9.0 days (8–10).

Due to the wide and partially overlapped clinical spectrum of both COVID-19 and PE, the differential diagnosis of these conditions can be demanding. Moreover, it is possible that the novelty of the situation and the lack of knowledge about this new infection led the clinicians to overlook the diagnosis of severe comorbidities, such as PE.

On this basis, we hypothesized that PE in COVID-19 patients could be misdiagnosed or underdiagnosed, determining a diagnostic delay that could affect the prognosis. The presenting signs and symptoms can be tricky and subtle, thus contributing to the delay. The identification of specific features of the PE related to COVID-19 could help the clinician to discriminate which patients should be promptly evaluated for PE.

The primary aim of our study was to assess the diagnostic delay by analyzing data from the clinical records of hospitalized COVID-19 patients with PE, in comparison to hospitalized non-COVID-19 patients with PE. The secondary aim was to identify a potential association between patient- or physician-related variables and the delay.

MATERIALS AND METHODS

Patient Population and Study Design

This was an exploratory, single-center observational retrospective study conducted in an academic, tertiary hospital in Pavia, Italy (San Matteo Hospital Foundation).

The study included all consecutive COVID-19 patients admitted to the San Matteo Hospital Foundation between February 2020 and May 2020, in which a diagnosis of PE was confirmed with angiographic computed tomography (CT). Patients below 18-year-old at the time of diagnosis were excluded. A control population of non-COVID-19 patients with a confirmed diagnosis of PE admitted at our hospital during the same time lapse in the previous year (February 2019–May 2019) entered into the study.

In each case, requested data were obtained from the local electronic records of the San Matteo Hospital Foundation, anonymized and then entered into a database. We reviewed the clinical history of each patient, looking for all the possible presenting signs, symptoms, and clues that were related to PE and COVID-19 onset, according to the present literature and expert opinion. To note, regarding COVID-19 patients, all data regarding the onset of symptoms were accurately collected at the time of, and during, hospitalization by the treating physicians. Asymptomatic patients with an incidental finding of PE (e.g., angiographic CT performed for oncological follow-up) were excluded. Furthermore, we reported relevant sociodemographic features, comorbidities, risk factors for thrombosis and outcomes (i.e., dead or discharged). The number of physicians involved in the diagnosis of PE, as well as the possible misdiagnoses, were also indicated. Among the blood tests, platelet count (PC), lactate dehydrogenase (LDH), and D-dimer at admission were recorded. If not performed at admission, laboratory tests were taken into account only if performed within the first 48 h.

For the purpose of the study, we considered two types of delay. The patient-related delay, defined as the time between the onset of the symptoms and the first medical examination, and the physician-related delay, defined as the time between the first medical examination and the final diagnosis of PE. The overall diagnostic delay was obtained by summing both patient-related and physician-related delay and expressed in days. The day of the diagnosis of PE was considered as the date of the pulmonary angiographic CT.

The study was performed as a clinical audit using routinely collected clinical data and as such is exempt from the need to require written informed consent. The study was approved by the local ethics committee (San Matteo Hospital Foundation; Protocol Number 2020-0072882).

Statistical Analysis

The RStudio (11) statistical package was used for all the descriptive and inferential statistics. Median and range were used instead of mean and standard deviation due to skewed data distributions. Non-parametric Wilcoxon-test was used to check the difference between continuous variables. Kaplan-Meier estimate was used to plot cumulative diagnosis probability in patients who died or were alive at discharge. Log-rank-test was used to assess the difference between survival curves. Pearson's correlation coefficient has been used to measure linear correlation between pairs of variables. Univariable and multivariable linear regression analyzes were used to find predictors of the diagnostic delay. For the univariate, the most frequent and important variables used in the current literature were analyzed. For the multivariate analysis, none authomatic procedure was used. Variables that were significant at univariate analysis were considered first, then additional variables have been tested since also those that are not significant at univariate analysis could show significance once combined with other ones. Moreover, we limit the set of the tested models to three variables, given our limited sample size, using the rule of thumb of around 10 cases for variable. Logarithmic transformation of the observed delay times was done in order to improve the times distribution normality. Given the relatively small sample size, we decided to test multivariate models with no more than three variables, to avoid overfitting.

RESULTS

The PE-associated COVID-19 population comprised 29 patients, with a median age at diagnosis of 62 years (range 29–82, M:F ratio = 3.8:1). Other sociodemographic features included in the study are shown in **Table 1**, which also show the same variables for the control population (non-COVID-19 patients).

In the COVID-19 population the median overall diagnostic delay was 19 days (range 1–47), the median patient-related and physician-related delay were, respectively, 3 days (range 0–10) and 14 days (range 0–46).

All patients showed COVID-19 related symptoms, being the most frequent clinical pictures fever with dyspnea (13 patients, 44.8%), fever with dyspnea and cough (five patients, 17.2%), fever with dyspnea and gastrointestinal symptoms (four patients, 13.8%).

Signs, symptoms and clues potentially related to PE are shown in **Table 2**.

Dyspnea was always present, with various degrees of severity. Seventeen out of 29 patients (58.6%) had DVT at the time of SARS-CoV-2 infection diagnosis. D-dimer levels were altered in 22 patients (75.8%). Other blood tests revealed thrombocytopenia in five patients (17.2%), thrombocytosis in one patient (3.5%). LDH was abnormal in 26 patients (89.6%).

Almost all the patients were first assessed by an emergency physician (27 patients, 93.1%). In 26 cases (89.6%) at least another physician was consulted in the diagnostic process. In the majority of cases, the reason for the achievement of the definite diagnosis was a persistent respiratory failure (16 patients, 55.2%). Other clues that led to the diagnosis were: increased levels of D-dimer (eight patients, 27.6%), compression ultrasonography screening (1 patient, 3.4%), incidental finding (four patients, 13.8%).

When present (18 patients, 62.1%), the misdiagnosis was mostly a worsening of the COVID-19 related pneumonia (13 patients, 44.8%). Further misdiagnoses are described in **Table 3**.

TABLE 1 | Sociodemographic characteristics of the 29 COVID-19 patients with PE and 23 non-COVID-19 patients with PE.

	COVID-19 (2020) *n* (%)	Non-COVID-19 (2019) *n* (%)
Age		
≥65	10 (34.5)	15 (65.2)
<65	19 (65.5)	8 (34.8)
Sex		
Female	6 (20.7)	12 (52.2)
Male	23 (79.3)	11 (47.8)
Smoking status		
Never smoked	13 (44.8)	11 (47.8)
Current smoker	5 (17.2)	7 (30.4)
Former smoker	11 (38)	5 (21.8)
Obesity (BMI ≥ 30)		
Yes	2 (6.9)	3 (13)
No	27 (93.1)	20 (87)
Years of education		
≤5	0	1 (4.3)
>5, ≤8	3 (10.3)	3 (13)
>8, ≤13	11 (38)	10 (43.5)
>13	15 (51.7)	9 (39.2)
Marital status		
Single or divorced	8 (27.6)	4 (17.4)
Married	17 (58.6)	15 (65.2)
Widowed	3 (10.3)	4 (17.4)
Cohabiting/partner	1 (3.5)	0
Exemption from healthcare taxes		
No	8 (27.6)	4 (17.4)
Yes	21 (72.4)	19 (82.6)
Income		
<1,000 €	14 (48.3)	13 (56.5)
≥1,000 €	15 (51.7)	10 (43.5)

PE, pulmonary embolism.
The included variables are in bold.

TABLE 2 | Symptoms, alterations, or clues that have prompted further work-up to confirm pulmonary embolism in COVID-19 patients.

	N (%)
Respiratory symptoms	
Dyspnea not requiring oxygen therapy	5 (17.2)
Dyspnea requiring oxygen therapy	9 (31)
Dyspnea requiring mechanical invasive ventilation	15 (51.7)
Cough	8 (27.6)
Hemoptysis	2 (6.9)
Heart symptoms	
Thorax pain	4 (13.8)
Palpitations	0
Syncope	0
Fever	26 (89.7)
Hematological alterations	
Increase in platelets number (>400 × 10^9/L)	1 (3.5)
Decrease in platelets number (<150 × 10^9/L)	5 (17.2)
Increase in LDH levels (>220 mU/mL)	26 (89.7)
Increase in D-dimer levels (>500 mcg/L)	11 (37.9)
Increase in D-dimer levels (>5,000 mcg/L)	11 (37.9)
Deep vein thrombosis	17 (58.6)

LDH, lactate dehydrogenase.

TABLE 3 | Misdiagnosis that led to diagnostic delay.

	N (%)
Respiratory diseases	
Worsening in COVID-19 pneumonia	13 (44.8)
Bacterial superinfection	2 (6.9)
COPD exacerbation	1 (3.5)
Heart diseases	
Acute pulmonary edema	2 (6.9)

COPD, chronic obstructive pulmonary disease.

Notably, only a few patients (10 patients, 34.4%) had previous thrombotic risk factors. The median number of comorbidities was 2.5 (range 0–10).

The outcome was positive (patient alive upon discharge) in 19 patients (65.5%) and negative (death) in 10 patients (34.5%).

The overall diagnostic delay depends mainly on the physician-related delay that is significantly higher than patient-related delay (14 days, range 0–46 vs. 3 days, range 0–10, $p < 0.0001$) (**Figure 1**).

Moreover, considering long diagnostic delay (≥10 days), the delay was significantly higher in patient who died ($p = 0.02$; **Figure 2**).

Univariable and multivariable analysis were performed to identify the factors that affected the diagnostic delay the most (**Table 4**).

A lower level of education was statistically associated with a longer patient-related delay, while the presence of fever with a longer physician-related delay. When the definitive diagnosis was based upon an increase in D-dimer levels, the physician delay remarkably decreased.

The variables that only showed a trend toward a statistically significant prolonged delay, though without reaching it, were female sex and single/divorced/widowed status for the patient-related delay, and the number of specialists involved in the diagnosis for the physician-related delay.

Age, monthly income, exemption from medical expenses, levels of LDH or PC, presenting symptoms (either COVID-19 symptoms or PE symptoms, including DVT), thrombotic risk factors and comorbidities did not influence the patient-related delay. Furthermore, no association was found between physician-related delay and level of instruction, gender, marital status, specialization of first medical consultant, comorbidities, thrombotic risk factors, misdiagnosis, blood tests.

According to the multivariable analysis, the key factors for the delay were the presence of fever, respiratory symptoms and high levels of LDH. When considered together, these factors remained extremely relevant and determined a prolonged delay. The correlation was significant both for the physician-related delay and for the overall diagnostic delay.

The control population included 23 patients with PE but without COVID-19. Sociodemographic characteristics are described in **Table 1**. There were no significant differences between the 2 groups, except from higher prevalence of male patients in the COVID-19 group ($p = 0.03$) and a slight older age in non-COVID-19 patients ($p = 0.05$). Among clinical and laboratory findings, three patients (13.0%) referred palpitations and 14 patients (60.8%) presented altered LDH levels. In 20 patients (86.9%) the specialist that first assessed the patient was an emergency doctor, who was also the physician that most frequently established the correct diagnosis (15 patients, 65.2%). The feature that led to the diagnosis in most of the patients (13 patients, 56.5%) was the persistence of respiratory failure.

The median overall diagnostic delay in this population was seven days (range 0–30). Of note, the median physician-related delay was only 4 days (range 0–30), while the median patient-related delay was 0 days (range 0–26).

No correlation was found between the diagnostic delay and level of education, marital status, gender, age at diagnosis, monthly income, exemption from medical expenses, LDH, PC and D-dimer, presenting symptoms including DVT, comorbidities, specialization of specialists involved in the diagnosis. Instead, a higher number of specialists corresponded to a longer physician-related delay.

Differently from the COVID-19 cohort, where the overall diagnostic delay was only physician-related, in the non-COVID-19 cohort, it was statistically correlated with both the physician-related delay ($p = 0.011$) and, even more, with the patient-related delay ($p < 0.0001$).

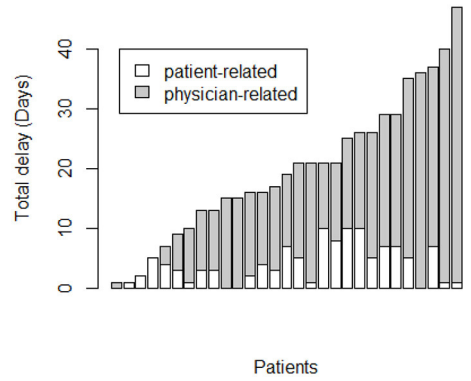

FIGURE 1 | Correlation between pulmonary embolism overall diagnostic delay (days) and physician-related delay.

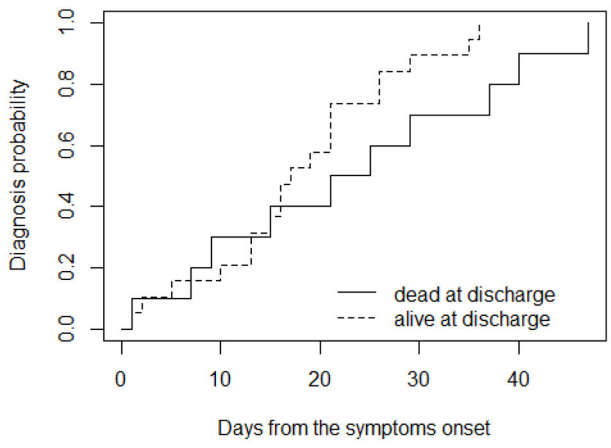

FIGURE 2 | Cumulative probability of receiving diagnosis over time, for patients grouped according to their outcome at (dead or alive) at discharge.

DISCUSSION

COVID-19 is a recently described disease, the possible clinical scenarios of which are still under investigation. Pulmonary embolism is a widespread, life-threatening condition that can

TABLE 4 | Univariable and multivariable analysis for the most relevant characteristics considered for overall, physician-dependent and patient-dependent diagnostic delay in COVID-19 patients affected by pulmonary embolism.

Diagnostic delay		**Univariable analysis**		**Multivariable analysis**	
Overall	**Median (dy; 25th−75th)**	**Difference in log (95%CI)**	***P*-value**	**Difference in log (95%CI)**	***P*-value**
Sex					
Female	20 (8.5–24.75)	0	0.431		
Male	17 (13–27.5)	0.34 (−0.50 to 1.19)			
Fever					
Yes	21 (15–29)	1.72 (0.95 to 2.45)	0.00016	1.56 (0.67 to 2.47)	0.0023
No	3.5 (1–7)	0			
Dyspnoea					
Mild	14 (6–20)	0			
Severe	25 (17.5–35.5)	0.94 (0.34 to 1.53)	0.00473	0.58 (0.06 to 1.10)	0.040
D-dimer elevation					
Yes	13 (8–15.25)	−0.7506 (−1.35 to −0.15)	0.0206		
No	21 (16–29)	0			
LDH					
Normal	29 (25–33)	0			
High	16.5 (13–26)	−0.6 (−1.85 to 0.65)	0.356	−0.59 (−1.12 to −0.05)	0.041
Physician-dependent					
Sex					
Female	12.5 (3–15.25)	0	0.20145		
Male	15 (10–21.5)	0.6316 (−0.31 to 1.58)			
Fever					
Yes	15 (12–22)	1.9517 (1.07 to 2.83)	0.00017	1.56 (0.54 to 2.58)	0.006
No	0 (0–2.5)	0			
Dyspnoea					
Mild	11.5 (1.5–14.75)	0			
Severe	16 (12–30)	0.9995 (0.30 to 1.69)	0.00889	0.53 (0.11 to 0.94)	0.02
LDH					
Normal	15 (15.75–25.25)	0			
High	20.5 (10–20.75)	−0.000570 (−0.00296 to 0.00096)	0.6	−0.001793 (−0.00296 to 0.00096)	0.03
DVT					
Yes	13 (10.5–15.5)	0.0733 (−0.98 to 1.12)	0.892		
No	13 (9–20)	0			
Risk factor VTE					
Yes	12.5 (2.5–15)	−0.6520 (−1.2 to −0.05)	0.033		
No	15.5 (12–28)	0			
Physician specialization					
ED	0	−2.5185 (−3.66 to −1.37)	0.000238		
Internal medicine	14 (7–17)	−0.3699 (−1.51 to 0.77)	0.532308		
Pulmonology	14 (9.75–17.25)	0.1225 (−0.88 to 1.13)	0.813249		
Infectious diseases	13.5 (11.5–15.5)	0			
ICU	18.5 (12–30)	0.4267 (−0.52 to 1.37)	0.386113		
Previous misdiagnosis					
No	13 (0–14)	(−39.7 to 52.7)	0.019273		
Interstitial pneumoniae	11.5 (10.5–15.5)	(−26.54 to 40.87)	0.001180		
Worsening i. p.	22 (13.5–33)	(−24.71 to 41.06)	0.000296		
Patient-dependent					
Sex					
Female	7 (2–7.5)	0	0.336		
Male	3 (1–5)	−0.3315 (−0.69 to 0.62)			

(Continued)

TABLE 4 | Continued

Diagnostic delay		Univariable analysis		Multivariable analysis	
Overall	**Median (dy; 25th–75th)**	**Difference in log (95%CI)**	***P*-value**	**Difference in log (95%CI)**	***P*-value**
Years of education		(−0.62 to 0.22)	0.361		
≤5					
≤8	8 (6–9)	0			
≤13	2 (1–4.5)	−0.8661 (−1.78 to 0.05)	0.07		
>13	3 (1–6)	−0.6970 (−1.59 to 0.19)	0.14		
Dyspnoea					
Mild	2 (1–4.5)	0			
Severe	5 (2–7)	0.28 (−0.26 to 0.81)	0.322		
D-dimer					
D-Dimer <5,000 mcg/L	3 (1–5)	0			
D-Dimer ≥5,000 mcg/L	5 (2.75–7)	0.55 (−0.04 to 1.13)	0.07		

DVT, deep vein thrombosis; ED, emergency department; ICU, intensive care unit; LDH, lactate dehydrogenase; VTE, venous thromboembolism; i.p., interstitial pneumonia.

complicate COVID-19. Both pathologies may exhibit unspecific and partly overlapping signs and symptoms, with a consequent diagnostic delay. Our study sought to investigate this diagnostic delay, identifying any characteristics that may early identify patients who need to be evaluated for this important comorbidity.

The median overall diagnostic delay was 19 days for the PE-COVID-19 cohort, while in non-COVID-19 patients was 7 days.

The presence of a significant diagnostic delay in patients with PE has previously been demonstrated in other papers (8–10). However, our study, which specifically analyzed patients with PE and COVID-19, showed that the diagnostic delay is even greater in the case of SARS-CoV-2 infection. The median overall delay in PE-COVID-19 patients was 19 days, twice as long as the delay reported by Bulbul et al. and Wallen et al. (8.4 and 9 days, respectively) (8, 10) and the quadruple compared to the delay described by Elliott et al. (4.8 days) (9).

Analyzing the two different components of the PE-COVID-19 delay, physician and patient-related, respectively, it emerged how the delay was almost exclusively attributable to the physicians. The main feature that led physicians to diagnostic delay was the presence of fever. This is quite understandable since fever is a non-specific symptom that is frequent in various pathological conditions including infections, particularly COVID-19. In addition, fever is a rare clinical presentation of PE (12). Even if it is difficult to prove it, we can also speculate on the fact that the state of emergency and the novelty of this condition have negatively influenced the work of physicians. According to current knowledge, it is certainly a mistake not to consider PE as a possible diagnosis just for the presence of fever. This retrospective study analyzed COVID-19 patient's management in the first period of the pandemic, when the correlation with thrombotic phenomena was still based on few studies.

The physician-related delay was instead greatly decreased when the clue that led to the diagnosis was an increase in the D-dimer values. This is also understandable, given that this data is considered to be more specifically indicative of PE, or generally VTE (13–15). Therefore, it seems reasonable to support the D-dimer screening in COVID-19 patients, both at admission and during hospitalization.

The patient-related delay was significantly increased in patients with a low level of education. Probably the lack of instruction made it possible for the patients not to identify some clinical characteristics as dangerous and indicative of a pathological condition.

Even if it was not statistically significant, the presence of DVT conducted the patient to quickly look for a medical examination. Definitely, DVT often has visible, typical and disabling signs and symptoms.

During the COVID-19 epidemic, people were found to be afraid of going to hospital, even in the presence of alarm symptoms, due to a potential infectious risk. This is supported by the dramatic increase of out-of-hospital cardiac arrests that were noticed in Lombardy during the highest peak of infections (16). This may have caused an important patient-related diagnostic delay for various diseases, including non-COVID-19 related diseases.

Fortunately, a longer diagnostic delay was not statistically related to a worse outcome, although there was a tendency, which was observed for longer delays. Even if one study reported a higher in-hospital mortality rate in patients with a diagnostic delay >3 days (17), most of the other studies are in line with our results (18–21). Also, all hospitalized COVID-19 patients were given thromboprophylaxis with heparin, and this could have improved the final outcome.

Dyspnea as the main clinical symptom has been previously associated with a shorter time to diagnosis (19). In other cases, this association was not found (10), or even the presence of dyspnea led to a longer diagnostic delay (22). In our series, dyspnea was reported in all the patients; therefore, it is difficult to understand its role in the diagnostic delay. Thus, its meaning remains controversial in the differential diagnostic process.

Notably, the presence of pre-existent risk factors for VTE did not reduce the delay so much, while this association was reported in many other studies (17, 18, 22, 23).

In non-COVID-19 patients the median diagnostic delay was only 7 days, even lower than the median diagnostic delay in the general patient population (8–10). This can reasonably exclude a systematic medical error in the diagnosis of PE in our hospital. Although the delay in this cohort was statistically related to a delay of both the physician and the patient, the latter was the component that affected the delay the most.

The statistical analysis revealed that the overall delay in COVID-19 patients was significatively longer than in non-COVID-19 patients. Moreover, the correlation between the overall delay and the physician-related delay was more pronounced for the COVID-19 patients.

Some differences in the 2 groups of the study must be mentioned. As expected, male were more represented in COVID-19 cohort. The age of the non-COVID-19 patients tended to be higher than that of COVID-19 patients, and probably with a greater sample size a definite statistical significance would have been reached. This is due to the fact that SARS-CoV-2 has affected people of varying ages, including the youngest (24). PE, on the other hand, is generally typical of an elderly population (12, 25, 26). In previous reports, an older age was associated with a longer diagnostic delay for PE in non-COVID-19 patients (19, 27).

Interestingly, there was a symptom of PE that we observed only in non-COVID-19 patients, that was the presence of palpitations. It is conceivable that the SARS-CoV-2 bradycardising action played a role in this difference (28).

Among blood tests, LDH levels tended to be more frequently altered in COVID-19 patients than in non-COVID-19 patients (89.6 and 60.8%, respectively, $p = 0.02441$). As an indicator, among other things, of severe infection, it is justifiable that LDH presented high levels in the course of SARS-CoV-2 infection. Literature reports increased LDH levels as a common evidence in COVID-19 (29, 30).

The negative impact of high LDH levels in the physician's perception of the risk of PE it is comprehensible, since it led the physician to focus on the infectious side of the disease and to interpret any other symptoms of pulmonary embolism as related to a particularly severe picture of COVID-19.

LDH values, together with fever and respiratory symptoms, are the three independent variables that in the multivariate analysis were found to be fundamental in prolonging the diagnostic delay. All three of these features, especially when present simultaneously, can be indicative of respiratory infection (29, 31, 32), and have therefore been misunderstood in SARS-CoV-2 related pneumonia. In fact, it is not surprising that a worsening in COVID-19 pneumonia was the most common misdiagnosis in our cohort.

Conversely, heart diseases were improperly diagnosed in a small number of patients, while in other studies were a frequent confounding factor for the diagnosis of PE (19).

The differences in the expression of PE between the two populations could be due to a different pathogenesis of the thrombotic event. There is evidence in the literature of local vasculitic damage at the basis of thrombotic phenomena during SARS-CoV-2 infection (33, 34). Although more than a half of the COVID-19 patients had DVT, it is possible that vasculitic damage represented, if not the cause, at least a contributing factor in the development of pulmonary embolism, generating different clinical characteristics.

We acknowledge the many limitations of this study. First, this is a single center study, conducted in a tertiary hospital in Northern Italy, thus only COVID-19 patients with a severe clinical pattern that required hospitalization have come to our attention. The delay in asymptomatic patients or patients with mild symptoms that were treated in other settings, or other geographical and climatic areas remains undefined. Also, the diagnostic approach in the emergency department and in the in-patient departments follows guidelines common to the whole hospital, so it is possible that different results have been achieved in other medical centers. A relevant feature in the evaluation of the COVID-19 patients in our hospital was to use mainly chest radiography and ultrasound as instrumental examinations. CT was not used in the first instance because of management and infectious problems. A wider use of this imaging technique would probably have reduced the delay. Second, the sample size of the study is limited, compared to the wide prevalence of both PE and COVID-19. It would certainly be interesting to integrate the data with those of other centers in order to have a greater statistical significance. Third, since COVID-19 is a new pathological condition, there is an understandable lack of knowledge that can contribute to a medical error. This is obviously a common limitation among studies about COVID-19, but it is also the reason that makes them critically important in the path toward the optimal SARS-CoV-2 management. Indeed, our data should be cautiously interpreted in the light of this specific setting, which is that of hospitalized patients with COVID-19, representing a high pre-test probability of having PE. More studies are needed for assessing generalizability of our results.

In conclusion, although its exploratory nature, this is the first study that analyzes the diagnostic delay of PE in patients affected by COVID-19, its confounding factors and potential effects on outcome. While during the past years in our center the delay was almost totally patient-related, during pandemic some COVID-19 features (mainly fever, worsening dyspnea, and persistent increased D-dimer levels) have turned away physicians from the right differential diagnosis. Another error to highlight is the scarce use of chest CT in the imaging diagnostic protocol of COVID-19 pneumonia at the time of hospital admission in our center.

Persistence of high D-dimer values over the time, contrary to what is known by literature, could be maybe a spy of a thromboembolic condition. This could be related to the different genesis of thrombosis. Indeed, in COVID-19, pulmonary inflammation induces endotheliitis and hyperactivation of coagulation cascade, causing likely local over time protracted thrombogenesis (35, 36), and rise of D-dimer values (37, 38), compatible with pulmonary thrombosis extension and persistent inflammation. However, more data are needed to define whether D-dimer value could be related to both early diagnosis and worst prognosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRCCS Policlinico San Matteo Foundation. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ADS, FM, and MR designed and coordinated the study, interpreted data, and wrote the manuscript. SQ performed the statistical analysis. All the other authors followed up patients, locally collected data, and reviewed the paper for final approval. ADS, FM, and MVL reviewed the paper and made final critical revisions for important intellectual contents. FM, MR, SQ, FF, MVL, and ADS significantly participated in the drafting of the manuscript or critical revision of the manuscript for important intellectual content and provided approval of the final submitted version.

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Corrigendum: Diagnostic Delay of Pulmonary Embolism in COVID-19 Patients

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Estimating the Instantaneous Asymptomatic Proportion With a Simple Approach: Exemplified With the Publicly Available COVID-19 Surveillance Data in Hong Kong

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Background: The asymptomatic proportion is a critical epidemiological characteristic that modulates the pandemic potential of emerging respiratory virus, which may vary depending on the nature of the disease source, population characteristics, source–host interaction, and environmental factors.

Methods: We developed a simple likelihood-based framework to estimate the instantaneous asymptomatic proportion of infectious diseases. Taking the COVID-19 epidemics in Hong Kong as a case study, we applied the estimation framework to estimate the reported asymptomatic proportion (rAP) using the publicly available surveillance data. We divided the time series of daily cases into four stages of epidemics in Hong Kong by examining the persistency of the epidemic and compared the rAPs of imported cases and local cases at different stages.

Results: As of July 31, 2020, there were two intermittent epidemics in Hong Kong. The first one was dominated by imported cases, accounting for 63.2% of the total cases, and the second one was dominated by local cases, accounting for 86.5% of the total cases. The rAP was estimated at 23.1% (95% CI: 10.8–39.7%) from January 23 to July 31, and the rAPs were estimated at 22.6% (95% CI: 11.1–38.9%) among local cases and 38.7% (95% CI: 9.0–72.0%) among imported cases. Our results showed that the rAPs of local cases were not significantly different between the two epidemics, but increased gradually during the first epidemic period. In contrast, the rAPs of imported cases in the latter epidemic period were significantly higher than that in the previous epidemic period.

Conclusion: Hong Kong has a high rAP of imported COVID-19 cases and should continue to strengthen the detection and isolation of imported individuals to prevent the resurgence of the disease.

Keywords: COVID-19, likelihood-based framework, instantaneous asymptomatic proportion, Hong Kong, epidemic

INTRODUCTION

An atypical pneumonia case in early December 2019 caught the attention of medical institutions and was later confirmed to be novel coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (1, 2). Since early December 2019, the disease has spread rapidly around the world, with many countries and regions reporting an exponential increase in confirmed cases. In the face of tensions all over the world, the World Health Organization announced that the COVID-19 outbreak was considered as a Public Health Emergency of International Concern since January 31, and eventually classified it as a pandemic on March 11, 2020 (3). As of August 16, 2020, 216 countries and territories had reported more than 21 million confirmed cases, including 760,000 deaths (4). While these numbers are horrifying, they are only a fraction of those infected.

Most of the COVID-19 infections appear to have two outcomes, some become severely ill or even fatal (symptomatic infections), while others show no symptom (asymptomatic infections) (5). In other words, asymptomatic infected individuals are defined as those who have positive RT-PCR testing outcome without any symptom. The asymptomatic COVID-19 infections have been frequently reported since January 2020 and take a large ratio of the total COVID-19 cases (6–8). Several studies showed that the viral load of asymptomatic individuals is similar to that of symptomatic cases, which suggested that asymptomatic individuals can also promote the spread of the disease (9, 10). In parallel, He et al. (11) showed that infectiveness of asymptomatic cases was 25% of that related to the symptomatic ones. Moreover, Day et al. (12) showed that the majority (from 50 to 75%) of people infected with COVID-19 were asymptomatic, but represented "*a formidable source*" of contagion. On the other hand, a previous study reported that asymptomatic individuals can still transmit the pathogen even 14 days later after they become infectious (5).

Based on these evidences mentioned above, we can see that it is fundamental to estimate the proportion of asymptomatic cases, further evaluate the impact of it on the disease burden and the effectiveness of the control interventions, and finally provide the decision-making basis in controlling the spread of the diseases (13–17). At present, many studies have estimated the asymptomatic proportion of total COVID-19-infected cases at different sites by observational studies or mathematical models (18–25). These estimated proportions were raw rates or assumed to remain constant over time. However, asymptomatic proportion may vary depending on the nature of the disease source, population characteristics (e.g., age structure, sex, health status, immune status, and genetic characteristics), pathogen–host interaction, and environmental factors. At the same time, in several countries or regions, the COVID-19 epidemics resurge and have a second wave of peak after a brief respite. It remains unknown whether the instantaneous asymptomatic proportion will change during this process (6).

The main purpose of this study is to develop a simple likelihood-based but generalized framework to estimate the instantaneous asymptomatic proportions for uncovering the features of COVID-19, thereby providing insights into understanding the spread of epidemics. Taking the epidemics in Hong Kong as a case study, we demonstrate the estimation framework by using the publicly available COVID-19 surveillance data.

METHODS

Estimation Framework

We denote the time interval between symptoms onset (if symptomatic) and being confirmed as τ, and let $f(\tau)$ be the probability distribution function (PDF) of τ. That is, if one case is reported on date t who becomes symptomatic eventually, the value of $f(\tau)$ is considered as the relative likelihood of symptoms onset on date $(t + \tau)$.

We assume that all symptomatic cases will be confirmed (most likely in Hong Kong), while confirmed cases can be symptomatic, pre-symptomatic, or asymptomatic at the time of reporting. Thus, the term τ need not necessarily be positive; i.e., negative values are also possible theoretically. Hence, we consider all the confirmed cases as the "pool" of symptomatic cases, and we model this candidate pool as a time-varying function denoted by $\Phi(t)$ on date t. On date t, the ith case, who is reported on date υ_i, contributes $f(\tau = \upsilon_i - t)$ to $\Phi(t)$. For the contribution from all reported cases, $\Phi(t)$ is summated as in Equation (1).

$$\Phi(t) = \sum_i f(\tau = \upsilon_i - t) \tag{1}$$

Hence, the reported asymptomatic proportion (rAP), i.e., the asymptomatic proportion among reported cases, on date t is calculated by $\mathrm{rAP}_t = 1 - \alpha_t/\Phi_t$. Here, α_t is the observed number of cases with symptoms onset on date t, and Φ_t is the discretized $\Phi(t)$ on date t.

Given the infection time of one case (as condition), the onset time of this case is conditionally independent from each other case. Thus, to construct the likelihood profile, we model α_t as a binomial process with sizes at Φ_t (rounding to the closest integer) and successful probabilities at rAP_t to be estimated. As such, by fitting to the daily number of symptomatic cases time series, the rAP_t can be estimated by using the maximum likelihood estimation approach. The 95% confidence intervals (95% CI) of rAP_ts are calculated by using the profile likelihood estimation framework with a cutoff threshold determined by a Chi-square quantile (26), as well as previously adopted in (27–32).

COVID-19 Surveillance Data in Hong Kong

For demonstration, we used the publicly available COVID-19 surveillance data from January 23 to August 8, 2020 in Hong Kong as an example to construct the instantaneous rAPts series. The daily reported number of COVID-19 cases and date of onset were collected from https://www.coronavirus.gov.hk/eng/index.html. A laboratory-confirmed case was defined if the patient had a positive test of SARS-CoV-2 virus by the real-time reverse-transcription-polymerase-chain-reaction (RT-PCR) assay or high-throughput sequencing of nasal and pharyngeal

TABLE 1 | Summary of cases and the estimated asymptomatic proportions in the reported imported and local COVID-19 confirmed cases (rAP) at different stages of the epidemic in Hong Kong.

Stage	Period	# of cases			Daily cases	rAP	
		Total	Imported	Local		Imported	Local
(**I**)	Jan 23–Mar 7	110	32 (29.1%)	78 (70.9%)	2.4	51.9% (5.7–94.6%)	54.6% (2.3–99.1%)
(**II**)	Mar 8–Apr 3	753	476 (63.2%)	277 (36.8%)	27.9	27.3% (6.6–60.1%)	22.4% (2.6–64.1%)
(**III**)	Apr 4–Jun 30	343	287 (83.7%)	56 (16.3%)	3.9	68.0% (15.8–98.5%)	59.4% (9.9–98.8%)
(**IV**)	Jul 1–Jul 31	2,066	279 (13.5%)	1,787 (86.5%)	66.6	56.4% (13.2–91.6%)	22.2% (11.9–36.2%)
Pooled est.	Jan 23–Jul 31	3,272	1,074 (32.8%)	2,198 (67.2%)	17.1	38.7% (9.0–72.0%)	22.6% (11.1–38.9%)

The estimates are showed in "point estimate (95% CI)" format.

swab specimens (33). Only laboratory-confirmed cases were included in this study.

We divided the time series of daily cases in Hong Kong into different stages of the epidemic by examining the persistency of the epidemic. In this study, we considered the criterion that the epidemic persists with the daily number of cases larger than 5 for three consecutive days, but does not persist otherwise. Following this criterion, we have the following four stages of the epidemic, and they included:

- stage (**I**): from January 23 to March 7, with sporadic cases,
- stage (**II**): from March 8 to April 3, with an epidemic peak,
- stage (**III**): from April 4 to June 30, with sporadic cases, and
- stage (**IV**): from July 1 to July 31, with another epidemic peak.

Based on the estimated instantaneous asymptomatic proportion, we summarized the pooled asymptomatic proportions during the four different stages. To avoid the estimation inaccuracy due to reporting delay, we excluded the data from August 1 to August 8, 2020, and conducted the estimation using the remaining dataset.

To set up the initial conditions of the model framework in Equation (1), we initialized the PDF $f(\cdot)$ by a gamma distribution. Although τ can be negative theoretically, the situation when the report is prior to the symptoms onset rarely occurs in Hong Kong (only 1 out of a total of 3,067 symptomatic cases). Thus, for simplification, we model $f(\cdot)$ as PDF defined all positive values, which will not affect our main conclusions. We fitted gamma distribution $f(\cdot)$ to the observed time intervals between symptoms onset and being reported, and the parameters of $f(\cdot)$ are estimated by using the maximum likelihood estimation. We estimated the mean at 6.0, 7.2, or 5.7 days, and standard deviation (SD) at 4.3, 6.6, or 3.4 days for all, imported, or local COVID-19 cases, respectively. These estimates are implemented to set up $f(\cdot)$ in Equation (1) for further rAP estimation.

RESULTS

By July 31, a total of 3,272 cases were reported in Hong Kong, of which 67.2% were locals and 32.8% were imported, respectively. In particular, the first two cases were reported on January 23, 2020, and both were imported. In stage (**I**), all cases were imported in the first week, after which the local transmission emerges and the local cases gradually dominate the total cases, with local cases accounting for 70.9% of the total cases (78 out of 110 cases). In stage (**II**), the daily number of COVID-19 confirmed cases increased rapidly and reached the peak of 44 new cases on March 19. After that, the daily number of cases gradually declined till April 4, 2020, when it dropped to below 5. A total of 753 confirmed cases were reported in stage (**II**), most of them were imported, accounting for 63.2% of the total cases. In stage (**III**), only sporadic new cases emerged every day. A total of 343 cases were reported within about 3 months, of which the vast majority (83.7%) were imported. From July 1, 2020, the epidemics in Hong Kong entered its stage (**IV**), during which the number of daily cases increased rapidly again with a peak of 122 cases on July 7, 2020, and then declined gradually. The epidemic intensity of stage (**IV**) was 2.7-fold, i.e., 2,066 vs. 753 total number of cases, higher than that of stage (**II**). Different from the previous stages, local cases dominated the epidemic in stage (**IV**), accounting for 86.5% of the total cases (1,787 out of 2,066 cases), while only 13.5% of the cases were imported (**Table 1** and **Figure 1**).

In Hong Kong, the pooled rAP was estimated at 23.1% (95% CI: 10.8–39.7%) from January 23 to July 31, and the rAPs were estimated at 22.6% (95% CI: 11.1–38.9%) among local cases and 38.7% (95% CI: 9.0–72.0%) among imported cases. In stage (**I**), the rAPs fluctuated considerably, especially in local cases. After entering stage (**II**), the rAPs were low at the beginning, but increased gradually as the epidemic progressed. At this stage, the increasing trends of rAPs of local and imported cases were similar, but the one for local cases was generally lower than that for imported cases. In stage (**III**), asymptomatic individuals were mainly imported, while the local cases were negligible. The rAPs were relative volatile in stage (**III**), similar to those in stage (**I**). In stage (**IV**), the rAPs of local cases were relatively stable, maintaining around 22%, while the rAPs of imported cases fluctuated greatly, reaching higher than 60% in about half of the time period (**Table 1** and **Figure 1**).

DISCUSSION

We developed a simple likelihood-based framework to estimate the instantaneous asymptomatic proportion of infectious

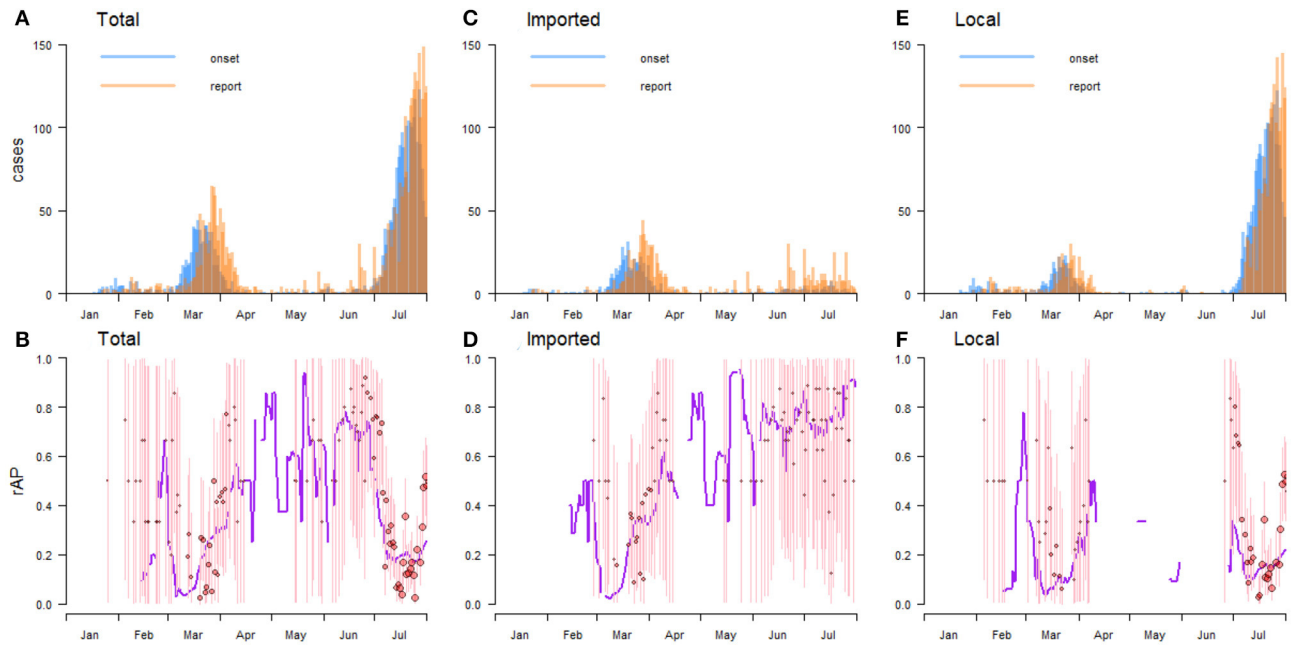

FIGURE 1 | The daily number of total **(A)**, imported **(C)**, and local **(E)** COVID-19 confirmed cases in Hong Kong. The daily reported asymptomatic proportions (rAPs) among the total **(B)**, imported **(D)**, and local **(F)** cases. In panels **(B,D,F)**, the red circles represent the daily rAP, where the circle size represents the sample size, the pink vertical bar represents the 95% confidence interval (95% CI), and the purple curve represents the crude rAP.

diseases and used the publicly available COVID-19 surveillance data in Hong Kong as an example for demonstration.

As an international metropolis, Hong Kong has a high population mobility. Imported cases account for a significant proportion of reported cases, particularly during the first epidemic. We found that the pooled rAP estimation of imported cases (38.7%) appears higher than that of local cases (22.6%). Several potential factors led to this result. Firstly, imported cases are mainly from the United Kingdom and the United States (34), and the asymptomatic rate reflects the comprehensive level of the importing countries. Some representative studies have shown that the asymptomatic infection rate is around 40% (35, 36), which is consistent with our estimated rAP at 38.7% (95% CI: 9.0–72.0) among imported cases. Secondly, imported cases are mainly returned from overseas study or tourism. They are mostly young people, with a large proportion aged from 15 to 24 (11, 34). However, elder people appear more likely to develop severe symptoms, as shown in the epidemic on the Diamond Princess cruise ship (24, 37). Thirdly, asymptomatic infections rarely seek medical advice and thus are less likely ascertained. However, during the outbreak, the Hong Kong government quarantined all arrivals, which can lead to more stringent testing and quarantine for imported than local individuals. As such, asymptomatic individuals in imported cases can be more fully captured.

The time series of the COVID-19 epidemic in Hong Kong included in our study was divided into four stages, of which stages (**II**) and (**IV**) were two discontinuous epidemic periods. The trend of rAPs, proportion of imported cases, and rAPs of imported cases changed greatly between the two epidemic periods. During an epidemic, the testing coverage and the level of contact tracing should be constantly increased, which results in a significant increase of the detection rate of asymptomatic infected individuals (38), that is, leads to a gradual increase in instantaneous asymptomatic rates of both imported cases and local cases. Most Hong Kong residents who studied or traveled abroad returned on or before the first epidemic period (34). With the development of the epidemic, public awareness may gradually increase. COVID-19 infected cases with symptoms will choose to travel less, hence less likely to import into Hong Kong. Meanwhile, the suspension of most airlines and shipping to Hong Kong with a strengthened quarantine rate and the spread of portable devices such as thermometers have also contributed to the less imported cases. These factors played important roles in reducing the proportion of imported cases and increasing the rAPs of imported cases.

There were sporadic daily cases in stages (**I**) and (**III**). At these two stages, a slight change in the number of asymptomatic infected individuals could cause a drastic fluctuation of the rAPs; consequently, the confidence intervals of estimation are relatively large.

The data-driven rAP depends highly on the precise ratio of asymptomatic infected individuals and symptomatic cases. We found that the reporting of asymptomatic individuals may have significantly influenced the scale of case data, as

symptomatic cases are less likely to be incorrectly identified (or under-ascertainment rate to be relatively low). Therefore, on one hand, the under-ascertainment in asymptomatic individuals can result in an underestimation of the rAPs. On the other hand, if we assume the asymptomatic proportion ranging from 27.8 to 30.8% among clinically diagnosable COVID-19 cases as estimated in previous studies (19, 21), an average under-ascertainment rate of asymptomatic individuals in Hong Kong ranging from 22.0 to 32.5% is calculated backwardly.

For another aspect, asymptomatic COVID-19 cases may have important contributions to secondary infections (39). They can unknowingly spread the virus and are more likely to produce asymptomatic offspring, bringing severe battles for epidemic prevention and control (40). In this study, we proposed an analytical approach to estimate the instantaneous asymptomatic proportion of infectious diseases and, as a case study, to reveal the temporal patterns of COVID-19 transmission and spectrum. We believe that our study can bring an insight into understanding the transmission of COVID-19. It should be pointed out that our study still has several limitations. Firstly, our estimates rely on total and timely reporting of asymptomatic infected individuals. Alternatively, an overdispersion setting in the likelihood distribution can be incorporated to resolve inaccurate deterministic scenarios. Secondly, as a data-driven analysis, our estimates rely on the consistency of the statistical framework and reported COVID-19 case data.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.chp.gov.hk/files/pdf/local_situation_covid19_en.pdf.

ETHICS STATEMENT

The ethical approval and individual consents were exempted as the aggregated data used in this study are from public domain.

AUTHOR CONTRIBUTIONS

All authors conceived and conducted the research, wrote the draft, critically revised the manuscript, and approved the submission.

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SYSTEMATIC REVIEW
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Post-COVID-19 Syndrome: The Persistent Symptoms at the Post-viral Stage of the Disease. A Systematic Review of the Current Data

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Whilst the entire world is battling the second wave of COVID-19, a substantial proportion of patients who have suffered from the condition in the past months are reporting symptoms that last for months after recovery, i. e., long-term COVID-19 symptoms. We aimed to assess the current evidence on the long-term symptoms in COVID-19 patients. We did a systematic review on PubMed, Web of Science, EMBASE, and Google Scholar from database inception to February 15, 2021, for studies on long-term COVID-19 symptoms. We included all type of papers that reported at least one long-term COVID-19 symptom. We screened studies using a standardized data collection form and pooled data from published studies. Cohort cross-sectional, case-report, cases-series, case-control studies, and review were graded using specific quality assessment tools. Of 11,361 publications found following our initial search we assessed 218 full-text articles, of which 145 met all selection criteria. We found that 20.70% of reports on long-term COVID-19 symptoms were on abnormal lung functions, 24.13% on neurologic complaints and olfactory dysfunctions, and 55.17% on specific widespread symptoms, mainly chronic fatigue, and pain. Despite the relatively high heterogeneity of the reviewed studies, our findings highlighted that a noteworthy proportion of patients who have suffered from SARS-CoV-2 infection present a "post-COVID syndrome." The multifaceted understanding of all aspects of the COVID-19 pandemic, including these long-term symptoms, will allow us to respond to all the global health challenges, thus paving the way to a stronger public health.

Keywords: COVID-19, long-term symptoms, persistent symptoms, long-term sequalae, virus

INTRODUCTION

As of March 2021, about 117 million people worldwide have been diagnosed with COVID-19, with more than 2.6 million deaths (1). COVID-19 is caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a heterogeneous virus that manifests itself with a wide spectrum of symptoms, from asymptomatic to life-threatening and fatal disease

(2–7). Interstitial pneumonia is one of the most common features of SARS-CoV-2 and can be complicated by acute respiratory distress syndrome (ARDS), a disease related with high mortality, particularly in elderly people with multiple comorbidities (2, 3). As the pandemic of COVID-19 continues, numerous additional symptoms, such as fever, dry cough, shortness of breath, fatigue, myalgias, nausea/vomiting or diarrhea, headache, weakness, rhinorrhea, anosmia/ageusia, and many laboratory abnormalities, i.e., lymphopenia and elevated inflammatory markers (e.g., erythrocyte sedimentation rate, C-reactive protein, ferritin, tumor necrosis factor-α, IL-1, and IL-6) have been reported (2, 3). Other critical and severe complications of COVID-19 can include impaired function of the heart, brain, lung, liver, kidney, and coagulation system (4–7).

Most of the infected patients completely recovered after COVID-19 infection. However, a substantial proportion of patients who have been infected with SARS-CoV-2 continue to have symptoms long past the time that they recovered from the initial phases of COVID-19 disease. Clinicians worldwide called these long-term effects of COVID-19 "Long-Haul COVID-19" or "Long-term COVID-19" (8–11). In detail, "long-term COVID-19" defines those individuals who have had SARS-CoV-2 infection but do not recover completely over a period of a few weeks (commonly 2–3 weeks) (8–11). Based on the COVID-19 Symptom Study, a study carried-out on more than 4 million people in the US, UK, and Sweden, in which people enter their ongoing symptoms on a smartphone app, around 10% of patients who have tested positive for SARS-CoV-2 virus remain unwell beyond 3 weeks, and a smaller proportion for months (8). Thus, it is becoming clear, that some people who had a SARS-CoV-2 infection, even those described as "mild," continue to suffer from persisting or cyclical symptoms. However, because COVID-19 is a novel disease, to date, there is not yet consensus on the definition of post-COVID-19 symptoms. Since long-term symptoms and complications have been described for other highly homologous human coronaviruses, i.e., Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS), to date, it is unknown whether lessons from MERS and SARS are applicable to COVID-19 and the critical question is: "*Do persistent symptoms at the post-viral stage of the disease constitute a post-COVID-19 syndrome (long-term COVID-19) and what are the main persistent symptoms in patients that might cause such a syndrome?*" (12–14). The obvious answer is in research, but to date we do not know what to tell patients when they are asking about the course and prognosis of their ongoing complaints and potential long-term symptoms. Finding a concrete answer to these questions would also provide more information on the COVID-19 disease and enable comprehensive and targeted care to be given to survivors through the development of preventive and effective treatments. Although we are aware that it is too early to completely answer these questions, we believe that some general predictions are now possible, and would help to implement the right public health measures in particular after the pandemic has subsided. Thus, to give a complete overview on the persistent symptoms at the post-viral stage of COVID-19, we carried out a systematic review of the current data considering all types of papers evaluating individual persistent symptoms in mild, moderate, and severe/critical COVID-19 patients. Realizing the long-term sequelae of COVID-19 is imperative for understanding the complete history of disease, truly predicting the growing effect of the disease beyond hospitalization and mortality and defining whether inpatient or post-discharge-specific rehabilitation should be evaluated.

METHODS

Eligibility Criteria

The PICO model was used to formulate the questions for this study: (1) studies that considered patients with long-term COVID-19 symptoms (Population), (2) studies where the primary aim was to evaluate long-term COVID-19 symptoms in mild, moderate, severe, and critical patients that have a follow-up of at least 14 days (Interventions), (3) studies with or without a control group (Comparisons), (4) studies that reported the long-term COVID-19 symptoms (Outcomes). Studies conducted up to February 15, 2021 were included in this review if they met the PICO criteria.

Search Strategies

Our systematic review involved a search conducted on February 15, 2021. We performed the review according to PRISMA statement (15). The search was carried out on PubMed, Web of Science, EMBASE, and Google Scholar databases to identify all type of papers on the long-term symptoms of COVID-19. The search was conducted combining the terms COVID-19, persistent symptoms, long-term symptoms, chronic symptoms, enduring symptoms, permanent symptoms. The combination of free-vocabulary and/or MeSH terms for the identification of studies in PubMed, Web of Science, EMBASE, and Google Scholar were reported in **Table 1**. Reference lists of relevant articles were searched for other potentially appropriate publications.

Inclusion and Exclusion Criteria

Papers of any design evaluating individual persistent symptoms in mild, moderate, severe, and critical COVID-19 patients that have a follow-up of at least 14 days were included in this review.

Exclusion criteria included: unpublished reports, unspecified date/location of the study or suspicion of duplicate reporting, coronavirus strains other than COVID-19, unreported long-term COVID-19 symptoms, and studies that only hypothesize post-COVID-19 sequelae.

Study Selection and Data Extraction

Possible relevant articles were screened using the title and abstract by one reviewer (FS) and articles that did not meet the inclusion criteria were excluded. After screening the title and abstract, articles were submitted to a public reference manager (Mendeley v.1.17.9) to eliminate duplicates. Subsequently, the remaining full-text articles were examined by two reviewers (FS and FV). Any disagreement was resolved through discussion until a consensus was reached, or with the involvement of a third reviewer (MF).

The following items were extracted from each cohort study, cross-sectional, case-report, cases-series, case-control studies, if available: author, study type, study country, and period, patient characteristics (numbers, gender, age), COVID-19 severity (mild, moderate, severe, and critical), hospitalization, ICU admission, baseline COVID-19 symptoms, method of evaluating long-term COVID-19 symptoms, follow-up, and long-term COVID-19 symptoms.

Risk of Bias Assessment

Two reviewers (FS and FV) independently assessed the methodological quality of cohort, cross-sectional, case-reports, case-control, case-series studies, and reviews. Disagreements regarding the methodological quality of the studies were discussed between the two reviewers. If consensus was not reached, a third reviewer (MF) arbitrated. Cohort and Cross-Sectional Studies were assessed by Quality Assessment Tool for Observational Cohort and Cross-Sectional Studies from the National Heart, Lung, and Blood Institute (NIH) (16). Case-control studies were assessed by the quality assessment criteria of The Quality Assessment Tool for Case-Control Studies from NIH (16). The methodological quality of case-series and case-reports were assessed by the quality assessment tool proposed by Murad et al. (17). Finally, reviews were assessed by the Quality Assessment Tool for Systematic Reviews and Meta-Analyses from NIH (16). No bias evaluation was performed for letters, commentary, editorial, news articles, survey, practice, communications, and medical hypothesis.

RESULTS

Study Selection and Characteristics

The initial literature search retrieved 11,361 studies. Of those, 3,132 studies were identified using PubMed, 2,776 using Web of Science, 2,073 using EMBASE and 3,380 using Google Scholar. After screening the title and abstract 315 articles were run through Mendeley to eliminate duplicate articles. The resulting 218 full-text articles were then reviewed to establish whether the publication met the inclusion criteria and 139 were considered eligible. From the reference lists of the selected articles 6 additional publications were found. Of the 145 articles eligible for this review 47 were cohort studies (22 retrospective and 25 prospective), 11 cross-sectional, 2 case-control, 3 case-series, 14 case-reports, 10 review, 16 letters to Editor (of which 6 reported a cohort study, 1 reported a cross-sectional study, 1 reported a case-report, and 3 reported surveys), 3 commentary, 2 reply to commentary, 1 correspondence, 6 editorial, 18 survey (social media, interview, phone application), 1 opinion, 1 brief communication that reported a retrospective cohort study, 1 clinical update and 1 view point, 1 practice, 6 news articles, and 1 medical hypothesis. Search strategy and study inclusion and exclusion criteria are detailed in **Figure 1**.

Risk of Bias Assessment

Of the 145 articles eligible for the review, we found 54 cohort studies (28 prospective, 26 retrospective), six of which were published as letters and one as brief communication, and 12 cross-sectional studies, one of which was published as letters. Using the NIH Quality Assessment Tool for Observational Cohort and Cross-Sectional Studies (16), we rated three prospective studies, one ambidirectional cohort study and two cross-sectional studies at a "good" quality rating and 60 studies at a "fair" quality rating (**Supplementary Material 1**). For the 60 cohort and cross-sectional studies at a "fair" quality rating, the principal missing quality assessment criteria were sample size justification, blinded assessors to the exposure of participants, and missing data on key potential confounding variables measured and adjusted statistically for their impact on the relationship between exposure(s) and outcome(s). Concerning the two case-control studies found, one was at a "good" quality rating and it did not specify only if outcome assessors did not know whether participants were exposed/unexposed, while the other was at a "fair" quality rating (**Supplementary Material 1**). For the case-control at "fair" quality rating, data on sample size justification, random selection of cases and controls, measures of exposure/risk across all study participants and on blinded assessors of exposure/risk were not reported. The methodological quality of the three case-series and of the 15 case-reports, one of which was published as letters, assessed by the tool proposed by Murad et al. (17), showed that 17/18 studies were at a "good" quality rating (**Supplementary Material 1**). For the two case-reports rated at a "fair" quality rating, the missing quality assessments criteria were the not adequately ascertained outcome, the lack of alternative causes that may explain the observation, and the absence of sufficient and specific details to describe the case. The quality assessment of reviews showed that 1/10 reviews was at a "good" quality rating while all the others were at a "poor" quality rating (**Supplementary Material 1**). The "poor" quality rating was because none of them include a comprehensive search of potentially relevant articles and did not use explicit criteria in the selection of articles. The research designs and study characteristics were not appraised, data were not synthesized, and results were not interpreted using a predefined systematic approach.

Long-Term Symptoms of COVID-19

Of the 145 eligible papers, 30 were on persistent lung symptoms (20.70%), 35 were on persistent neurological and olfactory dysfunctions (24.13%), and 80 were on widespread persistent symptoms (55.17%) (**Table 1**). **Table 1** was split-up based on long-term lung symptoms, long-term neurological and olfactory symptoms, and widespread long-term symptoms.

Persistent Lung Symptoms and Dysfunctions

While SARS-CoV-2 was detected in many organ systems, the lungs seem to be the main organs affected by the virus (105–107). Abnormal lung functions and structural changes were reported up to 6 months after hospitalization in mild-to-critical COVID-19 patients (25, 27, 28, 32, 34, 36, 39, 41), also with diffuse alveolar damage, desquamation of alveolar epithelial type II cells, fibrine exudation, hyaline membranes, scattered interstitial inflammation, monocytes, and macrophages

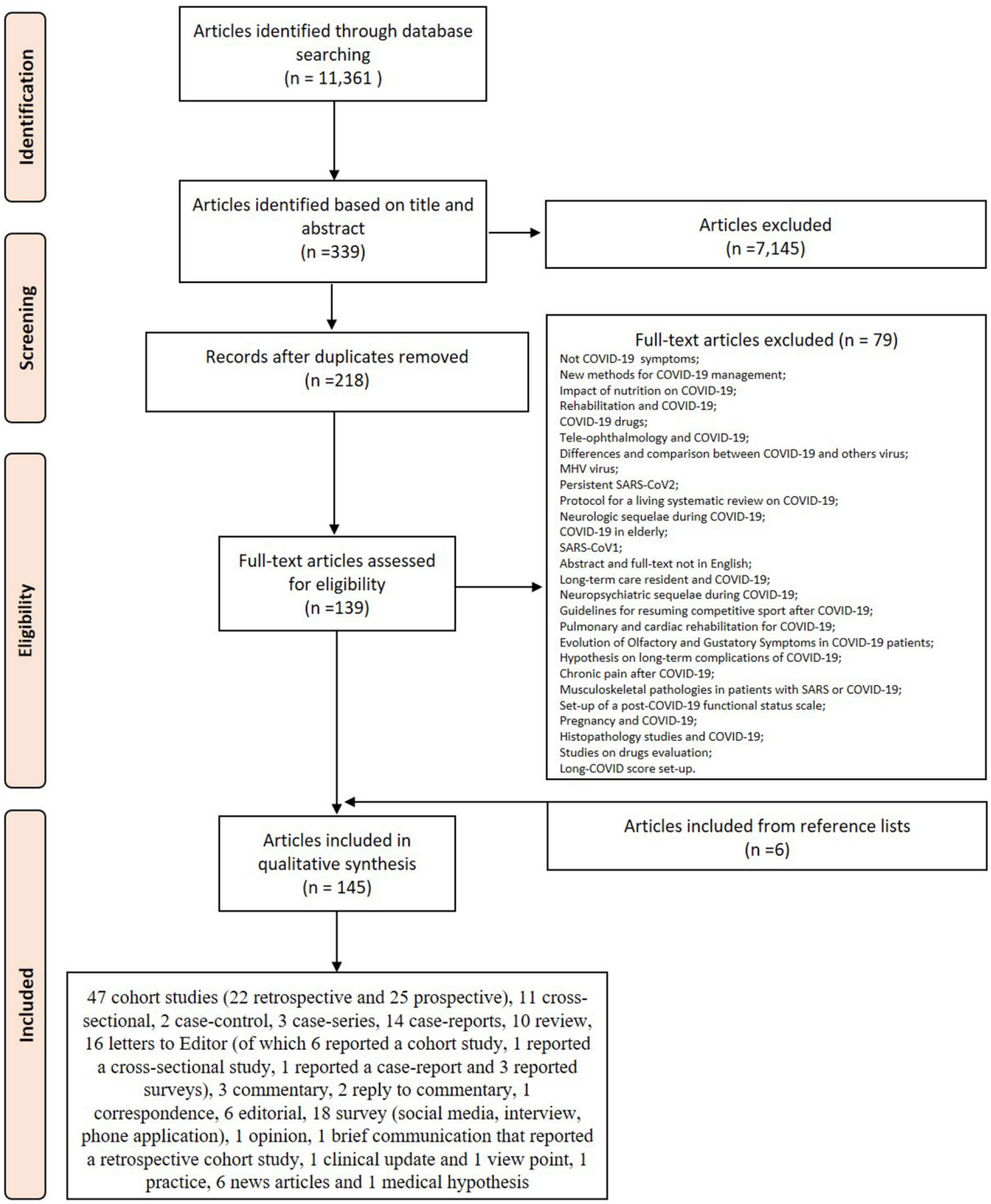

FIGURE 1 | PRISMA flowchart for the study selection.

(23, 24, 34). Several authors reported that these persistent lung symptoms and dysfunctions correlated with prior COVID-19 severity (19, 20, 24, 25, 27, 36, 38, 41). In this context, Han et al. in a prospective study evaluating 114 severe COVID-19 patients showed lung fibrotic-like changes in 35% patients up to 6 months after infection (24). Differently, Latronico et al. showed that since residual abnormal chest-X ray findings were detected in about 70% of critically ill COVID-19 patients at 3 months, very few of them (∼12%) had persisting respiratory symptoms at 6 months (27). An anecdotal study by Zhu et al. also reported long-term abnormal airway function for up to 11 months in a severe COVID-19 patient (44).

TABLE 1 | Cohort (perspective and retrospective), cross-sectional, case-report, cases-series and case-control studies on long-term lungs symptoms, long-term neurological and olfactory symptoms, and widespread long-term symptoms.

References	Study type	Study country and period	Patients characteristics (numbers, gender, age)	COVID-19 severity	Hospitalization	ICU admission	Baseline COVID-19 symptoms	Method of evaluating long-term COVID-19 symptoms	Follow-up	Long-term COVID-19 symptoms
Persistent lungs symptoms and dysfunctions										
Bellan et al. (18)	Prospective	• Novara, Italy • March 1 and June 29, 2020	• 238 patients: • 96 females • 142 males • Mean age: 61 (50–71)	Mild to-severe	Yes	28 patients	Fever, cough, dyspnea, ageusia, anosmia, diarrhea, arthralgia, myalgia	D_{LCO}, score for posttraumatic stress symptoms and for functional impairment	120 days	D_{LCO} reduced to less than 80% of the estimated value in 113 patients and less than 60% in 34 patients. Functional impairment in 53 patients
Chun et al. (19)	Retrospective	New Haven, CT	• 61 patients • 44% females 56% males • Mean age: 53 (43–62)	• 13 mild • 30 non-critical • 18 critical	30 patients	18 patients	Dyspnea and cough	Pulmonary function tests, plasma biomarker profiling	45–67 days	Dyspnea (69%), cough (58%). Pulmonary function declined as acute COVID-19 severity increased and not correlate with symptoms. LCN2, MMP-7, HGF were higher in ICU subjects and inversely correlated with pulmonary function
Daher et al. (20)	Prospective	• Aachen, Germany • February–May 2020	• 33 patients • 11 females • 22 males • Mean age: 64 ± 3	Severe	Yes	No	• Increased D-dimer • LDH activity and CRP, ferritin, and IL-6	Body plethysmography, D_{LCO}, blood gas analysis (ABG), 6-min walk test (6MWT), echocardiography, laboratory tests	45 days	Reduced D_{LCO} and 6MWT, and persistent fatigue and dyspnea in most patients.
Ding et al. (21)	Retrospective	• Wuhan, China • February–March 2020	• 112 patients: • 61 females • 51 males • Mean age: 55.8	NR	Yes	NR	Fever, dry cough, fatigue, chest distress, dyspnea, myalgia	CT scan	28 days	Abnormalities in 98.1 % of lungs CT scans (ground-glass opacities, crazy-paving pattern, consolidation and linear opacities)
Frija-Masson et al. (22)	Retrospective	• Paris, France • 4 March−1 April 2020	• 50 patients: • 22 females • 28 males • Age ≤ 85	• 12 mild • 17 moderate • 16 severe • 5 not classified	Yes	8 patients	Respiratory symptoms	Spirometry, functional residual capacity, total lung capacity, D_{LCO} (single breath real-time CO/NH_4)	30 days	Impaired lung function in 54% of patients (restriction and/or altered D_{LCO}), with a mix of restrictive and low diffusion patterns

(Continued)

TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Hall et al. (23)	Retrospective	• London, UK • May 2020	• 200 patients: • 38.5 females • 61.5% males • Mean age: 54.8 ± 15.0	Moderate to-severe	89 patients	77 patients	NR	Dual energy CT or high-resolution CT, ventilation-perfusion scanning, spirometry, echocardiography and ECG	30–45 days	40% of patients with cardiorespiratory cause of breathlessness, i.e. persistent parenchymal abnormality pulmonary embolism, cardiac complications
Han et al. (24)	Prospective	• Hubei, China • December 25, 2019 - February 20, 2020	• 114 patients: • 34 females • 80 males • Mean age: 54 ± 12	Severe	Yes	NR	Pneumonia	CT scan	175 ± 20 days	Lung fibrotic-like changes in 35% patients, while in 65% patients complete radiological resolution (38%) or residual ground-glass opacification or interstitial thickening (27%)
Heiss et al. (25)	Case-reports	Erlangen, Germany	• 1 male • 60-year-old	Severe	Yes	No	Peripheral, multilobar areas of ground-glass Opacity (GGO)	CT scan MRI	90 days	Residual pulmonary changes with patchy, peripheral GGOs, and consolidations
Hu et al. (26)	Retrospective	• Wuhan, China • 1 January 2020–28 February 2020	• 46 patients: • 19 females • 27 males • Mean age: 39.17	• 36 mild/moderate • 10 severe	Yes	NR	Fever, cough, myalgia, fatigue, vomiting, or diarrhea	CT scan	31 days	Lung lesions completely absorbed only in 28.57 % of patients
Latronico et al. (27)	Prospective	• Brescia, Italy • February–June 2020	• 59 patients • Median age: 54–64	Critical	Yes	Yes	Acute respiratory distress syndrome	X-ray, spirometry	90–180 days	Chest X-ray and pulmonary function altered in 70% of patients at 3 months; few patients had persisting respiratory symptoms at 6 months
Liang et al. (28)	Prospective	Wuhan, China	• 76 patients: • 55 females • 21 males • Mean age 41.3 ± 13.8	• 69 mild/general • 7 severe/critical	Yes	9 patients	NR	Standard questionnaire; pulmonary function tests (total lung capacity -TLC, D_{LCO}, carbon monoxide diffusion constant (D_{LCO}/VA)	90 days	42% of patients with pulmonary function abnormalities

(Continued)

TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Liao et al. (29)	Retrospective	• Guangzhou, China • January 22,–April 10, 2020	• 158 patients: • 22 females • 88 males • Mean age: 48.0 ± 17.7	• 14 mild • 110 moderate • 34 severe	Yes	3 patients	fever, fatigue, diarrhea, polypnea, anorexia	Peripheral blood analyses (inflammatory cytokines expression), CT scan	60 days	Persistent elevation of IL-6 associated with persistent pulmonary lesions
Manckoundia et al. (30)	Case-report	Dijon, France	49-year-old man	Mild	No	No	Asthenia, fever, dry cough, dysgeusia, headache	General practitioner consult	90 days	Non-inflammatory tracheal hypersecretion
Mo et al. (31)	Cross-sectional	• Guangzhou, China • February–March 2020	• 110 patients: • 55 females • 55 males • Mean age: 49.10	• 24 mild • 67 moderate • 19 severe	Yes	NR	NR	Spirometry, D_{LCO}	20 ± 6 days in mild cases; 29 ± 8 days in moderate cases; 34 ± 7 days in severe cases	D_{LCO} anomalies in 47.2% of patients, total lung capacity in 25.0%, forced expiratory volume in 1 s (FEV1) % in 13.6%, forced vital capacity (FVC) % in 9.1%, FEV1/FVC in 4.5% and small airway function in 7.3% of patients
Moreno-Perez et al. (32)	Prospective	• Alicante, Spain • February–April 2020	• 277 patients: • 47.3% females • 52.7% males • Median age: 62.0	• 34.3% mild • 65.7% severe	182	NR	NR	Spirometry, chest radiology	70 - 98 days	Spirometry alterations present in 9.3% patients, while in radiographs in 18.9%
Ramakrishnan et al. (33)	Retrospective	• Atlanta, USA • April, 2020	• 107 patients: • 26 males • 81 females • Mean age: 55	NR	NR	NR	Fever, cough, smell or taste alteration	Lung auscultation, ECG	30 days	10% of with dyspnea and fatigue
Shah et al. (34)	Prospective	• Vancouver, Canada • March-May 2020	• 60 patients: • 32% females • 68% males • Median age: 67	NR	Yes	NR	Dyspnoea, cough	Pulmonary function testing (PFT), 6 min walk test (6MWT), high-resolution CT of the chest	90 days	More than half of patients with lung function and chest imaging abnormalities

(Continued)

TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Sonnweber et al. (35)	Prospective	Innsbruck, Austria	• 109 patients: • 44 females • 65 males • Mean age: 58	• 22 mild • 34 moderate • 35 severe • 18 critical	87 patients	18 patients	NR	CT scan, serum biomarkers	60 days	Iron deficiency in 30% of patients, anemia in 9%. Increased inflammation markers levels, such as IL-6 and C-reactive protein in anemic patients. 38% of patients with hyperferritinemia associated with severe lung pathologies
Tabatabaei et al. (36)	Retrospective	Kashan, Iran	• 52 patients: • 20 females • 32 males • Mean age: 50.17 ± 13.1	Severe/critical	Yes	11 patients	Fever, fatigue, dyspnea, GGO, consolidation, and mixed pattern	CT scan, serum biomarkers	90 days	42.3% with residual pulmonary disease. General poor health status in the domains of functional impairment (64%), fatigue (69%), QoL (72%)
Trinkmann et al. (37)	Prespective	• Heidelberg, Germany • March–June 2020	246 patients: Mean age: 48 ± 15	Mild to-severe	20 patients	2 patients	Olfactory loss, cough, pyrexia, dyspnoea, sore throat, rhinitis, thoracic pain, limb pain, cephalgia, fatigue	Spirometry and body-plethysmography	68 ± 16 days	Lower lung function even in younger SARS-CoV-2 convalescents with few comorbidities
Truffaut et al. (38)	Retrospective	• Brussels, Belgium • March–June 2020	• 22 patients: • 6 females • 16 males • Mean age: 54.6 ± 10.9	Severe	Yes	Yes	NR	Pulmonary function test (PFT), 6-min walking distance test (6MWDT), dyspnoea (modified Medical Research Council (mMRC)	90 days	55% of patients with restrictive pattern ± altered D_{LCO}. 65% with a 6MWDT below 80% and 52% were free from exertional dyspnoea according to mMRC scale
van den Borst et al. (39)	Prospective	• Nijmegen, The Netherlands • 23 April - 15 July 2020	• 124 patients • 50 females • 74 males • Mean age: 59 ± 14	• 27 mild • 51 moderate • 26 severe • 20 critical	Yes	Yes	NR	CT scan Clinical Frailty Scale (CFS) Pulmonary function tests (D_{LCO}, TLC)	90 days	90% of patients with residual pulmonary parenchymal abnormalities

(Continued)

TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
van Gassel et al. (40)	Retrospective	March–May 2020	48 patients	Severe	Yes	Yes	Severe pneumonia	Pulmonary function testing (PFT), i.e. spirometry, lung volumes, D_{LCO} adjusted for Hb, chest high-resolution CT (HRCT) imaging, and 6-minute-walk test (6-MWT)	90 days	Reduced total lung capacity and diffusion capacity in 23 and 36 participants, respectively, but no airway obstruction on PFT. Ground-glass opacities in 89% of cases. Signs of reticulation, bronchiectasis, bronchiolectasis in 67% of cases
Weerahandi et al. (41)	Prospective	• New York, USA • April 15, 2020,	• 152 patients: • 57 females • 95 males • Mean age: 62	Severe	Yes	101	NR	Patient-Reported Outcomes Measurement Information System (PROMIS®) Dyspnea Characteristics instrument	30–40 days	Shortness of breath in 74% of patients; 35.1% patients require home oxygen after hospital discharge
Yao et al. (42)	Case-report	• China • January 27, 2020	• 1 female • 78-year-old	Mild	Yes	No	Multiple patchy shadows in both lungs	Lungs biopsy	14 days	Diffuse alveolar damage, extensive desquamation of proliferative type II alveolar epithelial cells, exudative monocytes and macrophages
Zhao et al. (43)	Retrospective	• 3 tertiary hospitals of Henan Province, China • 20 January−24 February 2020	• 55 patients: • 23 females • 32 males • Mean age: 47.74	• 4 mild • 47 moderate • 4 severe	Yes	NR	Gastrointestinal symptoms, headache, fatigue, dyspnea, cough, sputum, olfactory, and gustatory dysfunctions	CT scan, pulmonary function test	90 days	Abnormalities of pulmonary function and chest radiography in three quarters of patients. Higher D-dimer level at admission predict impaired D_{LCO} 3 months after discharge
Zhu et al. (44)	Case-report	• Hubei, China • January 2020	30-year-old male	Severe	Yes	NR	Dry cough, fever, emphysema in both upper lungs, with ground glass density at the edge	Chest CT, laboratory examination results, lung function examination, sleep monitoring, sex hormones, sperm morphology and activity	11 months	Abnormal airway function, cough, chest pain, chest tightness, and shortness of breath, unstructured sleep apnea hypopnea syndrome, and nocturnal sleep hypoxemia

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TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Persistent neurological symptoms and olfactory dysfunctions										
Boscolo-Rizzo (45)	Prospective	Treviso, Italy	183 patients	Mild	NR	No	Fever, dry cough or coughing up mucus, loss of appetite, felt tired, altered sense of smell or taste	Interviews	60 days	18.6% of patients with altered sense of smell or taste
Caronna et al. (46)	Prospective	• Barcelona, Spain • 28 March−22 April 2020	• 130 patients: • 66 females • 64 males • Mean age: 53.9	Mild-to-severe	80%	8.5%	Headache, fever, malaise, myalgia, dizziness, cough, dyspnea, chest pain, expectoration, odynophagia, loss of smell/taste, diarrhea	Neurological assessment	45 days	74.6 % of patients had headache. At follow-up 37.8% of these had persistent headache (50% with no previous headache history)
D'Ascanio et al. (47)	Case-control	• Santa Croce Hospital AORMN, Fano-Pesaro, Italy • 1 February–April 24, • 2020	• 43 COVID-19 patients • 25 healthy controls	Mild	20 patients	No	Anosmia, hyposmia, headache	A 7-question survey instrument, subjective olfactory dysfunction	30 days	Resolution of anosmia or hyposmia in ∼85% of patients
Dani et al. (48)	Case-series	London, UK	• 6 female patients • Age: 26–50 years	NR	No	No	Gastrointestinal symptoms, upper respiratory tract symptoms, chesty cough, flu-like symptoms	Echocardiogram	21 days	Orthostatic intolerance syndromes (orthostatic hypotension, vasovagal syncope, postural orthostatic tachycardia syndrome)
Fjaeldstad et al. (49)	Retrospective	• Denmark • 22 April−4 May 2020	• 109 patients: • 79 females • 30 males • Mean age: 39.4	Mild	No	No	Fever, headache, fatigue, dyspnea, cough sputum, olfactory, gustatory loss	Subjective chemosensory function	> 30 days	28% and 20% of patients not experienced improvement respectively of their olfactory and gustatory function, whereas 44% and 50% fully recovered olfactory and gustatory loss respectively

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TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Galal et al. (50)	Cross-sectional	• Aswan, Egypt • 18 July−31 August 2020	• 430 patients: • 274 females • 156 males • Mean age: 37.4 ± 12.6	Mild-to-critical	103 patients	20 patients	Myalgia, fever, restriction of daily activities, memory loss	A 4-point Likert scale	30 days	Myalgia (60.0%), arthralgia (57.2%), restriction of daily activities (57.0%), sleeping troubles (50.9%), nervousness and hopelessness (53.3%), anorexia (42.6%), chest pain (32.6%), gastritis (32.3%), cough (29.3%) and dyspnea (29.1%)
Gallus et al. (51)	Retrospective	• Sassari, Italy • April-May 2020	• 48 patients: • 37 females • 11 males • 37 (77%) Mean age: 45	Mild	No	No	Fever, dyspnea, cough, thoracic pain, asthenia, myalgia, diarrhea, conjuntivitis, general malaise, sore throat, headache, cutaneous rash, hypo-anosmia, hypo-ageusia	Tonal pure tone audiometry, a vHIT and SHIMP test	14 days from the second negative swab	8.3% patients reported hearing loss, 4.2% tinnitus, 8.3% dizziness, 2% spinning vertigo, 2% dynamic imbalance, 6.3 static imbalance
Guedj et al. (52)	Case-report	Marseill, France	• 54-year-old man • 62-year-old man	• 1 severe • 1 moderate	Yes	Yes	Acute respiratory distress syndrome, anosmia or ageusia	Whole-body ^{18}F-FDG PET	30 days	Hypometabolism of the olfactory/rectus gyrus on the two patients
Hellmuth et al. (53)	Case-report	San Francisco, CA, USA	• 33-year-old woman • 56-year-old woman	Mild	No	No	Neck pain, fatigue, fever, cough, myalgias, and non-migrainous headaches, cognitive symptoms	Cerebrospinal fluid and blood analyses, MRI	• 149 days • 72 days	• Deficits in working memory and digit span backwards with high average attentional skills • Word finding difficulties, inefficient learning, and decreased organization leading to missed deadlines
Lim et al. (54)	Case-report	UK	55-year-old woman	Mild	Yes	No	Fever, myalgia, cough, breathlessness, anosmia, ageusia, headache	CT scan, MRI, Addenbrooke's Cognitive Examination-III	52 days	Persistent psychotic symptoms

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TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Lu et al. (55)	Prospective	• Fuyang No.2 People's Hospital, China • January - February 2020	• 60 patients: • 26 females • 34 males • Mean age: 44.10 • 39 age and • sex-matched non COVID-19 controls	• 47 mild • 12 severe • 1 critical	Yes	NR	Fever, cough, gastrointestinal symptoms, neurological symptoms	Diffusion tensor imaging (DTI), 3D high-resolution T1WI sequences	90 days	68.33% of patients with disruption to micro-structural and functional brain integrity during infection and 55% of them maintain the same symptoms after 90 days
Mendez et al. (56)	Prospective	• Valencia, Spain • March - April	• 179 patients • 74 females • 105 males • Mean rage: 22–81	Mild-to severe	Yes	34 patients	NR	Standardized instruments evaluating neurocognitive function, psychiatric morbidity, and QoL	60 days	58.7% presented at least moderate neurocognitive decline, 39.1% psychiatric morbidity, and ∼40% had poor QoL
Moein et al. (57)	Prospective	• Tehran, Iran • 21 March−3 May, 2020	• 82 patients: • 28 females • 45 males • Mean age: 45.53	• 58 mild • 30 moderate • 12 severe	Yes	No	Fever, cough, breathlessness, headache, myalgia, shivering, sweating, gastrointestinal symptoms, malaise, tinnitus, bloody sputum	40-item University of Pennsylvania Smell Identification Test (UPSIT)	40–60 days	96% of patients with smell loss during infection. At follow-up, the test scores of 63% of the retested patients were normal. However, the mean UPSIT score at that time continued to remain below that of age- and sex matched healthy controls
Negrini et al. (58)	Case-series	• Milan, Italy • 3 March−8 April, 2020	• 9 patients: • 3 females • 6 males • Mean age: 60	• 4 mild/moderate • 5 severe	Yes	5 patients	NR	Mini-Mental State Examination (MMSE) test	30 days	General cognitive decay in 33.3% of patients, with a specific decline in attention, memory, language, and praxis abilities. The cognitive decay appears to be associated with the length of stay (in days) in ICU
Novak et al. (59)	Case-report	Boston, USA	64-year-old woman	NR	No	No	Cough, dyspnea	CT scan	20 days	Probable orthostatic hypoperfusion syndrome and painful small fiber neuropathy in post- COVID disease.

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TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Panda et al. (60)	Prospective	• New Delhi, India • 23 April−29 June 2020	• 225 patients: • 63 females • 159 males • 3 transgenders • Mean age: 34.96	• 145 mild • 80 asymptomatic	No	No	Otolaryngologic symptoms, fever, cough, dyspnea, gastrointestinal symptoms	Ear, Nose and Throat (ENT) symptoms evaluation	28 days	96% of the patients regaining ENT function at follow-up
Pilotto et al. (61)	Retrospective	• Brescia, Italy • February–April 2020	• 165 patients: • 50 females • 115 males • Mean age: 64.8 ± 12.6	Moderate-to severe	Yes	NR	NR	Montreal Cognitive Assessment (MoCA) score	180 days	Fatigue (34%), memory/attention (31%), sleep disorders (30%). 37.4% of patients with neurological abnormalities, i.e. cognitive deficits (17.5%), hyposmia (15.7%), postural tremor (13.8%)
Pritza et al. (62)	Retrospective	• Thessaloniki, Greece • March–April 2020	• 90 patients: • 37 females • 53 males • Mean age: 55.8 • ± 17.3	• 45 mild • 35 moderate • 10 severe	Yes	10 patients	Olfactory and gustatory dysfunction	Questionnaires	61 days	8.57 % patients with persistent hyposmia
Raahimi et al. (63)	Case-report	Portsmouth, UK	46-year-old man	Severe	Yes	Yes	Sensory loss in his feet, progressing to gait unsteadiness and distal lower limb weakness	Cerebrospinal fluid analysis, ECG, CT scan, MRI, spirometry	90–150 days	At 90 days intermittent neuropathic pain and paraesthesia in distal limbs were present. At 150 days improvement in nerve function, with normalizing distal motor latencies
Sampaio Rocha-Filho et al. (64)	Case-report	Recife, Brazil	40-year-old woman	Mild	No	No	Diarrhea, cough, fatigue, myalgia, anosmia, facial pain, headache	MRI, intracranial magnetic resonance angiography	85 days	Persistent anosmia and headaches
Tobechukwu et al. (65)	Case-report	Red Bank, USA	46-year-old woman	Mild	Yes	No	Fever, chest pain, vomiting, cough, confusion	X-ray, CT scan. MRI	90 days	Delirium and allucinations

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TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Ugurlu et al. (66)	Retrospective	• Çorum, Turkey • March–June 2020	• 42 patients: • 23 female 19 male Mean age: 41.2 ± 14.6	Mild	No	No	Fever, cough, dyspnea, diarrhea, sore throat, nasal drip, nasal obstruction, headache	Brief smell identification test	90 days	Full recovery in 85.7% of patients. Olfactory dysfunction persisted in 14,3% of patients
Vaira et al. (67)	Prospective	• University • Hospital of Sassari, San Paolo Hospital in Milan, and Bellaria-Maggiore Hospital in • Bologna, Italy	• 138 patients: • 70 females • 68 males • Mean age: 51.2	Mild-to-severe	Yes	NR	Chemosensitive dysfunction	Self-administered olfactory and gustatory psychophysical tests in outpatients, Connecticut Chemosensory Clinical Research Center orthonasal olfaction test in hospitalized patients	60 days	5.8 % with moderate to severe olfactory dysfunction, 4.3 % with significant taste disorder. Four patients with combined chemosensitive dysfunctions, 4 patients with isolated smell impairments and two patients with isolated taste disorders
Yan et al. (68)	Cross-sectional	• California, USA • 9 March–April 29, 2020	46 patients	NR	NR	NR	NR	10-point scale score for sense of smell	16 days	Olfactory dysfunction reported by 23 patients (17 reported no loss, 5 were unreachable, 1 died). At follow up 78% of patients with chemosensory dysfunction
Widespread persistent symptoms										
Abdallah et al. (69)	Case-report	• Philadelphia, USA • March 2020	30-year-old man	Mild	No	No	Chest pain, fever, anosmia	X-ray, CT scan	8 months	Chest pain, dyspnoea, and fatigue, intercostal neuralgia
Arnold et al. (70)	Prospective	• Southmead Way, Bristol • 30 March and 3 June 2020	110 patients	27 mild 65 moderate 18: severe	Yes	No	NR	Chest radiograph, spirometry, exercise test, bloods, and health-related quality of life (HRQoL) questionnaires	83 days	Most (74%) patients with persistent symptoms (notably breathlessness and excessive fatigue) with reduced HRQoL
Buonsenso et al. (71)	Cross-sectional	• Rome, Italy • March–November 2020	• 129 children: • 62 females • 67 males • Mean age: 11 ± 4.4	Mild-to severe	6 patients	3 patients	NR	Questionnaire	162.5 ± 113.7 days	35.7% had 1 or 2 symptoms and 22.5% had 3 or more. 52.7% had at least one symptom 120 days or more after diagnosis. Fatigue, muscle and joint pain, headache, insomnia, respiratory problems and palpitations are the main reported symptoms

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TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Buselli et al. (72)	Case-report	Azienda Ospedaliero-Universitaria Pisana, Pisa, Italy	50-year-old woman	Mild	No	No	Dry cough, asthenia, myalgia, diarrhea, fever, dyspnea, headache, fatigue, dysphonia	Pneumology examination, CT scan, neurological examination with brain scan, cardiology examination with echocardiograph, pulmonary ultrasound and ENT specialist examination	≥ ys	Persistent fatigue and dysphonia
Carfi et al. (73)	Retrospective	• Fondazione Policlinico Universitario • Agostino Gemelli IRCCS, Rome, Italy • 21 April – 29 May, 2020	• 143 patients: • 53 females • 90 males • Mean age: 56.5	• 21 mild • 104 moderate • 18 severe	Yes	18 patients	Fatigue, dyspnea, joint pain, chest pain, cough, anosmia, sicca syndrome, rhinitis, red eyes, dysgeusia, headache, sputum production, lack of appetite, sore throat, vertigo, myalgia, diarrhea	EuroQol visual analog scale	60 days	At follow-up, only 12.6% of patients with no COVID-19 related symptom, while 32% had 1 or 2 symptoms and 55%had 3 or more. Main persistent symptoms were fatigue (53.1%), dyspnea (43.4%), joint pain, (27.3%) and chest pain (21.7%).
Carvalho-Schneider et al. (74)	Prospective	• Tours University Hospital, France • 17 March−3 June, 2020	• 150 patients: • 84 females • 66 males • Mean age: 49	Non-critical	Yes	No	Dyspnea, fever, weight loss, chest pain, headache, asthenia, myalgia, gastrointestinal symptoms, anosmia, ageusia	Clinical algorithm	30 and 60 days	At 30 days 68% of patients with at least one symptom and 66% at 60 days. Anosmia/ageusia: 28% at 30 days, 23% at 60 days. Dyspnea: 36.7% at 30 days, 30% at 60 days. Asthenia: 50% at 30 days, 40%) at 60 days. Persistent symptoms at 60 days significantly associated with age 40–60, hospital admission and abnormal auscultation at symptom onset
Chen et al. (75)	Cross-sectional	• 12 Hospitals in Wenzhou, Zhejiang, China • 17 January−20 March, 2020	• 361 patients: • 175 females • 186 males • Mean age: 47.22	• 327 mild • 34 severe	Yes	NR	NR	Chinese version of Short-Form 36-item questionnaire (SF-36)	30 days	Health-related quality of life (HRQoL) was poor among COVID-19 patients at follow-up

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TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Cirulli et al. (76)	Prospective	• Nevada, USA • April–September 2020	233 patients	Mild	8 patients	No	Fever, headache, asthenia, fatigue, diarrhea, ageusia, dry cough, chest pain, bone and joint pain, red eyes, dizziness, anorexia	Self-reported short and long-term symptoms	30 and 90 days	43.4% of patients with symptoms longer than 30 days, 24.1% with at least one symptom after 90 days. Long-term symptoms were anosmia, ageusia, difficulty concentrating, fatigue, dyspnea, memory loss, confusion, headache, heart palpitations, chest pain, pain with deep breaths, dizziness, and tachycardia
D'Cruz et al. (77)	Prospective	June–July 2020	• 119 patients: • 45 females • 74 males • Mean age: 58.7 ± 14.4	Severe	Yes	Yes	Pneumonia	X-ray, CT scan, clinical outcomes, symptom questionnaires, mental health screening, physiologic (4MGS and STS) al testing	51–67 days	Persistent fatigue (68%), sleep disturbance (57%) and breathlessness (32%), post-traumatic stress disorder (25%), anxiety (22%) and depression (18%). 4MGS was slow in 38% and 35% desaturated by ≥4% during the STS test
Erçalik et al. (78)	Retrospective	• Istanbul, Turkey • March–May 2020	• 206 patients: • 105 females • 101 males • Mean age: 56.24 ± 16.99	• 153 mild • 48 moderate • 5 severe	Yes	Yes	Fever, cough, dyspnea, runny nose	Pain assessment using a numeric rating scale	45.99 ± 14.64 days	40.7% of the patients had chronic pain for at least 3 months before COVID, and this rate increased to 82.5% during COVID and to 55.1% after COVID
Galván-Tejada et al. (79)	Case-control	• Zacatecas Mexico • July–September 2020	• 219 patients: • 141 recovered • 78 controls • 51% females • 49% males • Mean age female: 39.14 Mean age male: 39.01	NR	Yes	NR	NR	Questionnaire	60 days	Chills, dyspnea, anosmia or dysgeusia, nausea or vomiting cough, red eyes as persistent symptoms in COVID-19 patients

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TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Hosseini et al. (80)	Case-report	Qom, Iran	48-year-old man	Mild	No	No	Fever, chills, weakness, lethargy, myalgia	Laboratory Tests, CT scan, ECG	30 days	Persisten advanced atrioventricular block
Huang et al. (81)	Prospective	Wuhan, China between January−7 May 2020	• 1733 patients • 48% females • 52% males • Median age: 57.0	Mild to-severe	Yes	76 patients	NR	Questionnaires, physical examination, blood tests, CT scan, 6-min walking test	186 days	Fatigue or muscle weakness (63%), sleep difficulties (26%) were the most common symptoms. Anxiety or depression was reported among 23% of patients
Isoldi et al. (82)	Prospective	• Latina, Italy • April–June 2020	• 15 children: • 7 females • 8 males • Median age: 12.2	Mild	No	No	Fever, hyperemia of the pharynx (53.3%), abdominal swelling, tender to the touch (33.3%), active conjunctival injection (6.7%)	Laboratory (blood, urine, feces) tests, ECG	180 days	Two patients with hyperfiltration exhibited high blood pressure levels at diagnosis, and persistence of a prehypertension at 6-month follow-up
Iqbal et al. (83)	Cross-sectional	• Karachi, PAK • September–December 2020	• 158 patients: • 87 females • 71 males • Mean age: 32.10 ± 12.42	• 112 mild • 33 moderate • 13 severe	Yes	13 patients	NR	Questionnaire	20–90 days	Fatigue (82.9%), poor sleep quality (56.3%), anxiety (53.2%), dyspnea (50%), joint pain (47.5%) were the most prevalent post-discharge manifestation
Jacobs et al. (84)	Prospective	• New Jersey, USA • 22 March–April 16	• 183 patients • 38.5% females • 61.5% males • Mean age: 57 • 61.5% male	• 160 mild • 23 severe	Yes	23	Fatigue, shortness of breath, cough, lack of taste, muscular pain, diarrhea, lack of smell, production of phlegm, headache	PROMIS® instruments to identify symptoms and quality of life parameters	35 days	Fatigue (55.0%), dyspnea (45.3%), muscular pain (51%), lower odds rating general health (41.5%), quality of life (39.8%), physical health (38.7%), mental health (43.7%) and social active role (38.7%)
Khalaf et al. (85)	Cross-sectional	• Assiut, Egypt • August–October 2020,	• 538 patients • Mean age: 41.17 ± 14.84 • 45.9 females • 54.1% males	• Mild-to-severe • (61.3% mild, 31% moderate, 7.6% severe)	51.3% of patients	6.5% of patients	NR	Online questionnaire	83 days	Fatigue (59.1%), sense of fever (46.5%), anorexia (24.3%), diarrhea (24.3%), loss of taste and smell (22.3%), headache (21.4%), cough (20.8), dyspnea (21%)

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TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Ludvigsson et al. (86)	Case-report	• Stockholm, Sweden • October 2020	• 5 children • Mean age: 12 • 4 girls • 1 boy	Mild	No	No	Fever, dyspnea, abdominal pain, upper respiratory symptoms, dizziness, extreme fatigue, cough, lost taste and smell, headache, abdominal pain, diarrhea, nausea, norexia	NR	6–8 months	Fatigue, dyspnoea, heart palpitations or chest pain, headaches, difficulties concentrating, muscle weakness, dizziness, sore throats
Mandal et al. (87)	Cross-sectional	London, UK	• 384 patients • Mean age: 59.9 • 38% females • 62% males	Mild-to-critical	Yes	54 patients	NR	CT scan, blood tests, 11-point (0–10) scale score	54 days	Persistent breathlessness (53%), cough (34%) fatigue (69%), depression (14.6%), elevated d-dimer (30.1%) and C reactive protein (9.5%), abnormal chest radiographs (38%)
Mahmud et al. (88)	Prospective	• Dhaka, • Bangladesh • June–August 2020	• 355 patients • 148 females • 207 males • Mean age: 39.8	• 221 mild • 93 moderate 41 severe	Yes	Yes	Fever, cough, respiratory distress, anosmia, anorexia headache, lethargy	Telephonic interview	At least 30 days	46% of patients developed long-term symptoms. Post-viral fatigue (70%) was the most prevalent symptom. Post-COVID features are significantly higher among female
Martin et al. (89)	Retrospective	• USA • March–September 2020	9,989 patients	Mild- to severe	Yes	NR	NR	Electronic health records	90–180 days	Persistent neuropsychiatric, pulmonary, metabolic, and coagulopathic phenotypes
Pellaud et al. (90)	Retrospective	• Fribourg, Switzerland • March–April 2020	• 196 patients: • 77 females • 119 males • Mean age: 70	• Mild • Moderate • Severe/Critical	Yes	49 patients	NR	Data collected by electronic health records or by telephone	30 days	Among 117 patients discharged from hospital within 30 days after the beginning of symptoms, 63% reported persistent symptoms. The main persistent symptoms are asthenia (67%), respiratory symptoms (56%), anosmia/dysgeusia (10%)

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TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Petersen et al. (91)	Retrospective	• Tórshavn, Faroe Islands • 22 April–16 August 2020	• 180 patients • Mean age: 39.9 ± 19.4 • 98 females • 82 males	Mild-to-moderate	8 patients	No	Fatigue, fever, headache, chills, and loss of smell and taste	Questionnaire	125 days	53.1% reported persistence of at least one symptom, 33.3% reported one or two symptoms and 19.4% three or more symptoms. Most prevalent persistent symptoms: fatigue, loss of smell and taste, arthralgias
Raman et al. (92)	Prospective	Oxford, UK March–May 2020	• 58 patients: • 24 females • 34 males • Mean age: 55 ± 13	Moderate to-severe	Yes	21 patients	Fever, malaise, shortness of breath, cough, dysgeusia, anosmia, diarrhea, chest pain, headache, vomiting	MRI of the brain, lungs, heart, liver, kidneys, 6-minute walk (6MWT) test, spirometry, cardiopulmonary exercise test (CPET), questionnaires, blood tests	60–90 days	• 64% of patients experienced breathlessness and 55% fatigue. MRI, abnormalities in lungs (60%), heart (26%), liver (10%), and kidneys (29%). • Impaired cognitive performance and reduced six-minute walk distance
Rosales-Castillo et al. (93)	Retrospective	March–May 2020	• 118 patients: • 44.1 females • 55.9% male • Mean age: 60.16	Mild to-severe	Yes	7.6% of patients	Fever, cough, dyspnoea, diarrhea, ageusia, myalgia, anosmia, chest pain, headache, expectoration	Physician consultation	50 days	62.5% of patients reported persistence of symptoms: dyspnoea (31.4%), asthenia (30.5%), myalgia (13%), cough (5%), anosmia (1.7%), and ageusia (1%)
Saeed et al. (94)	Case-report	Lahore	• 48-years-old-woman • 42-years-old-woman • 32-years-old-woman • 37-years-old- woman	Mild	No	No	Dry cough, fever, abdominal discomfort and diarrhea	Dermatological consulting	60–90 days	Hair shedding: telogen effluvium

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TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Saiful Islam et al. (95)	Cross-sectional	• Bangladesh • September–October 2020	• 1,002 patients: • 422 females • 580 male • mean age = 34.7 ± 13.9	Mild to-severe	208 patients	NR	Fever and fatigue	Online questionnaire	30 days	20% of patients reported persistent symptoms. The most reported persistent symptoms were diarrhea (12.7%) and fatigue (11.5%). 48% of participants had moderate to severe depression
Smane et al. (96)	Retrospective	• Riga, Latvia • July 2020	• 30 children: • 13 females • 17 male • Mean age: 9.2	• 5 asymptomatic • 24 mild • 1 moderate	No	No	Fever, rhinorrhoea, cough	Physician assessment	101 days	70% patients completely free of any COVID-19-related symptoms, while 30% had at least one symptom (fever, joint pain, headache, anosmia, ageusia, microhaematuria)
Sofian et al. (97)	Case-series	• Arak, Iran • February–April 2020	• 10 patients • 9 females • 1 male	Mild-to moderate	No	No	Fever, dry cough, nasal congestion, weakness, high diaphoresis, loss of smell, fatigue	CT scan	60 days	Dry cough, headache, severe sweating, shivering, loss of smell, mild on/off fever, and diarrhea, weight loss
Stavem et al. (98)	Cross-sectional	• Lørenskog, Norway • Until 1 June 2020	• 451 patients: • 253 females • 198 males • Mean age: 49.7	Mild	No	No	Fever, loss of smell, headache, dry cough, myalgia, chills, dyspnea, sore throat, gastrointestinal manifestations	Mixed-mode survey	117 days	53 % of woman and 67 % of men with no persistent symptoms. Fatigue and dyspnoea are common about 60 days
Sykes et al. (99)	Retrospective	Hull, UK	• 134 patients: • 46 females • 88 males • Median age: 58	Mild to-severe	Yes	20% patients	Breathlessness, myalgia anxiety, fatigue, low mood, sleep disturbance	X-ray, standardized clinical assessment, questionnaires for dyspnea, and quality of life	113 days	86% of patients reported at least one residual symptom: breathlessness (60%), anxiety (47.8%), extreme fatigue (39.6%), low mood (37.3%), and sleep disturbance (35.1%). Females reported most residual symptoms including anxiety, fatigue, and myalgia

(Continued)

TABLE 1 | Continued

Family	Common name	Species	Chromosome number (2n)	Ploidy	1C (Mbp)	Glyphosate Resistant	TSR	NTSR	Genome	References
Taboada et al. (100)	Prospective	• Santiago, Spain • March–April 2020	• 91 patients: • 32 females • 59 males • Mean age: 65.5	Critical	Yes	All patients	Myalgia, asthenia, insomni, arthralgi, cough, anosmia, chest pain	Questionnaire	180 days	Decrease in quality of life in 67% of patients (56% mobility, 37% usual activities, 13% self-care, 48% pain/discomfort, 46% anxiety/depression). Dyspnoea on exertion (57%), asthaenia (37%), myalgia (37%), and arthralgia (29%). Only 16% of patients were completely free of persistent symptoms
Townsend et al. (101)	Retrospective	Dublin, Ireland	• 128 patients • 54% females • 46% males • Mean age: 49.5 ± 15	Mild-to critical	71 patients	18 patients	Fatigue	Chalder Fatigue Score (CFQ-11), markers of peripheral immune activation and circulating pro-inflammatory cytokines	72 days	52.3% of patients reported persistent fatigue. No association between fatigue and COVID-19 severity, laboratory markers of inflammation, pro-inflammatory molecules
Townsend et al. (102)	Cross-sectional	• Dublin, Ireland • March–May 2020	• 153 patients: • 57 females • 96 males • Median age: 48	Mild-Critical	Yes	19 patients	NR	X-ray	75 days	Persistent abnormal x-rays of either persistent infiltrate or atelectasis in 19% of patients. 62% patients had not returned to full health, while 47% met the case definition for fatigue
Varghese et al. (103)	Retrospective	• Münster, Germany • June–September 2020	• 116 patients • 17 females • 99 males • Mean age: 41	NR	10 patients	No	Cough, anosmia, fatigue, fever, myalgia, headache	Laboratory measurements, attending physicians document symptoms	22–102 days	• At 3 months of follow-up persisting symptoms were fatigue (54%), dyspnea (29%), and anosmia (25%), lymphopenia (12%) • Lymphopenia in the later follow-up range of 80–102 days
Ya-Wen An et al. (104)	Cross-sectional	• Guangdong, China • February–May 2020	• 46 patients: • 20 females • 26 males • Mean age: 46.8 ± 15.3	• 36 non-severe • 10 severe	Yes	Yes	Fever, weak blocked or watery nose haryngeal symptoms muscle or joint pain chest distress dizziness or headache gastrointestinal symptom	Blood routine, blood biochemistry, urine routine, stool routine, and chest CT scans	60 days	Extremely low outlier ratio of total protein, albumin, and globulin

However, because is a single case, this research does not provide conclusive evidence. A small cohort of critically ill COVID-19 patients also showed alteration in the diffusing capacity of the lung for carbon monoxide (D_{LCO}) for up to 3 months (20, 27, 38, 40). Persistent D_{LCO} impairment was also detected in non-critical COVID-19 patients that also presented shortness of breath and dyspnea up to 4 months after infection (18, 22, 33, 43). Unlike the above cited studies which analyzed exclusively critically ill patients and two case-reports that analyzed solely mild COVID-19 patients (30, 42) all other studies analyzed heterogeneous cohorts of patients, i.e., from mild to severe. Lower lung functions were detected in 246 mild-to-severe SARS-CoV-2 convalescents patients with few comorbidities up to 2 months after infection (37). Widespread lung damages in mild-to-severe COVID-19 patients were further confirmed by numerous papers and by an Editorial where it was underlined that "*months after infection with SARS-CoV-2, some people are still battling lung damage*" (108–110), with more than one-third of them that having pulmonary tissue death and visible scars up to 6 months after symptoms onset (18, 21, 26, 35, 109, 110). In a news feature article it was reported that these lung damages lessened with time, 88% of patients had visible damage up to 6 weeks after infection, but 2 months after symptom onset this number had fallen to 56% (109). By examining retrospectively a cohort of 158 mild-to-severe COVID-19 patients, it was shown that these persistent pulmonary damages were also associated with a persistent elevation of IL-6 up to 2 months after infection (29). At the same follow-up, Chun et al., evaluating 61 prevalently non-critical COVID-19 patients, highlighted also higher levels of Lipocalin 2, suggesting that COVID-19 patients may have an ongoing neutrophil activation that could be amenable to targeted therapy (19). Sonnweber et al., evaluating a cohort of 109 patients with mild-to-critical COVID-19, showed that severe lung pathologies were also significantly associated with persisting hyperferritinemia that was present in ∼38% of patients (35). Other authors evaluated the lung abnormalities by CT scans at different stages of SARS-CoV-2 infection (21, 26). Ding et al., analyzing retrospectively a cohort of 112 COVID-19 patients at different stages of the disease, showed that the frequency of crazy-paving pattern, consolidation, and linear opacities peaked at 10–14 days (62.7%), 15–21 days (75.0%), and at 22–28 days (83.1%) and decreased thereafter (21). However, at more than 28 days of follow-up 98.1% of CT scans still showed abnormalities. Similarly, Hu et al., evaluating 46 patients with mild-to-severe COVID-19 who had an isolated pulmonary lesion on the first positive CT, highlighted the presence of reticular patterns from the 14 days after symptoms onset in 45% of patients. At 22–31 days, the lesions were completely absorbed only in 28.57% (26). Mo et al. also noted pulmonary anomalies in a cohort of 110 discharged COVID-19 cases, 24 mild cases, 67 cases of pneumonia and 19 cases of severe pneumonia (31). The duration from onset of disease to pulmonary function test was 20 ± 6 days in mild cases, 29 ± 8 days in pneumonia cases and 34 ± 7 days in cases with severe pneumonia (110). Anomalies were noted in D_{LCO} (47.2%), total lung capacity (25.0%), forced expiratory volume in 1 s (FEV1) (13.6%), forced vital capacity (FVC) (9.1%), FEV1/FVC (4.5%), and small airway function (7.3%) (31).

Persistent Neurological Symptoms and Olfactory Dysfunctions

Despite SARS-CoV-2 primarily affecting lungs, numerous data supported the neuro-invading potential of SARS-CoV-2 and, according to the first-hand evidence by Mao et al., ∼36.4% of COVID-19 patients presented neurological symptoms (5, 111). Additionally, conditions such as hypoxia, encephalitis, and stroke, all present in severe COVID-19 patients, can produce both long-term neurological symptoms and permanent neurocognitive impairment (52, 58, 112, 113). In fact, a case-series by Negrini et al. associated the long-term neurological symptoms and general cognitive decay to the length of stay in the ICU (58). Despite the long-term neurological symptoms and the general cognitive decay being associated to severe/critical COVID-19 patients, in this review we did not find any studies based solely on critically/severely ill patients. On the other hand, we found a retrospective study and several case reports on mild COVID-19 patients (51, 53, 54, 65). Gallus et al., evaluating retrospectively 48 mild COVID-19 patients, underlined that 8.3% patients reported hearing loss, 4.2% tinnitus, 8.3% dizziness, 2% spinning vertigo, 2% dynamic imbalance, and 6.3% static imbalance at about 1 month of follow-up (51). Several anecdotal reports in mild COVID-19 patients also detected persistent deficits in memory and psychotic symptoms during up to 5 months of follow-up (53, 54, 65). In addition to these studies, all the others found in this review analyzed heterogenic populations of patients with COVID-19, from mild to severe. In this context, a recent editorial and a systematic review provided a detailed overview into the spectrum of mental disorders that can occur during the intermediate and long-term phases of COVID-19 in mild-to-critical patients (114, 115). The most frequent neurological long-term symptoms in these patients were myalgia, arthralgia, sleeping troubles, and headache (46, 50, 61, 116). Additionally, a general cognitive decay, i.e., deficit in attention and calculation, short-term memory, constructional apraxia, and written language, was also observed during up to 6 months of follow-up (61). At 2 months of follow-up 58.7% of 179 mild-to-severe COVID-19 patients presented a moderate neurocognitive decline while 39.1% of patients also showed psychiatric morbidity (56). At a longer follow-up of 6 months, Pilotto et al., analyzing retrospectively 165 moderate-to-severe COVID-19 patients, showed that these long-term symptoms persisted in about 37% of patients (61). Also, symptoms consistent with orthostatic hypoperfusion syndrome and painful small fiber neuropathy were reported at short (3 weeks) and long (up to 3 months) follow-ups in two case-reports and in a small case-series (48, 59, 63). In a "*Long-Haul COVID*" communication, Nath et al., summarizing symptoms reported after mild-to-severe COVID-19, also highlighted persistent symptoms that overlapped with those patients with myalgic encephalomyelitis/chronic fatigue syndrome (117). In addition to the long-term neurological symptoms Lu et al. prospectively examined the presence of brain micro-structural changes in 60 mild-to-critical COVID-19

patients reporting presence of alterations in 50% of recovered patients after 3 months (55). Anecdotal evidence also showed the presence of long-term impairment of the brain structures in two COVID-19 patients highlighting hypometabolism of the olfactory/rectus gyrus in both patients (52).

Since SARS-CoV-2 can affect neuronal cells by both direct and indirect mechanisms, this can lead to various neurological manifestations also including anosmia and hypogeusia. Anosmia and hypogeusia are present both in mild/moderate cases and in severe cases of COVID-19 (45, 47, 49, 57, 60, 62, 64, 66–68, 118–122). As long-term COVID-19 symptoms, anosmia, and hypogeusia were evaluated in severe COVID-19 patients only in one protective study (67). The study evaluated 138 COVID-19 patients at 2 months of follow-up showing that 5.8% of patients had moderate to severe olfactory dysfunction, while 4.3% had a significant taste disorder (67). A greater number of studies evaluated olfactory and gustatory disfunctions in mild COVID-19 patients (45, 47, 49, 64, 66, 68, 118). Using a retrospective questionnaire Fjaeldstad evaluated olfactory and gustatory loss in 109 mild COVID-19 patients (49). At ∼1 month after symptoms onset since the chemosensory loss, participants reported relatively low recovery and improvement rates. For participants with olfactory loss, only 44% were fully recovered, whereas 28% had not yet experienced any improvement of symptoms (49). After gustatory loss, 50% had fully recovered, whereas 20% had not yet experienced any improvement. At a longer follow-up of 2 months after symptoms onset, Otte et al. evaluating through a questionnaire 91 mild COVID-19 patients for olfactory function, showed that 45.1% of patients were hyposmic while 53.8% showed an olfactory performance within the normal range (118). In the same way, at 2 months of follow-up, Boscolo-Rizzo et al. evaluated prospectively 183 mildly symptomatic COVID-19 patients showing that 18.6% presented altered sense of smell or taste (45). Interestingly, Ugurlu et al. in a cohort of mild COVID-19 patients showed persistent olfactory dysfunction in 14.3% of patients up to 3 months after symptoms onset (66). At the same follow-up, a long-term anosmia was also reported in a case-report of a 40-year-old woman with a mild COVID-19 diagnosis (64). Differently, other studies analyzing mild and asymptomatic COVID-19 patients for smell and taste disturbance reported resolution of anosmia up to 1 month after diagnosis (47, 60). Comparable results were also reported at the same follow-up by Konstantinidis et al. evaluating mild/moderate COVID-19 patients (119). Finally, Paolo et al., analyzing 75 mild-to moderate COVID-19 patients through a questionnaire reported olfactory and dysgeusia recovery within an average of 17 days, also finding a significantly decrease in viral load (120).

Finally, other studies evaluating heterogenous populations of mild-to-severe COVID-19 patients further confirmed persistent loss of smell up to 6 months after symptom onset (57, 61, 62, 64, 121, 122). Moein et al. in a prospective study on 82 mild-to-severe COVID-19 patients showed smell loss in ∼37% of patients at 2 months of follow-up (57). At the same follow-up, a prospective study on 138 patients and a retrospective study on 90 mild-to-severe patients showed persistent hyposmia in 5–8% of patients (57, 67). Lastly, Pilotto et al., by examining retrospectively 165 patients detected the presence of hyposmia in ∼15% of patients at up to 6 months of follow-up (61).

Widespread Persistent Symptoms

Numerous research groups reported widespread persisting symptoms in COVID-19 patients for up to 6 months after SARS-CoV-2 infection (70, 75, 123–142). They also described practice on the management of post-acute COVID-19 and performed comprehensive analyses of health-related quality of life (70, 75, 123). Furthermore, numerous editorials, reviews, news articles, clinical updates, narrative interviews, and focus groups have been published to explore what it is like to live with long-term COVID-19, also trying to emphasize the putative pathophysiology, risk factors, and treatments (124–142). Two cohort studies on severe/critical COVID-19 patients reported persistent physiological impairment and decrease in quality of life in more than half of the patients at up to 6 months of follow up (77, 100). Taboada et al. showed that at 6 months of follow-up only 16% of patients were completely free of persistent symptoms (100). However, in a Multistate Health Care Systems Network, Tenforde et al. reported that among 270 interviews conducted on COVID-19 patients, also among persons with milder outpatient illness, 14–21 days after symptoms onset, the 35% of patients had not returned to their usual state of health (143). In this context, Pellaud et al., examining the outcomes of 196 consecutively mild-moderate COVID-19 patients, 1 month after onset of symptoms, showed that among the 60% of patients that returned home, 63% reported persistent symptoms (90). Two months after symptom onset, evaluating 150 mild/moderate COVID-19 patients, Carvalho-Schneider et al. showed that about 66% of patients presented at least one symptom (74). Similarly, evaluating the long-term COVID-19 symptoms in 233 mild COVID-19 patients Cirulli et al. highlighted that ∼24% of patients had at least one symptom also after 3 months (76). These results were also confirmed by an online survey of doctors conducted by the *British Medical Association* (144). They reported that of 3,729 doctors who answered a question about patients' persistent symptoms after COVID-19, a third said that they had seen or treated patients with long-term COVID-19 symptoms (144). Davido et al. also reported that since mid-May they evaluated an average of 30 individuals per week for whom COVID-19 symptoms have not completely subsided, essentially young women (sex *ratio* 4:1) around 40 years old with no relevant medical history (145–147). Additionally, it was reported that female sex (mean age 47.22) is also a risk factor for poor health-related quality of life in Chinese COVID-19 patients (75). Also, Sudre et al. analyzing 4,182 incident cases of non-severe COVID-19 who logged their symptoms prospectively in the COVID-19 Symptom Study App showed that women aged 50–60 were at greatest risk of developing "long-COVID" (148). Patients described symptoms in every part of the body which were sometimes severe or fluctuating (149, 150). Paul Garner, a professor at Liverpool School of Tropical Medicine and Co-ordinating Editor of the Cochrane Infectious Diseases Group, wrote on the 95th day after symptoms onset in the *British Medical Journal Opinion* (151). He said "*I am unable to be out of bed for more than*

three hours at a stretch…I have ringing in the ears, intermittent brain fog, palpitations, and dramatic mood swings" (151). Other people also described similar complaints in the same journal (152–154). The science journalist Linda Geddes also discussed data from the Irish Centre for Vascular Biology in Dublin that reported COVID-19 patients being discharged from hospital, only to return several weeks later not only with widespread symptoms but also with deep vein thrombosis or blood clots on the lungs (155).

The main widespread reported long-term symptoms in COVID-19 patients were chronic fatigue, dyspnea, shortness of breath, chest pains, headache, loss of smell/taste, muscle, and joint pain, followed by depression, anxiety, insomnia, and itchy body, heart palpitations, tachycardia, anorexia, tingling fingertips, and brain fog (69, 70, 72, 77, 84, 85, 87, 91, 97, 98, 101, 103, 123, 138, 145–147, 150, 156–159). However, it was reported that the number of widespread long-term symptoms were higher for COVID-19 patients who were initially more ill (77, 100). D'Cruz et al. and Taboada et al., analyzing prospectively two cohorts of 119 and 91 severe/critical COVID-19 patients, respectively, showed the presence of dyspnoea on exertion (57%), asthaenia (37%), myalgia (37%), and arthralgia (29%) up to 2 months after symptoms onset and a general decrease in quality of life (mobility, usual activities, self-care, pain/discomfort, anxiety/depression) in 67% of patients at up to 6 months of follow-up (77, 100). However, these widespread long-term symptoms were not only present in severe COVID-19 patients, but also in patients who had mild and moderate disease (72, 76, 80, 94, 98, 146, 159). Carvalho-Schneider et al., in a prospective study on 150 mild/moderate COVID-19 patients at 2 months of follow-up, highlighted dyspnea and asthenia, respectively, in 30 and 40% of patients (74, 98). Similar results were also obtained in a cross-sectional study on 451 mild COVID-19 patients (98). In addition to these symptoms in a prospective study by Cirulli et al. symptoms such as difficulty concentrating, fatigue, memory loss, confusion, headache, heart palpitations, chest pain, pain with deep breaths, dizziness, and tachycardia were detected at 3 months of follow up (76). Fatigue, dyspnea, and heart dysfunctions in mild COVID-19 patients were also reported in several case-reports (69, 72, 80) at up to 8 months of follow-up. In addition, a case-report on three women also reported telogen effluvium, temporary hair shedding, as a long-term COVID-19 symptom 3 months after getting the infection (94). Several studies analyzing all together mild-to-severe COVID-19 patients also confirmed these long-term widespread symptoms (73, 78–81, 83, 88, 89, 92, 93, 95, 99, 102–104, 160–162). In a large cohort of 355 mild-to-severe COVID-19 patients Mahmud et al. detected that about 46% of patients developed long-term symptoms at 1 month of follow-up and that post-COVID features were significantly higher among the female gender with fatigue as the main long-term symptom (88). Similarly, persistent fatigue was also reported in about 12% of patients by examining a cohort of 1,002 mild-to-severe COVID-19 patients (95). At a longer follow-up, Rosales-Castillo et al. and Townsend et al. confirmed persistence of fatigue as the main long-term symptom in a cohort of mild-to-severe COVID-19 patients (93, 102). Banda et al., analyzing 150 *tweets* from moderate-to-severe COVID-19 patients, reported that the 10 most commonly long-term symptoms after COVID-19 were chronic fatigue (62%), dyspnea (19%), tachycardia/palpitations (13%), chest pain (13%), sleep disorders (10%), cough (9%), headache (7%), and joint pain, fever, and unspecified pain by 6% each (160). This analysis also matches clinician-collected data reported by an Italian study (73). The study followed 143 hospitalized mild-to-severe patients who were discharged from the hospital after COVID-19 and that had two negative test results for SARS-CoV-2 (73). At an average of 2 months after initial onset of symptoms, "*only 12.6% were completely free of any COVID-19-related symptom, while 32% had 1 or 2 symptoms and 55% had 3 or more*" (73). Also, in this case the most common symptoms were chronic fatigue (53.1%), dyspnea (43.4%), joint pain (27.3%), and chest pain (21.7%) (73). Authors also observed that individuals who had an initial symptom of dyspnea were more likely to develop long-term symptoms (73). These results were also confirmed by a case-control study that examined 141 mild-to-moderate COVID-19 patients and 78 controls at 2 months of follow-up (79). At the same follow-up a retrospective study on 206 mild-to-moderate COVID-19 patients also detected chronic pain in ∼40% of the patients (78). A particular cross-sectional study on 46 mild-to-severe COVID-19 patients also described an extremely low outlier *ratio* of total protein, albumin, and globulin at 2 months of follow-up, underlying a persistent abnormal liver function (104). At the same follow-up lymphopenia, elevated D-dimer, and C reactive protein were also detected and associated to persistent fatigue, dyspnea, and anosmia (87, 103). Fatigue and dyspnea were also the two most prevalent persistent symptoms 3 months after a SARS-CoV-2 infection in hospitalized and non-hospitalized patients (83, 92, 103, 161). Furthermore, at the same follow-up, Raman et al. also reported abnormalities in heart (26%), liver (10%), and kidneys (29%) (92). Dyspnea (42%), associated with chronic fatigue (55%), loss of memory (34%), concentration and sleep disorders (28 and 30.8%, respectively), was likewise reported in 120 COVID-19 patients (relatively non-severe) analyzed by questionnaire, 100 days after initial symptoms onset (162). In was also shown that these long-term symptoms persisted for up to 6 months, with fatigue or muscle weakness and sleep difficulties as the most common symptoms (81, 99). At 6 months, by examining 9,989 mild-to-severe COVID-19 patients, persistent neuropsychiatric, pulmonary, metabolic, and coagulopathic phenotypes were also reported (89).

Recent data reported several of these widespread long-term symptoms, i.e., fatigue, dyspnea, chest pains, muscle and joint pain, headache, insomnia, and palpitations, also in children and adolescent up to 6–8 months of follow-up (71, 86, 96). At 6 months of follow-up high blood pressure levels and persistence of a prehypertension were also detected in ∼13% of mild COVID-19 children (82). Examining a larger cohort of children, it was also described that ∼53–70% of these patients had at least one symptom 100 days or more after COVID-19 diagnosis (71, 82, 96, 163). Given these emerging data, recently, Hertting et al. in an editorial on *Acta Paediatrica* underlined the need to have more research and studies on the long-term effects of COVID-19 in children and adolescents (164).

DISCUSSION

Although we are aware that there are no long-term data on large numbers of COVID-19 patients with persistent symptoms and with comparison groups, and that an analysis in a field as engaging as COVID-19 can never be updated, this review allowed us to outline that a noteworthy number of patients present long-lasting *sequelae*, up to 6 months, in the post-COVID time. These long-term symptoms are not only present in severe COVID-19, but also in mild and moderate patients. In addition, recent preliminary data also underlined the presence of long-term COVID-19 symptoms on children and adolescents. Some clinical studies and survey questionnaires also highlighted a potential high-risk factor for long-term COVID-19 in the female gender; women's risk of developing long-term COVID-19 seems to be double that of men among patients aged between 40 and 50. After the age of 60 the risk level of long-term COVID between male and female should become similar. This pattern appears to be like that of autoimmune diseases that are more common in female through menopause to become similar between male and female after age 60 (165). Thus, it is possible that these gender differences, as well as other aspects of the disease, may be due to a different immune system response during and after COVID-19. However, currently, it is not yet clear whether this data reflects the population of people with long-term COVID-19 and which is the full spectrum of the duration and severity of long-term symptoms in these patients.

What emerges from this review is that the most common reported symptoms after COVID-19 are abnormal lung functions prevalently with persistent dyspnea, general neurological decay, smell and taste disturbances, and chronic fatigue. Other common symptoms include joint pain and chest pain. These symptoms may linger or recur for weeks or months following initial recovery. In detail, for patients with mild-to-moderate COVID-19 the more common long-term symptoms are chronic fatigue, anosmia/ageusia, dyspnea, but also difficulty in concentration, memory loss, and confusion. These symptoms seem to be present in a higher percentage of patients who were initially more ill. In critical-to-severe COVID-19 patients' supplementary long-term symptoms are lung fibrotic-like changes up to 6 months after infection and a high reduction in diffusing capacity of the lung for D_{LCO} that frequently required oxygen uses also after hospital discharge. Likewise, the general cognitive decay, despite also being present in mild-to-moderate COVID-19 patients, also appears to be more closely related to critical-to-severe forms of COVID-19. Considering the whole overview of widespread long-term symptoms reported in this review the one undeniably most prevalent in mild-to-critical COVID-19 patients is chronic fatigue. This is in line with past research that highlighted high levels of post-infectious fatigue for survivors of epidemics such as SARS and Ebolavirus (166, 167). Moreover, fatigue has been related with infections, such as mononucleosis, that occur outside of an epidemic or pandemic scale (167). Currently, it is not clear why chronic fatigue and the other long-term complications persist in some COVID-19 patients. However, most researchers and clinicians agree that the long-term COVID-19 symptoms are associated with the coronavirus' ability to trigger a massive inflammatory response. Thus, it will be mandatory to analyze cytokine networks in patients who recover from COVID-19 to evaluate whether the "cytokine storm" present during the disease persists and contributes to these long-term complications.

The main strength of this study is that it highlights multiple long-term symptoms which may hinder return to pre-COVID-19 infection functional status. However, despite this finding a weakness of our review was that while some studies included in this review focused on a single population of infected COVID-19 patients, i.e., mild/moderate and severe/critical, numerous studies included heterogeneous populations, from mild to critical, not taking into account disease severity as well as preexisting co-morbidities, treatment regimens, mean ages, gender, and other aspects. This bias can lead to alterations in the data evaluation and analysis, which potentially affect the results. Data from prospective designs, developed by evaluating homogeneous populations of COVID-19 patients able to consider their characteristics prior to and during infection, might provide new and detailed information into predisposing factors that lead to long-term COVID-19 symptoms. Another bias that should be considered is that despite the fact that in some studies the long-term COVID-19 *sequelae* were evaluated through clinical visits and/or specific instrumental analyzes, many others have used self-administered questionnaires and scores, telephone/online interview, and phone applications. This is because, to date, assessing the patient in the hospital is difficult due to the entry restrictions into the COVID-19 departments. On the other hand, checking and evaluating them at home presents almost insurmountable logistical problems during an emergency health situation like the one we are facing. However, this type of self-assessment highlights bias in the detection of symptoms as patients may have psychological and emotional involvement due to the disease itself.

At this stage, a detailed analysis and understanding of all the aspects associated with long-term COVID-19 are mandatory to mitigate against the potential persistent symptoms identified in the current review. Future studies should assess: (1) the full range of disorders associated with COVID-19 and their long-term manifestations; (2) the underlying associations between viral spread, associated pro-inflammatory changes, and long-term disease pathogenesis; (3) the duration and extent of long-term symptoms in relation to the resolution of the disease; (4) the association between disease severity and long-term dysfunctions; (5) the effect of specific antiviral therapies and/or interventions on long-term symptoms; (6) why symptoms persist or recur; and (7) the potential late effects of COVID-19 on children/adolescents. Another important point that should be assessed is SARS-CoV-2 levels (detection, load) in patients and how this relates to long-term symptoms. To date, it is not clear whether the initial viral load, *per se*, may meaningfully impact long-term symptoms, particularly in mild-to-moderate COVID-19 patients. Information relating to SARS-CoV-2 detection and viral load at different time points of infection will help the clinical interpretation of long-term symptoms of COVID-19. Similarly, there is a need for further studies to provide robust data on the association between viral shedding and long-term COVID-19. Despite has reports that the median

duration of viral shedding goes from 12-to-20 days, there is evidence that ongoing viral shedding in SARS-CoV-2 may be prolonged in the feces compared to respiratory secretions (168–170). The persistent fragments of viral genes, though not infectious, may still be triggering a violent immune overreaction that could explain the symptoms persistence in COVID-19-free patients. Alternatively, even if the virus is cleared, the immune system could continue to be overactive or perturbed, analogous to the long-term debilitation after glandular fever (165). A greater understanding of these last points could improve the knowledge not only of the causes of long-term symptoms but also on the immune system involvement and on transmission risk.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FS and MF designed the review. FS and FV performed the literature search and collected and assembled the data. FS, FV, and MF analyzed the obtained articles. FS, FV, ML, and MF wrote the paper. MF, LM, and ML revised the manuscript critically. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.653516/full#supplementary-material

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Prediction of Re-positivity for Coronavirus Nucleic Acid Among COVID-19 Patients in the Recovery Phase

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Background and Objectives: Although the pathogenesis and treatment of coronavirus disease 2019 (COVID-19) have been gradually revealed, the risk for re-emergence of coronavirus nucleic acids in recovered patients remains poorly understood. Hence, this study evaluated the risk predictors associated with re-positivity for virus nucleic acid.

Methods: Between February 1 and March 20, 2020, we retrospectively reviewed the clinical epidemiological data of 129 COVID-19 patients who were treated at Zhongxiang People's Hospital of Hubei Province in China. Subsequently, a risk prediction model for the re-positivity of virus nucleic acid was developed, and a receiver operating characteristic (ROC) curve was drawn for further validation.

Results: In this study, the rate of re-positivity for virus nucleic acid was 17.8% (23/129) where all re-positivity cases were asymptomatic. The median time interval from nucleic acid re-positivity to discharge after being cured again was 11.5 days (range: 7–23 days). Multivariate logistic regression analysis showed that leukocytopenia [odds ratio (OR) 7.316, 95% confidence interval (CI) 2.319–23.080, $p = 0.001$], prealbumin $<$ 150 mg/L (OR 4.199, 95% CI 1.461–12.071, $p = 0.008$), and hyperpyrexia (body temperature $>39^{\circ}$C, OR 4.643, 95% CI 1.426–15.117, $p = 0.011$) were independent risk factors associated with re-positivity. The area under the ROC curve was 0.815 (95% CI, 0.729–0.902).

Conclusion: COVID-19 patients with leukocytopenia, low prealbumin level, and hyperpyrexia are more likely to test positive for virus nucleic acid after discharge. Timely and effective treatment and appropriate extension of hospital stays and quarantine periods may be feasible strategies for managing such patients.

Keywords: COVID-19, re-positivity, virus nucleic acid, risk predication, re-emergence of coronavirus nucleic acids

INTRODUCTION

In early December 2019, the first case of unexplained coronavirus pneumonia was reported in Wuhan, China (1), which was followed by an outbreak worldwide. In January 2020, Ren et al. (2) led the completion of whole-genome sequencing of the coronavirus and confirmed a homology of more than 85% with bat severe acute respiratory syndrome (SARS)-like coronavirus (bat-SL-CoVZC45). Therefore, the International Virus Classification Committee (ITCV) named it as SARS coronavirus 2 (SARS-CoV-2), which was then officially named as coronavirus disease 2019 (COVID-19) (3). With increasing research being conducted concerning pathogen transmission and mechanisms (4, 5), continuous update of guidelines on treatment and diagnosis (6, 7), and its prevalence has now been controlled and effectively mitigated in China.

Serious dangers concerning the frequent emergence of test re-positivity of virus nucleic acid in recovered COVID-19 patients have been a widespread concern (8–10). Some studies revealed that this rate ranges from 3.3 to 30.8% (9–13). Yuan et al. (13) reported that young patients (<18 years old) had much higher re-positivity rates (30.8%) than those aged ≥18 years (9.5%). However, its mechanism remains unclear and necessitates further research. Of note, most re-positive patients do not show infectivity, which excludes the possibility of simple viral relapse or secondary infection (13–15). A few recent studies have proposed that virology, the detection of specimens, or the patient' s condition might be potential reasons for test re-positivity of virus nucleic acid (16–18).

In this study, we retrospectively analyzed clinical epidemiological data of 129 COVID-19 patients, and evaluated the risk factors associated with re-positivity for virus nucleic acid. Similarly, prompt and effective treatment and appropriate extension of hospitalization and quarantine period may be feasible strategies for patient management.

MATERIALS AND METHODS

Patients

This retrospective study, which complied with the Declaration of Helsinki, was approved by the Ethics Committee of Zhongxiang People's Hospital (ZXRY20200420) and the Ethics Committee of The Affiliated Hospital of Inner Mongolia Medical University (WZ 2020033).

Based on the novel coronavirus pneumonia diagnosis and treatment scheme (Trial Version 4), COVID-19 patients do not only require a clear epidemiological history and clinical manifestations but also must meet at least one of the following conditions: (1) positivity for coronavirus nucleic acid on real-time fluorescent reverse transcription-polymerase chain reaction (RT-PCR); (2) a viral gene sequence showing high homology with the novel coronavirus; and (3) positivity for serum novel coronavirus-specific IgM and IgG antibodies. Furthermore, exclusion criteria were as follows: death due to COVID-19 ($n = 1$) or other diseases [acute cardiovascular disease ($n = 2$), acute renal failure ($n = 1$)], loss to follow-up after being transferred to another hospital ($n = 1$), and serious loss of clinical data ($n = 1$). Finally, 129 COVID-19 cases were included in our study (**Figure 1**).

Study Design

Between February 1 and March 20, 2020, we retrospectively analyzed the demographic, clinical, and epidemiological data of 129 COVID-19 patients admitted to our institution. Laboratory findings, radiological results, and therapy course were independently obtained from a prospectively maintained database in Zhongxiang People's Hospital.

Patients were divided into two groups: those with re-positivity ($n = 23$) and without re-positivity ($n = 106$) for the virus nucleic acid group. The differences in sex, age, comorbidities, white blood cell count, body temperature, and prealbumin levels between the two groups were compared, and a risk prediction model was established via multivariate analysis. A receiver operating characteristic (ROC) curve was drawn to validate the model.

Chest computed tomography (CT) results were reviewed by two physicians (Shu-fen Zhu, Pei-lin Duan) and a radiologist (Jin-kuang Li). Leukopenia was defined as a white blood cell count $< 4 \times 10^9$/L. Hyperpyrexia was defined as body temperature above 39°C. The follow-up outcomes were collected through electronic medical records or telephone interviews by referral physicians or patients with a deadline of until March 20, 2020.

SARS-CoV-2 Nucleic Acid Testing

RT-PCR was used to detect novel coronavirus nucleic acids. The detection equipment were GeneRotex96 automatic nucleic acid extraction and Gentier96E real-time fluorescent quantitative PCR instruments (Tianlong Technology Co., Ltd., Xi'an, China). The extraction reagent used was the virus DNA/RNA extraction kit (magnetic beads method) (Tianlong Technology Co., Ltd., Xi'an, China). The detection reagent was the SARS-CoV-2 nucleic acid detection kit (PCR probe method) (Da'AN Gene Co.,

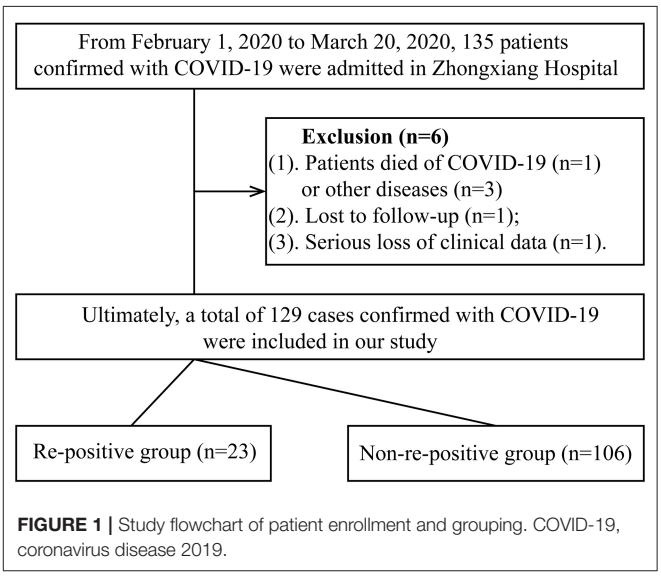

FIGURE 1 | Study flowchart of patient enrollment and grouping. COVID-19, coronavirus disease 2019.

Ltd., Zhongshan, China and Shengxiang Biotechnology Co., Ltd., Hunan, China). The target genes were the ORF1ab and N genes in the SARS-CoV-2 genome, and the positive reading criteria were described in the reagent kit packaging. Specimen sampling, nucleic acid preparation, and amplification were performed strictly using the kit instructions. In case of suspicious results, re-sampling and review were required.

Clinical Cure Standards for COVID-19 and Re-positivity of Nucleic Acid and Serum Antibody

The standard clinical cure for COVID-19 patients was in reference to the Novel Coronavirus Pneumonia Diagnosis and Treatment Scheme (Trial Version 4): (1) normal body temperature for more than 3 days; (2) significant improvement of respiratory symptoms; (3) notable absorption of inflammation on pulmonary imaging; and (4) two consecutive negative for SARS-CoV-2 nucleic acid tests (sampling interval of at least 24 h). When the patients met these criteria, they were quarantined in a designated area to continue isolation in observation and rehabilitation treatment for at least 14 days. During this period, upper respiratory specimens and blood specimens were collected for a SARS-CoV-2 nucleic acid and serum antibody test on day 14.

Statistical Analyses

Continuous variables were summarized as mean ± standard deviation (SD) or median plus interquartile range (IQR), and categorical variables were expressed as frequencies and percentages. In the univariate analysis, continuous and categorical variables were assessed via Student's *t*-test (for those

TABLE 1 | Clinical epidemiological characteristics and a comparative analysis between the re-positivity and non-re-positivity group.

	Total ($n = 129$)	Re-positivity ($n = 23$)	Non-re-positivity (106)	*P*-value
Age (years), mean ± SD	51.6 ± 14.0	48.9 ± 10.1	52.2 ± 14.7	0.299
Sex, male, *n* (%)	69 (53.5)	11 (47.8)	58 (54.9)	0.176
Comorbidities, *n* (%)				
Diabetes	9 (7.0)	0 (0.0)	9 (8.5)	0.361
Hypertension	16 (12.4)	4 (17.4)	12 (11.3)	0.485
CCD	9 (7.0)	1 (4.3)	8 (7.5)	0.585
CRD	6 (4.7)	1 (4.3)	5 (4.7)	0.939
CKD	7 (5.4)	1 (4.3)	6 (5.7)	0.801
Malignant diseases	5 (3.9)	1 (4.3)	4 (3.8)	0.897
Antibiotics, *n* (%)	92 (71.3)	19 (82.8)	73 (68.9)	0.187
Glucocorticoid, *n* (%)	40 (31.0)	6 (26.1)	34 (32.1)	0.629
Bilateral pneumonia, *n* (%)	83 (64.3)	19 (82.6)	64 (60.4)	0.044
Fever, *n* (%)	87 (67.4)	18 (78.3)	69 (65.1)	0.222
37.3–38.0°C	35 (27.1)	5 (21.7)	30 (28.3)	0.521
38.1–39.0°C	28 (21.7)	4 (17.4)	24 (22.6)	0.782
>39.0°C	24 (18.6)	9 (39.1)	15 (14.2)	0.014
Leukocyte($\times 10^9$/L), mean ± SD	4.9 ± 2.0	3.4 ± 0.9	5.3 ± 2.1	< 0.001
Leukopenia, *n* (%)	54 (41.9)	18 (78.3)	36 (34.0)	< 0.001
Neutrophil ratio (%), mean ± SD	65.8 ± 12.3	68.0 ± 7.8	65.3 ± 13.1	0.207
Lymphocyte ratio (%), mean ± SD	23.7 ± 11.0	21.0 ± 8.1	24.2 ± 11.5	0.121
Monocyte ratio (%), mean ± SD	6.3 ± 3.5	7.2 ± 3.2	6.1 ± 3.5	0.163
CRP (mg/L), mean ± SD	33.5 ± 24.5	29.0 ± 13.7	34.9 ± 26.8	0.168
ESR (mm/H), mean ± SD	28.8 ± 29.4	28.9 ± 37.5	28.8 ± 27.5	0.981
PCT (ng/ml), mean ± SD	0.5 ± 0.6	0.3 ± 0.2	0.5 ± 0.7	0.112
LDH (U/L), mean ± SD	215.7 ± 86.3	207.2 ± 48.6	217.3 ± 92.0	0.632
Albumin (g/L), mean ± SD	41.4 ± 21.6	39.7 ± 5.8	41.7 ± 23.7	0.685
Prealbumin (mg/dl), mean ± SD	21.9 ± 11.8	16.1 ± 5.5	23.2 ± 12.4	< 0.001
Prealbumin < 15mg/dl, *n* (%)	38 (29.5)	13 (56.5)	25 (23.6)	0.002
ALT (U/L), mean ± SD	93.3 ± 105.9	76.2 ± 62.0	96.7 ± 112.4	0.430
AST (U/L), mean ± SD	13.6 ± 11.3	14.7 ± 9.4	13.4 ± 11.7	0.620
CK (U/L), mean ± SD	27.3 ± 20.1	28.5 ± 20.6	27.0 ± 20.0	0.747

SD, standard deviation; CCD, chronic cardiovascular disease; CRD, chronic respiratory disease; CKD, chronic kidney disease; ESR, erythrocyte sedimentation rate.
Leukocyte (normal range 4–10); neutrophil ratio (normal range 50–70); lymphocyte ratio (normal range 20–40); monocyte ratio (normal range 3–8); CRP (C-reactive protein; normal range < 4.0); ESR (normal range 0–20); PCT (procalcitonin; normal range 0–0.5); LDH (lactate dehydrogenase; normal range 109–245); prealbumin (normal range 15–40); albumin (normal range 35–55); ALT and AST (alanine aminotransferase and Alanine aminotransferase; normal range 0–40); CK (creatinine kinase; normal range 18–198).

with a normal distribution) or Wilcoxon signed-rank test (for those with an abnormal distribution) and chi-squared test, respectively. A $p < 0.05$ was considered statistically significant.

Binary logistic regression was performed to develop the risk prediction model for re-positivity of SARS-CoV-2 nucleic acid, and the ROC curve was used to validate the model. The Statistical Package for the Social Sciences (SPSS, version 23.0; IBM Corp., Armonk, NY, USA) was used for data analysis.

RESULTS

Epidemiological History of COVID-19 Patients

A total of 129 COVID-19 patients were retrospectively analyzed. The following findings were gathered: 65 cases (51.9%) with travel or living histories in or around the Wuhan epidemic area; 42 cases (32.6%) with clear contact histories with COVID-19 patients; 10 cases (7.8%) living with COVID-19 patients in the same building, but denying a history of contact; five cases (3.9%) working in the same company as a COVID-19 patient; and five cases (3.9%) with no clear history of contact. Hypertension was found in 16 cases, diabetes in nine cases, chronic cardiovascular disease in nine cases, chronic kidney disease in seven cases, chronic respiratory diseases in six cases [bronchial asthma ($n = 1$), bronchiectasis ($n = 1$), chronic bronchitis ($n = 1$), chronic obstructive pulmonary disease ($n = 3$)], and malignant diseases in five cases (**Table 1**). The time interval from discharge to re-positivity for coronavirus nucleic acid and serum antibody was 14 days.

Demographic and Clinical Characteristics of COVID-19 Patients

All 129 patients were diagnosed with COVID-19 based on a positive nasopharyngeal swab nucleic acid test. The rate of re-positivity for virus nucleic acid was 17.8% (23/129) with a mean age of 51.5 ± 14.2 years. The proportion of male (54.3%) and female (45.7%) cases was approximately equal. Pneumonia, suggested by radiography, was detected in 109 cases (84.5%), where 80 (62.0%) of them presented with bilateral pneumonia. There were 87 cases (67.4%) with fever on admission, mainly of low or medium severity. During hospitalization, antiviral drugs (ribavirin, peginterferon, and/or abidor) and immunomodulatory drugs (thymosins) were administered for all COVID-19 patients. Similarly, there were 71 (70.5%) and 40 patients (31.0%) who received antibiotics and glucocorticoids during hospitalization, respectively.

Predictive Factors Associated With Re-positivity for Virus Nucleic Acid: Findings of Univariate and Multivariate Analyses

The results of the univariate analysis for possible predictive factors associated with the re-emergence of virus nucleic acids are summarized in **Table 2**. The following four variables were determined to be significant risk factors according to a univariate analysis ($p < 0.05$): leukopenia, prealbumin < 150 mg/L, hyperpyrexia, and bilateral pneumonia. In the binary logistic regression analysis, leukopenia [odds ratio (OR) 7.316, 95% confidence interval (CI) 2.319–23.080, $p < 0.001$], prealbumin < 150 mg/L (OR 4.199, 95% CI 1.461–12.071, $p = 0.035$), and hyperpyrexia (OR 4.643, 95% CI 1.426–15.117, $p = 0.035$) were independent risk predictors associated with re-positivity for SARS-CoV-2 RNA.

On this prediction model, an ROC curve was drawn to validate the model. The area under the ROC curve was 0.815 (95% CI 0.729–0.902), which suggested that the model had moderate to good predictive accuracy (**Figure 2**).

Clinical Outcomes After Re-positivity for Coronavirus Nucleic Acid

All re-positive patients were asymptomatic, and the emergence of new pulmonary infiltration or consolidation was not revealed on chest CT. Among the 23 re-positive cases, five were discovered during a community medical examination after isolation had been lifted. The family members of these five patients were also quarantined, and all coronavirus nucleic acid tests were found to be negative during this period. These patients were transferred to the hospital for a second period of 14 days, in which RT-PCR of the blood, nasopharyngeal swabs, and anal swabs were performed

TABLE 2 | Predictive factors associated with re-positivity for coronavirus nucleic acid in a multivariate analysis.

	OR	95% CI	*P*-value
Leukopenia	7.316	2.319–23.080	0.001
Hyperpyrexia	4.643	1.426–15.117	0.011
Prealbumin < 15mg/dl	4.199	1.461–12.071	0.008

OR, odd ratio; CI, confidence interval.

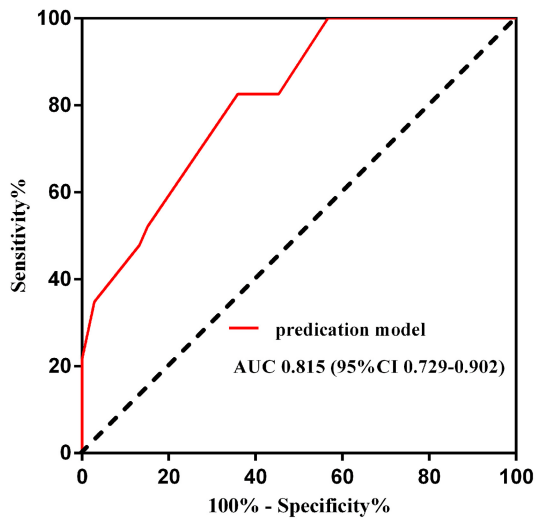

FIGURE 2 | Receiver operating curve of the predication model for NCR. AUC, area under the curve; CI, confidence interval.

on days 1, 4, 7, and 14. Furthermore, the coronavirus nucleic acid tests of five patients turned negative on day 4, 10 patients turned negative on day 7, and eight patients turned negative on day 14.

DISCUSSION

Most COVID-19 patients had a favorable prognosis under "Management of Diagnosis and Treatment of Novel Coronavirus Pneumonia Scheme Trial Version 4." However, the emergence of test re-positivity of SARS-CoV-2 nucleic acid in recovered patients will exacerbate the global situation.

A large study in South Korea showed that 292 (3.3%) out of 8,922 recovered COVID-19 patients showed re-positivity of virus nucleic acid post-discharge, although there was no detailed description concerning whether all of the recovered patients had been tested or whether only the symptomatic ones had been tested after discharge (11). Yuan et al. (13) reported that the re-positivity rate of patients <18 years old (30.8%) was much higher than that of patients ≥18 years old (9.5%). In this study, the re-positivity rate was 17.8% (23/129), which was close to the average level reported in previous studies (range: ∼14.5–16.7%) (9, 10, 12, 14). To avoid false negatives as much as possible, all patients ($n = 129$) underwent two consecutive SARS-CoV-2 nucleic acid tests (sampling interval of at least 24 h) before discharge.

Hyperexia and low serum prealbumin levels were determined to be independent risk factors associated with test re-positivity of SARS-CoV-2 RNA. Similarly, He et al. (19) reported that the severity of cytokine inflammatory storm was directly related to the severity of disease, as the long-term maintenance of a body temperature above 39°C (hyperpyrexia) is a symptom of serious infection. Mahallawi et al. (20) further demonstrated a remarkable pro-inflammatory cytokine response during the acute phase of human MERS-CoV infection. The expression of IFN-γ, TNF-α, IL-15, and IL-17 secreted by pro-inflammatory Th1 and Th17 cells differed significantly between patients with and without this infection. Thus, hyperpyrexia leading to an increased risk of re-positivity of SARS-CoV-2 nucleic acid may be achieved by inducing a cytokine inflammatory storm. Regarding the role of prealbumin in re-positive patients, it was an acute negative time reactive protein similar to albumin, of which the level was significantly lower in COVID-19 patients with a poor prognosis than in those with a good prognosis (21). More time may be required for patients with low serum prealbumin levels to completely eliminate SARS-CoV-2. This may somehow explain why a low serum prealbumin level was associated with test re-positivity for nucleic acid.

Leukopenia was another independent risk factor for re-positivity for nucleic acid. Guo et al. (22) indicated that leukocytes, especially lymphocytes (CD3+, CD4+, and CD8+), were significantly reduced in patients who died of viral pneumonia compared with survivors. Changes in the peripheral blood leukocyte count and lymphocyte subsets may play an important role in the pathogenesis of SARS-CoV-2 infection (23). Furthermore, several related studies have also shown that compared with patients with mild COVID-19 infection, the memorability and cytotoxicity of CD8+T cells in severe patients had significantly reduced (1, 20, 24). Lymphocyte apoptosis, immunological injury, and bone marrow suppression may be critical mechanisms leading to lymphopenia observed in severe COVID-19 cases (25, 26). However, few studies have demonstrated a relationship between lymphopenia and re-positivity for coronavirus nucleic acid. A comprehensive analysis of the results showed that the clinical manifestations of hyperpyrexia, leukopenia, and low prealbumin levels indicated the tendency for severe SARS-CoV-2 infection. The lymphocyte count in patients with re-positivity was lower than those without re-positivity, although not to a significant degree. This may have been due to the small sample size; hence, large-scale multicenter trials are suggested.

Since all discharged patients followed strict self-isolation protocols, reinfection was relatively unlikely to have led to re-positivity for SARS-CoV2 nucleic acid. All patients with re-positivity were asymptomatic and showed no signs of new pulmonary infiltration on chest CT. Furthermore, none presented with infectivity, and nearly all patients' viral nucleic acid tests turned negative again within a relatively short period (14). The causes of re-positivity of viral nuclear acid might be a false negative nucleic acid test at the time of discharge, or the viral load after treatment is below the lower limit of nucleic acid test. During the isolation period after discharge from the hospital, the viral load increased again, and the nucleic acid re-positivity occurred. Additionally, a recent study (13) suggested that re-positivity for SARS-CoV-2 RNA might be considered a process of virus shedding, so the re-positive patients were not infectious. However, different findings were shown in several case reports (27, 28), where infectivity was still detected in re-positive patients who had shown multiple negative nasopharyngeal swab tests. Therefore, as there is no clear evidence that re-positive patients cannot transmit the disease, these patients should be followed up scientifically and strictly isolated after discharge to avoid the risk of disease transmission.

However, several limitations must be considered as well. First, this study was retrospective in nature, which is inevitably susceptible for selective bias, observational bias, and confounding bias. Prospective studies concerning COVID-19 are necessary, and propensity score matching (PSM) might be a feasible way to balance out the biases in a retrospective study. Second, this study was conducted at a single center, and the small sample size makes it difficult to generalize the results. Furthermore, we preliminarily demonstrated that leukopenia was an independent risk factor related to re-positivity for SARS-CoV-2 nucleic acid and have yet to subdivide this factor into specific types of white blood cells, such as lymphocytes and neutrophils. To address these shortcomings, further multi-center, large-scale studies are needed.

Immunoglobulin G (IgG) and immunoglobulin M (IgM) are two specific serum antibodies for SARS-CoV-2 infection. As the main antibody during the humoral immunity, IgG has a high affinity for the pathogens and is widely distributed in the body, which is also the main force of the body to fight infection. The peak of IgG secretion appears later in infection, but it lasts for a long time. Therefore, IgG is commonly regarded as the main antibody for serological diagnosis and monitoring after

TABLE 3 | Antibody detection of SARS-CoV-2 viral RNA re-positive patients.

	Sex	Age (years)	COVID-19 IgG+IgM (S/CO)	COVID-19 IgM (S/CO)
Case 1	Male	68	26.06	1.35
Case 2	Female	35	25.51	1.31
Case 3	Male	30	138.61	6.35
Case 4	Female	44	5.61	**0.44**
Case 5	Male	48	82.03	11.70
Case 6	Male	58	46.10	1.29
Case 7	Female	56	122.27	2.78
Case 8	Female	61	28.98	1.14
Case 9	Male	50	32.49	1.22
Case 10	Female	52	39.95	**0.27**
Case 11	Female	38	169.28	6.53
Case 12	Female	43	89.83	16.91
Case 13	Male	43	4.03	**0.23**
Case 14	Female	48	21.14	**0.63**
Case 15	Male	52	27.15	4.12

IgM, immunoglobulin M; IgG, immunoglobulin G; S/CO, sample/cut off; S/CO < 1 was a negative result; S/CO ≥ 1 was a positive result for the antibody (IgG+IgM and IgM). Bold indicates results that are S/CO < 1.

vaccination. However, IgM appears in the early stage of pathogen infection and disappears shortly after acute infection (29). In this study, due to the limited conditions of serum antibody at the early stage of COVID-19 pandemic, viral antibody tests were only performed in a part of re-positive cases ($n = 15$), who were older than other re-positive patients (details in **Table 3**). The serum antibodies (IgG and IgM) of all re-positive patients were significantly increased, especially those of IgG, which indicated that those re-positive patients were in the recovery stage. Moreover, in the follow-up epidemiological investigation, it was found that after all COVID-19 patients ($n = 129$) were cured and discharged from the hospital, no new cases of COVID-19 were reported in Zhongxiang City.

In summary, COVID-19 patients with leukopenia, low serum prealbumin levels, and hyperpyrexia are more likely to show re-positivity for coronavirus nucleic acid after discharge than others. Although this study was retrospective, single center (Zhongxiang People's Hospital), and had a small sample size ($n = 129$), but with timely and effective treatment, the appropriate extension of hospitalization and the quarantine period may be feasible strategies for managing such patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Zhongxiang People's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

S-fZ, BS, J-kL, J-cH, J-hL, X-yL, C-wX, HS, and P-lD participated in the clinical treatment. YC, S-wX, X-xW, and S-wL wrote the original draft. P-fL, B-bG, and J-fW undertook validation, writing, review, and editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Review of Current COVID-19 Diagnostics and Opportunities for Further Development

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Diagnostic testing plays a critical role in addressing the coronavirus disease 2019 (COVID-19) pandemic, caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Rapid and accurate diagnostic tests are imperative for identifying and managing infected individuals, contact tracing, epidemiologic characterization, and public health decision making. Laboratory testing may be performed based on symptomatic presentation or for screening of asymptomatic people. Confirmation of SARS-CoV-2 infection is typically by nucleic acid amplification tests (NAAT), which requires specialized equipment and training and may be particularly challenging in resource-limited settings. NAAT may give false-negative results due to timing of sample collection relative to infection, improper sampling of respiratory specimens, inadequate preservation of samples, and technical limitations; false-positives may occur due to technical errors, particularly contamination during the manual real-time polymerase chain reaction (RT-PCR) process. Thus, clinical presentation, contact history and contemporary phyloepidemiology must be considered when interpreting results. Several sample-to-answer platforms, including high-throughput systems and Point of Care (PoC) assays, have been developed to increase testing capacity and decrease technical errors. Alternatives to RT-PCR assay, such as other RNA detection methods and antigen tests may be appropriate for certain situations, such as resource-limited settings. While sequencing is important to monitor on-going evolution of the SARS-CoV-2 genome, antibody assays are useful for epidemiologic purposes. The ever-expanding assortment of tests, with varying clinical utility, performance requirements, and limitations, merits comparative evaluation. We herein provide a comprehensive review of currently available COVID-19 diagnostics, exploring their pros and cons as well as appropriate indications. Strategies to further optimize safety, speed, and ease of SARS-CoV-2 testing without compromising accuracy are suggested. Access to scalable diagnostic tools and continued technologic advances, including machine learning and smartphone integration, will facilitate control of the current pandemic as well as preparedness for the next one.

Keywords: COVID-19, diagnostics, clinical, *in-vitro* assay, molecular test, serologic test, antigen test

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INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) (1), has dominated the attention of clinicians, researchers, policymakers and communities worldwide. COVID-19 represents the third major spill-over of a coronavirus from animals to humans during the last two decades (2), with greater global impact than the previous coronavirus outbreaks in 2003 (SARS-CoV) and 2012–2015 and 2020 (Middle East Respiratory Syndrome Coronavirus/MERS-CoV). Transmission of SARS-CoV-2 may have been enhanced by spread from asymptomatic and mildly symptomatic individuals, as opposed to SARS-CoV and MERS where patients tended to be sicker and less mobile, thus resulting in a higher basic reproduction number (R_0) for SARS-CoV-2 (3–6). First reported in China, SARS-CoV-2 spread globally within months, with the Americas, South Asia, and Europe being most severely affected to-date. As of end-March 2021, there were more than 125 million confirmed cases and over 2.7 million deaths, reflecting a global case fatality rate of 2.19% (7), compared to 8,096 total cases and 774 confirmed deaths for SARS, and 2,521 total cases with 866 confirmed deaths for MERS (3). As of Feb 21, 2021, U.S. deaths from COVID-19 had surpassed the death toll of its citizens from World War II, the Korean War, and the Vietnam War combined (8).

In response to the rapidly evolving COVID-19 pandemic, a variety of testing approaches have been employed based on local testing capacities, public health resources, and epidemiology. Large-scale testing, in combination with contact tracing and broad public health control measures, has proven effective in containing SARS-CoV-2 in South Korea and Taiwan (9–11). However, resource limitations in some regions and poor external validation of newly developed diagnostic assays create challenges for successful containment and mitigation (12). The ever-expanding list of diagnostics under the U.S. Food and Drug Administration (FDA)'s emergency use authorization (EUA) also contributes to the confusion around test selection, as performance characteristics, infrastructure requirements, and global availability vary. We herein review available COVID-19 diagnostic approaches, with a focus on their underlying principles and indications, and explore ways in which application of these diagnostics might be improved.

Diagnostic approaches to COVID-19 can be divided into two broad categories: Clinical diagnostics and *in vitro* diagnostics (12–14). Clinical diagnostics include symptoms, laboratory markers not specific to SARS-CoV-2, and imaging, all of which may raise suspicion of COVID-19 but do not provide definitive evidence (13). *In vitro* diagnostics consist of nucleic acid amplification tests (NAATs) and serologic antibody and antigen-based assays, which are specific to SARS-CoV-2 and are broadly applicable in the different settings of clinical care, public health, or epidemiologic investigations (15). *In vitro* diagnostic assays are recommended by the U.S. CDC and U.S. NIH for people who have symptoms of COVID-19, close contact (within 6 feet) with a confirmed case, have participated in higher risk activities where social distancing is not possible, or who have been referred for testing by a healthcare provider or health department (16). Individuals without symptoms or exposure risks are not currently prioritized for testing but may be screened for other reasons such as public health monitoring, active surveillance, or compliance with state and local plans (15, 16).

CLINICAL DIAGNOSTICS

Clinical diagnostics for COVID-19 include the initial assessment of possible COVID-19 related symptoms and exposure history. These should be considered in the context of the SARS-CoV-2 incubation period, which is estimated to be up to 14 days from exposure, with a median of 4–5 days (17–19). Eleven common symptoms of COVID-19 are noted by the U.S. CDC: fever or chills, cough, dyspnea, fatigue, muscle pain, headache, new loss of taste or smell, sore throat, congestion or runny nose, nausea or vomiting, and diarrhea (20). Hospital admission data suggests that fever and cough are the most frequent manifestations (17, 21, 22), and the WHO interim guidance updated on August 7th, 2020, emphasized recent anosmia or ageusia as specific for COVID-19 (23, 24). These observations may be related to high expression of the SARS-CoV-2 host receptor angiotensin-converting enzyme 2 (ACE2) in the nasopharynx (24, 25) or spike protein mutations (D614G) that augment local replication (26).

More recent data also suggest that conjunctivitis, dermatologic findings (maculopapular and vesicular lesions), and multisystem inflammatory syndrome in children (MIS-C), which clinically resembles Kawasaki disease, are associated with infection (27–29). Acute strokes and myocardial infarctions have also been reported, indicating multi-organ involvement that is being further evaluated in several studies (30, 31). A clinical prediction model based on eight factors (cough, fever, contact with a confirmed case, gender, age 60+, headache, sore throat, and shortness of breath) independent of RT-PCR has been developed by the Israeli Ministry of Health, with 87.30% sensitivity and 71.98% specificity (32). Validation of the model in a larger cohort is needed to improve generalizability and evaluate the need for inclusion unique COVID-19 symptoms such as anosmia and ageusia.

Radiography may also support clinical suspicion of COVID-19, and chest CT scanning has been used as a complementary approach for early diagnosis and evaluation of disease progression. CT scan findings are variable and can include multiple bilateral ground-glass opacities in the peripheral lower lung zones (33), which are also seen in patients with SARS-CoV and MERS-CoV infections (34, 35). In 1,014 patients in Wuhan, China, who underwent both RT-PCR testing and chest CT scanning, a "positive" chest CT scan for COVID-19 (per consensus of two radiologists) had a sensitivity of 97% when using RT-PCR as the reference, though specificity was only 25% (36). False-positive CT scan interpretation is not unexpected since findings overlap with other causes of pneumonia (37). Additional studies highlight chest radiograph findings (hazy opacities, consolidation, or horizontal linear opacities) (23, 38) and point-of-care ultrasound pathology (thickened pleural lines, fused B lines, comet-tail artifact or consolidation patterns with or without air bronchograms) (23, 39) as common features of

COVID-19. Nonetheless, COVID-19 patients may not show radiographic abnormalities (38, 40).

Laboratory biomarkers, like radiography, are non-specific for COVID-19 but may also contribute to clinical suspicion of the disease. Reliance upon widely available markers was especially common early in the pandemic, when specific testing capacity was extremely limited (17, 41). Common laboratory findings amongst COVID-19 patients include leukopenia, lymphopenia, elevated aminotransaminase levels, elevated lactate dehydrogenase (LDH) levels, and elevated inflammatory markers (e.g., ferritin, C-reactive protein, and erythrocyte sedimentation rate) (22, 42). Correlation between laboratory findings, disease severity, comorbidities and complications continue to be investigated (43). High D-dimer levels, severe lymphopenia, increased neutrophil to lymphocyte ratio, marked thrombocytopenia, hypoalbuminemia, elevated IL-6, procalcitonin, cardiac troponin I, and serum amyloid A are associated with critical illness or mortality in COVID-19 (44–48). However, these non-specific biomarkers may also be elevated in other infectious diseases such as dengue fever, typhoid fever, or influenza (49, 50).

Artificial intelligence (AI) has also shown promise for automated detection of COVID-19 via pattern recognition algorithms and may potentially reduce emergency department workloads (51). Radiology has been an early adopter of AI for disease detection. In one multisite study, AI deep learning on CT images was able to distinguish COVID-19 from other causes of pneumonia (AUC = 0.87 and 0.88) (52). AI systems based on chest X-ray images showed a sensitivity of 94.8% (53) and accuracy of 96% (54) for prediction of COVID-19 pneumonia. Radiologic data alone may not be suitable for ruling out COVID-19, especially during early disease. Machine learning that integrates chest CT findings with clinical symptoms, exposure history and laboratory testing shows promise for rapid COVID-19 diagnosis (55). An AI model achieved an AUC of 0.92, with sensitivity of 84.3% and specificity of 82.8% (55). Machine learning integration with a smartphone-based application has been proposed for COVID-19 self-testing using breathing or cough sounds; it recognizes acoustic patterns to diagnose COVID-19 early (56, 57). Real-world data should be collected to validate this approach.

AI deep learning has also been used to analyze species specificity of volatile organic compounds (VOCs) by breath-biochemistry, potentially providing a species level biological fingerprint for the pathogen (58). Sensitivity of breath-analyzer tests for COVID-19 ranges from 82.4 to 100% and specificity 54–90% (59–61). False-positives are influenced by diet, humidity, and background contamination (59, 61). Despite its relatively low specificity and need for validation, AI-based breath tests could become a quick, low-cost, and non-invasive triage tool for excluding COVID-19 in the future (60).

IN-VITRO DIAGNOSTICS: MOLECULAR TESTING

SARS-CoV-2 infection is confirmed by detection of SARS-CoV-2 RNA using NAAT (62). For detecting RNA viruses like SARS-CoV-2, Reverse Transcription quantitative PCR (RT-PCR) is recommended as the most sensitive NAAT method (63, 64). Conventional NAAT begins with RNA extraction from respiratory specimens, followed by RT-PCR, in which the purified total RNA (viral RNA and the host RNA) is reverse transcribed into complementary DNA (cDNA) first by reverse transcriptase, followed by cDNA aliquots undergoing qPCR to exponentially amplify the target gene of interest (15, 63, 65). This two-step assay usually takes 3.5–4.0 h and requires three reagent kits: one for the RNA extraction, one for cDNA synthesis, and another for the amplification and detection of the target nucleic acid, as well as specialized lab equipment (15). Throughout the pandemic, labs have faced global shortages of diagnostic reagents, particularly for RNA extraction, and personal protective equipment (PPE) for personnel at risk of exposure in the lab (66). Simplification of NAAT by removing the RNA extraction step is being explored (67). Reports suggest that skipping RNA extraction by simple direct heating of specimens for 5 min at 98°C results in sensitivity and specificity comparable with standard methods (68). Others have successfully processed fresh undiluted samples at 99°C for 5 min (69) or 70°C incubation for 10 min (66) without an RNA extraction step. However, optimization of analytical sensitivity across specimen types remains one of the greatest challenges.

Systems that automate nucleic acid extraction, purification, amplification and detection are available. These provide rapid, high-throughput results with minimal hands-on time (HoT) and less contamination (70–72). The Cobas® SARS-CoV-2 6,800 and 8,800 systems (Roche Molecular Diagnostics, Pleasanton, CA, USA) have sample throughputs ranging from 96 results in 3 h to 384 results (6,800 system) or 1,056 results (8,800 system) in 8 h (70, 73). Overall agreement with standard RT-PCR is up to 99.6% (74). Abbott Molecular (Des Plaines, IL, USA) has also developed a high-throughput, fully automated assay that runs on the m2000 system. This system processes up to 96 samples simultaneously and reports 470 test results in ∼24 h, with high sensitivity (93%) and specificity (100%) for detecting SARS-CoV-2 in clinical samples compared to the SARS-CoV-2 RT-PCR assay developed by the U.S. CDC (75).

Three other automated sample-to-answer assay platforms developed during the pandemic are the Hologic Panther Fusion SARS-CoV-2 assay (Hologic, Inc., San Diego, CA), the Hologic Aptima SARS-CoV-2 assay (Hologic, Inc., San Diego, CA), and the BioFire Defense COVID-19 test (BioFire Defense, Salt Lake City, UT) with throughputs of 335, 275, and 72 samples in 8 h, respectively (72, 76). The Fusion and BioFire automate all aspects of nucleic acid testing including sample preparation, nucleic acid extraction and PCR amplification using nested multiplex PCR, while the Aptima assay uses target capture and Transcription Mediated Amplification (TMA) for the isolation and amplification of SARS-CoV-2 RNA (77–79). Despite slight differences in SARS-CoV-2 target regions and NAAT method, they showed comparable clinical performance for detection of SARS-CoV-2 in NP swabs. Compared to the consensus result (positive for ≥2 of 3 NAATs), the Fusion and BioFire assays had a positive percent agreement (PPA) of 98.7%, followed by the Aptima assay at 94.7%. All 3 assays demonstrated 100% negative percent agreement (NPA), suggesting high specificity (76).

Laboratories facing reagent shortages have sometimes implemented multiple platforms to augment specimen processing capacity. As different platforms employ different techniques and expertise, simultaneous use of diverse platforms could result in inadvertent errors. Additional personnel may also be required, which can create undesirable crowding. And inefficiencies in processing can occur as technicians multitask between analyzers, resulting in increased turnaround time (TAT) (80). Unfortunately, availability of automated RT-PCR for high-throughput platforms remains critically limited, especially for low- and middle-income countries (81).

Specimens for Molecular Testing

SARS-CoV-2 NAAT is most commonly performed on upper respiratory samples. The U.S. CDC recommends that swabs be obtained from the nasopharynx (NP), oropharynx (OP), nasal mid-turbinate, or anterior nares. Wash or aspirate from the nares or NP is also appropriate (82). Samples should be collected by health care providers using a flocked swab with an aluminum or plastic shaft to enhance collection and release of cellular material. Swabs containing calcium alginate or wooden shafts are known to contain PCR inhibitory substances that can lead to false-negative results and should be avoided (63, 83). Swab specimens should be placed into universal transport medium (UTM) immediately after collection to preserve viral RNA (84). Comprehensive data is unavailable for comparing performance of different upper respiratory specimens, though some studies suggest that NP swabs are more sensitive and accurate than OP swabs (85, 86). Compared with standard NP specimens, less invasive nasal swabs (87) and nasal-mid turbinate specimens (88) may cause less discomfort and greater compliance, though at the expense of diagnostic accuracy. Upper respiratory samples have been the leading candidates for home testing thus far.

Due to a global swab shortage, discomfort associated with NP collection, need for trained healthcare personnel, and risk of aerosol droplet production, there is great interest in alternatives to NP specimens. Saliva is a leading candidate, as SARS-CoV-2 RNA is reliably detected within the first week of symptom onset (89). Saliva testing demonstrates similar sensitivity to NP specimens for the detection of SARS-CoV-2 during hospitalization (90). Salivary viral load also correlates with other biological markers such as LDH and may provide information about the clinical evolution of COVID-19 (91). In response to resource shortages and long testing delays, specimen pooling has been used as a large-scale testing strategy (92). Pooling is most efficient when SARS-CoV-2 infection incidence is low, as demonstrated in a study where testing capability was increased at least 69% when one positive swab was mixed with four negative SARS-CoV-2 specimens (93). Use of alternative specimens and modification of testing approaches to increase throughput should be further evaluated to ensure that performance compared to gold-standard RT-PCR is not compromised.

Lower respiratory tract specimens (tracheal aspirates, bronchoalveolar lavage (BAL), fibrobronchoscopic brush biopsy, or sputum) are also valuable for diagnostic testing, as they demonstrate higher positivity rates than upper respiratory specimens, especially later in disease course (94). A non-invasive Exhaled Breath Condensate (EBC) technique that samples respiratory droplets from the lower respiratory tract is being explored for COVID-19 molecular testing. However, EBC should only be used as an adjunct, as opposed to replacement, for NP RT-PCR due to inconsistent results thus far (95). Non-respiratory samples such as blood, feces, urine, semen, or cerebrospinal fluid (CSF) have been used, though their interpretation remains controversial (96–101). Infectious virus has been isolated from urine and feces, but the presence of RNA in non-respiratory specimens does not necessarily correlate with COVID-19 severity, local symptoms (e.g., diarrhea or urinary tract symptoms), or mode of transmission (98–101).

Stool has been considered for COVID-19 testing. SARS-CoV-2 RNA was detected in stool in 48.1% of patients during the course of illness but persisted longer than in respiratory samples (102). In a recent systematic review and meta-analysis, the mean duration of SARS-CoV-2 RNA shedding was 17.0 days (95% CI 15.5–18.6; 43 studies, 3,229 individuals) in the upper respiratory tract, 14.6 days (95% CI 9.3–20.0; seven studies, 260 individuals) in lower respiratory tract, and 17.2 days (95% CI 14.4–20.1; 13 studies, 586 individuals) in stool (103). An earlier study highlighted two COVID-19 cases with positive stool before pharyngeal specimens (102), suggesting that stool may be an alternative to respiratory specimens for early virus discovery in individuals unable to provide respiratory samples, such as infants (104). Stool as a source is consistent with the virus being found in wastewater, where it is presumed to survive several days. During the March–April 2020 Paris COVID-19 outbreak, SARS-CoV-2 levels in waste-water tracked the increase of regional COVID-19 cases observed (105). Thus, sewage–waste-water monitoring could be a non-invasive surveillance strategy (63, 106).

SARS-CoV-2 was also found in 15.8% of semen samples from 38 men with COVID-19 (107), and RNA has been detected in CSF despite its absence in NP swabs in a COVID-19 patient with meningitis/encephalitis (108). Lastly, it has been postulated that COVID-19 begins with circulating viremia before progressing to pneumonia (109), but the presence of SARS-CoV-2 RNA in blood remains unclear (99). Detection of SARS-CoV-2 in blood has ranged from 1 to 8%, and its presence may be associated with increased clinical severity (94, 99, 110). Systematic analysis (108 individuals) showed mean duration of SARS-CoV-2 RNA shedding in serum was 16.6 days (95% CI 3.6–29.7), and the maximum shedding duration was 60 days (103). However, one small study was unable to culture virus from 27 RT-PCR-positive serum samples (111). Correlations between specimen types in which SARS-CoV-2 is detected and organ system manifestations should be further explored.

Technical Aspects of Molecular Testing

Isolation of RNA is the initial step of the RT-PCR assay and critical for the assay's reproducibility and biological relevance (63). Unlike DNA, RNA is highly susceptible to degradation; sample storage, handling, and RNA isolation must follow optimized protocols to minimize degradation at each step (63, 112). After RNA purification, reverse transcription is conducted using different primers, including oligo-dT, random, or gene-specific, depending on the type of RNA, cDNA yield, and

specificity (113). Both the SARS-CoV-2 viral RNA and human RNA (host control RNA such as RNase P) are reverse transcribed; the same cDNA can be used for qPCR (63).

One-step and two-step RT-PCR assays are commercially available. In a one-step assay, reverse transcription and PCR amplification are consolidated into one reaction utilizing a single tube and buffer for RT and PCR steps. In a two-step assay, the reactions are done sequentially in separate tubes with independently optimized buffers (65, 114). One-step RT-PCR can provide rapid and reproducible results, is suitable for high-throughput diagnosis, and may reduce risk of cross-contamination and human error by limiting sample management (12). On the other hand, the more time consuming two-step RT-PCR offers superior sensitivity and lower detection limits (115).

RT-PCR should target highly conserved and abundantly expressed genes of SARS-CoV-2 (62). Positive and negative controls are also important for quality assurance (63). Samples spiked with synthetic SARS-CoV-2 RNA or previously validated positive samples may serve as positive controls (63). Internal "house keeping" control (IC) reactions such as human RNase P mRNA should be included to minimize false negatives associated with technical errors (63, 116). Failure to detect the RNase P gene may indicate improper RNA extraction, RNA degradation/loss, insufficient human cellular material, or reagent or equipment malfunction (63).

Different institutions rely on varying numbers of SARS-CoV-2 gene targets and different target regions. Gene targets include structural proteins, which have higher sensitivity for coronavirus detection, and species-specific SARS-CoV-2 accessory genes (104). Use of multiple PCR targets helps to avoid false-negatives associated with mutations in the primer site, especially mismatches at the 3' end (117, 118). The structural spike (S), nucleocapsid (N), non-structural RNA-dependent RNA polymerase (RdRp), and the open reading frame ORF1ab are the most commonly targeted genes (15).

The U.S. FDA and CDC recommend assays detecting viral nucleocapsids N1 and N2 and human RNase P genes as the primary targets and internal control (IC), respectively (119). A cycle threshold (Ct) value of <40 for all target genes is defined as a positive test, while a Ct value <40 for only one of the two nucleocapsid proteins is considered indeterminant and requires confirmation by retesting (15). This approach differs from the WHO assay, which employs the Charité, Berlin, two-step assay algorithm to confirm infection: step one screens for the envelope (E) gene of subgenus Sarbecovirus, and step two screens for the RdRp gene, which is highly specific for SARS-CoV-2 and does not cross-react with other coronaviruses (120). China CDC recommends the use of specific primers and probes in the N gene regions and the ORF1ab, which encodes a replicase polyprotein 1ab required for viral RNA replication and transcription. Infection is considered confirmed when both targets are positive (37). Other countries have adopted different viral targets for PCR detection: the Pasteur Institute of Paris targets two regions within the RdRp gene; the National Institute of Health, Thailand, and the National Institute of Infectious Disease, Japan mainly uses the N gene; and Hong Kong health authorities target ORF1b-nsp14 and the N gene (114, 121).

Recent studies comparing performance of RT-PCR assays using different target regions have shown that N and E gene primer-probe assays are more sensitive than RdRp based assays (116, 122–124). The lower sensitivity of the RdRp based assay may be due to a mismatch in the reverse primer (122). However, these findings could be confounded by use of different PCR systems, relatively small sample size, and lack of phylogenetic analysis (116). To improve diagnostic efficiency and reliability, duplex or multiplex real-time RT-PCR tests have been developed. These allow simultaneous detection of two or more target sequences via specific fluorescent-labeled probes (65). For instance, the FDA emergency use authorized Abbott RealTime SARS-CoV-2 assay is a dual target RT-PCR assay that detects RdRp and N genes; the TaqPath^{TM} COVID-19 Combo Kit by Life Technologies (Thermo Fisher Scientific, Inc.) employs quantitative recognition of ORF1ab, N, and S genes simultaneously (125, 126). However, the CDC and WHO recommend separating internal control reactions as opposed to multiplexing them in the same PCR reaction with SARS-CoV-2 target genes because relatively high levels of human RNase P RNA compared to SARS-CoV-2 viral RNA may reduce sensitivity of SARS-CoV-2 target genes when multiplexed in one reaction (63).

Like all diagnostic tests, false-negative results can occur with RT-PCR. False negatives have been reported to occur in ∼30% (range 10–40%) of patients with COVID-19 (15). Contributing factors may include (a) collecting the sample when the viral load is low (e.g., early after exposure and before the peak associated with symptom onset, or late in disease course), (b) sample collection technique resulting in reduced quality or quantity, (c) inadequate preservation of the unstable RNA virus, as specimens may degrade without appropriate transport medium or storage, and (d) technical limitations of the RT-PCR test (3, 15, 127–130). One pooled analysis found the probability of a false-negative result ranged from 100% on day 1 after infection to 21% on day 9 to 66% on day 21 (129). False-negative results might be addressed by adjusting the timing of swab collection and repeat testing in the context of high suspicion (12). Positive stool PCR tests with negative pharyngeal swabs have been reported in patients with predominantly GI symptoms. Thus, anal sampling has been considered when there are concerns that NP testing may be falsely negative (131–133). Interpretation of anal specimens should take into account that prolonged nucleic acid does not necessarily reflect presence of infectious virus. Furthermore, testing should not be eschewed to improve rates of case detection, but must be tailored to public health needs.

Test sensitivity may be impacted by natural mutations in the primer region, which could result in false-negatives (134). Based on sequence analysis of the SARS-CoV-2 genomes submitted to the Global Initiative on Sharing All Influenza Data (GISAID) database, viral mutation was highest in the China-CDC-N primer regions compared to other primer sets (https://www.gisaid.org/) (135). Though this does not necessarily mean that a primer would fail to bind, it reveals variability of the target region. It is unclear whether primers for SARS-CoV-2 should be updated regularly as with influenza. One study reported association between a C-to-U transition at position 26,340 of the SARS-CoV-2 genome and failure of the Cobas SARS-CoV-2 E gene RT-PCR

in eight patients (118). Another report showed deletion in S-gene positions 69 and 70 in the Variant of Concern (VOC) 202012/01 or B.1.1.7 causes S-gene target failure (SGTF) in at least one RT-PCR–based diagnostic assay, the ThermoFisher TaqPath COVID-19 assay, and may serve as a means pf identifying infection with this variant (136). These findings highlight the need for ongoing assessment of RT-PCR targets.

Viral RNA detection by RT-PCR does not demonstrate the presence of infectious virus, and patients who have recovered can be persistently PCR-positive but non-infectious, which is confusing for quarantine and control (137). Cell culture is a more accurate indicator of viability and contagiousness but must be performed in Biosafety Level 3 (BSL-3) facilities and is not routine (101, 138). Studies have shown that RT-PCR Ct values correlate strongly with the ability to cultivate virus (139–141). However, Ct value cut-offs differ between studies and depend on the PCR system used. Variation across PCR test runs, low viral copy number, and poor sampling collection may engender differences in absolute Ct values (142–144). Some studies have shown that the probability of culturing virus declines to 8% in samples with Ct > 35 by RT-PCR targeting RdRp gene (141), while other studies have concluded that patients with Ct > 33–34 by LightCycler Multiplex RNA Virus Master kit RT-PCR system targeting E gene are not contagious (140). Others have even provided data showing no virus growth in samples with Ct > 24 of E gene amplification by RT-PCR (139). Duration of illness negatively affects the viability of SARS-CoV-2 in specimens, as isolates have resulted in no growth when collected after day 8 of illness despite ongoing high viral loads by RT-PCR (97, 139). Surrogate methods to identify infectious virus, such as the detection of sub-genomic RNA (sgRNA) are being evaluated (144). Additional large-scale studies will be useful for the optimization of strategies to detect infectious virus, which would be helpful for guiding isolation polices.

Point of Care Molecular Diagnostic Tests

COVID-19 cases are typically confirmed by centralized RT-PCR testing in certified labs, which requires expertise, specialized equipment, and well-developed specimen management infrastructure. Due to the burden of large-scale testing suddenly placed on most labs, results may take a week or longer to be returned. This has spurred significant interest in reliable PoC molecular tests that produce rapid results (<1 h) (81), as they facilitate timely patient management decisions. At least two cartridge-based PoC assays have been developed to-date and granted an EUA from the U.S. FDA (72, 145, 146).

Xpert® Xpress SARS-CoV-2 test (Cepheid, Sunnyvale, CA), the most popular PoC test thus far, provides qualitative detection of the virus in ∼45 min using the GeneXpert benchtop system. This PoC NAAT for upper respiratory specimens requires <1-min HoT for sample preparation and targets the N2 and E genes of SARS-CoV-2. The Xpert® Xpress SARS-CoV-2 test demonstrated 100% agreement with in-house RT-PCR assays, with a lower limit of detection (LOD) of 8.26 copies/mL (147). Just as the GeneXpert Assay for tuberculosis (TB) is used for the detection of both wild-type and rifampicin-resistant TB (148), it is anticipated that Xpert COVID-19 could be further developed to detect mutations of SARS-CoV-2 which might impact prevention and treatment approaches.

The second PoC molecular assay under a U.S. FDA EUA is the ID Now COVID-19 test (Abbott Diagnostics Scarborough, Inc., Scarborough, ME). This automated test qualitatively detects SARS-CoV-2 RNA from upper respiratory specimens. ID Now COVID-19 uses an isothermal nucleic acid amplification test (INAAT) based on Nicking Enzyme-Assisted Reaction (NEAR) technology (149) to amplify the RdRp gene in 5–13 min, with a LOD of 125 genome equivalents/ml according to the manufacturer (150). However, the test is limited to only one sample per run, and it showed a sensitivity of only 80.4% and a specificity of 95.9% in a diagnostic confirmation study (151). The lower PPA occurred more frequently in specimens with low viral load or collected in universal or viral transport media (VTM), which may dilute the sample and decrease sensitivity. Therefore, the manufacturer recommends the use of freshly collected specimens for optimal performance (152, 153). However, a small study reported low PPA of ID Now compared with Xpert® Xpress irrespective of use of dry nasal swabs or swabs in VTM, which raises concerns about the suitability of ID Now as a confirmatory diagnostic (150). Due to its suboptimal sensitivity, several institutions have abandoned Abbott ID NOW for POC COVID-19 testing. The U.S. FDA also recommends confirming all negative Abbott ID NOW SARS-CoV-2 results with a sensitive molecular test (154).

Another cartridge-based PoC that has received the Europe CE mark is CovidNudge (DnaNudge, UK), a fully-automated multiplex RT-PCR system with a sample-to-answer run-time of <90 min. This assay uses dry NP swabs and targets seven SARS-CoV-2 gene regions (RdRp1, RdRp2, E-gene, N-gene, N1, N2, and N3) and a validated positive control host gene (RNase P), which reduces the false-negative testing rate caused by insufficient sampling. The overall sensitivity is 94% (95% CI 86–98), with an overall specificity of 100% (99, 100), and LOD 250 viral copies/swab (155, 156). However, since each unit can process only one cartridge at a time (maximum of 15 tests per machine per day), the assay has relatively low throughput and may require multiple processing units (Nudgebox) in a clinical setting (157). Prospective studies are required to assess the effectiveness of CovidNudge with non-NP/OP specimens and in comparison with other standard tests.

Truenat (Molbio Diagnostics, India) was recently developed by Indian scientists via adaptation of a test used for pulmonary tuberculosis (158). This chip-based portable PCR is intended to facilitate quick and affordable molecular pathogen detection by low infrastructure health facilities in developing countries (63). The Truenat Beta CoV E-gene screening assay and Truenat SARS- CoV-2 RdRp gene-confirmatory assay have demonstrated concordance with the reference standard RT-PCR (159). In a small validation study, this PoC assay exhibited 100% sensitivity and specificity and no cross-reactivity with other respiratory pathogens with LOD 486 copies/mL (160, 161). Although the technology lacks the throughput of the conventional PCR, its affordability, portability, ease of use, and test interpretation make it attractive for screening and confirmation of SARS-CoV-2 in developing countries (63).

BioFire® Respiratory Panel 2.1 (RP2.1) (BioFire Diagnostics, Biomérieux, France) is another widely used testing platform. This PoC test uses a closed disposable system containing the reagents necessary for sample preparation, reverse transcription, polymerase chain reaction (PCR), and detection of nucleic acid from multiple respiratory pathogens based on a single NP specimen. Runs take ∼45 min (72). RP2.1 was created by adding primers for the membrane (M) and spike (S) genes of SARS-CoV-2 to the existing FDA-cleared and CE-marked BioFire® Respiratory Panel 2 (RP2); RP2.1 can detect 22 viral and bacterial respiratory pathogens with LOD 500 copies/mL for SARS-CoV-2 (162, 163). A study comparing the BioFire RP2.1 with Roche Cobas, Hologic Fusion, and conventional RT-PCR for detection of SARS-CoV-2 demonstrated 98% PPA and 100% NPA in residual NP swab specimens (163). As RP2.1 detects spike genes, a hotspot for mutation, utility of this PoC test for detection of variants should be routinely assessed.

The cobas® Liat® SARS-CoV-2 and influenza A/B test (Roche Molecular Systems, Inc., Pleasanton, CA) has received emergency use authorization (EUA) for identification and differentiation of SARS-CoV-2, influenza A virus, and influenza B virus. This PoC test is a multiplex RT-PCR and provides results in ∼20 min (72). For SARS-CoV-2, the test utilizes two target gene regions (ORF1a/b and N) with LOD 12 copies/mL (164). A multisite U.S. study demonstrated excellent test agreement (100% PPA and 97.4% NPA) between the Liat and high-throughput Cobas® 68/8800 tests (165). The Liat is advantageous in that it simultaneously tests for influenza and SARS-CoV-2, allowing differentiation between multiple respiratory viruses that co-circulate (165). Given influenza's ability to exacerbate SARS-CoV-2 infection, early and rapid detection of SARS-CoV-2 and Influenza co-infection may reduce associated morbidity and mortality (166).

Nanomaterial-based biosensors have been developed as a potential PoC approach. These alternatives to viral RNA extraction and SARS-CoV-2 sequence detection use biosensors functionalized with nucleic acid hybridization (167, 168). The GenMark ePlex SARS-CoV-2 (GenMark Diagnostics, Carlsbad, CA), a PoC test based on the eSensor technology (169, 170), is a "True Sample-to-Answer Solution" that targets the N gene. It uses a combination of electrowetting and GenMark's eSensor technology for extraction, amplification, and detection. The technology relies on competitive DNA hybridization and electrochemical detection (155, 171). While the ePlex SARS-CoV-2 Test only detects 1 viral target, the ePlex Respiratory Pathogen Panel 2 (ePlex RP2 Panel) simultaneously detects 16 respiratory viral targets and two bacterial targets (155, 169, 172). The sample-to-result time for both tests is under 2 h, with SARS-CoV-2 LOD 750 genomic copies/mL for ePlex and 250 genomic copies/mL for ePlex RP2 Panel (155, 169, 172).

Simplexa™ COVID-19 Direct assay (Diasorin Molecular LLC, Cypress, CA) is another PoC test available under U.S. FDA Emergency Use Authorization (173). The system consists of the Simplexa™ COVID-19 Direct assay, the LIAISON® MDX (with LIAISON® MDX Studio Software), the Direct Amplification Disc (DAD), and associated accessories. A 50-μl volume of Simplexa COVID-19 Direct kit reaction mix (MOL4150) is added to the "R" well of the 8-well DAD followed by addition of 50 μl of non-extracted NP specimen to the "SAMPLE" well. The assay runs for ∼90 min (155, 173, 174). It targets the ORF1ab and S genes and has a LOD for NP specimen of 500 copies/mL (173). The Simplexa and ePlex assays have similar HoT and TAT, based on processing 8 samples per disc on the DiaSorin LIAISON MDX and 6 cartridges per tower in the GenMark ePlex (174). A study evaluating the analytical and clinical performance of the Simplexa, ePlex, Hologic Fusion, and modified CDC conventional RT-PCR showed comparability ($\kappa \geq 0.96$). PPA was 100% (51/51) for Simplexa, Hologic Fusion and conventional RT-PCR and the ePlex PPA was 96% (49/51) compared to the consensus result (positive for ≥3 of 4 NAATs). An NPA of 100% (53/53) was observed for ePlex and Simplexa; NPA ranged from 98% (52/53) for conventional RT-PCR to 96% (51/53) for Hologic Fusion (174).

While both PoC platforms and automated high-throughput systems (e.g., Hologic Fusion) out-performed conventional RT-PCR in hands-on and manual workflow steps, the high-throughput system is more appropriate for high-volume testing since pipetting of specimen into lysis tubes can be labor-intensive and time-consuming, thus may increase TAT. Both PoC and high-throughput assays are suitable for facilities with low to moderate testing volume and need for rapid results (174). Further studies are needed to determine their performance in comparison with gold standard RT-PCR and clinical utility, especially with regards to emerging variants.

Other Methods of Viral RNA Detection

Although conventional RT-PCR is currently the gold-standard in SAR-CoV-2 diagnosis, it can be time-consuming, laborious, and require specialized equipment and trained personnel (63). Loop-mediated isothermal amplification (LAMP) combined with reverse transcription (RT-LAMP) has been developed as an alternative (114). RT-LAMP is a highly specific assay that employs DNA polymerase and 4–6 primers that bind distinct target regions of the genome; it allows direct detection of SARS-CoV-2 genes such as ORF1ab, S, E, and/or N gene (175–178). RT-LAMP isothermally (60–65°C) amplifies DNA fragments of interest, thus does not require expensive thermal-cyclers or real-time PCR (179). Detection is based on photometric measurement of turbidity resulting from magnesium pyrophosphate precipitation that occurs as a by-product of amplification. This method enables real-time monitoring of results using colorimetric or fluorescent dyes (43, 180). Since RT-LAMP needs only heating and visual monitoring and has a sample-to-result time of around 1 h, it is an attractive possibility for low-cost field deployment. Furthermore, it might be adapted to smartphones and used as a personal PoC diagnostic (63, 181, 182). Several studies have shown promising RT-LAMP results in SARS-CoV-2, with detection accuracy ranging from 89.9 to 100% (63, 175–177). However, RT-LAMP is challenged by low specificity due to presence of multiple pair primers that may increase non-specific byproduct formation (183). False-negative RT-LAMP results have also been observed for specimens with Ct values above 35 due to low viral RNA; this causes inefficient amplification of the target sequence (175, 183). Sensitivity and

specificity of RT-LAMP assays should be evaluated against a range of SARS-CoV-2 viral loads for validation and optimization. LAMP has recently been coupled with nanopore sequencing and CRISPR-based detection platforms (explained below) to boost accuracy and performance (183, 184).

Along with isothermal amplification, another category of nucleic acid tests that could be used for SARS-CoV-2 is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) based method. Use of CRISPR for infectious disease applications has been garnering significant interest over the past few years (185). CRISPR belongs to a family of palindromic nucleic acid repeats found in bacteria, which are recognized and cut by a unique set of effector enzymes known as the CRISPR-associated (Cas) proteins (186). The Cas enzymes are exceptionally sensitive and specific as they can be programmed to identify and cut SARS-CoV-2 RNA sequences (12). Two companies, Sherlock Biosciences and Mammoth Biosciences, are independently exploring these platforms. The Specific High-sensitivity Enzymatic Reporter unlocking (SHERLOCK) assay uses Cas13 (187), and the DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) assay uses Cas12a (188). Cas13a and Cas12a have "collateral cleavage" activity triggered by target-dependent binding between the Cas-guide RNA complex (CRISPR complex) and the target sequence. This event activates the nuclease enzyme activity of the Cas, followed by cleavage of the nucleic acid reporter and generation of a detectable signal (178). Cas13 and Cas12a are activated upon binding to target nucleic acids, RNA and DNA, respectively, where they excise reporter RNA sequences and cut a quenched fluorescent probe to generate a fluorescence signal (187–189). Both tests are low-cost, can be performed in 1 h (188, 190, 191), and have been granted U.S. FDA EUA status (72, 192, 193). The SHERLOCK test demonstrated a sensitivity of 93.1% and a specificity of 98.5% (191), while the DETECTR assay demonstrated 95% positive predictive agreement and 100% negative predictive agreement (188), which makes both strong rapid diagnostic candidates. Another CRISPR/Cas13a system developed by Chinese researchers demonstrated sensitivity approaching a single copy and was highly specific compared to sequencing-based metagenomic and RT-PCR-based assays in a clinical cohort. With reaction TAT of only 40 min after nucleic acid preparation (30 min of DNA amplification by Reverse-transcription Recombinase Polymerase Amplification/RT-RPA and 10 min of Cas reaction), CRISPR is a promising alternative to conventional RT– PCR, particularly in the setting of infrastructure constraints (194). Nonetheless, emerging CRISPR-based methods require careful validation and field testing (195).

Another approach, droplet digital PCR (ddPCR), has been developed to detect SARS-CoV-2 and measure viral load, which facilitates surveillance of inter and intra-case variability (196). ddPCR is based on partitioning the sample into thousands of micro-reactions of defined volume (197). Compared with conventional quantitative PCR, ddPCR has the advantages of being able to perform absolute quantification by using principles of sample partitioning and Poisson statistics. This approach overcomes normalization and calibrator issues associated with qPCR and thus increases precision. ddPCR is also more sensitive for detecting low target copies and relatively insensitive to potential PCR inhibitors (198). Recent studies have reported higher sensitivity and robustness of ddPCR than RT-PCR for detection and quantification of SARS-CoV-2 RNA from purified RNA and crude lysate samples in UTM (196, 199). Digital droplet assays which enable detection and quantification with limited sample processing could potentially be used for monitoring clinical course and convalescence (199).

Genomic sequencing does not play a part in routine SARS-CoV-2 laboratory diagnosis; however, this technique is essential for phyloepidemiological evaluation of changes in the viral genome over time and to trace transmission patterns (67). Sequencing protocols based on Sanger and next-generation sequencing (NGS) (e.g., Illumina and MinION/Nanopore) are being applied to rapidly generate genome sequences (200–202), with the promise that data will inform diagnostic development, epidemiologic investigations, host-virus interactions, viral evolution, pathogenesis, and prevention and treatment targets (67). NGS can also be used to evaluate the host microbiome and co-infection with certain pathogens, which may influence how SARS-CoV-2 infection manifests and results in secondary infections (200). Studies using NGS are sparse in part due to the high cost and the tendency to employ NGS for research purposes as opposed to clinical management (12). In light of ongoing evolution of the SARS-CoV-2 genome, sequencing applications are essential for identifying mutations that may be associated with increasing transmissibility and/or virulence, evading detection by current diagnostics, and escaping antiviral treatment or immunity (203). As of March 2021, several Variants of Concern have been identified as more transmissible (e.g., Variant B.1.1.7 from the U.K.), increasingly resistant to neutralization by monoclonal antibodies, and less susceptible to vaccine induced immunity (e.g., Variant B.1.351 from South Africa, P.1 lineage from Brazil, and Variant B.1.526 from New York City containing the Spike-E484K mutation) (204–207). Given the SARS-CoV-2 genome's evolving nature, genomic surveillance should be conducted at levels that allow early temporospatial identification of new variants.

As of March 23, 2021, the U.K. (N = 307,233; 36.50%) and the U.S. (N = 200,425; 23.81%) accounted for the majority of all published genomic sequences (N = 841,700) in the GISAID database (7, 135). However, the proportion from reported COVID-19 cases of those two countries (the U.K. = 307,233/4,301,925 = 7.1%; and the U.S. = 200,425/30,576,962 = 0.7%) still lag behind Iceland (4,172/6,119 = 68.2%), Australia (17,674/29,211 = 60.5%), New Zealand (1,211/2,462 = 49.2%), Denmark (50,545/226,777 = 22.3%), and Taiwan (173/1,007 = 17.2%)—the five countries with the highest current proportion of reported sequences (7, 135, 208, 209). Hong-Kong has a sequence reporting rate of 11.0% (1,254/11,398) and documented the world's first confirmed COVID-19 reinfection using whole-genome analysis (7, 89, 135). Genomic surveillance by the South Korea Disease Control and Prevention Agency from January 2020 to January 2021 showed that amongst 2,488 COVID-19 cases, including 648 from abroad, Variant B.1.1.7 and B.1.351 were only identified from international travelers. This supports

the efficacy of South Korea's rapid implementation of non-pharmaceutical public health interventions, such as quarantining incoming travelers, for preventing dissemination of SARS-CoV-2 variants (210, 211). Further strengthening of global sequencing capacity will facilitate ending the current pandemic and early detection and management of future outbreaks (208, 212).

Over 300 tests for SARS-CoV-2 NAAT/molecular testing are currently described in FIND (Foundation for Innovative New Diagnostics), a diagnostics resource center established in collaboration with WHO to accelerate development and access to diagnostics as part of the global response to COVID-19. This foundation verifies test LODs using cultured viral stocks from clinical isolates, quantifies using an E-gene standard, and evaluates clinical performance using samples from individuals suspected to have COVID-19 that were tested by in-house PCR. Results are available online at: https://www.finddx.org/covid-19/sarscov2-eval-molecular/. Many molecular and serological PoC tests have also been granted EUAs from the U.S. FDA. Information on these assays can be found at: https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas. **Figure 1** shows a conceptual overview of COVID-19 molecular testing approaches. Consideration of the pros and cons of each method should guide clinical applications (213).

IN-VITRO DIAGNOSTICS: ANTIBODY ASSAYS

Serologic measurement of specific antibodies can be used to assess prior exposure to SARS-CoV-2 and infer potential immunity to the virus. As a diagnostic tool, antibody serology is particularly useful for patients with delayed clinical presentation, typically at least 2 weeks after illness onset (214), who may be missed by NAAT. A report from Singapore demonstrated the utility of antibody measurement in assessing an initially PCR-negative individual who linked two infection clusters (215). Serological data is particularly useful for epidemiologic purposes, such as estimation of the attack rate, R_0, and case fatality rate (216), and to evaluate the impact of control measures (lockdowns, broad testing, and other policies). Antibody evaluation can also facilitate identification of plasma donors and assessment of vaccine immunogenicity, especially in elderly or otherwise immunocompromised people (214, 216, 217). Cross-reactivity between antibodies to SARS-CoV-2 and other endemic human coronaviruses (CoVs) may enable design of pan-coronavirus therapeutics or vaccines (218, 219). Serological surveillance may also identify potential zoonotic disease transmission from wild-life reservoirs, such as bat-borne coronavirus and influenza virus (e.g., G4 genotype H1N1) (220, 221). However, in a pandemic context where early diagnosis is essential for patient management and outbreak control (222), antibody assays are suboptimal due to delayed seroconversion and performance variability, therefore are not the preferred frontline test (223).

Antibody Assay Platforms

Currently marketed platforms for serologic evaluation of antibodies include lateral flow immunoassays (LFIA), enzyme-linked immunosorbent assays (ELISA), and chemiluminescent immunoassays (CLIA). These assays rely on similar principles but differ in the method of antibody-antigen binding detection (224). LFIAs, which are small, portable, and suitable for qualitative PoC assessment, result in the appearance of a colored line following the addition of specimen to the strip (225). ELISAs may be qualitative or quantitative and may involve several manual steps, increasing their time to results. Well-plates pre-coated with SARS-CoV-2 spike or nucleocapsid protein are incubated with patient sera, and if antibodies are present, an antibody-antigen complex forms resulting in a downstream fluorescent-based readout (226, 227). CLIAs, also known as chemiluminescent microparticle immunoassays (228), are automated assays that rely on the mixing of patient samples with magnetic, protein-coated microparticles and generate a light-based, luminescent readout (229, 230).

SARS-CoV-2 proteome-based microarrays have more recently been developed for the automated detection of antibodies (231). In contrast to the conventional techniques described above, which test a single target antibody in a single reaction, protein microarrays employ proteome-wide characterization of antibodies in a high-throughput format to generate a more systematic description of antibody binding and viral antigens (232). A similar platform is VirScan, a programmable phage-display immunoprecipitation and sequencing technology platform that was developed in 2015 to explore antibody responses across the human virome. VirScan has been adapted for use with SARS-CoV-2 by employment of a coronavirus oligonucleotide library of 56-mer peptides tiling every 28 amino acids across the proteomes of 10 coronavirus strains, and 20-mer peptides tiling every 5 amino acids across the SARS-CoV-2 proteome. VirScan requires one drop of blood and scans over 1,000 virus strains. A machine learning model trained on VirScan data predicted SARS-CoV-2 exposure with 99% sensitivity and 98% specificity. This type of approach could be very useful for understanding past exposure epidemiology, though it is not yet widely available or suitable for acute diagnosis (233). Biosensors that use polyaniline nanofibers-coated optical fibers for serological measurements are also in development and could eventually be used in a plug-and-play format (234). A microfluidic ELISA system has also been proposed for detection of COVID-19 antibodies via a lab-on-chip platform. Plasma is separated using a microfluidic device and subsequently, antibodies are detected in the separated plasma using a semi-automated on-chip ELISA. Although the automated system is simpler to use than manual ELISA, performance of this platform still needs to be evaluated (235).

In general, LFIAs have lower sensitivities but comparable specificities to ELISAs and CLIAs. In a recent meta-analysis of 40 studies, the pooled sensitivity of IgG or IgM ELISA was 84.3% (95% confidence interval 75.6 to 90.9%), LFIA was 66.0% (49.3 to 79.3%), and CLIA was 97.8% (46.2 to 100%). Pooled specificities ranged from 96.6 to 99.7% (223),

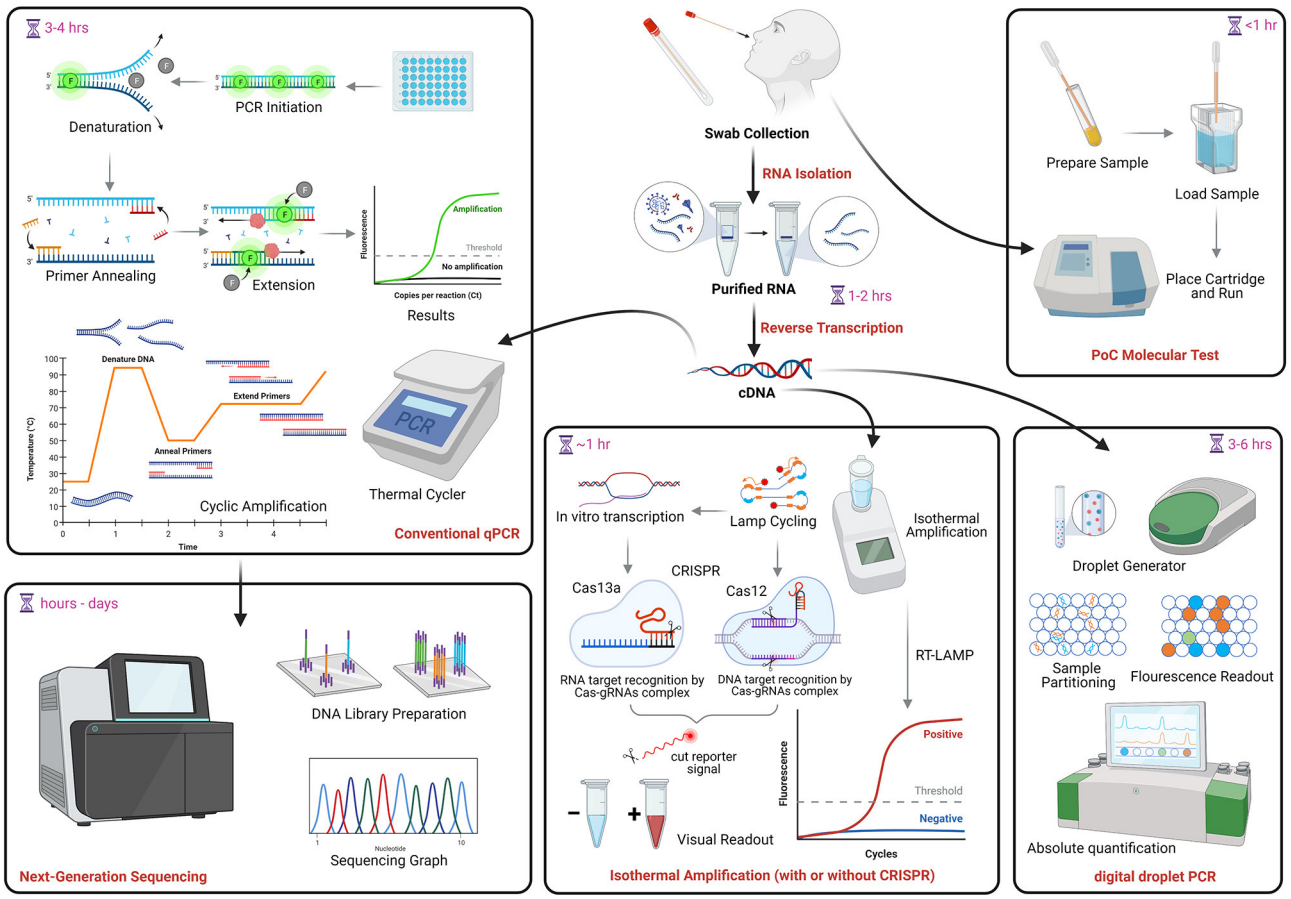

FIGURE 1 | COVID-19 molecular testing. NAAT begins with RNA extraction followed by reverse transcription into complementary DNA (cDNA). The same cDNA can be used for conventional qPCR, RT-LAMP, which can also be coupled with CRISPR technology, and droplet digital PCR. PoC assays (uppermost right) use direct specimen and cartridge-based tests to produce rapid results. The PCR amplification product may be used to generate viral genome sequences (lowermost left). NAAT, nucleic acid amplification tests; qPCR, quantitative polymerase chain reaction; PoC, point of care; LAMP, Loop-mediated isothermal amplification; CRISPR, clustered regularly interspaced short palindromic repeats. Image created in Biorender.com.

consistent with a previous report (224). The low sensitivity of LFIA in this analysis may be related to the use of whole blood, and the use of serum for LFIA and ELISA is likely to increase sensitivity (223). There is high variability in performance amongst commercially available LFIAs (236). This may be related to differences in validation protocols (237, 238), with some studies using archived pre-COVID emergence samples (239–241) and others using PCR negative samples as negative controls (241–243). Validation of immunologic assay techniques following a universal protocol would be very helpful in determining the comparative performance of the assays.

Spike and Nucleocapsid Protein-Based Antibody Assays

The SARS-CoV-2 spike and nucleocapsid proteins are the primary viral antigens used in currently available antibody assays (244, 245). The spike protein (S) is located on the surface of the virus, where its receptor-binding domain (RBD) attaches to the host ACE2 receptor to facilitate viral entry (246). S is highly immunogenic, and the neutralizing activity of anti-S antibodies has made them the focus of therapeutic and prevention strategies (247). The nucleocapsid protein (N) plays a crucial role in viral replication and assembly (248). N is abundantly expressed during infection, is highly immunogenic, and induces antibody production earlier than S (249). The N gene is reportedly more conserved and stable than S, with 90% amino acid homology and fewer mutations over time, making it a strong candidate for inclusion in vaccines against SARS–CoV-2 (250). However, studies of S, N, and associated antibodies show different results in terms of the superiority of N (251) over S (226). One study has suggested that an S-based assay is more cross-reactive with endemic human coronavirus antibodies than an N-based assay (248). Further studies are needed to characterize antibody dynamics and determine which antigen(s) should be used for monitoring and surveillance purposes.

A major limitation of currently available S-based assays is that they measure total binding antibodies (BAbs) (252) as opposed to neutralizing antibodies (NAbs) alone. Since not

all BAbs block infection, these assays do not actually reflect antibody inhibition of SARS-CoV-2 infection, even though some studies have shown that anti-RBD IgG titers correlate with NAbs titers (253, 254). Ideally, assays should specifically assess NAbs as an indicator of protective immunity to facilitate serodiagnosis, evaluation of convalescent plasma therapy, and vaccine development (255). NAbs are conventionally measured by the plaque reduction neutralization test (PRNT) (97), which requires handling infectious SARS-CoV-2 in a specialized BSL-3 containment facility, is labor-intensive, and requires 2–4 days to complete. These limitations make PRNT impractical for large scale applications (252). The pseudovirus-based Virus Neutralization Test (pVNT) utilizes a genetically-modified pseudovirus that mimics SARS-CoV-2 yet is safe to handle and can be evaluated in a BSL2 laboratory (256). Since the broad application of pVNTs is limited by the need for virus and cell culture facilities, the surrogate VNT (sVNT) has been developed to detect NAbs without the need for live virus or cells. sVNTs use purified RBD from the S protein and purified ACE2 to mimic the virus-host interaction in an ELISA plate well. sVNTs can be performed in 1–2 h under BSL-2 conditions and demonstrate 99.93% specificity and 95–100% sensitivity compared with conventional PRNTs (252). Unfortunately, comparative studies of sVNT and PRNT have not clearly defined the sVNT cut-off value in relation to the conventional PRNT titer, though excellent concordance was observed in a small study (257). Further validation between the two assays and using other virus clades is needed to ensure sVNT robustness.

Several point mutations (e.g., Spike-E484K and Spike-S477N) have demonstrated ability to escape neutralization by convalescent sera and monoclonal antibodies (258). Thus, the impact of mutations on SARS-CoV-2 antibody assays should be monitored. Mutations may alter an assay's ability to detect key antibodies, including those to viral spike protein or nucleocapsid. Ongoing evaluation is in progress (259).

Kinetics of SARS-CoV-2 Isotype Antibodies

Accurate interpretation of serologic testing depends on both antigen specificity and the antibody isotype detected (138). Of the five isotypes, IgM, IgG, and IgA are the primary testing targets (260). IgM is generally produced first because it is expressed on the surface of Naïve B cells prior to isotype switching (261), though IgG conversion prior to and simultaneous with IgM has been seen with COVID-19 (97). The antigen-binding sites of IgM pentamers is not highly specific (262, 263), with one study demonstrating occurrence of SARS-CoV-2 IgM ELISA false-positivity due to mid-to-high levels of rheumatoid factor IgM (22/36 false-positive results). A urea dissociation test was shown to reduce the false-positive rate (264). Low-level cross-reactivity of both IgM and IgG against N and S2-containing antigens from other betacoronaviruses (e.g., SARS, MERS, HKU1, OC43) has been demonstrated in SARS-CoV-2 convalescent blood specimens, although discrimination between COVID-19 cases and negative control is much greater for IgG antibodies than for IgM antibodies (265). In general, IgG is more specific and may appear later in infection (266). IgG is a high-affinity monomer that can directly neutralize microbes as part of the humoral immune response and can be transferred transplacentally from mother to fetus (267, 268). Mucosal IgA responses also play a critical role in blocking viral invasion and replication at mucosal surfaces where SARS-CoV-2 may enter (269, 270). Human breast milk from women exposed to SARS-CoV-2 antigens may contain IgA that protects the infant from infection (271, 272). The role of serum IgA is less clear, but reports suggest it is involved in formation of immune complexes that amplify inflammatory responses (273, 274). Sterlin et al. showed early SARS-CoV-2–specific humoral responses were dominated by IgA antibodies. These were more potent than IgG in neutralizing SARS-CoV-2, highlighting the potential role of IgA during early SARS-CoV-2 infection (275).

Variable kinetics of COVID-19 antibodies have been demonstrated. SARS-CoV-2 IgM may appear and peak earlier than (276, 277), simultaneously with, or after IgG (97, 278). IgA has been detected earlier than IgM or IgG but was found to be cross-reactive with other coronaviruses (279, 280). In a Cochrane Database systematic review of 54 cohorts with 15,976 samples, pooled results for all isotypes showed low sensitivity during the first week after onset of symptoms, rose in the second week, and peaked in the third week. Data on sensitivity of tests beyond 35 days post-symptom onset are inconclusive (281). Serologic antibody testing is useful as a complement to RNA testing, particularly in the later stages as PCR positivity decreases by 2 weeks after symptom onset (282). Antibody kinetics in the setting of COVID-19 re-infection merit further exploration (283).

Antibody Responses and Disease Severity

Variability in kinetics of SARS-CoV-2 isotype antibodies may be associated with illness severity, age, and comorbidities (276, 284, 285). One study found that IgM and IgG antibodies showed similar kinetics in both non-ICU and ICU patients, with the authors concluding that early class switching of IgM to IgG might predict better outcomes (285). Most studies of antibody responses have occurred in hospitalized COVID-19 patients with moderate to severe illness. Studies in asymptomatic and mildly ill patients have been limited (281), though one study of asymptomatic patients showed SARS-CoV-2 IgG levels (median S/CO, 3.4; IQR, 1.6–10.7) to be significantly lower than in the symptomatic group (median S/CO, 20.5; IQR, 5.8–38.2), with 40% of asymptomatic patients becoming seronegative during early convalescence. One interpretation of these data is that asymptomatic individuals had a weaker adaptive humoral immune response to SARS-CoV-2 infection (278). Other studies have reported later appearance and lower titers of IgA, IgG, and IgM in mild or moderate cases compared to severe cases (260, 277, 281).

Durability of antibodies and how they correlate with immunity are currently unclear (286). A longitudinal population-based study of over 9,000 community residents in Wuhan, China showed that IgG and neutralizing antibodies were relatively stable for at least 9 months, regardless of symptom presence (287). A Danish study observed ∼80% protection from re-infection during a second surge (∼6 months after initial infection) amongst people with PCR positivity, compared to those who were PCR negative. Protection associated with prior

infection decreased to 47% amongst people 65 years or older, supporting prioritization of vaccination for seniors (288). Long term, adequately powered COVID-19 cohort studies are needed to better characterize antibody kinetics as well as correlates of immunity.

IN-VITRO DIAGNOSTICS: ANTIGEN TESTING

SARS-CoV-2 antigen testing is another type of serologic assay that is attractive as a potential PoC diagnostic. Antigen-based diagnostics detect protein fragments on or within the virus, rather than viral nucleic acids, in specimens collected from NP swabs or nasal cavity (178). This type of testing can detect active infections within 15 min compared to hours with RT-PCR. Therefore, a highly sensitive method that directly detects viral antigens in clinical samples would be a great asset in in the containment of transmission during early infection (289). Viral proteins should be detected by antigen-capture methods (e.g., antibodies, aptamers) which are routinely used for other viral assays, such as human immunodeficiency virus (HIV) and hepatitis B virus (290). Based on previous experience with antigen testing in SARS and MERS, the N protein is considered an excellent target for a diagnostic sandwich assay using monoclonal antibodies. N protein is secreted abundantly during replication and has low cross-reactivity with other human CoVs, such as OC43 and 229E (227, 291). Interestingly, one study that measured serum N protein levels using ELISA in SARS-COV-2 infected patients showed a positivity rate of 76% before antibody was detected, implying that the detection of N protein in serum might be useful for early diagnosis. Although the results are encouraging, this was a very small study (292). Further studies are needed to confirm the results and determine whether infected patients have a higher incidence of viremia in the early stages or whether over-expressed N protein from the lung virus is spilling into the blood.

The widely available SARS-CoV-2 antigen kits use two main approaches: (1) the immunochromatographic (ICT) assay based on colloid gold conjugated antibodies that result in visible colored bands to reflect positivity and (2) the fluorescence immunochromatographic assay (FIA) that provides results via an automated immunofluorescence reader (290). Another approach developed to detect SARS-CoV-2 specific antigen uses nanotechnology in biosensor devices. A field-effect transistor/FET-based biosensing device and fiber-optic absorbance biosensor/P-FAB platform have been developed to detect S and N protein from SARS-CoV-2, respectively (293, 294). Preliminary evaluation suggests these devices are highly sensitive and require no or minimal sample pre-processing (293); however, additional external validation is needed before they can be incorporated into clinical practice.

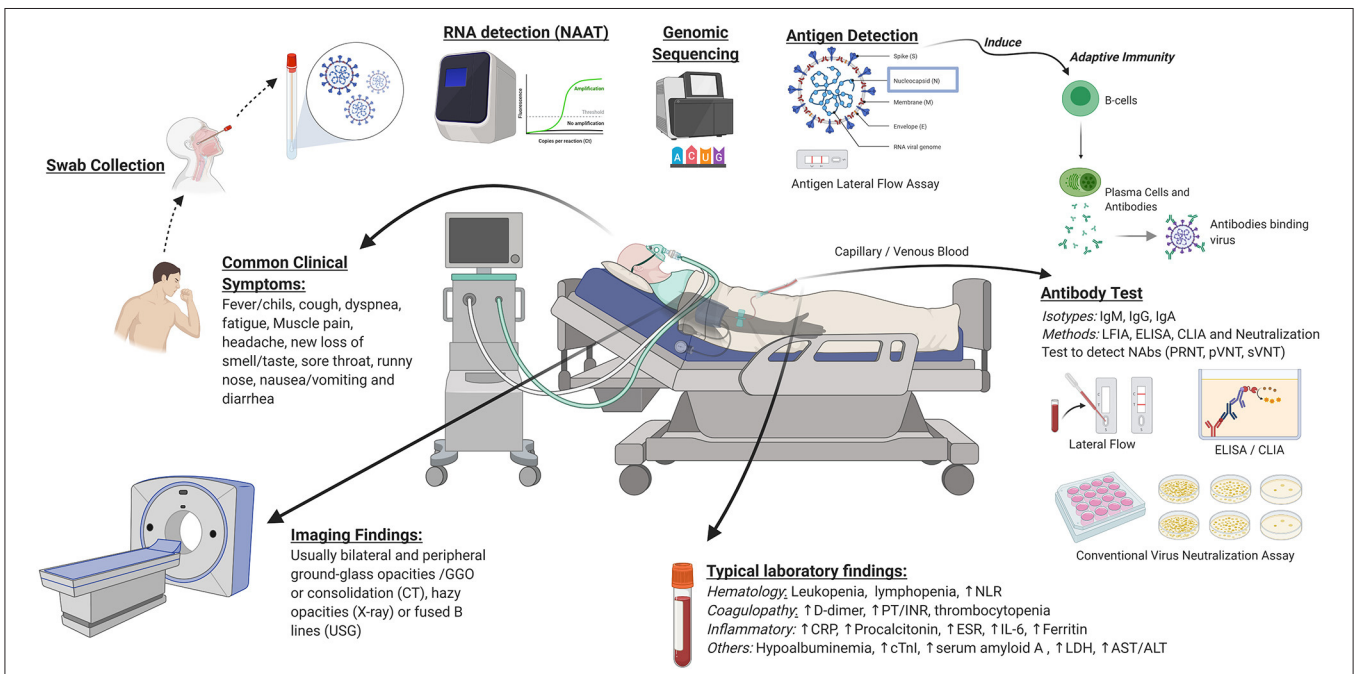

FIGURE 2 | Clinical and *in vitro* diagnostics for COVID-19. Clinical diagnostics consist of common clinical symptoms, imaging findings, and laboratory markers. *In vitro* diagnostics include molecular testing, antibody tests, and viral antigen detection. NAAT, nucleic acid amplification tests; PoC, point of care; CRISPR, clustered regularly interspaced short palindromic repeats; NAbs, neutralizing antibodies; PRNT, plaque reduction neutralization test; pVNT, pseudovirus-based virus neutralization test; sVNT, surrogate virus neutralization test; LFIA, lateral flow immunoassay; ELISA, enzyme-linked immunosorbent assay; CLIA, chemiluminescent immunoassay; NLR, neutrophil-lymphocyte ratio; PT/INR, prothrombin time and international normalized ratio; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IL-6, interleukin 6; cTnI, cardiac troponin I; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CT, computed tomography; USG, ultrasound sonography. Image was created in Biorender.com.

TABLE 1 | *In vitro* diagnostics for COVID-19 and potential areas for development.

In vitro diagnostic	Currently available assays	Brief description	Development areas
Molecular testing, NAAT	RT-PCR assays (conventional or automated). Alternative terminologies include rRT-PCR or RT-qPCR.	• NAAT detects the presence of viral RNA (62) • Purified RNA from clinical specimens is reverse transcribed into complementary DNA (cDNA), then added to a master mix containing target primers and a fluorophore-quencher probe. The RT-PCR process is carried out in a thermal cycler. The fluorophore-quencher probe is cleaved, generating a fluorescent signal that corresponds to the amplified product (63, 114) • While conventional NAAT begins from manual RNA preparation, followed by rRT-PCR; automated systems integrate RNA extraction, purification, amplification, and detection, resulting in rapid, high-throughput results and less contamination (70–72, 74)	• Pre-heating specimens to skip RNA extraction (66–69) • Accuracy with alternative, less invasive specimens (e.g., Saliva) in comparison with standard NP specimens (87–89, 91) • Lower respiratory specimens may provide benefit later in the disease course (94), while non-respiratory specimens may correlate with local symptoms (e.g., stool) or clinical severity (e.g., blood) (99, 103, 133) • Swab pooling to increase testing capacity (93) • Different PCR target regions may affect sensitivity (116, 122–124) • Monitoring effect of SARS-CoV-2 genome mutations on RT-PCR performance (118, 136) • One-step (consolidated RT and PCR) vs. two-step (separate RT and PCR) assays, and uniplex vs. multiplex RT-PCR (63, 65, 114) • Subgenomic RNA and/or Ct value as the surrogate for infectious/live virus (139)
	PoC–Xpert® Xpress SARS-CoV-2	It targets the E and N2 SARS-CoV-2 genes, performed on an automated GeneXpert instrument. LOD 8.26 copies/mL and TAT is 45 min (146)	Further development of Xpert® to detect important SARS-CoV-2 mutations may be needed, as is done for TB (148)
	PoC–CovidNudge	It is based on a fully-automated multiplex RT-PCR targeting seven SARS-CoV-2 gene targets (RdRp1, RdRp2, E-gene, N-gene, N1, N2, and N3). LOD 250 copies/mL and TAT is 90 min (155, 156)	• CovidNudge has low throughput compared with RT-PCR (1 sample per run), multiple instruments may be needed depending on the clinical setting (157) • Studies have only assessed performance with NP/OP swabs (156). Further validation is warranted, and other sample types should be examined
	PoC–TrueNat	This chip-based portable PoC targets SARS-CoV-2 E and RdRP genes. LOD 486 copies/mL and TAT is <1 h (160, 161)	Despite affordability and portability, this technology is low throughput and further external validation studies are warranted (63)
	PoC–ID Now COVID-19	It is based on the Nicking Enzyme-Assisted Reaction (NEAR), which targets the SARS-CoV-2 RdRP gene. LOD 125 genome equivalents/mL and TAT is 5–13 min (149, 150)	Suitability of ID Now as a confirmatory test is uncertain due to a study suggesting low PPA, despite using freshly collected specimens as now recommended by the manufacturers (151, 152)
	PoC–BioFire® Respiratory Panel 2.1 (RP2.1)	It was created by adding primers targeting M and S genes of SARS-CoV-2 to the existing multiplexed BioFire® Respiratory Panel 2 (RP2), which can detect multiple pathogens in a single swab. LoD 500 copies/mL and TAT is 45 min (162, 163)	As RP2.1 detects spike genes, a hotspot for mutation, utility of this PoC test for detection of variants should be routinely assessed.
	PoC–cobas® Liat®	It identifies and differentiates SARS-CoV-2 (targeting ORF1a/b and N genes), influenza A and B virus via multiplex RT-PCR. LoD 12 copies/mL and TAT is 20 min (164)	Since it simultaneously tests for influenza and SARS-CoV-2, thus allowing differentiation between both viruses that may co-circulate in the annual flu season (165). Validation with other multiplexed assays is desired
	PoC–GenMark ePlex	It targets the N gene of SARS-CoV-2 and uses electrowetting and GenMark's eSensor technology based on competitive DNA hybridization and electrochemical detection. LoD 750 copies/mL and TAT is <2 h (155, 171)	The multiplex version (ePlex RP2 Panel) should be further validated with another multiplexed assay (e.g., BioFire® RP2.1 and Cobas® Liat) since NAAT methods differ between those assays
	PoC–Diasorin Simplexa™	It targets SARS-CoV-2 ORF1ab and S genes, can run 8 samples per disc; LoD 500 copies/mL and TAT ~90 min (155, 173, 174)	As it detects the spike gene, a mutation hotspot, utility for detection of variants should be routinely assessed
	RT-LAMP	It detects multiple SARS-CoV-2 genes, including ORF1ab, S, E, and/or N gene, using isothermal amplification, thus does not require thermal cycling (175–178). Real-time results are monitored with colorimetric or fluorescent dyes (43, 180)	• False positives may occur due to presence of multiple pair primers (183), while false-negatives may occur with low viral RNA (175, 183); indicates evaluation should be performed across a range of SARS-CoV-2 viral loads • Smartphone integration and combination with nanopore sequencing and CRISPR-based detection platforms may improve performance (183, 184, 313)

(Continued)

TABLE 1 | Continued

In vitro diagnostic	Currently available assays	Brief description	Development areas
	CRISPR	The guide RNA (gRNA) targets SARS-CoV-2 RNA sequences, which can be recognized by CRISPR-associated (Cas) proteins, result in collateral cleavage of the reporter probes and the appearance of a positive band on the paper strip (178, 187–189)	• Advantages in comparison to RT-PCR include rapid TAT and reduced equipment and reagent requirements (194) • Emerging CRISPR-based methods require validation and additional field testing (195)
	ddPCR	In this digital PCR, the sample is fractionated into thousands of droplets, and the PCR amplification of the template molecules occurs in each droplet, thus allowing for absolute quantification of genomic material (197)	ddPCR assays enable nucleic acid measurement and pathogen diagnosis with limited sample processing, therefore may have a role in monitoring viral load during the disease course and convalescence (199)
	NGS	Sequencing is used to determine the order of the bases within the genome. NGS has three general steps: DNA library preparation, clonal amplification of the library, and DNA sequencing by detecting emitted optical or chemical signals (67, 200)	• Cost is currently high • Potential high utility in genomic surveillance to monitor variants with increased transmissibility and/or virulence, ability to evade detection by current diagnostics, and ability to escape antiviral treatment or immunity (203)
Antibody assays	Serology Assay: • ELISA • CLIA • LFIA	• Antibody serology assays detect antibodies against SARS-CoV-2 (15) • ELISA uses plates pre-coated with viral antigens, such as Spike or Nucleocapsid protein (226, 227), and CLIA uses magnetic, protein-coated microparticles to detect antibodies (228). If the serum contains SARS-CoV-2 antibodies, antibody-protein complexes form and are bound with anti-human antibodies tagged with the enzyme to produce a light-based, luminescent readout (229, 230) • LFIA employs a similar method with sandwich ELISA, but the immunological reaction is carried out on the chromatographic paper by capillary action, results in the appearance of a colored line on the strip (225)	• Serological data is most useful for epidemiologic purposes and may facilitate identification of potential convalescent plasma donors and assessment of vaccine immunogenicity (214, 216, 217), although protective titer is not yet well-defined • Poor sensitivity of LFIA compared with ELISA/CLIA may be associated with use of capillary blood for PoC-LFIA test vs. serum/plasma use on ELISA/CLIA (223) • Possible cross-reactivity with other pathogens and/or rheumatoid factor (248, 264) • Unclear whether Spike Protein-based Assay vs. Nucleocapsid Protein-based Assay has better sensitivity (226, 248) • Seroconversion timing between antibody class varies across studies (276, 281) • Dynamic antibody profiling data between severity stages and the duration of antibody response are not well-established (278, 285) • Theoretical possibility that mutations will affect assay performance (259) • Variable accuracy of results amongst different commercially available kits (236)
	Neutralization Assay: • PRNT • pVNT • sVNT	• NAbs are specific for viral epitopes that mediate entry of the virus into a host cell; thus their presences indicate protective immunity (255) • Conventionally, NAbs were measured by PRNT, in which serial dilutions are incubated on a host cell monolayer for several days to determine final dilution titer at which virus plaque formation is inhibited (97) • pVNT has a similar method but uses other viruses pseudotyped with SARS-COV-2 Spike to mimic the infectious virus (256) • sVNT detects NAbs without the need for live viruses or cells. Using purified RBD from the S protein and the host cell receptor ACE2, this test mimics the virus-host interaction in an ELISA plate well (252)	• PRNT is labor-intensive, requires BSL-3 facility, and takes 2–4 days to complete; it is thus impractical for large scale applications (252). Pseudovirus is safer to handle in a BSL-2 laboratory, but still requires culture methodology (256) • Studies did not clearly define sVNT cut-off value in relation to conventional PRNT titer. Validation with different clades or emerging variants is needed to ensure its robustness (252) • Some studies showed positive correlation between the SARS-CoV-2 viral NAbs titer and the S-RBD–specific IgG, with a NAb titer of 1:80 approximately equivalent to a titer of 1:1,280 for S-RBD-specific IgG (253), or NAb titers 1:160 corresponds to anti-RBD titer $\geq$1:1,350 (254). Studies differ in specific assay used, so titers between studies may not be equivalent. NAb protective titer is not yet well-defined
Antigen assays	ICT and FIA assay	• Antigen-based diagnostics detect protein fragments on or within the virus (178). They mostly target the C-terminus of N gene/protein via a diagnostic sandwich assay using monoclonal Abs (259) • ICT uses colloid gold conjugated antibodies, resulting in visible colored bands, while FIA is usually read by the automated immunofluorescence reader (290)	• As antigen tests perform best in samples with high viral loads and during the first 5–7 days of symptoms (302), they may be useful for early diagnosis and interruption of transmission (307) • Validation studies needed for fresh vs. frozen swab samples (300), viscous vs. non-viscous specimens (298), NP vs. saliva samples (297) • Performance of antigen assay might be impacted by virus mutations (259)

NAAT, nucleic acid amplification tests; RT-PCR, real-time quantitative reverse transcriptase polymerase chain reaction; dsDNA, double-stranded DNA; NP, nasopharyngeal; PoC, point of care; LOD, limit of detection; TAT, turnaround time; LAMP, Loop-mediated isothermal amplification; CRISPR, clustered regularly interspaced short palindromic repeats; ddPCR, droplet digital PCR; NGS, next-generation sequencing; ELISA, enzyme-linked immunosorbent assays; CLIA, chemiluminescent immunoassays; LFIA, lateral flow immunoassays; Nabs, neutralizing antibodies; PRNT, plaque reduction neutralization test; pVNT, pseudovirus-based virus neutralization test; sVNT, surrogate virus neutralization test; RBD, receptor binding domain; BSL, biosafety level; ICT, immunochromatographic; FIA, fluorescence immunochromatographic assay.

Several publications on the validation of the antigen kit against the gold standard (PCR) using swab samples showed excellent specificity (99.5–100%) and varying overall sensitivity (11.7–68.8%), with higher viral loads associated with better sensitivity (289, 295–298). This is analogous to the performance of the influenza antigen test in the H1N1 pandemic, where specificity was excellent but sensitivity was low (46.7–53.3%). Suboptimal sensitivity is not unexpected, as low viral loads, consistent with low number of viable viruses and likely low infectiousness, would predispose to false negatives (299, 300). Possible antigen destruction on frozen or repository swab samples may also decrease accuracy (300). According to the manufacturer's instruction for use, nasopharyngeal samples must be fresh and should be tested as soon as possible after collection. Antigen test evaluations performed on leftover sample material after a delay of 1 h to 2 days and storage at 4°C were conducted alongside qRT-PCR (289, 296). These prolonged storage conditions, along with the dilution of samples in transport media, may have impacted assay sensitivity (298). Alternatives to nasopharyngeal swabs, such as sputum or saliva, could also contribute to the variability of results (297, 301).

Although more evidence is needed, data suggest Ag-RDTs are likely to perform well (91–100% sensitivity) in patients with high viral loads (Ct values $\leq$25 or $>10^6$ genomic virus copies/mL) (302), which usually appear in the pre-symptomatic (1–3 days before symptom onset) and early symptomatic phases of the illness (within the first 5–7 days of illness) (303–305). A recent study on community-dwelling subjects with mild respiratory symptoms showed the Ag Rapid Test had 100% specificity and sensitivity above 95% for nasopharyngeal samples when using Ct-values $<$ 32 cycles as the cut-off for RT-qPCR test positivity (306). In its September 11th, 2020, interim guidance, WHO recommends use of SARS-CoV-2 Ag-RDTs that meet the minimum performance requirements of $\geq$80% sensitivity and $\geq$97% specificity compared to a NAAT reference assay. Testing should be conducted by trained staff in strict accordance with the manufacturer's instructions and within the first 5–7 days following onset of symptoms (302). Patients who present more than 5–7 days after symptom onset are more likely to have lower viral loads and false-negative results with Ag-RDTs (302).

When performance is acceptable, rapid antigen tests can reduce transmission through early detection of highly infectious cases, enabling implementation of targeted isolation and tracking of infectious cases and contacts (307). The excellent specificity of these tests could support public health decisions (298), though the current suboptimal sensitivity suggests that antigen testing may be most useful as an adjunct to the gold-standard RT-PCR (301). According to the updated December 2020 WHO COVID-19 case definition, a person with a positive SARS-CoV-2 Ag-RDT AND who meets either the probable of suspect case definition (high pre-test probability) is classified as confirmed case without RT-PCR confirmation (308). This new case definition is particularly useful in countries with limited molecular NAAT testing. However, because Ag-RDTs can perform differently in manufacturers' trials than in the real world, they merit further comparative evaluation with a standardized validation protocol (309). Additionally, impact of evolving mutations on performance of Ag-RDTs should be anticipated, although it is less likely as most tests target the C-terminus of N gene, which is not a mutation-hotspot (259).

FUTURE DIRECTION

Availability of established diagnostic technologies has enabled researchers to rapidly adapt them to COVID-19 (114). Lessons from the 2002 SARS outbreak have guided development of COVID-19 detection strategies. Only 3 weeks elapsed from visualization of the virus using transmission electron microscopy to elucidation of the SARS-CoV-2 genetic sequence, while SARS-CoV took 5 months to be recognized (114, 310). This reflects the research community's tremendously accelerated response as well as increases in diagnostic capacity between 2002 and 2020, including accessibility of next-generation sequencing for rapid sequence determination (311). Nonetheless, the ever-expanding panoply of tests requires ongoing optimization. Many need further validation to ensure accuracy, speed, ease of use and broad deployability. Additional research on utility of these diagnostics for zoonotic surveillance may help with mitigation of future epidemics (312).

Control of epidemics requires extensive, ongoing surveillance, and rapid sharing of epidemiological data (313). Smartphones, usage of which has increased exponentially, including in sub-Saharan Africa, can be leveraged for this purpose as they possess connectivity, computational power, and hardware to facilitate electronic reporting, epidemiological databasing, and point-of-care testing (114, 314). Combining diagnostics tools with smartphone integration could support better management, curb transmission of infection and reduce mortality (114).

Safety of laboratory workers who conduct COVID-19 testing is also paramount. Concern for laboratory-associated infection is of particular concern in the setting of Personal Protective Equipment (PPE) shortages, improper microbiological techniques, lack of training, and inadequate decontamination protocols or biosafety measure (315), all of which are more likely to occur when systems are overwhelmed. Optimization of mechanisms to protect laboratory workers should occur in parallel with optimization of COVID-19 diagnostics.

CONCLUSION

Diagnosis of COVID-19 is based upon clinical and *in vitro* approaches. A summary of clinical and *in vitro* diagnostic approaches for COVID-19 is depicted in **Figure 2**. Basic principles of *in vitro* diagnostics and potential areas for development are listed in **Table 1**. Selection of the most appropriate diagnostic method depends upon the situation, including patient presentation, timing relative to disease course, laboratory infrastructure, available management options, public health needs, and research agendas. Clinical diagnostic evaluation and antibody and antigen-based assays can complement RT-PCR, the preferred confirmatory diagnostic for COVID-19. While antibody assays are mainly indicated for epidemiologic purposes due to delayed seroconversion, the

antigen-based assay may be indicated for rapid identification of highly infectious cases in disease course, which could reduce further transmission. Availability of diagnostic assays is rapidly expanding, as demonstrated by the ever-increasing list of assays granted EUA status by the U.S. FDA. Well-designed validation studies should be conducted to identify products with the best performance and to obtain the data necessary to support licensure. As early diagnosis is essential for patient management and outbreak control, development of rapid, scalable, and high-accuracy PoC assays should be prioritized. Highest priority should be assigned to cost-effective multiplexed PoC tests that identify multiple pathogens.

AUTHOR CONTRIBUTIONS

All authors contributed equally with respect to conception and design of the study, literature review and analysis, drafting, critical revision, editing, approval of the final version, and approve this manuscript for publication. NCI and NIAID collaborators contributed to design of the study, collection, analysis, and interpretation of data, and writing of the manuscript.

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COVID-19 Surveillance in the Primary Health Care Population of Qatar: Experience of Prioritizing Timeliness Over Representativeness When Sampling the Population

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SARS-CoV2 a new emerging Corona Virus Disease in humans, which called for containment measures by many countries. The current paper aims to discuss the impact of two different sampling methodologies when executing a drive through COVID-19 survey on the quality of estimated disease burden measures. Secondary data analysis of a pilot cross-sectional survey targeting Qatar's primary health care registered population was done. Two groups with different sampling methods were compared for estimating COVID-19 point prevalence using molecular testing for nasopharyngeal swabs. The first group is a stratified random sample non-proportional to size ($N = 260$). A total of 16 population strata based on age group, gender, and nationality were sampled. The second group is the Open invitation group ($N = 841$). The results showed that the two groups were obviously and significantly different in age and nationality. Besides, reporting of COVID-19 symptoms was more frequent in the open invitation group (28.2%) than the random sample (16.2%). The open invitation group overestimated the symptomatic COVID-19 prevalence rate by more than four times, while it overestimated the asymptomatic COVID-19 cases by a small margin. The overall prevalence rate of active COVID-19 cases in the open invitation sample (13.3%) was almost double that of the random sample (6.9%). Furthermore, using population sampling weights reduced the prevalence rate to 0.8%. The lesson learned here is that it is wise to consider the magnitude of bias introduced in a surveillance system when relying on convenient sampling approaches in response to time constraints.

Keywords: COVID-19, surveillance, sampling methods, quality, primary health care

INTRODUCTION

On 31st December 2019, Chinese national authorities reported an outbreak of pneumonia with unknown etiology (1). On the 12th of January 2020, National Health Commission in China associated the outbreak to a seafood market in in Wuhan (China) and shared the genetic sequence of the novel causative agent - a novel coronavirus (1).

Coronaviruses in the recent past have come to attention as pathogens of emerging respiratory disease outbreaks such as, Severe Acute Respiratory Syndrome (SARS) in 2002–3 and Middle East Respiratory Syndrome (MERS) in 2012–14. The newly identified coronavirus with its epicenter in Wuhan was labeled Severe Acute Respiratory Coronavirus 2 (SARS-CoV2) and is also known as 2019 novel coronavirus (2019-nCoV) and coronavirus disease 2019 (COVID-2019) (2).

SARS-CoV2 very quickly spread to other parts of China and the world. First imported cases were reported in Japan, Thailand and Republic of Korea between the 13–20th January (1). The first 1,000 cases were infected within 48 days a significantly higher rate compared to SARS and MERS which took 4 months and 2 and a half years, respectively (3). With 18 countries affected and as the outbreak continued to spread globally, the World Health Organization (WHO) declared it a Public Health Emergency of International Concern (PHEIC) on the 30th January 2020 (4). Eventually on March 11, 2020, the WHO declared the SARS-CoV2 outbreak a pandemic (5). Controlling the disease is still a priority worldwide with more than 116 million cases and 2,700 thousand deaths recorded until the 7th of March 2021 (6).

Primary care is the cornerstone of any health system. During pandemics, primary care is the frontline against emerging infectious diseases in communities. It provides infrastructure and plays a variety of key roles such as disease surveillance, diagnosis and treatment, prevention, patient education etc., (7). During the peak week of a pandemic, one can expect additional primary care visits (8). These present challenges and opportunities in primary care as the SARS-CoV2 continues to spread in the country. Among them is describing the extent of disease spread and population sectors most affected. Survey tools are needed to assess the disease burden (9). Such tools are subject to known, or at least anticipated to have biases which can threaten clinical and epidemiological studies (10).

In May 2020 the only available laboratory testing approach to screen for COVID-19 was using anasopharyngeal swab to analyze by reversed transcription polymerase chain reaction (rt-PCR). This laboratory approach was used to calculate a crude measure of population prevalence which is the fraction of positive tests in a cross-sectional time frame. Such a measure of disease frequency is always liable to distortion by ascertainment bias since tests are typically only ordered from symptomatic cases seeking health care, whereas, a large proportion of infected might show little to no symptoms. Contact tracing may reduce this distortion, but this will always depend on test availability and the capacity of surveillance health system (11, 12). It has been suggested that this capacity for rapidly identifying individuals infected with the virus can become more efficient by pooling (or combining) individual samples (30 to 100 samples) and testing them in a single group. Such a method can decrease the cost of screening contacts at the expense of reduced test sensitivity (13).

Sampling technique is the most important concept in survey studies, since it is impractical, uneconomical or feasible to test the whole population, even after considering pooling of individual samples as a method of cost reduction. The sample should represent the population for the survey results to have external validity. It is clear that random samples are superior to convenient ones for quantitative research studies. However, a pandemic like COVID-19 may call for desperate actions and serves as an excuse for using less stringent criteria in choosing survey samples without assessing the extent of bias introduced during the process (14). Containment measures may push for an expedited approach to epidemiologic info.

A survey was designed to estimate prevalence in Qatar's primary care registered population (15).The aim of this paper is to present the lessons learned from using two different sampling methodologies applied when executing the survey. In addition, it provides a snapshot of the COVID-19 outbreak in Qatar's primary care registered population after 3 months from the start of the COVID-19 pandemic.

METHODS

The current study is based on secondary data analysis for a two days cross-sectional pilot survey study executed on May 2020. The study protocol for the survey was designed by the Department of Clinical Research at Primary Health Care Corporation to generate epidemiologic data to plan and respond to the pandemic in Qatar.

Study Settings

Qatar, a peninsular Arab country that operates a universal publicly funded health care system accessible to Qatari national and expatriates who hold a valid health card. The primary healthcare service in Qatar are delivered by the Primary Health Care Corporation (PHCC), which is the largest primary care provider in the country with 27 health centers distributed across three geographical regions – North, Central and South.

Study Samples

The survey originally targeted a random sample of PHCC registered population (N = 1,063,243 as of May 2020 or ∼70% of the total population of Qatar) with only two working days assigned for data collection phase. This group is referred to as the "Random Sample Group" (RSG). The sampling method was a stratified random sample non-proportional to size. The stratifying factors were age group, gender and nationality representative of the overall PHCC registered population. A total of 130 individuals were randomly selected from each of 16 population strata. To adjust for non-response, 50% extra participants were added (n = 65). The final strata sample size will be 195 and the resulting total sample size will be 3,120.This sampling approach was used to ensure adequate representation for all population strata, while obtaining a representative summary measure for the reference population through proper

weighting at the analysis stage. The details of the survey protocol are published elsewhere (15).

During the first day of the data collection phase of the survey a low response rate of around 10% was observed and a decision was made to send an open invitation to all the PHCC registered population to attend on the second (last) day of the pilot survey. The Open Invitation Group (OIG) was recruited during the second and final day of the survey. SMS messages were sent to every individual in the target population (PHCC registered population) providing them with the opportunity to be tested for COVID-19 on the next day if they register themselves on a designated web site.

Study Locations

One PHCC health center from each of the three regions in Qatar were identified as a study location - Al Thumama (South), Leaibab, and Al Waab. The health centers were set up to facilitate drive through testing of participants. This setup of test locations allowed equal chances for the invited residents from each of the three principal regions of Qatar to access them.

Invitation

The study was conducted over 2 days (5th and 6th of May). A national campaign to publicize the study was initiated 2 days prior its launch using social media and newspapers. RSG Participants were also sent an SMS message inviting them 2 days in advance. The SMS message included a link to a questionnaire survey to accept or decline the invitation. All participants were invited to attend a study location in the same region as the health center they were originally registered.

Data Collection

Data collection at study locations was undertaken as a drive through. Participants were seated in their cars and queued to be attended by a data collector. Data collection was undertaken as a 4-step process, steps 1–3 by a data collector and step 4 by a trained nurse.

- Step 1: Verify participants' identification details.
- Step 2: Confirm participant was invited by SMS or not.
- Step 3: Administer a questionnaire to collect information on their age, gender, nationality, and COVID-19 symptoms.
- Step 4: Provide a nasopharyngeal swab.

Laboratory rtPCR Test Procedure

The nasal and throat swabs were labeled and transported from the study location to the referral laboratory for the state of Qatar's at the end of each shift. RNA was extracted and isolated prior to amplification using the rtPCR (reverse transcription polymerase chain reaction) test. Each assay was validated for cycle threshold (CT) value interpretation using the manufacturer's instructions. Test results were reported as negative or positive (16).

Data Analysis

All data was subject to quality assurance. For the purposes of this study, point prevalence was defined as the number of active SARS-CoV2 infections (identified by RT-PCR) over the total sample size. Chi-square test of independence was used to assess the statistical significance of associations between nominal or ordinal scale variables. *P*-value less than the 0.05 level of significance was considered statistically significant. All statistical analyses were done using survey commands in SSPS (version 23).

Sampling weights are the inverse of the likelihood of being sampled. The purposes of weighting the summary prevalence estimate of the population at the analysis stage was to compensate for non-response and the unequal probabilities of selection. The sampling fraction and the response rates in each population strata were used as sampling weights (17). Please refer to **Supplementary Material** for further details of calculations.

Positive Test Results

All study participates were informed of their test results by SMS. All the participants who tested positive for SARS-CoV-2 were contacted by telephone by a team designated by the authorities to track their infection.

Ethical Considerations

The current study is based on anonymized secondary data analysis of a pilot survey study executed on the 4th and 5th of May 2020. The study presented a minimal risk of harm to its subjects since there was no direct interaction with study participants and the data was requested from the data custodian in PHCC with no personal identifiers. Overall, the study was conducted with integrity according to generally accepted ethical principles and was approved by the PHCC's independent ethics committee (PHCC/DCR/2020/05/051).

TABLE 1 | Comparing the two study samples by sociodemographic variables.

	Open invitation group *N* (%)	Random sample group *N* (%)	*P*
Age group (years)			<0.001
<18	25 (3.0)	0 (0.0)	
18–39	579 (68.8)	123 (47.3)	
40–59	216 (25.7)	106 (40.8)	
60–74	21 (2.5)	31 (11.9)	
Total	841	260	
Gender			0.91 [NS]
Female	161 (19.1)	49 (18.8)	
Male	680 (80.9)	211 (81.2)	
Total	841	260	
Nationality			<0.001
Qatar	110 (13.1)	56 (21.5)	
Other Arab countries	164 (19.5)	83 (31.9)	
Europe/North America/Australasia	21 (2.5)	11 (4.2)	
Southern Asia	456 (54.2)	96 (36.9)	
South-Eastern Asia	81 (9.6)	7 (2.7)	
Eastern-Central Asia	0 (0.0)	5 (1.9)	
Rest of Africa	9 (1.1)	2 (0.8)	
Total	841	260	

TABLE 2 | The difference in relative frequency of selected symptoms between the two study groups.

Symptoms/complaints in the last 2 weeks	Open invitation group ($n = 841$)		Random sample group ($n = 260$)		*P*
	N	%	N	%	
Fever 38°C or higher	63	7.5	8	3.1	0.011
Sore throat	87	10.3	12	4.6	0.005
Cough	109	13.0	10	3.8	<0.001
Chills	4	0.5	1	0.4	1 [NS]
Fatigue	18	2.1	3	1.2	0.43 [NS]
Muscle ache	22	2.6	4	1.5	0.31 [NS]
Runny nose	34	4.0	9	3.5	0.67 [NS]
Shortness of breath	23	2.7	2	0.8	0.06 [NS]
Wheezing	5	0.6	2	0.8	0.67 [NS]
Chest pain	23	2.7	3	1.2	0.14 [NS]
Other respiratory symptoms	25	3.0	3	1.2	0.10 [NS]
Headache	68	8.1	12	4.6	0.06 [NS]
Nausea/vomiting	6	0.7	0	0.0	0.35 [NS]
Abdominal Pain	4	0.5	4	1.5	0.1 [NS]
Diarrhea	9	1.1	0	0.0	0.13 [NS]
Loss of sense of smell	15	1.8	1	0.4	0.14 [NS]
Loss of sense of taste	9	1.1	1	0.4	0.47 [NS]
At least one symptom (in the last 2 weeks)	237	28.2	42	16.2	<0.001
Complaints requiring medical attention	9	1.1	3	1.2	1 [NS]

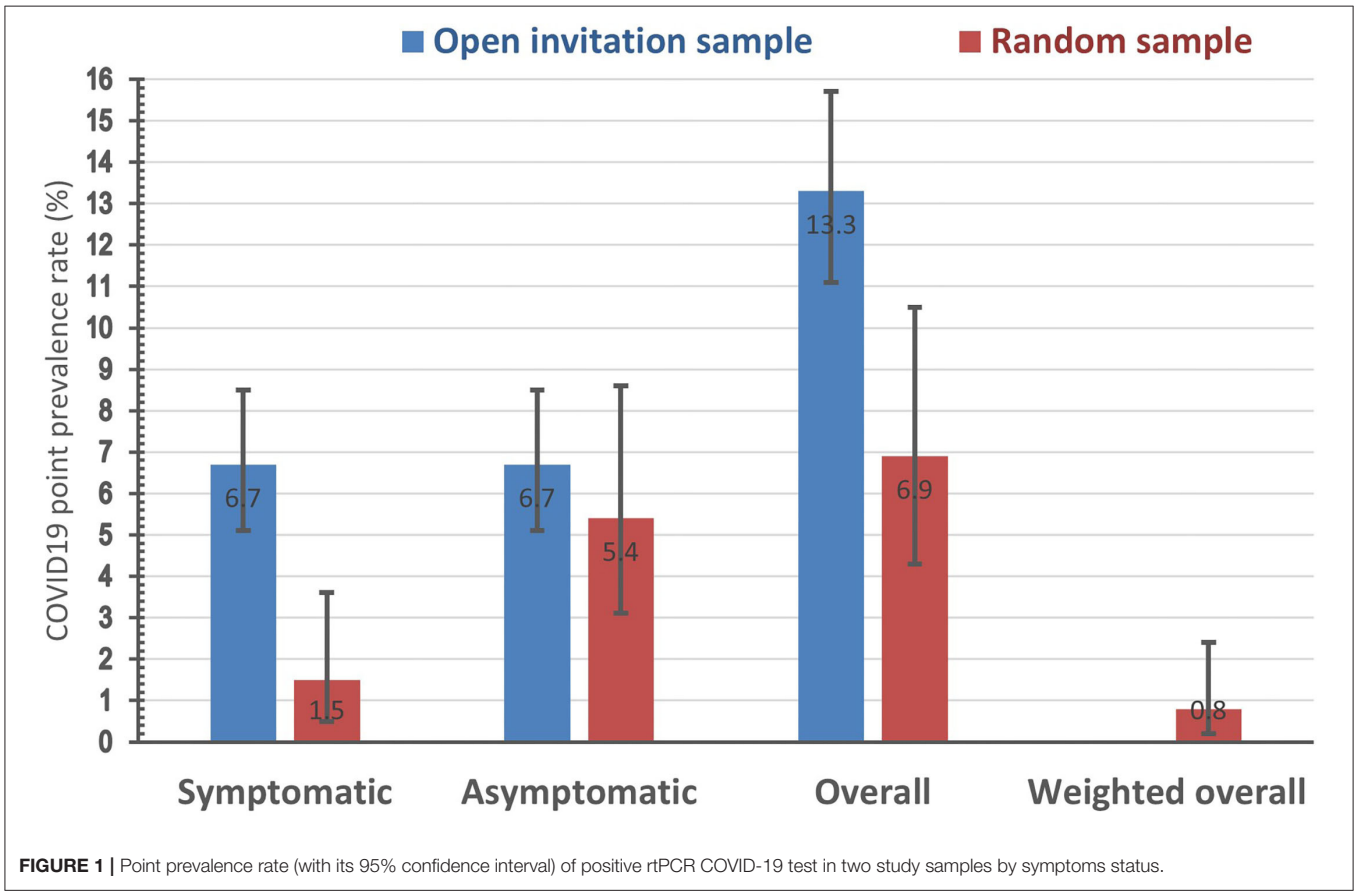

FIGURE 1 | Point prevalence rate (with its 95% confidence interval) of positive rtPCR COVID-19 test in two study samples by symptoms status.

TABLE 3 | Estimated period prevalence rates of Qatar population.

	Confirmed cases (PCR positive)*	PCR tests performed	Population size	Test yield (PCR test positivity rate)	Period prevalence estimate (%)
Cumulative counts since the start of pandemic (29/2/2020) till the last day of the current survey (5/5/2020)	17,142	109,762	2,807,805	15.6	0.6

** Note: These are national figures of COVID-19 positive PCR test results. They include the cases discovered in the current study survey.*

RESULTS

The results presented in this section were based on the analysis of 841 individual in the open invitation group and 260 individual in the random sample group. As shown in **Table 1**, there was an obvious and statistically significant difference in age distribution between the two study groups. The random sample being older in age than the open invitation. Gender distribution was however not different with females constituting less than one fifth of the two study groups. The composition of the groups according to nationality was also significantly different. Qataris and other Arab localities being less represented in the open invitation group, while Southern Asia and South-Eastern Asia nationalities were over-represented in the same group compared to random samples.

A history of contact with suspected or confirmed case in the last 2 weeks was significantly more frequent in the open invitation group (32%) compared to random sample group (13.3%). In addition, almost all the reported symptoms were more frequent in the open invitation group. Three of the symptoms, namely: fever 38°C or higher, sore throat and cough were significantly more frequent among the open invitation group compared to the random sample. The proportion of symptomatic subjects with at least one symptom in the last 2 weeks was also significantly higher in the open invitation group (28.2%) compared to random sample (16.2%), **Table 2**.

The prevalence rate of symptomatic COVID-19 cases was more than four times higher in the open invitation sample (6.7%) compared to the random one (1.5%). However, that of asymptomatic cases was only marginally higher in the open invitation sample (6.7%) compared to random one (5.4%). The overall prevalence rate of active COVID-19 cases in the open invitation sample (13.3%) was almost double that of the random sample (6.9%). The ratio of asymptomatic to symptomatic COVID-19 cases the random sample group was 3.6, while it was exactly 1 for the open invitation sample. The crude (unweighted) overall population prevalence rate in the random sample was 6.9%, while the weighted estimate after adjustment for the sampling fraction in of the 12 population strata available for analysis (the four strata of those younger than 18 years had a null value as none of these strata were respondents) is only 0.8% (with a 95% confidence interval ranging between 0.2 to 2.4%), **Figure 1**.

The cumulative prevalence rate of all positive COVID-19 PCR test from the time when the first case was recorded in Qatar on 29/2/2020 until the last day of the current study survey is 0.6%, **Table 3**.

DISCUSSION

As the demand for accountability increased in the recent time the quality of data and reported figures has become crucial for public health program's performance. According to MEASURE Evaluation "data must be of high quality if they are to be relied upon to inform decisions on health policy, health programs, and allocation of scarce resources" (18). Among the important elements of data quality is relevance, accuracy, comparability, and timeliness (19). The first three of these elements can only be assured by using a random sample. In addition, using mathematical modeling to measure bias is an established method in research (12), but the current study is among few that provides an opportunity to measure it directly in a real life example comparing the results provided non-random sample (OIG) to the random one (RSG).

The COVID-19 survey was planned as a sentinel surveillance to be repeated at regular intervals on a representative batch of nasopharyngeal specimens, which is strongly advised by WHO as a strategy to identify and estimate community cases and inform planning especially in a primary care setting (20). A probabilistic sampling in a determined population is the method of sampling advocated that organization in the context of COVID-19. Disease positivity rates obtained from surveillance is subject to distortion with under-ascertainment of cases being the most important. This type of bias is especially disturbing in pandemics of new diseases with wide variation in clinical features as this can impact the implementation of public health policy and risk awareness (20). Interestingly, the current study showed an inverse type of bias affecting the surveillance system that was tested in primary health setting, that is an over-estimation of the point prevalence rate driven by the open invitation sample. This type of convenient non-probability sample was used in a COVID-19 population survey in Iceland, where a total of 10,797 persons received open invitations and another 2,283 invited in a random sample selection. The Icelandic study which was executed during March and April of 2020 showed that random sampling was associated with a lower proportion of positive PCR test results for COVID-19 (0.6%) compared to the open invitation group (0.8%) (21).

The current study in Qatar showed an obvious and statistically significant difference in age group and nationality representations between the random sample and open invitation group. A history of contact with suspected or confirmed case in the last 2 weeks, which is clearly an important risk factor for testing positive for COVID-19 was almost three times more frequent in the open invitation group. This difference may

serve as an explanation for the overestimation bias caused by the open invitation group in a time where testing COVID-19 was not available for personal motives. Having a contact history motivates an individual to seek for COVID-19 testing and increase the probability of responding to an open invitation for testing COVID-19, since the test is not available upon personal request. The second argument that may serve as an explanation for the over-estimation bias caused by the open invitation group in the current study is the higher frequency of reporting fever, sore throat and cough among that group that motivated those individuals to favorably reply to the invitation sent. Similarly, this may give some clue to the equal ratio of asymptomatic and symptomatic COVID-19 cases detected in the open invitation group, while asymptomatic individuals constituted the majority in the random sample group.

The stratified random sample non-proportional to size was used in this study to facilitate logistics required for a short study period, therefore a weighted summary estimate of the point prevalence rate was calculated which further reduced the prevalence rate from 13.3% in the open invitation group to 6.9% in the crude unweighted random sample to only 0.8% in the weight random sample estimate. This weighted prevalence estimate of active COVID-19 cases (defined at that time as any individual with a positive COVID-19 PCR test) in the current survey is still bigger than the 0.6% population period prevalence rate covering the 2 months period of COVID-19 from its first reported case on 29/2/2020 till the last day of the current survey. One can argue that this difference is expected to be larger after considering the underestimation bias possibly introduced by the small response rate in the 0.8% prevalence figure and the overestimation bias in the period prevalence introduced by including some cases that are currently recovered.

The current study has its own limitations also. The random sample group represented a response rate of <10% for the targeted sample size. This was actually the reason behind opting to include an open invitation group. The strata of children 10–17 years old was completely missing from the random sample. Its worth noting that only two sample strata out of the total 12 available in the random sample showed positive COVID cases. These were Non-Qatari Males aged 18–39 years and 40–59 years. All the remaining strata showed no positive COVID-19 cases. These two strata had a higher opportunity for detecting positive cases, because they contained more tested people (they accounted for 62% of the completed random sample size of 260). One possible explanation for this finding is that COVID-19 is still localized in certain population subgroups and not widespread in the community at the time of executing the pilot survey. However, the high non-response rate in the random sample and the small sample size might bias such a conclusion. In addition, the COVID-19 cases included in the calculation of prevalence estimates were only those diagnosed using the random sample or the open invitation group during the 2 days of survey activities. The daily reported COVID-19 cases that present themselves to the health system or are captured by case finding screening activities are not part of the figures reported in this manuscript. Another possible source of bias introduced in the current survey is the effect of the infectious disease clustering in selected residential areas, which may have affected even the random sample because of the large non-response rate.

CONCLUSION

The current study emphasized the importance for a robust sampling method in survey studies and the huge implications of sampling methodology on calculating COVID-19 prevalence estimates, which can inform critical decisions.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study were requested from PHCC (Data custodian). As per PHCC rules that protect patient's privacy and confidentiality and the Confidentiality Agreement signed by the study team with PHCC the database can not be shared with external entities and can not be publicly available. Requests to access these datasets should be directed to researchsection@phcc.gov.qa.

AUTHOR CONTRIBUTIONS

HA/Q, MS, and HA: assisted in developing the study proposal and wrote parts of the first draft. In addition to reviewing the final version of the submission. AA: assisted in developing the study proposal, performed statistical analysis and wrote the study results section and discussion, and in addition to reviewing the final version of the submission. AZ, HK, TM, and SV: assisted in developing the study proposal and reviewed the final version of the submission. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.654734/full#supplementary-material

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Vagus Nerve Stimulation: A Potential Adjunct Therapy for COVID-19

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The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes coronavirus disease 2019 (COVID-19) through excessive end organ inflammation. Despite improved understanding of the pathophysiology, management, and the great efforts worldwide to produce effective drugs, death rates of COVID-19 patients remain unacceptably high, and effective treatment is unfortunately lacking. Pharmacological strategies aimed at modulating inflammation in COVID-19 are being evaluated worldwide. Several drug therapies targeting this excessive inflammation, such as tocilizumab, an interleukin (IL)-6 inhibitor, corticosteroids, programmed cell death protein (PD)-1/PD-L1 checkpoint inhibition, cytokine-adsorption devices, and intravenous immunoglobulin have been identified as potentially useful and reliable approaches to counteract the cytokine storm. However, little attention is currently paid for non-drug therapeutic strategies targeting inflammatory and immunological processes that may be useful for reducing COVID-19-induced complications and improving patient outcome. Vagus nerve stimulation attenuates inflammation both in experimental models and preliminary data in human. Modulating the activity of cholinergic anti-inflammatory pathways (CAPs) described by the group of KJ Tracey has indeed become an important target of therapeutic research strategies for inflammatory diseases and sepsis. Non-invasive transcutaneous vagal nerve stimulation (t-VNS), as a non-pharmacological adjuvant, may help reduce the burden of COVID-19 and deserve to be investigated. VNS as an adjunct therapy in COVID-19 patients should be investigated in clinical trials. Two clinical trials on this topic are currently underway (NCT04382391 and NCT04368156). The results of these trials will be informative, but additional larger studies are needed.

Keywords: COVID-19, cytokine storm, inflammation, non-drug therapy, vagus nerve stimulation, neuromodulation, outcome

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) faced currently worldwide includes in its pathophysiology an excessive inflammatory phase called "cytokine storm" that is closely linked to its high mortality (1, 2). During sepsis, the host response to a pathogen is mediated by the interaction between

pathogen-associated molecular pattern and their receptors located on innate immune cells (3). This interaction leads to activation of the innate immune cell, release of inflammatory cytokines, and recruitment of further cells of the immune system (4). When this immune response is exaggerated, excessive inflammation may lead to end tissue damage. All major organs may be affected during sepsis including altered hypothalamic–pituitary–adrenal and altered cardiovascular responses (5, 6). Disruption of the hypothalamic–pituitary–adrenal axis may translate in patients with sepsis into cardiovascular and organ dysfunction and an increase in the risk of death (5, 6). Impaired heart rate variability and high concentrations of circulating catecholamines and impaired sympathetic modulation are common findings of septic and septic shock patients, reflecting dysfunction of the medullary autonomic centers (7) and suggesting that central autonomic regulatory impairment contributes to circulatory failure (8–10). Clinical patterns concordant with these hypotheses have been documented in COVID-19 patients and support the hypothesis of a potential contribution of a dysfunction in autonomic tone to the cytokine release syndrome and related multiorgan damage in COVID-19 (11–17).

Specific treatment for COVID-19 is unfortunately lacking (18, 19). However, given the high mortality rate and economic damage to date, great efforts are being made worldwide to produce successful drugs (20). Particularly, pharmacological strategies that restore inflammatory control or inhibit cytokine release are being evaluated (21–23). Several drug therapies targeting this excessive inflammation, such as tocilizumab, an interleukin (IL)-6 inhibitor, corticosteroids, programmed cell death protein (PD)-1/PD-L1 checkpoint inhibition, cytokine-adsorption devices, and intravenous immunoglobulin have been identified as potentially useful and reliable approaches to counteract cytokine storm in COVID-19 patients (1, 2, 18, 24–32). Little attention is currently paid for non-drug therapeutic strategies targeting inflammatory and immunological processes that may be useful for reducing COVID-19-induced complications and improving patient outcome (33–35).

VAGUS NERVE STIMULATION A POTENTIAL ADJUNCT THERAPY IN COVID-19

Modulating the activity of cholinergic anti-inflammatory pathways (CAPs) described by the group of KJ Tracey (36–44) has indeed become an important target of therapeutic research strategies for inflammatory diseases and sepsis (37, 38, 45–47). In fact, the CAP pathways innervate the spleen through the efferent vagus nerve and the splenic nerve relay and act on macrophages by transforming adrenergic stimulation into a cholinergic signal by the T cells of the spleen, which plays an anti-inflammatory effect (48).

About 80% of the vagus nerve is composed of afferent sensory fibers carrying information from the periphery to the brain (49). Within the central nervous system, the vagus primarily projects to the nucleus of the solitary tract (NTS), releasing excitatory neurotransmitters (glutamate and aspartate), inhibitory neurotransmitter (gamma-aminobutyric acid), acetylcholine, norepinephrine, and neuropeptides implicated in signal transduction (49). In turn, the NTS has widespread efferent pathways to the parabrachial nucleus, reticular formation, basal forebrain, amygdala, hippocampus, hypothalamus, dorsal raphe, cerebellum, and spinal cord (50). NTS projections to brainstem nuclei (locus coeruleus and dorsal raphe magnus) modulate serotonin and norepinephrine release to the entire brain (51). Through efferent and afferent fibers, the vagus nerve plays a role in maintaining cardiovascular homeostasis and in modulating inflammation (52). The autonomic nervous system regulates the production of cytokines, through interactions with the hypothalamic–pituitary–adrenal axis, leading to the release of anti-inflammatory glucocorticoid hormones. Vagal efferent fibers also release acetylcholine (Ach), which, by interacting with α7-subunit-containing nicotinic receptors found in tissue macrophages, and dendritic cells, inhibit the release of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα), IL-1β, IL-6, and IL-18 (36, 53). Inflammatory reflex signaling, which is enhanced by electrically stimulating the vagus nerve, significantly reduces cytokine production and attenuates disease severity in animal models of inflammatory diseases and in experimental models of sepsis (36, 54–57). Electrical stimulation of the vagus nerve attenuates inflammation in a variety of pathological conditions with little side effects (36, 58, 59). Recently, Meneses and colleagues demonstrated that vagus nerve stimulation attenuates the inflammatory response in the central nervous system induced by peripheral lipopolysaccharide challenge in rats (60). Kohoutova and colleagues recently demonstrated that vagus nerve stimulation attenuates multiple organ dysfunction in a porcine model of sepsis (61). These findings suggest that VNS could be a promising adjunctive therapy targeting inflammatory pathways in COVID-19 patients. VNS might attenuate sepsis-related inflammatory processes leading to endothelial activation, impaired microcirculation, multiorgan failure, and death. VNS may also exhibit favorable cardiovascular effects during sepsis, including antiarythmogen, decreased myocardial oxygen consumption, and improved diastole (62). Vagus nerve stimulation has a favorable safety track record. Implanted VNS devices have been used for decades to treat refractory partial-onset seizures and severe recurrent refractory depression with confirmed safety and only mild to moderate side effects that are predictable improve over time (63–65). More recently, non-invasive transcutaneous vagus nerve stimulation devices (t-VNS) have been developed and commercialized (66). Evidence from preclinical models (61, 67) as well as from several clinical reports (47, 68) is accumulating (68–72). Boezaart and Botha reported a drastic reduction of two COVID-19 patients treated with t-VNS (69). Non-invasive VNS as adjunct therapy in COVID-19 patients might alleviate organ dysfunction and improve patients' outcome. Randomized controlled studies assessing the effectiveness of non-invasive vagus nerve stimulation as adjunct therapy to current best medical practice for COVID-19 are needed (72). Two studies

evaluating the efficacy of non-invasive VNS in COVID-19 patients are now on going using the gammaCore® non-invasive vagal nerve stimulation device. The gammaCore® (electroCore, Inc., Basking Ridge, NJ) is handheld and requires no surgery or implants. The device is applied by healthcare providers or patients to the skin at the neck over the vagus nerve to deliver periodic doses of VNS non-invasively. Tariq Cheema and colleagues are conducting a prospective, randomized, controlled investigation designed to assess the reduction in respiratory distress in a COVID-19 population: the SAVIORII study NCT04382391. The primary objective is to reduce initiation of mechanical ventilation in patients with COVID-19 compared to the control group. Secondary objectives are to evaluate cytokine trends/prevent cytokine storms, evaluate supplemental oxygen requirements, decrease mortality of COVID-19 patients, and delay the onset of mechanical ventilation. The second ongoing clinical trial using the same device is conducted by Carlos Tornero and colleagues NCT04368156: the SAVIOR study. The SAVIOR study is a prospective, randomized, controlled study assessing vagus nerve stimulation in COVID-19 respiratory symptoms (72). The primary outcome measures were incidence of changes in specific clinical events such as the proportion of subjects requiring mechanical ventilation, days to onset of mechanical ventilation, oxygen support requirements, O_2 saturation, pain levels, PaO_2/FiO_2, coagulation, laboratory measurements related to circulating cytokines and inflammation, live discharge from the hospital, patient length of stay, mortality, need for intensive care, shortness of breath, device-related serious adverse events, and adverse events. The results of these trials will be informative, but additional, larger, studies are needed.

DISCUSSION

COVID-19 remains a major healthcare issue worldwide. Excessive inflammation and its end organ consequences are key elements in the pathogenesis of COVID-19-induced multiple organ dysfunction (19, 26, 32). Specific treatment for COVID-19 is unfortunately lacking. Several promising pharmacological strategies aimed at modulating inflammation in COVID-19 are being evaluated worldwide. However, little attention is currently paid for non-drug therapeutic strategies targeting inflammatory and immunological processes, which may be useful for reducing COVID-19-induced complications and improving patient outcome (33–35). Vagal neurostimulation has a wide field of therapeutic benefit for patients and should be combined with the best current medical strategies (15, 17, 69, 70). Vagus nerve stimulation attenuates inflammation both in experimental models and preliminary data in man. The development non-invasive vagal nerve stimulation (t-VNS), a non-pharmacological adjuvant, may help reduce the burden of COVID-19 and deserve to be investigated. The aim of this paper is to promote the emergence of original studies assessing non-invasive VNS as an adjuvant treatment for the management of COVID-19.

AUTHOR CONTRIBUTIONS

EA and DA conceived the manuscript. EA, NH, GB, RB, and DA were involved in early discussions and mapping the concepts that led to this paper and wrote the first draft of the manuscript. All authors read and critically reviewed drafts of the manuscript.

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Health Issues and Immunological Assessment Related to Wuhan's COVID-19 Survivors: A Multicenter Follow-Up Study

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Background: Currently, a large number of hospitalized coronavirus infectious disease-2019 (COVID-19) patients have met the clinical discharge criteria and have been discharged. Little is known about the sequelae and herd immunity, two important factors influencing the life quality and safety of COVID-19 survivors.

Methods: Discharged COVID-19 patients from four medical facilities in Wuhan, China, were followed in order to record and investigate possible post-COVID-19 sequelae and herd immunity. After hospital discharge, patients reported to Fangcang shelter hospitals for an initial 14-day period of mandatory clinical monitoring. After release from these shelter hospitals, patients returned home for self-quarantine. Real-time quantitative PCR (RT-qPCR) was used for severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) detection. Colloidal gold-based immunochromatographic strip assay (ICGSA) was used for anti-SARS-CoV-2 immunoglobulin G (IgG) and immunoglobulin M (IgM) antibody testing. The data for this study are derived from case reports, medical records, and self-reports.

Results: A total of 3,677 COVID-19 survivors [median age = 59 years, interquartile range (IQR) = 47–68, range = 10–98; 55.5% female] who were released from four hospitals in Wuhan, China, between January 18 and March 29, 2020 were followed for a median of 144 days (IQR = 135–157). During follow-up, 976 (26.5%) patients had at least one post-COVID-19 sequela. The incidence of post-COVID-19 sequelae among elderly COVID-19 survivors (age ≥60 years) was slightly increased compared to that of young COVID-19 survivors (age <60 years; relative risk = 1.05, 95% CI = 1.02–1.10, $p = 0.007$). During follow-up, a dramatic reduction of anti-SARS-CoV-2 IgG (88.0%, 95% CI = 84.2–90.4) and IgM (93.2%, 95% CI = 88.5–96.4) antibodies was observed. Among these COVID-19 survivors, 1.2% ($n = 45$) retested positive for SARS-CoV-2 and 1.0% ($n = 37$) died during follow-up.

Of those who died during follow-up, 70.3% were male and all were negative for both IgG and IgM, except for one person who was IgG-positive.

Conclusions: Our study documents significant post-COVID-19 sequelae that impair functions of multiple organ systems in COVID-19 survivors, suggesting that the long-term effects of this disease will negatively impact survivors' quality of life, continue to strain health care systems, and result in extended periods of lost productivity. Furthermore, female gender and anti-SARS-CoV-2 immunity may play an essential role in the survival after COVID-19 infection.

Keywords: post-COVID-19, SARS-CoV-2, mortality, hospital discharge, post-COVID-19 sequela, physical and psychological symptoms, antibody test, IgG and IgM

INTRODUCTION

The coronavirus infectious disease-2019 (COVID-19) pandemic continues to affect people worldwide. As of the end of July 2020, there are more than 14 million confirmed cases, with more than 0.6 million deaths (1). While the numbers of cases and deaths are expected to rise, a larger number of COVID-19 patients have recovered and have been discharged from medical facilities worldwide (1). However, little is known about post-COVID-19 sequelae among the discharged patients and related potential risk factors. Wuhan, China, was the first city to experience the emergence of COVID-19 (2). The central government launched timely public health strategies for virus control, including mandatory curfews and face coverings. At the medical facility level, COVID-19 testing was implemented and strict hospital discharge criteria were developed, including a mandatory 14-day period of post-hospital discharge clinical monitoring at regional shelter hospitals. M [a]any medical facilities continued to follow COVID-19 patients after primary hospital discharge, including the time periods during and after their stay at secondary shelter hospitals. We utilized these long-term follow-up data to investigate both the physical and psychological symptoms, including severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) immune recognition, among a large cohort of COVID-19 survivors released from four medical facilities in Wuhan.

METHODS

COVID-19 Survivors Studied

This study investigated post-infection sequelae among all patients with confirmed COVID-19 infection who were discharged from four hospitals in Wuhan, China, between January 18 and March 29, 2020. These government-designated COVID-19 hospitals included Wuhan No.1 Hospital, Wuchang Hospital, Zhongshang Hospital, and Hubei Province Hospital. The hospitals were mandated to treat all infected patients regardless of disease severity (i.e., mild, severe, and critical). Standard hospital discharge criteria (3) included: (1) absence of fever for more than 3 days; (2) radiological evidence of significant resolution of pneumonia via CT scan; and (3) two sequential negative SARS-CoV-2 real-time quantitative PCR (RT-qPCR) tests on nasopharyngeal/oropharyngeal swab samples with at least a 24-h interval between sampling.

Early in the disease outbreak, little was known regarding the clinical characteristics, disease course, and mortality of COVID-19 infection, or the dynamics and range of viral transmission. Thus, all hospitalized patients who met the discharge criteria were immediately transferred to a Fangcang-like medical facility for a mandated 14-day period of clinical monitoring (4). The date of primary hospital discharge is the start of the follow-up. The last day of follow-up for the COVID-19 survivors included in this study was July 24, 2020. The data for this study are derived from case reports, medical records, and self-reports. This study was approved by the institutional ethics board of Wuhan No.1 Hospital, China (no. [2020] 6). Informed consent was obtained from each participant.

Fangcang-Like Medical Facility and Its Discharge Criteria

Fangcang is a public health concept that was instituted for the first time in China during the SARS-CoV-2 outbreak of 2020. It was highly efficient at the mobilization of medical resources and dramatically reduced the burden on local medical capacity (4). Fangcang shelter hospitals are large-scale, temporary hospitals that are rapidly constructed by converting public venues (e.g., exhibition centers and football stadiums) into medical facilities equipped for basic medical care, frequent patient monitoring, and rapid patient assessment and referral. To maximize medical resources and to contain the rapidly emerging COVID-19 epidemic, government-designated hospitals in China discharged all COVID-19 patients directly to a Fangcang shelter hospital for a defined period of clinical observation, typically 14 days. This clinical observation serves as the initial phase of follow-up. After this observation period in the Fangcang hospital, patients were discharged home to self-quarantine if they met the following criteria: (a) no recurrence of any clinical symptom including respiratory-related inflammation and (b) negative SARS-CoV-2 RT-qPCR test on nasopharyngeal/oropharyngeal swab samples after 14 days.

COVID-19 survivors who developed any clinical symptom or sign during monitoring at the Fangcang shelter hospitals

TABLE 1 | Severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) real-time quantitative PCR (RT-qPCR) test results.

ORF1ab	Gene N	Gene E	Result
+	+	+	Positive
+	+	−	Positive
+	−	+	Positive
+	−	−	Positive only when a repeated test shows the same result
−	+	−	Suspicious. Recheck after a certain period
−	−	+	
−	−	−	Negative

were immediately readmitted to hospitals. After discharge from Fangcang shelter hospitals, the community hospitals were responsible for clinical monitoring and diagnostic testings. When clinical symptoms occurred, or other medical circumstances required, or upon personal request, CT imaging and SARS-CoV-2 retesting were performed.

SARS-CoV-2 Real-Time Quantitative PCR Test

RT-qPCR for SARS-CoV-2 was performed on nasopharyngeal/oropharyngeal swabs for viral detection. Viral RNA was extracted from the patients' nasopharyngeal/oropharyngeal swabs, sputum, and other samples, which were placed in a 56°C incubator for 30 min to inactivate the virus. Primer probes targeted three genes of SARS-CoV-2: open reading frame 1ab (*ORF1ab*) and genes of nucleocapsid proteins N and E (**Table 1**). The PCR buffer, reverse transcriptase enzyme, DNA polymerase, and gene primers were mixed together and added to a 96-well plate. The extracted RNA samples were then added to the wells, the plate sealed, and RT-qPCR amplification was performed as follows: one cycle at 40–45°C for 10 min, followed by 95°C for 3 min. Then, DNA denaturation and amplification proceeded for 45 cycles at 95°C for 15 s and 55–58°C for 30 s. The test results of SARS-CoV-2 were reported as positive or negative when the cycle threshold values remained below 44 or exceeded 43, respectively. The results in **Table 1** were used to determine whether a sample was COVID-19-positive. This RT-qPCR test can detect SARS-CoV-2 nucleic acid standard substance within 400 copies/ml with a detection rate of 100%. Therefore, the sensitivity of the SARS-CoV-2 RT-qPCR is 400 copies/ml.

For each patient, this SARS-CoV-2 RT-qPCR was performed twice on nasopharyngeal/oropharyngeal swab samples obtained with at least a 24-h interval between samples. Both tests must be negative in order to meet the discharge criteria of the hospital, as noted above. Subsequently, this test was performed at least once during Fangcang-medical monitoring; afterwards, this test was performed when clinical symptoms reoccurred, or upon personal requests, or for other reasons such as entering medical facilities and community centers.

TABLE 2 | Sensitivity and specificity of the colloidal gold-based immunochromatographic strip assay (ICGSA).

Immunoglobulin test	Sensitivity (95% CI)	Specificity (95% CI)
IgM	87.1% (83.9–91.3)	99.7% (98.4–100)
IgG	88.3% (85.6–92.0)	99.4% (98.0–99.8)
Combination of IgG and IgM	91.6% (88.6–93.4)	99.2% (97.6–99.7)

Colloidal Gold-Based Immunochromatographic Strip Assay

The colloidal gold-based immunochromatographic strip assay (IGCSA) was used for immunoglobulin G (IgG) and immunoglobulin M (IgM) detection. In brief, the IgM and IgG test cards were numbered sequentially. Anticoagulated (citrated) blood samples were centrifuged for 5 min at 500 × *g* and 10 μl of plasma was added to the sample well for 10–15 min. A test was considered positive when lines for the patient sample and the positive control sample appeared simultaneously. Samples in which a line developed only for the positive control sample were regarded as negative. Tests in which only the patient sample, and not the positive control sample, was positive were deemed invalid, requiring another test. **Table 2** indicates the sensitivity and specificity of this assay for each immunoglobulin alone and in combination regarding SARS-CoV-2 detection in samples.

Statistical Analysis

Continuous variables were expressed as median (interquartile range, IQR) and compared with the Mann–Whitney *U*-test; categorical variables were expressed as number (percentage) and compared by Fisher's exact test. $P < 0.05$ was regarded as statistically significant.

RESULTS

Between Jan 18 and Mar. 29, 2020, 3,677 hospitalized COVID-19 patients met the clinical discharge criteria and were discharged from the aforementioned four hospitals in Wuhan, China. All these COVID-19 survivors were included in our analyses. The median age was 59 years (IQR = 47–68, range = 10–98; 54.1% female). The survivors were followed for a median of 144 days (IQR = 135–157, range = 117–188; **Table 3**) by four independent medical teams. Among this cohort, 2,331 (63.4%) survivors had mild, 1,239 (33.7%) severe, and 95 (2.6%) had critical condition during their initial hospitalization. During this initial hospitalization, 3,570 (97.1%) survivors received antiviral therapy, 3,026 (82.3%) antibiotic treatment, 2,401 (65.3%) corticosteroids, 1,540 (41.9%) interferon nebulization treatment, and 1,445 (39.3%) γ-immunoglobulin treatment. Three thousand and sixty-six (83.4%) survivors were given a standard oxygen therapy via nasal catheter, 467 (12.7%) received high-flow nasal cannula therapy, 173 (4.7%) had non-invasive mechanic ventilation, and 30 (0.8%) required invasive mechanic ventilation. The median time from symptom onset to hospital admission was 8.0 days (IQR = 6.0–11.0). The median length of initial hospitalization was 17.0 days (IQR = 11.0–25.0).

TABLE 3 | Clinical characteristics, retest positivity, and sequelae among discharged coronavirus infectious disease-2019 (COVID-19) patients.

Characteristics	All patients (IQR/%)	Retested positive group (IQR/%)	Deceased group (IQR/%)
No. of patients	3,677	45 (1.2)	37 (1.0)
Median age (years)	59.0 (47.0–68.0)	57 (50.0–64.0)	70.0 (56.0–79.0)
Gender			
Male	1,688 (45.9)	14 (31.1)	26 (70.3)
Female	1,989 (54.1)	31 (68.9)	11 (29.7)
Retest positivity	45 (1.2)	45 (100)	0
No. of new viral transmission	0	0	0
Severity (initial hospitalization)			
Mild	2,331 (63.4)	19 (42.2)	15 (40.5)
Severe	1,239 (33.7)	24 (53.3)	4 (10.8)
Critical	107 (2.9)	2 (4.5)	18 (48.6)
Treatment (initial hospitalization)			
Antiviral therapy	3,570 (97.1)	41 (91.1)	34 (91.9)
Antibiotic treatment	3,026 (82.3)	38 (84.4)	36 (97.3)
Corticosteroids	2,401 (65.3)	28 (62.2)	36 (97.3)
Interferon nebulization	1,540 (41.9)	15 (33.3)	5 (11.1)
γ-Immunoglobulin	1,445 (39.3)	1 (2.2)	3 (8.1)
Median time from symptom onset to admission (days, IQR)	8.0 (6.0–11.0)	8.0 (6.0–11.0)	10.0 (7.0–13.0)
Median time to hospitalization (days, IQR)	17.0 (11.0–25.0)	16.5 (11.0–24.0)	16.0 (10.0–23.0)
Median follow-up time (days, IQR)	144.0 (135.0–157.0)	150.0 (139.5–158.5)	33.0 (18.0–42.0)
Sequelae			
Pulmonary function	337 (9.2)		10 (27.0)
Shortness of breath	136	5	3
Cough/sputum	87	4	2
Pharyngitis/foreign body feeling	42	1	2
Dyspnea	30	7	3
Pulmonary fibrosis	21		
Lung damage	12		
Bronchitis	4		
COPD	3		
Hemoptysis	2		
Cardiac function	278 (7.6)		8 (21.6)
Chest pain/tightness	184	4	4
Palpitation	63	3	
Cardiac disease	14		2
Tachycardia	13	1	1
Angina pectoris	3		
Heart attack	1		1
Neurologic function	289 (7.9)		5 (13.5)
Insomnia	78	4	4
Joint pain/back pain/lumbago	71	3	
Fatigue	55		
headache/dizziness/poor memory	49		
Change of taste and smell	10		
Myalgia	8		
Impaired vision	5		
Leg numbness/finger stiffness	5		
Neuralgia	2		
Paralysis	2		

(Continued)

TABLE 3 | Continued

Characteristics	All patients (IQR/%)	Retested positive group (IQR/%)	Deceased group (IQR/%)
Tinnitus	2		
Confusion	1		1
Coma	1		
cerebral infarction	1		
Endocrine system	90 (2.4)		
Hair loss	67		
Bitter/dryness in mouth	12		
High blood sugar	6		
Diabetes	5		
Gastrointestinal function	42 (1.1)		1 (2.7)
Gastrointestinal complaints/poor appetite	31	3	1
Diarrhea	8		
Constipation	2		
Emesia	1		
Dermatological system	33 (0.9)		
Hidrosis	24		
Erythra	7		
Allergy	2		
Hepatic system	16 (0.4)		1 (2.7)
Hepatic insufficiency	8		1
Edema	7		
Antiadoncus	1		
Kidney function	12 (0.3)		1 (2.7)
Hypertension	6		
Kidney insufficiency	6		1
Various	66 (1.8)		5 (13.5)
Reduction of physical strength	64		5
Dryness/excessive secretion in eye	2		

TABLE 4 | Sequelae disposition by 10-year age intervals of discharged coronavirus infectious disease-2019 (COVID-19) patients.

Age intervals (years)	Sequelae Nr./Nr.		Relative risk ratio (95% CI, *p*-value)
	Male	Female	Male vs. female
10–19	2/10	0/4	1.25 (0.92–1.70, $p = 1$)
20–29	7/56	14/91	0.98 (0.85–1.10, $p = 0.81$)
30–39	46/183	61/206	0.94 (0.83–1.06, $p = 0.36$)
40–49	77/256	78/250	0.98 (0.88–1.10, $p = 0.84$)
50–59	88/353	163/473	0.87 (0.80–0.95, $p = 0.0036$)
60–69	117/452	185/592	0.92 (0.86–1.00, $p = 0.063$)
70–79	48/265	56/256	0.95 (0.88–1.04, $p = 0.323$)
80–89	12/95	19/112	0.95 (0.85–1.06, $p = 0.438$)
>90	3/18	0/5	1.2 (0.98–1.48, $p = 1$)

During follow-up, 976 (26.5%) COVID-19 survivors had at least one sequelae (median age = 57, IQR = 47.8–56.4, range = 17–92; 59.0% female), the most common being chest pain/tightness (184, 5.0%), shortness of breath (136, 3.7%), and cough/sputum (87, 2.4%) (**Table 3**). Three hundred thirty-seven (9.2%) survivors had sequelae affecting pulmonary function, 278 (7.6%) had sequelae related to cardiac function, and 289 (7.9%) and 90 (2.4%) had sequelae impairing the neurologic system and endocrine function, respectively. Sequelae disposition by 10-year age interval of all the 3,677 survivors is included in **Table 4**. The incidence of post-COVID-19 sequelae of elderly COVID-19 survivors (age ≥60 years, $n = 1{,}795$) was slightly increased compared to that of young survivors (age <60, $n = 1{,}882$) [relative risk ratio (RR) = 1.05, 95% CI = 1.02–1.10, $p = 0.007$]. However, gender did not significantly influence the incidence of post-COVID-19 sequelae (**Table 4**). Additionally, 173 (4.7%) survivors self-reported diverse psychological symptoms such as anxiety (103, 2.8%), depression (70, 1.9%), and emotional instability (37, 1.0%). One hundred and thirty-two (3.6%) survivors refused to report personal feelings. Eight hundred two (21.8%) survivors were assessed by mental health care specialists and were deemed to have a clinically defined psychological condition. The psychological conditions of 136 (3.7%) survivors improved after psychological therapy.

At the initial phase of the follow-up (days 1–45 post-hospital discharge), the results of the ICGSA for anti-SARS-CoV-2 viral immunoglobulins showed that 249 (6.8%) COVID-19 survivors

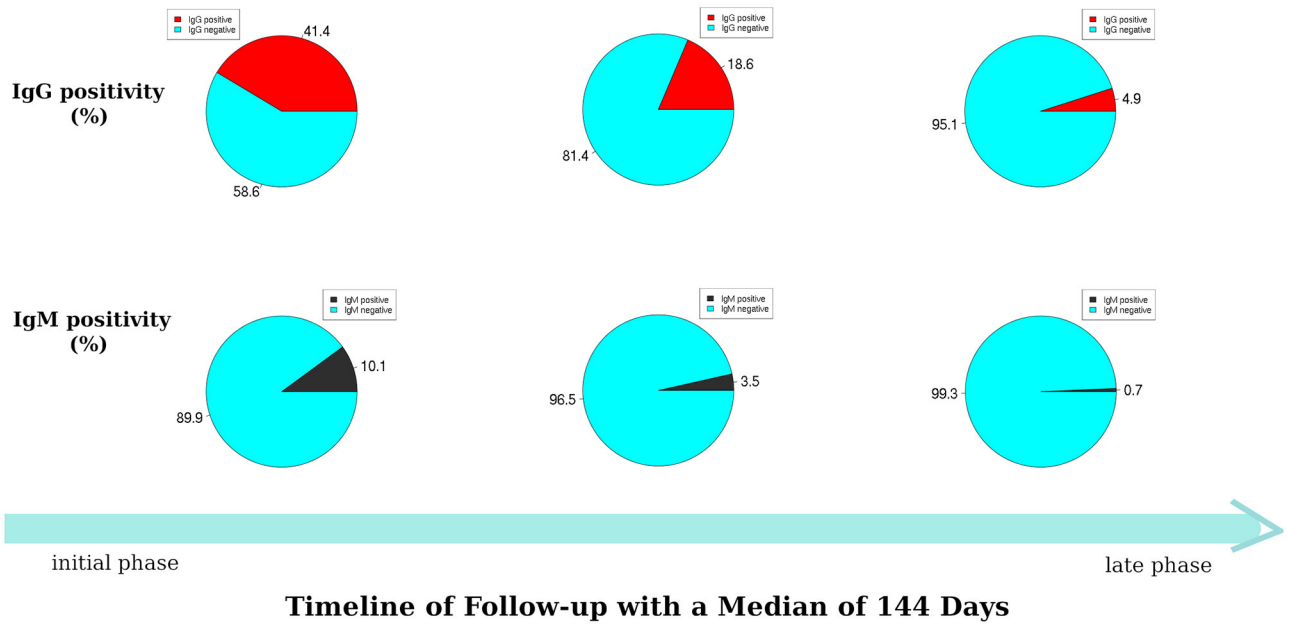

FIGURE 1 | Dynamic decline of immunoglobulin G (IgG) and immunoglobulin M (IgM) among 3,677 coronavirus infectious disease-2019 (COVID-19) survivors during follow-up.

were positive for both IgM and IgG, 1,274 (34.6%) were IgG-positive and IgM-negative, 121 (3.3%) were IgG-negative and IgM-positive, and 2,033 (55.3%) were negative for both IgG and IgM (**Figure 1**). At the late phase of the follow-up (days 100–150 post-hospital discharge), the IgG and IgM antibody positivity rates were reduced by 88.0% (95% CI = 84.2–90.4) and 93.2% (95% CI = 88.5–96.4), respectively. Specifically, only 25 (0.7%) survivors were positive for both IgG and IgM, 157 (4.3%) were IgG-positive and IgM-negative, none were IgG-negative and IgM-positive, and 3,495 (95.1%) were negative for both IgG and IgM (**Figure 1**).

During follow-up, 45 (1.2%) survivors retested positive for SARS-CoV-2 (median age = 57 years, IQR = 50–64, range = 25–81; 68.9% female; **Table 3**). None of these 45 was a health care worker, and none had taken medicine regularly after their initial hospital discharge. Of these, 25 survivors were immediately readmitted to hospitals and 20 remained at home under self-quarantine. Two of the 45 survivors had both IgG and IgM antibodies, 26 were IgG-positive and IgM-negative, two were IgG-negative and IgM-positive, and the remaining 15 were negative for both antibodies. The median duration between initial hospital discharge and retest positivity was 32.0 days (IQR = 28.0–40.0, range = 9–58; **Table 3**). Furthermore, 21 survivors in this retest-positive subgroup were asymptomatic, while 24 had at least one symptom associated with COVID-19, the most common being dyspnea, cough, and chest tightness. During their initial hospitalization, 19 of the 45 survivors had mild disease, 24 had severe condition, and two had critical condition. As of July 24, 2020, all 45 retest-positive survivors were alive. Twenty readmitted and retested positive survivors met the discharge criteria and were once again released to home quarantine. During follow-up, no new viral transmission was observed or reported.

During follow-up of the 3,677 COVID-19 survivors, 37 (1.0%) individuals died (median age = 70.0 years, IQR = 56.0–79.0, range = 31.0–98.0; 29.7% female; **Table 3**). None of the deceased was a health care worker. Thirty-one of the deaths were attributed to COVID-19, while six deaths were caused by comorbidities including diabetes, hepatobiliary tube cancer, heart attack, encephalorrhagia, epilepsy, and scurvy. The median duration from hospital discharge to death was 33.0 days (IQR = 18.0–42.0; **Table 3**). None of these deceased retested positive. Five of these 37 individuals had a worsened condition after hospital discharge and were therefore readmitted to the hospital; they died 4–15 days after readmission. The remaining 32 died at home. None of the deceased had taken any medicine regularly after initial hospital discharge and their psychological conditions had been stable. Except for one IgG-positive/IgM-negative individual, all other deceased individuals were IgG- and IgM-negative, indicating no immune system recognition of SARS-CoV-2. Within this deceased subgroup, 15 (40.5%) individuals had mild, four (10.8%) severe, and 18 (48.6%) had critical condition during their initial hospitalization. Also, during this initial hospitalization, 34 (91.9%) individuals were given antiviral treatments including arbidol, ganciclovir, oseltamivir, ribavirin, and hydroxychloroquine (**Table 3**), 36

(97.3%) individuals received antibiotics, and 36 (97.3%) were given corticosteroids.

DISCUSSION

To our knowledge, this study describes the longest duration of clinical follow-up in the largest cohort of discharged COVID-19 patients. Clearly, acute COVID-19 infection damages pulmonary function, but it has also been associated with the dysfunction of many other organ systems including the circulatory (5), cardiovascular (6), renal (7), gastrointestinal (8), endocrine (9), nervous (10), and skin (11) systems. In contrast, our study shows that diverse multi-organ functional impairments also occur well after hospital discharge in patients deemed recovered from primary acute infection. Specifically, during a mean follow-up time of 144 days, 976 (26.5%) of the COVID-19 survivors developed functional abnormalities of the cardiovascular, neurological, and endocrine systems. The risk of the development of such physical abnormalities was independent of age and gender. Furthermore, we show that 173 (4.7%) discharged patients had an associated psychological condition post-COVID-19 infection. These post-COVID-19 sequelae greatly impact the patients' long-term quality of life and will continue to strain the health care system.

Our study also reports a dramatic reduction of the anti-SARS-CoV-2 antibodies IgG and IgM during this long-term follow-up, which is consistent with the results of several recent studies (12–14). This result strongly suggests that adequate herd immunity may not develop or be maintained for a sufficient period to quell the pandemic. Such a finding supports the need to extend public health practices including social distancing procedures, face covering, and hygiene-based measures in order to limit viral transmission until an effective vaccine is developed and made widely available.

Our study describes a 1% overall mortality rate among Wuhan's COVID-19 survivors. Importantly, the majority of these deaths occurred in individuals who tested negative for both IgG and IgM. These results suggest that immune responses to SARS-CoV-19 infection may lower patients' risk of life-threatening post-discharge sequelae. The median age of this deceased group is 70 years, indicating that advanced age is not only a risk factor for death from primary COVID-19 infection but it also influences the mortality from post-COVID-19 sequelae. Fifteen of the 37 deceased persons had a mild course of COVID-19 during the initial hospitalization, indicating that the disease severity of primary COVID-19 infection is not the sole factor contributing to mortality in the post-COVID-19 period. Furthermore, the majority of deaths during follow-up were those of male survivors (26, 70.3%), implying that female gender could play an important role in survival during the post-COVID-19 period.

Lastly, we report a 1.2% rate of COVID-19 retest positivity among all COVID-19 survivors, with no new viral transmission. We cannot confirm whether or not retest-positive people are able to infect others; however, as long as the appropriate social health measures are practiced, reinfection via retest-positive people can likely be avoided.

Our study has some limitations. Being a national multicenter study, the findings must be further verified by international studies. Secondly, this study was not designed or able to determine the effects of treatment on the follow-up outcome. Thirdly, a recent study revealed the clinical characteristics of family members for COVID-19 infection (15). However, our study was not able to determine the post-COVID-19 sequelae-related genetic relationship. This warrants further investigation.

In summary, our results indicate that persistent and often severe morbidity is prevalent among COVID-19 survivors. Individuals with such post-viral sequelae may have reduced quality of life, including lost productivity, and may continue to strain health care systems. Closer follow-up of COVID-19 survivors with prompt medical intervention for developing sequelae may improve the long-term outlook for these individuals.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional ethics board of Wuhan No.1 Hospital, China (No. [2020] 6). Informed consent was obtained from each participant. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QM, FW, YY, GH, JL, CK, QZ, and SG contributed to the acquisition, analysis, or interpretation of data. QM, YY, JL, and AB drafted the manuscript. QM and JL did the statistical analysis. AB, FW, GH, LW, XY, and LZ contributed to the critical revision of the manuscript for important intellectual content. FW obtained funding. YY and GH gave administrative, technical, or material support. LW, XY, and JL helped with conception design and supervision. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Post-partum Coronavirus Disease 19 Like Pneumonia Before the COVID-19 Italian Pandemic Outbreak: A Case Report

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Here we present a case of severe post-partum pneumonia that we observed at the end of January 2020. Specimen of blood was cultured and revealed *Klebsiella pneumoniae* bacteremia. However, the course of infection was atypical and the recovery time particularly long. Subsequently emerged COVID-19 hallmarks suggested to re-evaluate the case. After a multidisciplinary consultation, we concluded that, considering the clinical and imaging characteristics, the most likely hypothesis was that the patient was affected by novel Coronavirus pneumonia. The present case supports the hypothesis that Coronavirus might have circulated in northern Italy for weeks before its official detection.

Keywords: COVID-19, coronavirus, pneumonia, *Klebsiella pneumoniae*, post-partum

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INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2), rapidly escalated to a pandemic in the span of 2 months and has compromised healthcare systems around the world (1). The virus was first confirmed to have spread to Italy on 31 January 2020, when two Chinese tourists in Rome tested positive for the virus. On February 21, 2019, the first Italian patient with Coronavirus COVID-19 was diagnosed, a 38-year-old man hospitalized at Codogno Hospital, Lodi, in northern Italy. Also, in northern Italy, on February 21, 2020, another outbreak of viruses was discovered in Vò Euganeo (Padua) and, in the Veneto region, the first death was reported, a 78-year-old man in a hospital in Padua. He was the first of a long series of deaths (2). As of 6 June 2020, Italy has 35,877 active cases, one of the highest in the world. Overall, there have been 234,801 confirmed cases and 33,846 deaths (a rate of 561 deaths per million population), while there have been 165,078 recoveries or dismissals. By 5 June, Italy had tested about 2,565,000 people. Due to the limited number of tests performed, the real number of infected people in Italy, as in other countries, is estimated to be higher than the official count (3).

Available epidemiological models failed to justify such a rapid growth in the number of infections. Still undemonstrated theories sustain that the new Coronavirus may have circulated in northern Italy for weeks before it was detected, seriously complicating efforts to track and control its rapid spread across Europe. Preliminary evidence suggested the virus could have been spreading below the radar in the quarantined areas. The real beginnings of the outbreak, which has spread

TABLE 1 | Blood test performed on January 25th, 2020.

Variable	Value	Reference range
White blood cells ($\times 10^9$/L)	16.61	3.50–10.50
Red blood cells ($\times 10^{12}$/L)	3.26	3.90–5.00
Hemoglobin (g/dl)	10.8	12.0–15.5
Hematocrit (%)	30.3	34.9–44.5
Mean corpuscolar volume (fl)	92.9	80–99
Mean cell hemoglobin (pg)	33.1	27–32
Mean corpuscular hemoglobin concentration (g Hb/dL RBC)	35.6	32.0–36.0
Platelets ($\times 10^9$/L)	145	130–400
Neutrophils ($\times 10^9$/L)	15.26	1.50–8.00
Eosinophils ($\times 10^9$/L)	0	0.00–0.50
Basophils ($\times 10^9$/L)	0.03	0.00–0.20
Lymphocites ($\times 10^9$/L)	0.65	0.70–5.00
Monocytes ($\times 10^9$/L)	0.67	0.10–1.00

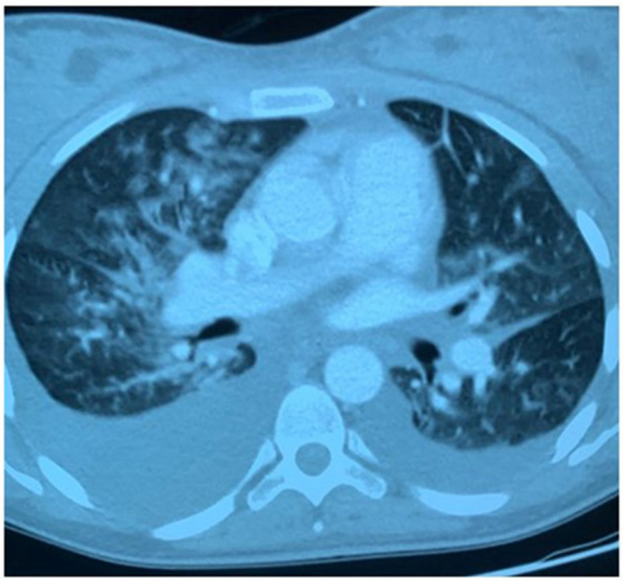

FIGURE 1 | Pulmonary computed-tomographic (CT) performed on January 26th, 2020: bilateral confluent and patchy ground-glass and consolidative pulmonary opacities, small, bilateral pleural effusions.

from Italy across Europe, were probably seeded at least two or 3 weeks before the first detection and possibly before flights between Italy and China were suspended at the end of January.

CASE REPORT

A 38-year-old primiparous woman was admitted to the Obstetrics and Gynecology Unit of the Humanitas S. Pio X Hospital in Milan (Italy) at 38 weeks 4 days of gestation because of oligohydramnios [Amniotic Fluid Index (AFI = 3 cm)] on January 23rd, 2020. The fetus was in cephalic presentation, with a heart rate of 134 beats per minute. The placenta was positioned anteriorly, the cervical length was 21 mm, and the umbilical artery appeared normal on Doppler examination.

During the previous 1.5 years, the patient had been well but unable to conceive. The patient was referred to an expert fertility consultant (PL-S), Director of the Humanitas Fertility Center in Rozzano (Milan, Italy). Transvaginal ultrasound showed bilateral ovarian endometrioma. Ovarian reserve resulted depleted [Antral follicle count (AFC) = 6; Anti-Müllerian Hormone (AMH) < 0.1 ng/ml]. She completed two cycles of *in vitro* fertilization (IVF); two high-quality embryos were transferred during each cycle. The second IVF attempt resulted in the present singleton pregnancy.

She was otherwise healthy and was a non-smoker taking no medications. After the admission, the patient was hydrated. Nevertheless, the ultrasound reevaluation confirmed oligohydramnios and the labor was induced with intravaginal prostaglandin E2. During labor, the cardiotocography tracing was characterized by reduced variability and showed repetitive variable decelerations suggesting a high probability of fetal hypoxia/acidosis according to the International Federation of Gynecology and Obstetrics (FIGO) consensus guidelines on intrapartum fetal monitoring (4). An emergency cesarean section was promptly carried out under combined spinal-epidural anesthesia on January 24th, 2020. Cefazolin was administered intravenously before incision of the skin. The infant was delivered 5 min after the skin incision. The 1 and 5-min scores were 8 and 10, respectively. The umbilical cord blood parameters were reassuring and didn't reflect a fetal hypoxic stress. Cesarean section was exempt from surgical complications.

Twenty hours after the c-section, mild dyspnea on exertion developed, associated with slight leg edema. On examination, the blood pressure was 135/72 mm Hg, the respiratory rate 16 breaths per minute, and the oxygen saturation 99% while the patient was breathing ambient air; the temperature was 37.6°C. An electrocardiogram (ECG) showed sinus rhythm at 118 beats per minute. Blood levels of total bilirubin, total protein, albumin, calcium, alanine aminotransferase, and aspartate aminotransferase were normal, as were tests of renal function; other test results are shown in **Table 1**. She was given broad spectrum antibiotics and strictly monitored. The day after (50 h after the c-section) dyspnea progressively worsened and a productive cough appeared. Body temperature was 38.2°C, the respiratory rate 16 breaths per minute, and the oxygen saturation 97% while the patient was breathing ambient air. Lung auscultation revealed bi-basal rhonchi and vesicular sounds bibasaly reduced. After a consultation with a pulmonologist, pulmonary computed-tomographic (CT) was planned and showed bilateral confluent and patchy ground-glass and consolidative pulmonary opacities, small, bilateral pleural effusions and no evidence of pulmonary embolism, aortic aneurysm, or pericardial effusion (**Figure 1**). Specimen of blood was cultured and revealed *Klebsiella pneumoniae* bacteremia.

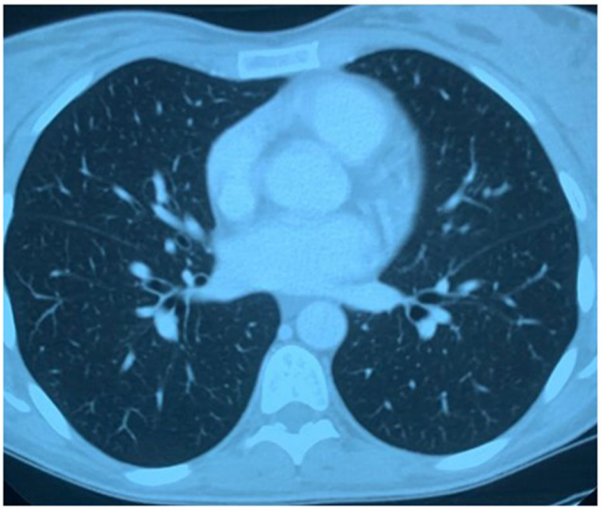

FIGURE 2 | Pulmonary computed-tomographic (CT) performed on February 13th, 2020: complete restoration of the physiological transparency of the lung fields without residual consolidation thickenings and thin bilateral basal fibrotic outcomes.

An infectious disease specialist consultation was required and a targeted antibiotic therapy with Metronidazole and Ceftadizime was started. After 4 days, the clinical picture showed no significant improvements. A new pulmonary CT was performed and confirmed the absence of benefits of the current treatment. The infectious disease specialist therefore decided to add the administration of Meropenem to the ongoing antibiotic therapy.

The clinical picture and the laboratory findings slowly and progressively improved. Pulmonary CT was repeated 20 days after the first day of hospitalization and demonstrated complete restoration of the physiological transparency of the lung fields without residual consolidation thickenings and thin bilateral basal fibrotic outcomes (**Figure 2**). The patient was discharged in good health after 21 days of hospitalization. Follow-up appointments were scheduled: physical examinations and laboratory tests demonstrated a complete resolution of the clinical picture.

DISCUSSION

Herein, we presented a case of severe post-partum pneumonia before the COVID-19 Italian pandemic outbreak. The responsible agent was considered *K. pneumoniae*. However, the evidence that subsequently emerged about the clinical and imaging features of women affected by COVID-19 suggested to reassess the case. After a multidisciplinary consultation, we concluded that the most likely hypothesis was that the patient was affected by novel Coronavirus pneumonia. Co-infection with *K. pneumoniae* probably inhibited the host immune system making the recovery process particularly prolonged.

The arguments in support of our thesis are: (1) the patient clinical characteristics compatible with those of COVID-19; (2) unresponsiveness to targeted antibiotic therapy; (3) CT findings: chest CT is considered the imaging method of choice in the diagnosis of COVID-19 infection; CT characteristics of the present case are consistent with the hallmarks of COVID-19 infection (5–7); (4) the imaging aspect is not the most typical for *K. pneumoniae*.

On the other hand, some clinical features that might question our interpretation must be disclosed. First of all, the observed lung insemination and bilateral pleurisy in the presence of clinical signs of fever, cough, and dyspnea could suggest a bacterial etiology. The hematogenous dissemination of *K. pneumoniae* supports this hypothesis. However, also a nosocomial gram negative bacterium infection cannot be excluded. Second, in COVID-19 infection, bilateral pleurisy is rare and ground grass infiltrates are predominantly sub-pleural. Finally, ceftazidime resistance could also appear for *K. pneumoniae* and metronidazole would have not affected gram-negative bacteria.

In the absence of the real time reverse transcription–polymerase chain reaction (real time RT–PCR) test for SARS-CoV-2 and the Immunoglobulin G (Ig G) virus-specific antibody detection for COVID-19, the certainty in diagnosis is obviously unattainable. However, the low level of accuracy of such tests that was feared in the early phase of the pandemic and the uncertainties about how long people who recovered would have had immunity dissuaded us from contacting the patient for confirmation tests. Furthermore, even if the serological tests were positive, it could not have been excluded that the patient had contracted the infection later without developing significant symptoms.

In conclusion, here we presented a case highly suspected for COVID-19 observed at the end of January 2020 supporting the hypothesis of a Coronavirus Italian spreading before the official outbreak.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patient/participant of this study provided her written informed consent for the analysis and publication of any her potentially identifiable images or data.

AUTHOR CONTRIBUTIONS

PL-S conceived the manuscript. ABus, ABul, SA, and AC collected data. ABus wrote the first draft. All authors revised the manuscript and approved the final version.

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Predicting Progression of COVID-19 Infection to Prioritize Medical Resource Allocation: A Novel Triage Model Based on Patient Characteristics and Symptoms at Presentation

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Background: The COVID-19 global pandemic has posed unprecedented challenges to health care systems all over the world. The speed of the viral spread results in a tsunami of patients, which begs for a reliable screening tool using readily available data to predict disease progression.

Methods: Multicenter retrospective cohort study was performed to develop and validate a triage model. Patient demographic and non-laboratory clinical data were recorded. Using only the data from Zhongnan Hospital, step-wise multivariable logistic regression was performed, and a prognostic nomogram was constructed based on the independent variables identifies. The discrimination and calibration of the model were validated. External independent validation was performed to further address the utility of this model using data from Jinyintan Hospital.

Results: A total of 716 confirmed COVID-19 cases from Zhongnan Hospital were included for model construction. Men, increased age, fever, hypertension, cardio-cerebrovascular disease, dyspnea, cough, and myalgia are independent risk factors for disease progression. External independent validation was carried out in a cohort with 201 cases from Jinyintan Hospital. The area under the curve (AUC) was 0.787 (95% confidence interval [CI]: 0.747–0.827) in the training group and 0.704 (95% CI: 0.632–0.777) in the validation group.

Conclusions: We developed a novel triage model based on basic and clinical data. Our model could be used as a pragmatic screening aid to allow for cost efficient screening to be carried out such as over the phone, which may reduce disease propagation through limiting unnecessary contact. This may help allocation of limited medical resources.

Keywords: COVID-19, pandemic, risk factor, nomogram, triage

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INTRODUCTION

In December 2019, Chinese and World Health Organization (WHO) health experts identified a growing number of pneumonia of unknown cause cases leading to substantial health issues for many citizens located in Wuhan, China (1, 2). Identified as a virus, infection caused severe respiratory syndromes and commonly used treatments were often ineffective. Today, we now know the cause to be a novel coronavirus known as the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), or COVID-19.

COVID-19 is the latest threat to global health. On March 11, 2020, WHO declared COVID-19 to be a global pandemic, as infection cases were reported in at least 114 countries (3). As of March 15, 2021, a total of more than 119,452,269 cases and 2,647,662 deaths were confirmed worldwide and the number of new cases was expected to increase (4). The high number of COVID-19 cases has overwhelmed health systems globally.

As health system resources are limited—even in highly developed countries—it is crucial to conduct clinical research to determine the best utilization of resources. For example, due to the limited number of medical professionals and equipment (e.g., N95 masks and respirators) available at such short notice, it is almost impossible to provide meticulous, high resource (e.g., intensive care level) health care for every single case of COVID-19 infection. However, this may be acceptable given the fact that initial epidemiologic studies demonstrated that most of COVID-19 cases were classified as mild (81% with non-pneumonia or mild pneumonia) and did not require intensive medical care (5). However, this important finding can only help if we can prioritize resources to those who need it most. Thus, it is important for front-line medical professionals to have a reliable and technically easy way to differentiate those at higher risk for severe and critical symptoms from those at lower risk.

Given that health care systems globally are overwhelmed with the exponential growth of COVID-19 cases and health care resources are limited, it is of extraordinary importance to allocate medical resources effectively and fairly (6). Decisions on resource allocation must be able to be made right at initial patient presentation in order to optimize resource use up front. Thus, it is of notable value to develop a triage model using only patient characteristics and clinical data (i.e., data readily available to medical professionals without the need for additional resources, such as laboratory, and/or imaging technology). In the present study, we create and validate such a novel triage model based on patient data from Chinese COVID-19 epicenter.

METHODS

Data Sources

This retrospective cohort study was approved by Research Ethics Commission of Zhongnan Hospital of Wuhan University (2020032), Jinyintan Hospital (KY-2020-50.01), and Zhejiang Provincial People's Hospital (2020QT068). The requirement of informed consent was waived due to its retrospective design. On March 10, 2020, all medical records of inpatients diagnosed with COVID-19 in Zhongnan Hospital of Wuhan University and Jinyintan Hospital, Wuhan, China were reviewed. Epidemiological, demographic, clinical symptoms, signs, and comorbidities information were extracted from electronic medical records. All data were examined by two of the listed authors (LNS and ZJ) independently to ensure accuracy.

Definitions

For this study, the severity of COVID-19 infection was defined according to the World Health Organization (WHO) interim guidance (7). Mild type infection is defined as cases where patients have non-pneumonia or mild pneumonia (5). Severe type infection is diagnosed when at least one of the following three diagnostic criteria is met: (1) respiratory distress (RR $\geq$ 30/min); (2) resting blood oxygen saturation =< 93%; or (3) arterial blood oxygen partial pressure (PaO2)/FiO2 =<300 mmHg. Critical type is diagnosed when at least one of the following three diagnostic criteria is met: (1) respiratory failure needing mechanical oxygenation; (2) shock; or (3) development of other organ failure, requiring intensive care unit (ICU) care. Fever was defined as axillary temperature of at least 37.3°C. Using these criteria, patient cases were divided into two groups: (1) mild, which could be treated via isolation at home or at the temporary hospital; and (2) severe/critical, which should be admitted for inpatient care within a hospital with full resources/equipment as soon as possible.

Statistical Analysis

Baseline characteristics of the patients of two groups (mild and severe/critical) were described using counts and percentages for categorical variables and medians with interquartile ranges (IQR) for continuous variables. Differences between groups were tested using the χ^2 test or Fisher's exact test for categorical variables and *t* test or Wilcoxon test for continuous variables, depending on the nature of the distribution. No imputation was made for missing data. To explore factors associated with severe/critical COVID-19 infection, univariate and multivariate logistic regression models were performed. Independent variables with $p < 0.05$ in univariate analyses were entered in a multivariate model, in which the predictors with $p < 0.05$ were further selected in the final multivariate logistic regression model. Sex and age were selected for all multivariate models for effect-adjustment purpose.

A nomogram was developed based on the final model. Internal and independent validations were implemented to evaluate the predictive performance of the derived nomogram, in terms of discrimination and calibration. For internal validation, we used bootstrap resampling with 1,000 samples to compute bias-corrected estimates. For independent validation, to account for potential discrepancy between the model-development dataset and independent-validation dataset, calibration plots were created for the original and recalibrated nomogram, with recalibration based on the intercept and slope framework as originally proposed by D.R. COX (8). Discrimination was assessed by the receiver operating characteristic (ROC) curve and area under the curve (AUC). Calibration was assessed by comparing observed severe/critical COVID-19 rates with predictions from the final model. All statistical analyses were

conducted by one of the authors listed as (JYZ), who works as an independent statistician and was not involved in patient care. All statistical analyses were performed using R software, version 3.6.2 (R Foundation for Statistical Computing), and a two-sided α of <0.05 was considered statistically significant for all tests.

RESULTS

Demographic Data and Symptoms

A total of 1,181 patients with suspected or confirmed COVID-19 infection were admitted to Zhongnan Hospital of Wuhan University between December 30, 2019 and March 10, 2020. Among these patients, 406 patients (34%) were excluded because they were assumed positive for COVID-19 infection based only on clinical symptoms and/or CT scan prior to testing kits becoming readily available (performed in Hubei Province only in certain period of time). Another 59 cases (5%) were excluded because of missing clinical data in electronic medical records. Ultimately, a final sample of 716 patients (61%) with confirmed COVID-19 infection was included as a training data set. Same set of data of a cohort of 201 patients with COVID-19 from Jinyintan Hospital was included for independent validation.

The basic epidemiological, demographic, clinical characteristics for the training set (medium age 55, 46.9% male) and the validation set (medium age 63, 49.3% male) were shown in **Table 1**. In the training set, 161 cases (22.5%) were diagnosed as severe/critical type, while in the validation set, 90 cases (44.8%) were diagnosed as severe/critical. In both set, patients in severe/critical group were significantly older than those in mild group ($p < 0.001$). Hypertension, diabetes and cardio-cerebrovascular disease were the most common comorbidities (**Table 1**). The most common symptoms on admission were fever, cough, dyspnea, and myalgia (**Table 1**).

Independent Risk Factor Identification

Univariate analysis identified that sex, age, presence of fever, current smoker, former smoker, alcohol consumption, hypertension, diabetes mellitus, cardio-cerebrovascular disease, dyspnea, cough, and myalgia were significantly associated with progression of COVID-19 from mild to severe/critical (**Table 2**).

Using the results of the univariate analysis, a multivariate logistic regression model was developed, which identified that sex (man), increased age, presence of fever, current smoker, hypertension, cardio-cerebrovascular disease, dyspnea, cough, and myalgia were independently associated with increased odds of progression of COVID-19 disease from mild to severe/critical. Woman sex was the only characteristic associated with decreased risk of disease progression (**Table 2**).

Nomogram Development

The probability of progressing from the mild to severe/critical group was assessed based on the results of the final multivariate logistic regression. The final multivariate logistic regression model for constructing the nomogram can be expressed as $\ln\left(\frac{P_{severe/critical}}{1-P_{severe/critical}}\right) = -4.91 + 0.36\ \text{male} + 0.03\ \text{age} + 0.66\ \text{fever} + 0.64\ \text{smoke} + 0.61\ \text{hypertension} + 1.41\ \text{cardio} - \text{cerebrovascular disease} + 0.81\ \text{dyspnea} + 0.54\ \text{cough} + 0.68\ \text{myalgia}$ where $P_{severe/critical}$ denotes the probability for a patient with COVID-19 to progress to severe/critical COVID-19.

Nomogram Construction and Validation

A prognostic nomogram for early recognition of those cases that would likely progress to severe/critical cases was constructed using the multivariate logistic regression results. Points were assigned to the identified factors according to the absolute maximum beta value based on the logistic regression model, given that the units are different for the continuous (age) and categorical predictors (sex, fever, smoke, hypertension, cardio-cerebrovascular disease, dyspnea, cough, and myalgia). Though with the smallest beta coefficient of 0.03, the calculated absolute maximum beta value (Beta $\times$ value range of the predictor) of age is $0.03 \times 89 = 2.67$, which means that it has the greatest impact on the probability of the event compared with the other seven predictors (**Figure 1**). As shown in the nomogram, patients with the following characteristics were more likely to progress to the severe/critical group: sex (man), older in age, presence of fever, current smoker, hypertension, cardio-cerebrovascular disease, dyspnea, cough, and myalgia. Summing all points led to a total score. Locating the total score on the nomogram scale, the risk of progressing to the severe/critical group could be determined at patient presentation.

Internal and External Independent Validation

To evaluate the discrimination of the model and to reduce overfitting bias, internal validation was performed using a bootstrapping technique with 1,000 resamples as qualified. **Figure 2** showed the internal validation of the nomogram using a receiver operating characteristic (ROC) curve with an area under the curve (AUC) of 0.787 (95% confidence interval [CI]: 0.747–0.827). We performed external independent validation of our nomogram as well, which demonstrated as AUC of 0.704 (95% CI: 0.632–0.777). The calibration curve showed excellent accordance between the nomogram prediction and the actual observation of severe/critical cases of COVID-19 (**Figure 3**). An external calibration plot for Jinyintan dataset based on the original nomogram and on the recalibrated nomogram is shown in **Figure 4**.

DISCUSSION

The COVID-19 global pandemic has caused great strain on the world's economies and health systems. Without a vaccine or therapeutic available, the number of confirmed cases continues to rise in many areas with many patients requiring hospitalization and a great deal of health care resources. However, health care resources are limited and optimizing their use is critical to successfully tackling this pandemic. In this present study, patient and clinical (non-laboratory) data on 917 patients from two different hospitals, Zhongnan Hospital of Wuhan University and Jinyintan Hospital, in Wuhan, China with confirmed COVID-19 infection were retrospectively reviewed. Step-wise multivariate logistic regression was used to identify risk factors for progression from mild to severe/critical disease. This information was

TABLE 1 | Characteristics of patients in the training and validation cohorts.

	Training Cohort (Zhongnan Hospital)				Validation Cohort (Jinyintan Hospital)			
	Mild (*N* = 555)	**Severe/critical (*N* = 161)**	**Total (*N* = 716)**	***p***	**Mild (*N* = 111)**	**Severe/critical (*N* = 90)**	**Total (*N* = 201)**	***p***
Sex				0.003				0.002
Female	311 (56.0%)	69 (42.9%)	380 (53.1%)		67 (60.4%)	35 (38.9%)	102 (50.7%)	
Male	244 (44.0%)	92 (57.1%)	336 (46.9%)		44 (39.6%)	55 (61.1%)	99 (49.3%)	
Age				<0.001				<0.001
N	555	161	716		111	90	201	
Median (IQR)	52 (38–62)	62 (54–72)	55 (41–65)		61 (50–68)	67 (58–73)	63 (53–70)	
Medical professionals				0.072				NA
No	325 (89.5%)	94 (95.9%)	419 (90.9%)		NA	NA	NA	
Yes	38 (10.5%)	4 (4.1%)	42 (9.1%)		NA	NA	NA	
Symptom to admission (Days)				0.933				0.001
N	547	158	705		111	90	201	
Median (IQR)	7 (3–15)	7 (3–15)	7 (3–15)		8 (4–13)	12 (7–16)	10 (5–15)	
Fever				0.006				0.275
No	195 (35.1%)	38 (23.6%)	233 (32.5%)		24 (21.6%)	14 (15.6%)	38 (18.9%)	
Yes	360 (64.9%)	123 (76.4%)	483 (67.5%)		87 (78.4%)	76 (84.4%)	163 (81.1%)	
Weight				0.489				NA
N	402	120	522		NA	NA	NA	
Median (IQR)	65 (57.5–73.0)	65 (60.0–74.0)	65 (58.0–73.0)		NA	NA	NA	
Height				0.64				NA
N	343	120	463		NA	NA	NA	
Median (IQR)	165 (160–170)	165 (160–170)	165 (160–170)		NA	NA	NA	
BMI				0.118				NA
N	342	118	460		NA	NA	NA	
Median (IQR)	23.5 (21.5–25.6)	24.1 (22.4–26.0)	23.7 (21.7–25.7)		NA	NA	NA	
Current smoker				<0.001				0.177
No	519 (93.9%)	132 (83.0%)	651 (91.4%)		108 (97.3%)	84 (93.3%)	192 (95.5%)	
Yes	34 (6.1%)	27 (17.0%)	61 (8.6%)		3 (2.7%)	6 (6.7%)	9 (4.5%)	
Former Smoker				0.002				NA
No	538 (97.3%)	146 (91.8%)	684 (96.1%)		NA	NA	NA	
Yes	15 (2.7%)	13 (8.2%)	28 (3.9%)		NA	NA	NA	
Alcohol consumption				0.018				0.054
No	493 (88.8%)	130 (81.8%)	623 (87.3%)		110 (99.1%)	85 (94.4%)	195 (97.0%)	
Yes	62 (11.2%)	29 (18.2%)	91 (12.7%)		1 (0.9%)	5 (5.6%)	6 (3.0%)	
Hypertension				<0.001				0.017
No	451 (81.4%)	91 (56.9%)	542 (75.9%)		79 (71.8%)	50 (55.6%)	129 (64.5%)	
Yes	103 (18.6%)	69 (43.1%)	172 (24.1%)		31 (28.2%)	40 (44.4%)	71 (35.5%)	
DM				0.016				0.156
No	509 (91.9%)	136 (85.5%)	645 (90.5%)		100 (90.1%)	75 (83.3%)	175 (87.1%)	
Yes	45 (8.1%)	23 (14.5%)	68 (9.5%)		11 (9.9%)	15 (16.7%)	26 (12.9%)	
COPD				0.233				0.114
No	542 (98.0%)	152 (96.2%)	694 (97.6%)		111 (100.0%)	88 (97.8%)	199 (99.0%)	
Yes	11 (2.0%)	6 (3.8%)	17 (2.4%)		0 (0.0%)	2 (2.2%)	2 (1.0%)	
Cardio-Cerebrovascular Disease				<0.001				0.228
No	535 (96.6%)	125 (78.6%)	660 (92.6%)		102 (91.9%)	78 (86.7%)	180 (89.6%)	
Yes	19 (3.4%)	34 (21.4%)	53 (7.4%)		9 (8.1%)	12 (13.3%)	21 (10.4%)	

(Continued)

TABLE 1 | Continued

	Training Cohort (Zhongnan Hospital)			*p*	Validation Cohort (Jinyintan Hospital)			*p*
	Mild (*N* = 555)	**Severe/critical (*N* = 161)**	**Total (*N* = 716)**		**Mild (*N* = 111)**	**Severe/critical (*N* = 90)**	**Total (*N* = 201)**	
Dyspnea				<0.001				0.001
No	434 (78.2%)	90 (55.9%)	524 (73.2%)		57 (51.4%)	25 (27.8%)	82 (40.8%)	
Yes	121 (21.8%)	71 (44.1%)	192 (26.8%)		54 (48.6%)	65 (72.2%)	119 (59.2%)	
Diarrhea				0.082				0.503
No	519 (93.5%)	144 (89.4%)	663 (92.6%)		106 (95.5%)	84 (93.3%)	190 (94.5%)	
Yes	36 (6.5%)	17 (10.6%)	53 (7.4%)		5 (4.5%)	6 (6.7%)	11 (5.5%)	
Myalgia				0.011				0.918
No	499 (89.9%)	133 (82.6%)	632 (88.3%)		104 (93.7%)	84 (93.3%)	188 (93.5%)	
Yes	56 (10.1%)	28 (17.4%)	84 (11.7%)		7 (6.3%)	6 (6.7%)	13 (6.5%)	
Cough				<0.001				0.738
No	288 (51.9%)	56 (34.8%)	344 (48.0%)		37 (33.3%)	28 (31.1%)	65 (32.3%)	
Yes	267 (48.1%)	105 (65.2%)	372 (52.0%)		74 (66.7%)	62 (68.9%)	136 (67.7%)	

BMI, Body Mass Index; DM, Diabetes Mellitus; COPD, Chronic Obstructive Pulmonary Disease.

TABLE 2 | Risk factors associated with developing severe/critical group COVID-19.

Variables	**Univariate**	***P***	**Multivariate**	***P***
	OR (95%CI)		**OR (95%CI)**	
Sex (ref: male)	0.588 (0.413–0.839)	0.003	0.696 (0.459–1.057)	0.089
Age (per year)	1.049 (1.036–1.063)	0.000	1.035 (1.019–1.051)	0.000
Medical professionals	0.364 (0.127–1.046)	0.061		
Fever	1.753 (1.171–2.624)	0.006	1.940 (1.204–3.126)	0.006
BMI (kg/m^2)	1.058 (0.991–1.130)	0.093		
Current smoker	3.122 (1.819–5.359)	0.000	1.894 (1.008–3.559)	0.047
Former smoker	3.194 (1.486–6.862)	0.003		
Alcohol consumption	1.774 (1.096–2.871)	0.020		
Hypertension	3.320 (2.273–4.850)	0.000	1.845 (1.157–2.942)	0.010
DM	1.913 (1.118–3.272)	0.018		
Cardio-cerebrovascular Disease	7.659 (4.228–13.875)	0.000	4.109 (2.086–8.093)	0.000
Dyspnea	2.830 (1.953–4.099)	0.000	2.244 (1.464–3.440)	0.000
Cough	2.022 (1.405–2.912)	0.000	1.723 (1.137–2.611)	0.010
Diarrhea	1.702 (0.929–3.119)	0.085		
Myalgia	1.876 (1.147–3.069)	0.012	1.981 (1.120–3.504)	0.019

Variables were transformed to their nature logarithms.
CI, Confidence Interval; OR, Odds Ratio; DM, Diabetes Mellitus.

utilized to produce a nomogram predictive model. Men, older in age, presence of fever, current smoker, hypertension, cardio-cerebrovascular disease, dyspnea, cough, and myalgia were all characteristics associated with higher risk for disease progression. Woman sex was the only protective factor. This information can help medical professionals and governments maximize the use of their medical resources by prioritizing patients with greater odds of progressing to severe/critical disease.

Given COVID-19 is a novel coronavirus that was only identified in December 2019, there is an overall paucity of literature to date. However, of the limited prior research, one previous study indicated that older age was an important independent variable associated with mortality in critical COVID-19 patients (9). While our study did not directly examine mortality, our research identified the importance of older age as a variable associated with the progression of COVID-19 disease from mild to severe/critical. The underlying mechanism causing age-related issues could be an age-dependent deficiency in B-cell and T-cell function and the dysfunction of viral elimination due to the excess production

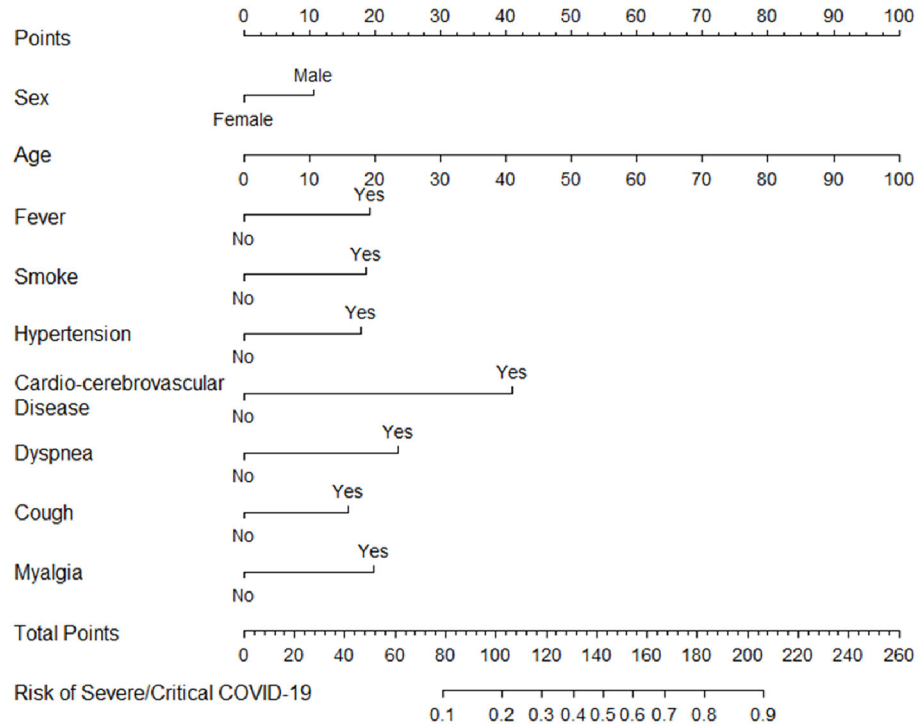

FIGURE 1 | Nomogram of probability to develop severe/critical COVID-19. To use the nomogram, draw an upward vertical line from each covariate to the points bar to calculate the number of points. Based on the sum of the covariate points, draw a downward vertical line from the total points line to calculate the probability of developing severe/critical COVID-19.

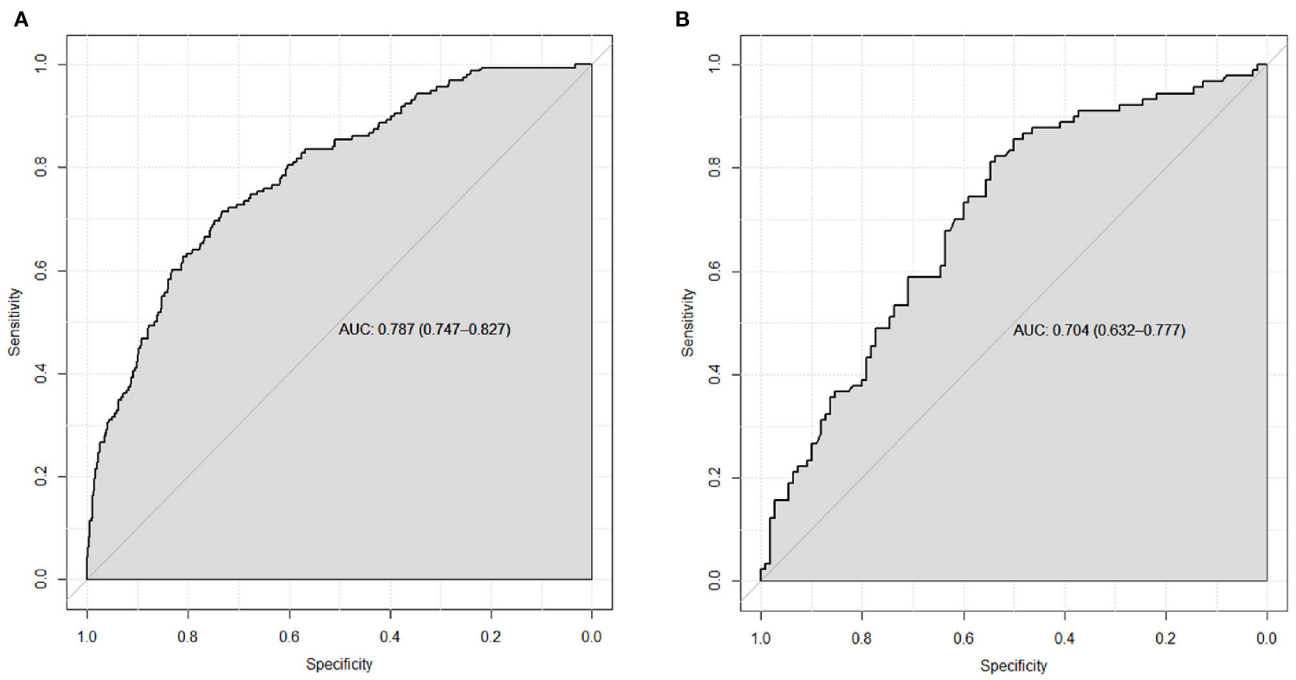

FIGURE 2 | (A) ROC curve for the nomogram based on the full Zhongnan Hospital dataset. The bias-corrected AUC is 0.772 based on internal validation using bootstrap resampling (1,000 patients) **(B)** ROC curve from an external, independent validation using the Jinyintan Hospital dataset. The estimate of AUC and its 95% confidence interval are shown in the plots. Key: ROC, receiver operating characteristic. AUC, area under the curve.

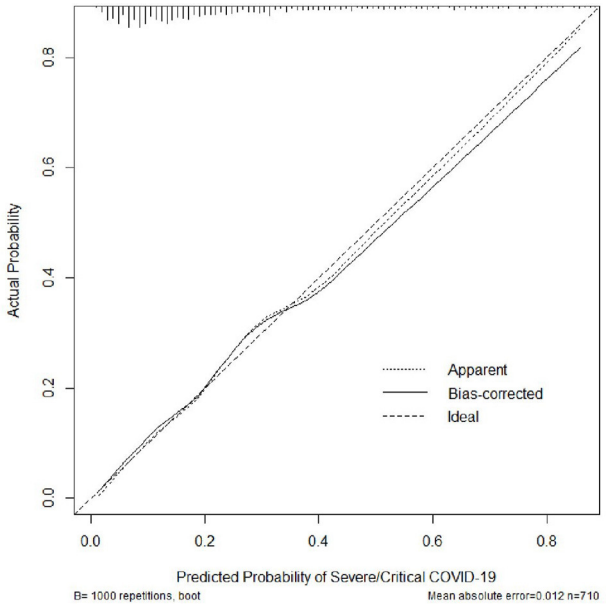

FIGURE 3 | Calibration plot for the nomogram. The bias-corrected (overfitting-corrected) estimates of predicted vs. observed values were obtained based on bootstrap resampling with 1,000 samples for internal validation purpose.

of type 2 cytokines, leading to prolonged pro-inflammatory responses (10).

In addition to age, sex is an important factor to consider. The limited literature to date reported that men account for a high proportion of COVID-19 cases, ranging from 58 to 67% (9, 11–13). One hypothesis as to why the literature from China suggests this disease predilection for men is that majority workers in Huanan Seafood Wholesale Market, where the disease appears to have originated, were men (14, 15). Intriguingly, our sample was nearly split evenly by sex. However, our results indicate that men with COVID-19 infection have higher risk for disease progression. While more severe disease in men is consistent with media reports, this is the first study, to our knowledge, that confirms this finding scientifically. However, additional research from other pandemic epicenters is warranted to further evaluate the impact of sex on disease progression and mortality (16).

Presenting clinical symptoms are also crucial elements of initial evaluation of patients with COVID-19 infection. One of the most common presenting symptoms of COVID-19 infection is fever (5, 14, 17). In our study, we found that 67.5% of the cases had fever at presentation. The impact of fever on poor clinical outcomes could be associated with IL-6, which is generally known as a strong pro-inflammatory cytokine and highly expressed in non-survivor groups with severe/critical disease in previous studies (9, 18). The other three symptoms that were independently associated with increased risk of disease progression were dyspnea, cough, and myalgia. Because of the impact of COVID-19 infection on the respiratory system, these were not unexpected finding; however, it remains important for front-line medical professions to consider these specific symptoms as alarming risk factors when treating patients who initially present for care with COVID-19 infection.

Comorbidities are also important to consider when evaluating risk factors for disease progression. Among all of the comorbidities analyzed, hypertension and cardio-cerebrovascular disease were associated with disease progression. Our results showed a significantly higher proportion of patients with hypertension in the severe/critical group than in the mild group (43.1 vs. 18.6%, $p < 0.01$). Hypertension was identified as a risk factor for disease progression, which is partially consistent with previous studies (9, 12, 14). Cardio-cerebrovascular disease was also significantly associated with higher risk of disease progression in our model. Despite its low incidence (7.4% in training cohort and 10.4% in validation cohort), cardio-cerebrovascular disease is of notable concern and medical professionals should be aware of such a diagnosis. Cardio-cerebrovascular disease is a well-known risk factor due to its strong association with all-cause dementia and depression and all-cause mortality (19–21). Our previous study also found that the cases with COVID-19 who was transferred to ICU had a higher proportion of cardio-cerebrovascular disease comorbidity (14).

There are several limitations of our study. First, our analysis included patients from only one country; therefore, the generalizability of our findings to other areas of the world is unknown. However, our findings scientifically verify many of the global media reports and can be considered by public health officials making resource utilization decisions. Second, we included all patients with confirmed COVID-19 infection at their time of initial presentation; however, we did not account for any difference in the duration of symptoms prior to presentation. Because all were aware of this concerning disease, we suspect many did not present with delay. Further, by including all who presented for care, we feel selection biased was reduced. Third, we did not include results from any laboratory or radiographic tests. Such information, could potentially provide additional insight as to factors associated with disease progression. However, our model provides an efficient and easy approach to triaging patients at initial presentation based strictly on patient characteristics, comorbidities, and symptoms. Further, this type of approach is of value in areas where medical supplies and resources are of substantial shortage. Lastly, due to our limited sample size and retrospective cohort study design, we believe a prospective, randomized clinical trial with larger sample size would be helpful to confirm our findings and/or validate new findings. However, given the overwhelming nature of this global pandemic, such a study design may be challenging to perform, especially as new and experimental interventions are being introduced nearly daily. Our study demonstrates the natural disease process for those not undergoing experimental therapeutic intervention.

Overall, we determined which factors are associated with progression of COVID-19 infection from mild to

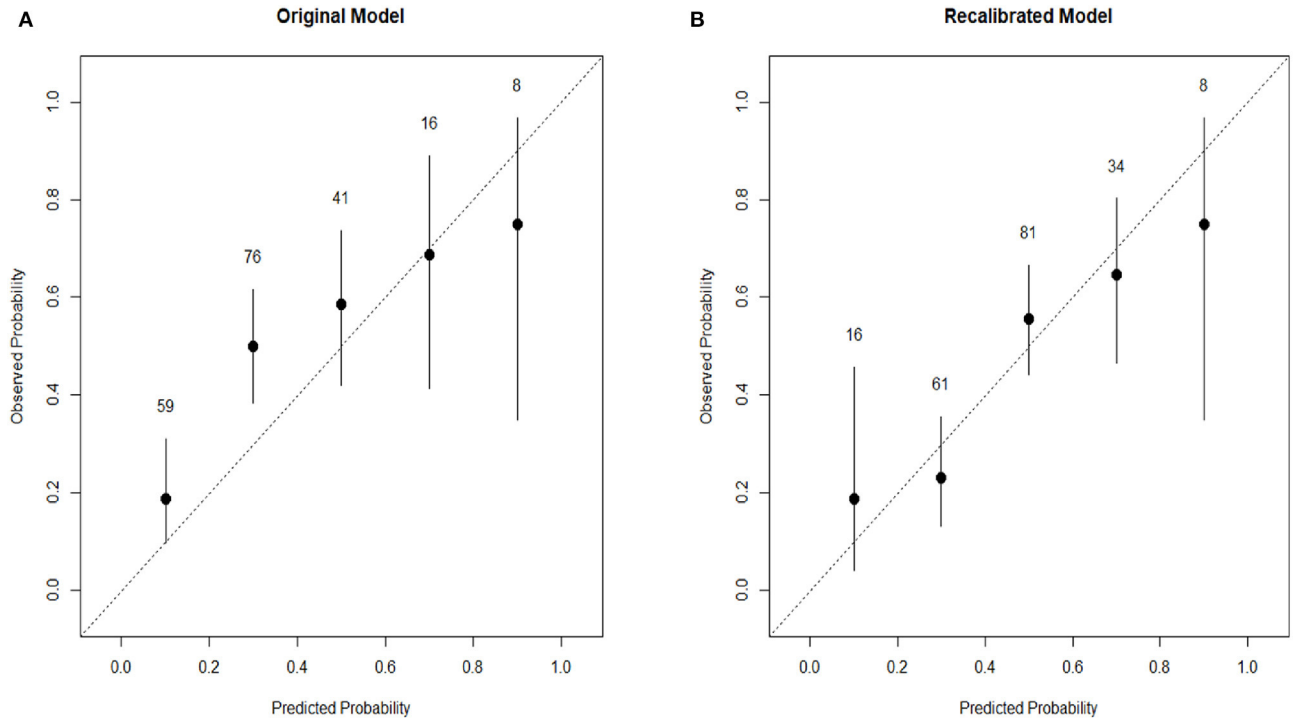

FIGURE 4 | (A) External calibration plot for Jinyintan dataset based on the original nomogram. **(B)** External calibration plot for Jinyintan dataset based on the recalibrated nomogram. The nomogram was recalibrated by the intercept and slope framework as originally proposed by D.R. Cox.[8] The plots are grouped into five bins based on their predicted probabilities, and then the bin prevalence (the ratio of plots in this bin with observed number of severe/critical COVID-19 vs. the total number of plots in this bin) is calculated for each bin. The confidence interval for each bin is also plotted, and the total number of plots is labeled above each the bin. Confidence intervals are calculated for the binomial bin counts using the F distribution.

severe/critical. Based on these results, a validated nomogram was developed to help triage patients at presentation and then externally validated. We believe our study findings could be applied in outpatient clinic or emergency department settings to better triage the growing number of newly confirmed COVID-19 cases during this global pandemic. This could help optimize resource utilization within health care systems globally, which is critical at this time of concerned shortages.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This retrospective cohort study was approved by Research Ethics Commission of Zhongnan Hospital of Wuhan University (2020032), Jinyintan Hospital (KY-2020-50.01), and Zhejiang Provincial People's Hospital (2020QT068). The requirement of informed consent was waived due to its retrospective design.

AUTHOR CONTRIBUTIONS

YT, XZ, LS, JZhe, JZha, HH, YZ, and MG conceived of the presented idea. JWa, MW, JWu, JZhu, RY, YJ, LC, DZ, HW, SC, RL, and JL collected clinical data for this study. JZhe, YW, and WT performed statistical analysis. JZha, HH, YZ, and MG encouraged YT, XZ, LS, and JZhe to investigate and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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The Association of Hypertension With the Severity of and Mortality From the COVID-19 in the Early Stage of the Epidemic in Wuhan, China: A Multicenter Retrospective Cohort Study

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Background: Hypertension may affect the prognosis of COVID-19 illness. We analyzed the epidemiological and clinical characteristics associated with the disease severity and mortality in hypertensive vs. non-hypertensive deceased COVID-19 patients.

Methods: We included all the deceased patients with laboratory-confirmed COVID-19 admitted to >200 health facilities in Wuhan between December 1 and February 24, 2020. The median survival time in COVID-19 patients with and without hypertension, the association of hypertension with the disease severity, and the risk factors associated with the COVID-19 mortality stratified by the hypertension status were assessed using the Kaplan-Meier survival analysis, logistic regression, and Cox proportional regression, respectively before and after the propensity score-matching (PS) for age and sex.

Results: The prevalence of hypertension in the studied 1,833 COVID-19 patients was 40.5%. Patients with hypertension were more likely to have severe COVID-19 illness than patients without hypertension; the PS-matched multivariable-adjusted odds ratio (95% CI) was 2.44 (1.77–3.08). Moreover, the median survival time in the hypertension group was 3–5 days shorter than the non-hypertension group. There was a 2-fold increased risk of COVID-19 mortality in the hypertension group compared with the non-hypertension group; the PS-matched multivariable-adjusted hazard ratio (HR) = 2.04 (1.61–2.72), and the significant increased risk of COVID-19 mortality in the moderate vs. mild COVID-19 illness was confined to patients with hypertension. Additionally, the history and the number of underlying chronic diseases, occupation, and residential location showed stronger associations with the COVID-19 mortality among patients with hypertension than patients without hypertension.

Conclusion: Hypertension was associated with the severity and mortality of COVID-19 illness.

Keywords: COVID-19, hypertension, severe, mortality, critical, risk factors

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INTRODUCTION

In the early of December 2019, a series of sudden unfathomable cases of a respiratory disease occurred and spread rapidly among the residences in China. This disease has been named "coronavirus disease 2019 (COVID-19)" by the World Health Organization, and the coronavirus was subsequently named by the International Committee on Taxonomy of Viruses as Severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) (1, 2). As of July 15, 2020, the cumulative number of confirmed cases worldwide was 12,964,863, and the cumulative death was 570,288 (3). A growing body of evidence is demonstrating that clinical comorbidities such as diabetes, hypertension, and cardiovascular diseases are highly prevalent among COVID-19 patients (4–13). Moreover, many patients with COVID-19 are critically ill and require care in the intensive care unit (ICU). Previous study suggested that the mortality rate and absolute mortality were high, hospital and ICU mortality rates were 12 and 27 per 1,000 patients-days (14). In China, the estimated mortality was 1.1% in non-severe COVID-19 patients and 32.5% in severe cases during the average 32 days of follow-up period, and severe male patients with complications may have a higher risk of death (4, 15, 16).

Hypertension was the most prevalent reported comorbidity in COVID-19 patients in Wuhan; the reported prevalence rates ranged from 15.0% (5, 9) to 36.5% (6). Suggestions were also made for some associations of the hypertension status with the severity of and mortality from the COVID-19 illness. In patients with the severe COVID-19 illness, one study reported 23.7% (5) prevalence rate of hypertension, while the rate was as high as 58.3% (10) in another study. The prevalence of hypertension was also higher in COVID-19 deceased patients; 34.0% vs. those who were discharged alive; 28.0% (7). The available systematic review and meta-analysis confirmed that hypertension was the most prevalent chronic morbidity in COVID-19 patients [17%; 95% confidence interval (CI):14–22%]. In that meta-analysis, the overall odds ratio (OR) of hypertension in patients with the severe COVID-19 illness, in reference to those with the non-severe illness, was 3.42 (95%CI: 1.88–6.22) (17). Most of the previous studies lacked the adjustment for factors that could confound the associations of hypertension with the risks of COVID-19 illness, the disease severity and the related mortality. Most importantly was the patients' age, which was suggested as the possible real contributing factor for the augmented risk among patients with hypertension, considering the high correlation between age and hypertension (7). Of the few studies that conducted multivariate adjustments, Huang et al., failed to document a significant association between the hypertension status and the COVID-19 severity or mortality in 225 patients using logistic regression models adjusted for age and sex (6). To the contrary, in a larger study which included 3,430 COVID-19 patients, of whom 1,128 had hypertension, by Zhang et al., hypertension was significantly associated with 41% higher risk of mortality due to COVID-19 after the adjustment for age, gender, and comorbid diabetes, cerebrovascular diseases, and chronic renal disease using the Cox proportional hazard regression (18).

As for the mechanism of COVID-19, the infection results in diverse symptoms and morbidity. Previous study suggests that severe COVID-19 pathophysiology includes destruction of lung epithelial cells, thrombosis, hypercoagulation, and vascular leak leading to sepsis. Specifically, COVID-19 risk factors mainly include cardiovascular disease, hypertension, and diabetes; for this population, the upregulation of the angiotensin converting enzyme-2 (ACE2) receptor is exploited by COVID-19 as the route of entry and infection. In the infection, viral envelope proteins bind to and degrade ACE2 receptors, preventing normal ACE2 function, which causes imbalances in ACE2 and induces an inflammatory immune response, known as a cytokine storm, both of which amplify comorbidities within the host (19). In the mechanism for severe COVID-19 infection, ACE2 is involved in modulating blood pressure and establishing blood pressure homeostasis (20). Several studies indicated that hypertensive patients are treated with drugs to reduce blood pressure mostly through ACE-inhibitors, that leads to increased ACE2 expression, used by the COVID-19 virus for human's cell entry (11, 21). Thus, hypertension and the severe COVID-19 infection seems to be closely associated.

To further explore the association between hypertension and the severity of the COVID-19 infection, detect the effect of hypertension on the risk of mortality in COVID-19 patients, in general, and across the different severity grades of COVID-19 infection, and to compare the other risk factors associated with the mortality in the two groups of COVID-19 patients (with and without hypertension), this study included 1,833 deceased COVID-19 patients admitted to more than 200 hospitals/community health centers in Wuhan during the early outbreak in 2019–2020.

METHODS

Data Sources

This multicenter, retrospective cohort study was conducted based on the Chinese Infectious Disease Reporting Information System. In this system, more than 200 hospitals and community health centers have admitted almost all the COVID-19 patients in Wuhan. Data of COVID-19 patients were collected from these hospitals, including the Wuhan Hospital of Traditional Chinese and Western Medicine, Wuhan Union Hospital and Tongji, Tongji Medical College of HUST, Renmin Hospital of Wuhan University, Zhongnan Hospital of Wuhan University, Wuhan Pulmonary Hospital, Wuhan Jinyintan Hospital, Wuhan Puren Hospital, Raytheon Mountain Hospital, Airborne Military Hospital of PLA, Central Theater General Hospital of PLA, etc. All identifiable personal information was deleted for privacy protection. The epidemiological and clinical data and the hypertension status of all patients with COVID-19 were obtained from the electronic medical records of each hospital in the system. The Ethics Committee of Medical Department of Wuhan University granted ethical approval of this study (Grant number: WHU2020-2020YF0031).

Study Population

The study population was all ($n = 1{,}833$) the deceased COVID-19 patients aged 18 years or more who were admitted to the designated hospitals between December 1, 2019 and February 24,

2020, with laboratory-confirmed COVID-19 infection according to the diagnostic criteria of the new coronavirus infection pneumonia diagnosis and treatment plan (trial fifth version) (22).

Study Variables

All identifiable personal information was deleted for privacy protection. We collected the hospital admission data on the socio-demographic (age, sex, location, and occupation), and clinical characteristics including classification of the disease severity (mild, moderate, severe and critical COVID-19 illness) (18), underlying chronic disease histories (diabetes, cardiovascular disease, cerebrovascular disease, respiratory disease, and cancer). The hypertension status was ascertained via the documented medical history of the patients. Hypertension was defined as systolic blood pressure of ≥140 mmHg, diastolic blood pressure of ≥ 90 mmHg, and/or being on antihypertensive medication (18). The time-related indicators included the dates of symptoms' onset, clinical diagnosis and death, from which we calculated the durations from the symptom' onset to clinical diagnosis, and from the symptom' onset to the endpoint (death).

Statistical Analysis

We conducted the statistical analyses twice, before and after propensity score matching for the patients' age and sex between patients in the two groups (with and without hypertension). Among total deceased COVID-19 patients, the patients with-hypertension were statistically matched (2:3.3) with patients without-hypertension according to propensity score matching without replacement (**Figure 1**). The logistic regression method was used to generate propensity score and matching was performed using the nearest neighbor algorithm with a caliper distance of 0.25. In practice, a wide variety of calipers is used and some studies recommended reducing the caliper from 0.25 standard deviations to 0.2 standard deviations to get the balanced groups (23–26). Previously, a caliper of 0.25 standard deviations based on the results of Cochran and Rubin (27) has been taken as a recommendation. A standardized mean difference, defined as the mean difference between the groups divided by the standard deviation of the control group was reported before and after propensity score matching (28). The variables with missing data were not included in analysis.

Further, the Kolmogorov-Smirnov test was used to test the distribution of the characteristics' variables. Continuous variables were expressed as medians and interquartile ranges (IQR), and categorical variables were described as the frequencies and percentages. The Mann-Whitney *U*-test was used to test the difference in the continuous variables and the Chi-square its Fisher exact tests were used to test the difference in the categorical variables between the two groups of deceased patients (with and without hypertension). The logistic regression analysis was used to estimate the ORs and the respective 95% CIs of hypertension in the moderate, severe, and critical COVID-19 illnesses in reference to the mild illness, and in the severe (severe/critical) vs. the non-severe (mild/moderate) groups of the COVID-19 illness. For total, non-severe, and severe COVID-19 groups, we used the Kaplan-Meier estimator to visualize the survival curves and to calculate the overall and the specific median survival times and their 95% CIs in total deceased patients and in each comorbidity, and compared them in patients with and without hypertension.

The Cox proportional hazard regression models adjusted for age, and sex in the unmatched data and matched for age and sex in the propensity score-matched data and adjusted for the other demographic and clinical factors were used to estimate the hazard ratios (HRs) and the respective 95% CIs of mortality from COVID-19 infection associated with the hypertension status among the total COVID-19 deceased patients, and among patients with different COVID-19 severity. Moreover, Cox proportional regression analyses, stratified by the hypertension status, were conducted to test the factors associated with the mortality and to compare the magnitude of the association in each factor among COVID-19 patients with and without hypertension before and after the propensity score-matching. The *p*-interaction between the hypertension status and each of the tested factors was estimated by adding a cross-product term of the dichotomous hypertension status and the target variable into the model. For all the Cox analyses, the time of follow-up was defined as the duration from the onset of symptoms to death with no censoring. There was no evidence of violations of the Cox proportional hazard assumptions as the *p*-value of the Schoenfeld residuals test were > 0.1 for all the used models. A two-sided $p < 0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS (version 24.0).

RESULTS

Characteristics of COVID-19 Patients With and Without Hypertension

Among the 1,833 patients diagnosed with and died from the COVID-19 illness in more than 200 health facilities in Wuhan, China between December 1, and February 24, 2020, and 742 (40.5%) patients had hypertension (**Table 1**). Of the total 1,833 patients, 1,211 (66.10%) were men, and the median age was 73 years (IQR, 66–80) in patients with hypertension patients and 72 years (IQR, 64–78) years in patients without hypertension. After the propensity score-matching for age and sex, the median age of COVID-19 patients was 71 years in the both the hypertension and non-hypertension groups. As for occupation, the frequency of retirees (57.40 vs. 44.30%) and housework and unemployment (20.50 vs. 15.80%) was significantly higher in patients with hypertension than patients without hypertension. When considering the geographical distribution, most cases were concentrated on central area in Wuhan, and there was no significant difference between the hypertension and non-hypertension groups. Notably, deceased COVID-19 patients with hypertension were more likely to have other comorbidities than those without hypertension, with an approximately double-fold increment for the presence of two or less other comorbidities, and almost a 7-fold increment for the presence of more than two other comorbidities among the hypertension group compared with the non-hypertension group, even after the propensity score-matching for the patients' age and sex. During the study period, the large bulk of deaths has occurred between February 1, and February 15, 2020, with more deaths in the hypertension

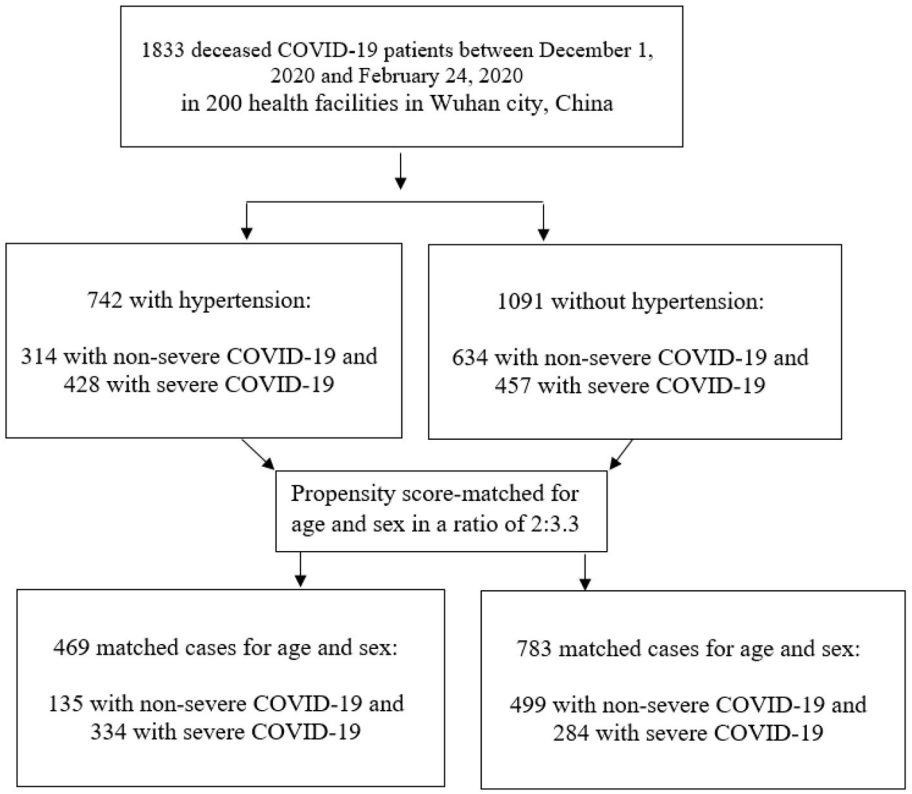

FIGURE 1 | Study flow chart.

group than in the non-hypertension group. We also observed that the median duration from onset to endpoint (death) was 15 days (IQR, 10–21) in the hypertension group and 17 days (IQR, 11–23) in the non-hypertension group; $p = 0.047$. The COVID-19 illness was critical in over a quarter of the COVID-19 patients with hypertension, while it represented not more than one-eighth of the non-hypertension group (**Table 1**).

Furthermore, hypertension was associated with the COVID-19 severity in both the unmatched and matched analyses; the age-and sex-matched multivariate-adjusted ORs (95%CIs) of hypertension in patients with moderate, severe and critical COVID-19 illness in reference to that in mild illness were 2.60 (2.32–3.40), 10.60 (6.10–17.31), and 35.02 (20.11–81.00); *p*-trend < 0.001; while the OR in severe (severe and critical) in reference to non-severe (mild and moderate) illnesses was 2.44 [95% CI: 1.77–3.08], matched for the age and sex, and adjusted for occupation, location, and the number of underlying diseases (**Table 2**).

Kaplan-Meier Survival Analysis in COVID-19 Patients With and Without Hypertension

The log-rank test results indicated a statistically significant shorter median (95%CI) survival time in patients with hypertension than those without hypertension (**Supplementary Figure 1**). The overall median (95% CI) survival time was 17.0 (16.4–17.6) days in the hypertension group, while it was 20.0 (19.1–22.9) days in the non-hypertension group. The respective median survival durations were 19.0 (18.1–19.9) vs. 20.0 (16.1–22.4) for the non-severe illness and 15.0 (14.1–15.9) vs. 17.0 (16.1–17.9) for the severe illness. The curves for the patients with each specified comorbidity showed 3–4 days shorter median survival times among patients with hypertension than among those without hypertension, for accompanying diabetes, cardiovascular disease, respiratory disease or cancer; with the exception of a longer median survival time for the accompanying cerebrovascular disease in patients with hypertension than those without hypertension. The same trends were found in the stratified analyses by the COVID-19 severity; however, the largest difference was observed for the severe illness, and was the 5 days shorter median survival time in the group of hypertension with cardiovascular disease; 11.0 (8.0–14.0) than that in the non-hypertension group with cardiovascular disease; 16.0 (13.0–19.0); *P* for Log-rank test = 0.01.

The Magnitude of the Association Between Hypertension and the Risk of COVID-19 Mortality in the Different Grades of Illness

Table 3 shows a double-fold higher risk of COVID-19 mortality in patients with hypertension in reference to those without hypertension; HR (95% CI) was 2.01 (1.79–3.13) after adjusting

TABLE 1 | The epidemiological, clinical, and social characteristics of deceased COVID-19 patients at the early stage of the epidemic in Wuhan, China (Overall and stratified by the patients' hypertensive status) before and after propensity score-matching for age and sex.

Parameters	Unmatched (before propensity score-matching)					Matched (after propensity score-matching)				
	All patients (*n* = 1,833)	**Patients without hypertension (*n* = 1,091)**	**Patients with hypertension (*n* = 742)**	***p*-value***	**Standardized**** difference**	**All patients (*n* = 1,252)**	**Patients without hypertension (*n* = 783)**	**Patients with hypertension (*n* = 469)**	***p*-value***	**Standardized**** difference**
Age, years M(IQR)	70 (63,79)	72 (64,78)	73 (66,80)	0.56	0.10	71 (64,81)	71 (65,81)	71 (63,80)	0.16	0.01
Gender										
Male	1,211 (66.10)	706 (64.70)	505 (68.1)	0.13	0.17	799 (63.80)	498 (63.60)	301 (64.20)	0.84	0.07
Female	622 (33.90)	385 (35.30)	237 (31.90)			453 (36.20)	285 (36.40)	168 (35.8)		
Occupation										
Retirees	858 (46.80)	462 (42.30)	396 (53.40)	<0.001	0.14	616 (49.20)	347 (44.30)	269 (57.40)	<0.001	0.04
Housework and unemployment	324 (17.70)	191 (17.50)	133 (17.90)			220 (17.60)	124 (15.80)	96 (20.50)		
Public servant	25 (1.40)	21 (1.90)	4 (0.50)			12 (1.00)	10 (1.30)	2 (0.40)		
Laborers	23 (1.30)	13(1.20)	10 (1.30)			13 (1.00)	7 (0.90)	6 (1.30)		
Cadres	35 (1.90)	24(2.20)	11 (1.50)			22 (1.80)	13 (1.70)	9 (1.90)		
Farmers and migrant workers	66 (3.60)	52 (4.80)	14 (1.90)			46 (3.70)	34 (4.30)	12 (2.60)		
Medical workers	21 (1.10)	14 (1.30)	7 (0.90)			14 (1.10)	8 (1.00)	6 (1.30)		
Other occupations	481 (26.20)	314 (28.80)	167 (22.50)			309(24.70)	240 (30.70)	69 (14.70)		
Location										
Central area in Wuhan	1,385 (75.60)	818 (75.00)	567 (76.40)	0.08	0.16	922 (73.60)	569 (72.70)	353 (75.30)	0.23	0.10
Sub urban area in Wuhan	288 (15.70)	186 (17.00)	102 (13.70)			220 (17.60)	137 (17.50)	83 (17.70)		
Out of city/other	160 (8.70)	87 (8.00)	73 (9.80)			110 (8.80)	77 (9.80)	33 (7.00)		
Clinical characteristics										
Diabetes	357 (19.50)	117 (10.70)	240 (32.30)	<0.001	0.13	254 (20.30)	107 (13.70)	147 (31.30)	<0.001	0.04
Cardiovascular diseases	330 (18.00)	114 (10.40)	216 (29.10)	<0.001	0.15	251 (20.00)	107 (13.70)	144 (30.70)	<0.001	0.03
Cerebrovascular diseases	174 (9.50)	51 (4.70)	123 (16.60)	<0.001	0.04	140 (11.20)	57 (7.30)	83 (17.70)	<0.001	0.08
Respiratory	338 (18.40)	142 (13.00)	196 (26.40)	<0.001	0.26	268 (21.40)	148 (18.90)	120 (25.60)	0.005	0.04
Cancer	343 (18.70)	150 (13.70)	193 (26.00)	<0.001	0.11	266 (21.20)	153 (19.50)	113 (24.10)	0.041	0.07
Other diseases**	590 (32.20)	288 (26.40)	302 (40.70)	<0.001	0.10	447 (35.70)	262 (33.50)	185 (39.40)	0.032	0.05
# of underlying disease										
None	629 (34.30)	629 (57.70)	0 (0.00)	<0.001	0.04	373 (29.80)	373 (47.60)	0 (0.00)	<0.001	0.09
≤2	889 (48.50)	403 (36.90)	486 (65.50)			639 (51.00)	334 (42.70)	305 (65.00)		
>2	315 (17.20)	59 (5.40)	256 (34.50)			240 (19.20)	76 (9.70)	164 (35.00)		
COVID-19 severity***										
Mild	597 (32.60)	418 (38.30)	179 (24.10)	<0.001	0.06	387 (30.90)	267 (34.10)	120 (25.60)	<0.001	0.04
Moderate	351 (19.10)	21 6(19.80)	135 (18.20)			247 (19.70)	232 (29.60)	15 (3.20)		
Severe	551 (30.10)	320 (29.30)	231 (31.10)			390 (31.20)	211 (26.90)	179 (38.20)		
Critical	334 (18.20)	137 (12.60)	197 (26.50)			228 (18.20)	73 (9.30)	155 (33.00)		

(Continued)

TABLE 1 | Continued

Parameters	Unmatched (before propensity score-matching)					Matched (after propensity score-matching)				
	All patients (*n* = 1,833)	**Patients without hypertension (*n* = 1,091)**	**Patients with hypertension (*n* = 742)**	***p*-value***	**Standardized**** difference**	**All patients (*n* = 1,252)**	**Patients without hypertension (*n* = 783)**	**Patients with hypertension (*n* = 469)**	***p*-value***	**Standardized**** difference**
Date of onset										
Dec-1 to Dec-31-2019	33 (1.80)	20 (1.80)	13 (1.80)	0.08	0.19	21 (1.70)	15 (1.90)	6 (1.30)	<0.001	0.06
Jan-1 to Jan-15-2020	272 (14.80)	161 (14.80)	111 (150)			184(14.70)	131 (16.70)	53 (11.30)		
Jan-16 to Jan-31-2020	1,159 (63.20)	672 (61.60)	487 (65.60)			787(62.90)	450 (57.50)	337 (71.90)		
Feb-1 to Feb-15-2020	346 (18.90)	219 (20.10)	127 (17.10)			244 (19.50)	173 (22.10)	71 (15.10)		
Feb-16 to Feb-24-2020	23 (1.30)	19 (1.70)	4 (0.50)			16 (1.30)	14 (1.80)	2 (0.40)		
Date of diagnosis										
Dec-1 to Dec-31-2019	3 (0.20)	3 (0.30)	0 (0.00)	0.001	0.08	3 (0.20)	3 (0.40)	0 (0.00)	0.001	0.05
Jan-1 to Jan-15-2020	32 (1.70)	24 (2.20)	8 (1.10)			22 (1.8 0)	18 (2.30)	4 (0.90)		
Jan-16 to Jan-31-2020	509 (27.80)	288 (26.40)	221 (29.80)			254 (28.30)	229 (29.20)	125 (26.70)		
Feb-1 to Feb-15-2020	1157 (63.10)	679 (62.20)	478 (64.40)			788 (62.90)	466 (59.50)	322 (68.70)		
Feb-16 to Feb-24-2020	132 (7.20)	97 (8.90)	35 (4.70)			85 (6.80)	67 (8.60)	18 (3.80)		
Date of death										
Jan-1 to Jan-15-2020	1 (0.10)	1 (0.10)	0 (0.00)	<0.001	0.05	0 (0.00)	0 (0.00)	0 (0.00)	<0.001	0.03
Jan-16 to Jan-31-2020	223 (12.20)	144 (13.20)	79 (10.60)			158 (12.60)	119 (15.2)	39 (8.30)		
Feb-1 to Feb-15-2020	1,229 (67.00)	662 (60.70)	567 (76.40)			844 (67.40)	470 (60.00)	374 (79.70)		
Feb-16 to Feb-24-2020	380 (20.70)	284 (26)	96 (12.90)			250 (20.00)	194 (24.80)	56 (11.90)		
Duration from onset to diagnosis, Median (IQR)	10 (6, 14)	10 (6, 15)	10 (7, 14)	0.47	0.22	10 (6, 14)	10 (6, 14)	10 (7, 14)	0.85	0.10
Duration from onset to endpoint (death), Median (IQR)	17 (12, 22)	17 (12, 23)	15 (10, 21)	0.031	0.02	17 (11, 22)	17 (11, 23)	15 (10, 21)	0.047	0.03

**The Mann Whitney U-test was used to test the difference in the continuous variables and the Chi square or Fisher exact test was used to test the difference in the categorical variables between the two groups (with and without hypertension).*

***Other diseases included: anemia, hypothyroidism, Parkinson's disease, prostatic hyperplasia, fractures, etc.*

****The severity categories were according to the diagnostic criteria of the new coronavirus infection pneumonia diagnosis and treatment plan (trial fifth version).*

*****The standardized mean difference, defined as the mean difference between the groups divided by the standard deviation of the control (without hypertension) group.*

TABLE 2 | The association between hypertension and the COVID-19 severity in the deceased COVID-19 patients using the logistic regression analysis before and after the propensity score-matching[a].

	Unmatched				Matched			
	Cases with hypertension/ all cases	**Univariate OR (95% CI)**	**Multivariate OR (95% CI)***	**Multivariate OR (95% CI)****	**Cases with hypertension/ all cases**	**Univariate OR (95% CI)**	**Multivariate OR (95% CI)***	**Multivariate OR (95% CI)****
Mild COVID-19 illness	179/597	1.00 (reference)	1.00 (reference)	1.00 (reference)	120/387	1.00 (reference)	1.00 (reference)	1.00 (reference)
Moderate COVID-19 illness	135/351	2.62 (1.50–2.77)	3.40 (2.23–5.14)	2.80 (2.10–4.04)	15/247	1.02 (0.51–2.87)	1.14 (1.12–5.14)	2.60 (2.32–3.40)
Severe COVID-19 illness	231/551	4.72 (3.60–10.85)	9.19 (5.29–15.96)	11.66 (6.19–16.31)	179/390	2.72 (2.10–2.85)	9.13 (5.21–13.26)	10.60 (6.10–17.31)
Critical COVID-19 illness	197/334	9.44 (5.16–23.79)	42.76 (20.53–89.03)	36.16 (23.13–67.00)	155/228	2.64 (2.26–3.09)	32.16 (23.43–79.04)	35.02 (20.11–81.00)
P-trend		<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
Non-severe COVID-19 illness***	314/948	1.00 (reference)	1.00 (reference)	1.00 (reference)	135/634	1.00 (reference)	1.00 (reference)	1.00 (reference)
Severe COVID-19 illness***	428/885	2.93 (2.62–3.04)	3.02 (2.56–3.10)	2.90 (2.00–3.10)	334/618	2.51 (2.17–2.91)	2.19 (2.09–2.76)	2.44 (1.77–3.08)

[a]*The adjustment for age and sex was conducted in all the multivariate models for the unmatched data, and the matching for age and sex was conducted in all the models for the matched analyses.*

**The estimated ORs (95% CIs) of hypertension after further adjustment for occupation and location.*

***The estimated ORs (95% CI) of hypertension after further adjustment for occupation, location and the other underlying diseases.*

****The non-severe COVID-19 illness included mild and moderate illnesses, while the severe COVOID-19 illness included severe and critical illnesses.*

TABLE 3 | Cox regression analysis of the association between hypertension and mortality in total COVID-19 patients and in different disease severities before and after the propensity score-matching[a].

	Unmatched					Matched				
	Person-days	**Cases, *n***	**Univariate HR (95% CI)**	**Multivariate HR (95% CI)***	**Multivariate HR (95% CI)****	**Person-days**	**Cases, *n***	**Univariate HR (95% CI)**	**Multivariate HR (95% CI)***	**Multivariate HR (95% CI)****
Mild COVID-19 illness	10,445	597	2.97 (2.12, 3.16)	2.49 (0.82,2.99)	2.77 (0.15, 3.01)	6,776	387	1.24 (1.21, 2.01)	1.49 (0.23, 2.89)	1.31 (0.11, 2.21)
Moderate COVID-19 illness	6,082	351	2.85 (2.68, 3.05)	2.24 (0.61, 2.98)	3.73 (2.55, 4.95)	4,181	247	1.92 (1.51, 2.20)	2.04 (0.71, 2.54)	2.72 (2.34, 3.95)
Severe COVID-19 illness	9,955	551	4.03 (3.87, 5.22)	3.99 (3.43, 5.18)	3.61 (2.99, 4.84)	6,942	390	3.07 (3.02, 4.16)	3.68 (3.44, 4.98)	3.21 (2.89, 4.01)
Critical COVID-19 illness	5,934	334	3.18 (2.95, 4.47)	2.10 (1.87, 3.40)	3.00 (2.76, 4.32)	3,924	228	2.13 (2.10, 3.54)	2.51 (1.87, 2.66)	3.01 (2.86, 3.56)
Total COVID-19 illness of any severity	32,416	1,833	2.73 (1.93, 2.96)	1.99 (1.80, 2.09)	2.01 (1.79, 3.13)	21,823	1,252	2.62 (1.83, 2.76)	1.90 (1.73, 2.47)	2.04 (1.61, 2.72)

[a]*The adjustment for age and sex was conducted in all the multivariate models for the unmatched data, and the matching for age and sex was conducted in all the models for the matched analyses.*

**The estimated mortality HRs (95% CIs) for patients with hypertension in reference to those without hypertension after further adjustment for occupation and location.*

***The estimated mortality HRs (95% CIs) for patients with hypertension in reference to those without hypertension after further adjustment for occupation, location and the other underlying diseases.*

****The non-severe COVID-19 illness included mild and moderate illnesses, while the severe COVOID-19 illness included severe and critical illnesses.*

for age, sex, and other demographic and clinical characteristics including the severity of COVID-19 illness, and similar findings were observed in the propensity score-matched models; HR= 2.04 (1.61–2.72). The higher risk of mortality in patients with hypertension in reference to that in patients without hypertension was statistically significant and was almost of the same magnitude (3.0- to 3.7- fold) in patients with moderate, severe and critical illnesses, but was less and statistically

insignificant, in case of the mild COVID-19 illness; HR (95% CI) was 2.77 (0.15–3.01) in the unmatched and 1.31 (0.11–2.21) in the matched analyses.

Risk Factors for COVID-19 Mortality Stratified by the Hypertension Status

Although the demographic and clinical risk factors of mortality in COVID-19 patients were the same in the hypertension and non-hypertension groups. However, it was obvious that the risk estimates were larger in the hypertension group. The advanced age and male gender were associated with the risk in the unmatched data in both the hypertension and the non-hypertension group (**Table 4**). However, we repeated these stratified analyses after matching for patients' age and sex of in those with and without hypertension, by the propensity score, in **Table 5**, and documented similar augmented associations in the hypertension group more than that in the non-hypertension group for the suburban location in Wuhan, chronic comorbidities and the number of underlying diseases. In this stratified analysis, considering the mild COVID-19 illness as the reference, the moderate illness was associated with the higher risk of mortality only among the patients with hypertension; HR = 1.15 (1.10–1.69) but in patients without hypertension; HR = 1.01 (0.02–1.06). The mortality risk estimates for the severe and critical COVID-19 illnesses in patients with hypertension (p-trend = < 0.001) were approximately double those in patients without hypertension (p-trend = 0.002) (**Table 5**).

DISCUSSION

The previous studies had found 15% (5, 9) to 36.5% (6) of COVID-19 patients had a previously diagnosed hypertension. The prevalence rate varied according to the age of the included patients in each study (7), and for example, common comorbidities were hypertension (40.8%) in elderly patients (29). Based on the recently published clinical and epidemiological characteristics of COVID-19 patients (4–12), several editorials and reviews, published in famous cardiology journals, pointed to the higher risk of COVID-19 infection, the more severe disease, and augmented mortality outcomes among the infected elderly (30–32). Accordingly, it was plausible to think that the higher prevalence of hypertension in COVID-19 patients is expected and doesn't necessarily imply that hypertension is causally related to the infection with, severity of, or mortality from the new coronavirus, since hypertension is highly prevalent in the elderly (31, 33, 34). However, there was no sufficient evidence to show that subjects with hypertension are more likely to be diagnosed with the severe COVID-19 illness or proceed to a poor clinical outcome including the death due to COVID-19 than those without hypertension, independent of the age or other confounding factors.

The analysis of the clinical data of 310 COVID-19 patients (113 with and 197 without hypertension, median (IQR) of age = 62 (49, 70) years, and 56% were males) admitted to two hospitals in Wuhan, suggested a tendency to develop severe inflammation, organ damage and poor prognosis in patients with hypertension than those without hypertension. However, after adjusting for the patients' age and sex, the increased odds of hypertension in those who had the COVID-19 severe illness vs. the non-severe illness, and in those who died due to COVID-19 vs. those who were discharged alive didn't reach the significance level; ORs (95% CIs) were 1.45 (0.93–2.63) and 1.26 (0.68–2.33), respectively (6). On the other hand, a larger study that included 3,340 COVID-19 patients (1,128 with and 2,302 without hypertension, median (IQR) of age = 64 (55, 69) years, and 53% were males) admitted to nine hospitals in Hubei), the HR (95%CI) of mortality due to COVID-19 after the adjustment for age, sex, and comorbid diabetes, cerebrovascular diseases, and chronic renal disease was 1.41 (1.03–1.94) in patients with hypertension in reference to those without hypertension (18).

In our study, we found that hypertension was prevalent in 40.5% of the patients whose age was older than that reported in the previous studies and the male sex represented two-thirds of the cohort sample. Hypertension was significantly associated with both the severity of and mortality from the COVID-19 illness, even after controlling for the patients' age and sex (by adjustment or propensity score-matching); the OR of hypertension in the patients with the severe illness was 2.4- to 2.9-fold higher than that in the patients with the non-severe illness, and the HR for mortality was as twice higher in patients with hypertension as that in patients without hypertension after the adjustment of the patients' occupation, location, and the number of other underlying diseases besides, the adjustment or matching for age and sex.

The exact mechanisms by which hypertension could associate with the risk of the COVID-19 infection, its severity, and the mortality outcomes warrant further biologic and clinical investigations. However, the suggested mechanisms were mainly concentrating on the high affinity of the SARS-COV-2 to the angiotensin-converting enzyme 2 (ACE2) receptors (13, 32, 35, 36). This was shown to facilitate the viral binding to the targeted epithelial cells of the lung, heart and other organs. The debate was about how the use of the angiotensin-converting enzyme inhibitors and the ACE2 blockers drugs, commonly used in patients with hypertension would affect the risks of COVID-19 infection, severity, and mortality. Some investigators suggested beneficial effects of those drugs via not only controlling the blood pressure levels, but also reducing the inflammatory response, and blocking the viral entry to the lung and cardiac cells, while others suggested deteriorating effects through the possible retrograde feedback mechanism, by which ACE2 receptors are upregulated after being blocked by those drugs leading to increased binding sites for SARS-CoV-2 and preferential COVID-19 infection (32). However, the current protocol of COVID-19 management does not recommend patients with hypertension who are taking these drugs to stop them, because there was no significant evidence to support an association between the administration of these drugs and the higher risk for severe COVID-19 infection (4, 17). It has been also hypothesized that the hypertension-related immoderate activation of renin-angiotensin-aldosterone system (37) might motivate the NADH/NADPH oxidase system (38), prompt a massive inflammatory response and cytokine storm (39), and stimulate vascular cell contraction and constriction

TABLE 4 | Multivariable Cox regression analysis for factors associated with the mortality in 1,833 COVID-19 patients stratified by the hypertension status before the propensity score-matching for age and sex.

	Patients without hypertension, *n* = 1,091			Patients with hypertension, *n* = 742		
	Person-days	**Cases, *n***	**Multivariate HR (95% CI)***	**Person-days**	**Cases, *n***	**Multivariate HR (95% CI)***
Age group (Ref: ≤40 years)						
Age (41–60)	4,229	221	1.02 (1.06, 2.37)	1,431	82	1.32 (1.62, 1.99)
Age (61–80)	11,749	661	1.08 (1.04, 1.60)	8,673	476	1.12 (1.04, 1.16)
Age (>80)	2,765	177	1.36 (1.26, 2.05)	2,938	181	1.92 (1.61, 2.05)
P-trend			0.034			0.013
Gender (Ref: female)						
Male	12,551	385	1.99 (1.87, 2.10)	905	505	2.99 (1.87, 3.12)
Occupation (Ref: other)						
Retirees	8,075	462	5.26 (2.14, 11.33)	6,897	396	7.56 (2.14, 9.93)
Housework and unemployment	3,631	191	4.18 (2.02, 12.06)	2,301	133	5.38 (2.32, 11.26)
Public servant	354	21	1.01 (0.01, 5.60)	38	4	1.03 (0.01, 6.80)
Laborers	209	13	2.38 (0.51, 8.13)	174	10	2.18 (0.13, 5.12)
Cadres	450	24	1.48 (1.73, 10.62)	187	11	1.78 (1.63, 9.61)
Farmers and migrant workers	871	52	3.11 (3.02, 12.75)	163	14	4.51 (2.23, 14.75)
Medical workers	242	14	2.07 (0.74, 11.36)	147	7	1.02 (0.34, 9.26)
Location (Ref: Central Wuhan)						
Sub urban area in Wuhan	3,278	186	13.21 (11.32, 21.23)	1,897	102	16.41(13.67, 26.95)
Out of city/other	1,526	87	4.06 (0.36, 4.43)	1,353	73	3.32 (0.37, 5.08)
Chronic diseases (Ref: No)						
Diabetes	2,107	117	3.25 (2.85, 4.60)	4,077	240	4.68 (2.57, 5.80)
Cardiovascular diseases	2,113	114	5.24 (4.01, 7.11)	3,892	216	7.17 (5.14, 8.22)
Cerebrovascular diseases	861	51	2.35 (1.70, 2.99)	2,117	123	2.88 (2.33, 3.89)
Respiratory diseases	2,643	142	5.08 (4.02, 6.37)	3,627	196	4.73 (4.12, 7.52)
Cancer	2,672	150	5.82 (5.62, 7.90)	3,632	193	6.10 (5.99, 6.42)
Disease severity (Ref: Mild)						
Moderate COVID-19 illness	3,648	216	1.01 (0.02, 1.06)	2,434	135	1.15 (1.10, 1.69)
Severe COVID-19 illness	5,836	320	1.28 (1.11, 1.67)	4,119	231	2.32 (1.92, 2.61)
Critical COVID-19 illness	2,557	137	1.10 (1.02, 1.70)	3,377	197	2.62 (2.22, 2.81)
P-trend			<0.001			<0.001
Disease severity (Ref: non- severe)						
Severe COVID-19	8,393	457	1.59 (1.40, 1.78)	7,496	428	2.59 (2.32, 3.02)
Number of underlying diseases (Ref: None)						
Number of under lying diseases ≤2	7,039	403	3.30 (1.06, 3.62)	8,575	486	4.21 (2.06, 5.91)
Number of under lying diseases > 2	1,113	59	2.58 (2.07, 3.91)	4,522	256	3.31 (2.81, 3.76)

**The model included all the variables in the table.*

The p-value for the interaction between the hypertension and various risk factors toward the risk of COVID-19 death were (0.031) for age group, (0.51) for gender, (0.64) for occupation, (0.05) for location, (0.004) for chronic diseases, (0.042) for the disease severity (4 categories), (0.040) for the disease severity (two categories), and (0.021) for the number of underlying diseases.

(40). COVID-19 patients with hypertension showed higher leukocytes count, aggressive radiological pulmonary injuries, and increased plasma levels of cytokines than patients without hypertension (6).

In our study, compared with COVID-19 patients without hypertension, patients with hypertension were more likely to have two or more chronic disease comorbidities and the most common ones were diabetes (32.3 vs. 10.7%), cardiovascular diseases (29.1 vs. 10.4%), respiratory diseases (26.4 vs. 13.0%), and cancer (26.0 vs. 13.7%). These findings were in line with those from the previous studies (4–12, 17, 37, 41). The higher prevalence of these comorbidities could add to the explanation of the higher risks of the more severe disease and mortality in patients with hypertension. A previous study indicated that the median time from COVID-19 illness onset (i.e., before admission) to death was 18.5 days (IQR, 15–22) (4); while in our study, it was shorter 17.0 (IQR, 12–22) days. The median survival time was significantly shorter in patients having those comorbidities besides hypertension than in those having these comorbidities without hypertension. This was more evident for

TABLE 5 | Multivariable Cox regression analysis for factors associated with the mortality in 1,252 COVID-19 patients stratified by the hypertension status after the propensity score-matching for age and sex.

	Patients without hypertension, $n = 783$			Patients with hypertension, $n = 469$		
	Person-days	**Cases, *n***	**Multivariate HR (95% CI)***	**Person-days**	**Cases, *n***	**Multivariate HR (95% CI)***
Occupation (Ref: other)						
Retirees	6,100	347	6.21 (3.22, 10.41)	4,626	269	6.45 (3.34, 10.33)
Housework and unemployment	2,229	124	5.12 (2.00, 11.07)	1,688	96	4.88 (3.32, 14.36)
Public servant	181	10	1.03 (0.01, 4.30)	20	02	2.05 (0.67, 7.30)
Laborers	91	07	2.48 (0.41, 7.00)	113	06	2.38 (0.13, 6.12)
Cadres	241	13	1.28 (1.13, 9.22)	148	09	1.68 (1.53, 9.71)
Farmers and migrant workers	601	34	3.01 (2.94, 11.15)	149	12	4.11 (2.43, 13.55)
Medical workers	109	08	2.36 (0.44, 13.66)	128	06	1.08 (0.64, 10.96)
Location (Ref: Central Wuhan)						
Sub urban area in Wuhan	2,427	137	12.31 (10.22, 20.33)	1,521	83	14.11 (12.17, 22.45)
Out of city/other	1,340	77	6.84 (0.76, 8.44)	589	33	5.62 (0.47, 7.04)
Chronic diseases (Ref: No)						
Diabetes	1,813	107	3.15 (2.65, 3.72)	2,455	147	3.68 (2.45, 4.91)
Cardiovascular diseases	1,892	107	4.94 (3.21, 7.11)	2,521	144	6.10 (4.74, 7.04)
Cerebrovascular diseases	977	57	2.04 (1.37, 2.83)	1,427	83	2.63 (2.17, 3.93)
Respiratory diseases	2,665	148	5.58 (3.13, 7.71)	2,138	120	4.43 (3.35, 6.76)
Cancer	2,661	153	5.11 (4.62, 8.01)	2,069	113	6.33 (4.89, 7.42)
Disease severity (Ref: Mild)						
Moderate COVID-19 illness	3,928	232	1.01 (0.02, 1.06)	253	15	1.15 (1.10, 1.69)
Severe COVID-19 illness	3,764	211	1.44 (1.16, 2.07)	3,178	179	2.41 (2.10, 2.82)
Critical COVID-19 illness	1,335	73	1.23 (1.14, 1.68)	2,589	155	2.52 (2.29, 3.04)
P-trend			0.002			<0.001
Disease severity (Ref: non- severe)						
Severe COVID-19	5,099	284	1.19 (1.10, 1.62)	5,767	334	2.39 (2.12, 2.91)
Number of underlying diseases (Ref: None)						
Number of under lying diseases ≤2	5,758	334	3.21 (1.30, 3.71)	5,225	305	3.52 (2.15, 5.61)
Number of under lying diseases > 2	1,352	76	2.68 (2.16, 3.43)	2,856	164	3.26 (2.69, 3.50)

**The model included all the variables in the table.*

The p-value for the interaction between the hypertension and various risk factors toward the risk of COVID-19 death were (0.74) for occupation, (0.12) for location, (0.013) for chronic diseases, (0.022) for the disease severity (4 categories), (0.030) for the disease severity (two categories), and (0.011) for the number of underlying diseases.

the patients diagnosed with the severe than those diagnosed with the non-severe COVID-19 illness. However, in 113 COVID-19 patients with hypertension, Huang et al. (6) had reported no statistical difference in the laboratory tests and clinical indices between patients with other comorbidities besides the hypertension ($n = 48$) and those without any other comorbidity ($n = 65$), and suggested a limited confounding role of these comorbidities in the association of hypertension with the severity of and mortality from the COVID-19 illness.

A systematic review demonstrated that older age (≥65 years old), male gender, hypertension, cardiovascular diseases, diabetes, chronic obstructive pulmonary disease and malignancies were associated with greater risk of death from COVID-19 infection (42). Another observation emerged in our stratified analyses by the hypertension status, was the augmented risk of mortality in the hypertension group than that in the non-hypertension group for all the associated factors with the COVID-19 mortality. For example, the increased risk with the advanced age and male sex in the unmatched analysis, and the persistently increased risk in the unmatched and matched analyses in different occupations, comorbidities, and the increasing number of underlying diseases was stronger in the hypertension group than the non-hypertension group. The moderate COVID-19 illness, in reference to the mild, was associated with higher mortality in patients with hypertension but not in those without hypertension. These above-mentioned factors were previously shown to be associated with the COVID-19 mortality in general (40); however, our findings of the stratified analyses by the hypertension status weren't verified by any previous studies, although systematic reviews and editorial concluded further that hypertension could significantly increase the risks of severity and fatality of SARS-CoV-2 infection (43–45).

Our study has several limitations. Owing to its retrospective design, the urgency of time, as well as the difficulties in obtaining the data, the current study lacks important dynamic clinical and

laboratory testing data. An example was the type and dosage of the used medication, nevertheless the anti-hypertension drugs. It was indicated that COVID-19 patients with hypertension who were treated with the angiotensin-converting enzyme inhibitors and the ACE2 blockers drugs were at lower risk of mortality when compared with those treated with other antihypertensive medication; the propensity score-matched and multivariable-adjusted HR was 0.30 (0.12–0.70) (18). Regarding the lack of clinical data, we could not adjust or match for the levels of factors such as C-reactive protein, creatinine, or cardiac troponins, these factors were markers for renal and cardiac injuries that were associated with the COVID-19 severity and mortality (46, 47); however, we have adjusted for the major comorbidities including cardiovascular diseases. Also, the diagnosis of hypertension was based on the medical history data, which might have led to some inevitable classification. Last, our analyses were based on a cohort of deceased COVID-19 patients; the patients' characteristics, the prevalence of hypertension, and the associations between the hypertension status and the disease severity could differ in the patients who were discharged alive after COVID-19 infection.

CONCLUSION

Analyzing the data of 1,833 deceased COVID-19 patients during the early epidemic of Wuhan city, China indicated that hypertension was prevalent in over 40% of the cases, and was more prevalent in patients of the severe illness than the non-severe illness. Hypertension was associated with the increased risk of mortality in COVID-19 patients independent of the age, sex, occupation, location, comorbidities, and the number of underlying diseases. The magnitude of the associations of the demographic and clinical characteristics of the patients with the risk of mortality in COVID-19 illness (advanced age, male sex, occupation, location, COVID-19 severity, and underlying comorbidities) was higher and the median survival time was shorter in patients with hypertension than in those without hypertension.

DATA AVAILABILITY STATEMENT

The datasets processed and analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Medical Department of Wuhan University (WHU2020-2020YF0031). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CY and QL collected the data. CY, JC, and SM conceptualized the design. SM did the data analysis. XL wrote the first draft of the paper. EE, KL, FW, FS, HW, and JB reviewed and provided comments on the first draft. SM and XL revised and prepared the final draft. All authors have reviewed and agree to publish the final manuscript.

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SUPPLEMENTARY MATERIAL

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Scalable modEls of Community rehAbilitation for Individuals Recovering From COVID:19 reLated illnEss: A Longitudinal Service Evaluation Protocol—"SeaCole Cohort Evaluation"

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Introduction: High levels of physical, cognitive, and psychosocial impairments are anticipated for those recovering from the COVID-19. In the UK, ∼50% of survivors will require additional rehabilitation. Despite this, there is currently no evidence-based guideline available in England and Wales that addresses the identification, timing and nature of effective interventions to manage the morbidity associated following COVID-19. It is now timely to accelerate the development and evaluation of a rehabilitation service to support patients and healthcare services. Nuffield Health have responded by configuring a scalable rehabilitation pathway addressing the immediate requirements for those recovering from COVID-19 in the community.

Methods and Analysis: This long-term evaluation will examine the effectiveness of a 12-week community rehabilitation programme for COVID-19 patients who have been discharged following in-patient treatment. Consisting of two distinct 6-week phases; Phase 1 is an entirely remote service, delivered via digital applications. Phase 2 sees the same patients transition into a gym-based setting for supervised group-based rehabilitation. Trained rehabilitation specialists will coach patients across areas such as goal setting, exercise prescription, symptom management and emotional well-being. Outcomes will be collected at 0, 6, and 12 weeks and at 6- and 12-months. Primary outcome measures will assess changes in health-related quality of life (HR-QOL) and COVID-19 symptoms using EuroQol Five Dimension Five Level Version (EQ-5D-5L) and Dyspnea-12, respectively. Secondary outcome measures of the Duke Activity Status Questionnaire (DASI), 30 s sit to stand test, General Anxiety Disorder-7 (GAD-7), Patient Health Questionnaire-9 (PHQ-9), Patient Experience Questionnaire (PEQ) and Quality Adjusted Life Years (QALY) will allow for the evaluation of outcomes, mediators and moderators of outcome, and cost-effectiveness of treatment.

Discussion: This evaluation will investigate the immediate and long-term impact, as well as the cost effectiveness of a blended rehabilitation programme for COVID-19 survivors. This evaluation will provide a founding contribution to the literature, evaluating one of the first programmes of this type in the UK. The evaluation has international relevance, with the potential to show how a new model of service provision can support health services in the wake of COVID-19.

Trial Registration: Current Trials ISRCTN ISRCTN14707226
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Keywords: COVID-19, rehabilitation, exercise, emotional well-being, digital health, NHS, independent sector

INTRODUCTION

Clinical Impact of COVID-19

In late 2019 a highly pathogenic novel coronavirus (CoV), severe acute respiratory syndrome (SARS)-CoV-2, emerged, causing a global pandemic with millions of cases worldwide (1). SARS-CoV-2 commonly attacks the respiratory system, leading to hospitalisation with many requiring breathing support advancing in some cases to intensive care support (2). Further complications include those meeting diagnostic criteria for acute respiratory distress syndrome (ARDS), anaemia, cardiac injury and secondary infection (2). COVID-19 is a highly infectious respiratory disease and as a result the COVID-19 pandemic has profoundly impacted the UK population resulting in strict measures to curtail the spread of infection. This disease was unknown in humans and most research has concentrated on the acute phase to reduce mortality. Acute treatment is largely symptomatic and supportive depending on the severity of infection. As of June 2020, there was no specific treatment or vaccination available. Indications show that COVID-19 will have a profound long-term impact on those infected as was previously seen following the MERS and SARS pandemics. MERS survivors showed significantly negatively impacted HR-QoL for up to 14 months post-virus (3) indicating that rehabilitation should be measured in months/years rather than weeks (4).

Data from previous pandemics such as those described, indicates a number of adverse side effects in recovering patients. Long-term ventilatory dysfunction and associated lung damage are common characteristic as are muscle weakness and fatigue (5, 6). Whilst less common, metabolic disorders, including hyperinsulinemia, insulin resistance, hyperglycemia, and type 1 or 2 diabetes were reported in recovering SARS patients (4).

Non-physical morbidity such as psychological morbidity and cognitive dysfunction are also common after a period of critical illness such as COVID-19. It has been reported that 1 in 10 critically ill patients develop severe psychological problems including anxiety, depression and post-traumatic stress disorder (PTSD) (7). Whilst there are a multitude on contributory mechanisms, the potential areas of comorbidity here all represent important target areas within rehabilitation.

It is anticipated that COVID-19 will hospitalise ∼150,000 people by the end of 2020. Of those individuals, it is suggested that 50% will require additional rehabilitation support in the community to support improvement in HR-QoL (8) and to reduce burden on NHS services.

Impact on Rehabilitation Services

The COVID-19 pandemic has acted as a sharp reminder as to the exceptional work of the National Health Service (NHS). As we move further through the pandemic patients are being medically discharged in growing numbers. As patients move out of the acute phase of care it is clear to see the impending burden facing rehabilitation services, described by the Chartered Society of Physiotherapy as an "tsunami of rehabilitation need" (8). Normal health and social care delivery in many countries, including the UK has been deferred in order to support the acute phase of COVID-19. Healthcare interventions aimed at improving or maintaining function such as falls prevention programmes, as well as well-established rehabilitation pathways such as cardiac and pulmonary rehabilitation, are unable to continue, with potential deleterious effects on function. These issues risk worsening health, physical and psychological function for vast numbers of people who may not have suffered from COVID-19 directly (9). As movement restrictions are lifted, the consequences of these indirect effects of the pandemic will become apparent. Prior to the COVID-19 pandemic, to meet the 18-week standard for newly referred patients and clear the backlog of patients who will have already waited longer than 18 weeks, the NHS would have needed to treat an additional 500,000 patients a year for the next 4 years. The pandemic is likely to make waiting lists grow further and the challenge will be even greater (10).

Alarmingly and as has been made clear by National Institute for Health and Care Excellence (NICE) there is currently no evidence-based guideline available in England and Wales that addresses the identification, timing and nature of effective interventions to manage the physical and non-physical

Abbreviations: ARDS, Acute respiratory distress syndrome; AUC, Area under the curve; CIMSPA, The Chartered Institute for the Management of Sport and Physical Activity; CK, Creatine kinase; COVID-19, Corona virus disease; CPD, Continued professional development; EQ-5D-5L, EuroQoL Five Dimension Five Level Version; GAD-7, General anxiety disorder assessment-7; H7N9, Avian influenza; HR-QoL, Health Related Quality of Life; ICU, Intensive care unit; IL-6, Interleukin-6; MERS, Middle East respiratory syndrome; NHS, National Health Service; PEQ, Patient experience questionnaire; PHQ-9, Patient health questionnaire-9; PTSD, Post-traumatic stress syndrome; QALY, Quality adjusted life years; SARS, Severe acute respiratory syndrome.

morbidity associated following COVID-19 (11). Progress is being made, with an initial framework devised for assessing early rehabilitation needs of COVID-19 patients, following intensive care treatment. Much more work is required to address the spectrum of needs, particularly for those that have not spent time in intensive care (12). Pulmonary rehabilitation has been shown to be successful in improving exercise tolerance and HR-QoL, and has been shown to reduce hospital admissions rates in patients with COPD (13, 14), yet despite the associated severe muscle wastage and deconditioning, on-going dyspnoea, sleep disorders and severe fatigue, memory problems, anxiety, depression, and post-traumatic stress disorder (15), rehabilitation is neither defined or guaranteed for those recovering from COVID-19.

New Models of Rehabilitation

A rapid expansion in rehabilitation services is necessary to support an increasing number of patients suffering from long-term complications of COVID-19. Given the level of urgency, a more diverse rehabilitation workforce is required to meet the scale of this challenge, using capacity and skills from sectors outside healthcare organisations. Specifically, improved capacity could be achieved by developing rehabilitation capabilities across the wider non-registered health care staff, including specialist trained exercise professionals, to help meet both demand and effective dose and progression of exercise (16).

The Australian healthcare system may provide a strong basis upon which to base a new model of rehabilitation support, utilising the expertise of exercise professionals. Inclusion of exercise professionals within the Australian healthcare sector has resulted in substantial healthcare cost savings with annual well-being gains of $7,967 and $11,847 per person with diabetes and cardiovascular disease, respectively, with a benefit-cost ratio of 9:1 and 6:1 (17).

Compelling data also exist for the cost effectiveness of exercise in the treatment of mental health, dementia and other common chronic diseases (17) The utilisation of exercise professionals to support clinical rehabilitation is something that has been long employed by Nuffield Health, rendering the Charity well-placed to mobilise and investigate the approach with a cohort of COVID-19 survivors. It should be made clear, that at present there is no formal accreditation pathway for clinical exercise physiologists.

Not only is the organisation of personnel key to new models of rehabilitation, but also the mode of delivery. Delivery modality of rehabilitation will be one of the most significant changes as we progress through the Post-COVID-19 phase. To reduce the number of "face to face" consultations and indeed resource strain, remote consultations including telephone and video platforms have evolved significantly to provide a continuity of care (18). Whilst previous uptake of digital rehabilitation options has been poor (19) this delivery approach has been shown to confer positive health and well-being outcomes in participants that showed strong adherence (19–21). As an example, "Activate Your Heart" is a well-established digital cardiac rehabilitation programme and was evaluated in several different locations. Data demonstrated that users' exercise capacity and HR-QoL improved after completing the programme (22).

The restrictive conditions associated with the COVID-19 lockdown and indeed social anxieties as restrictions are lifted, suggest that there will likely be a greater acceptance of digital healthcare from both a patient and clinician perspective (23, 24). Nuffield health has experience in delivering remote digital interventions for mental health, primary care and physiotherapy. The learnings from these areas will be built into the development of the remote COVID-19 rehabilitation programme. The digital component will be evaluated for both clinical effectiveness as well as acceptability from both the clinician and patient perspective.

Aims

The aim of the present evaluation is to implement and appraise a novel model of community rehabilitation for individuals recovering from COVID-19. The focus will be on the clinical effectiveness of the programme for improvements in HR-QoL and suppression of COVID-19 related symptoms. The specific research questions include the following:

- Is HR-QoL improved and are symptoms related to COVID-19 reduced at 6 and 12 weeks post-intervention and are benefits retained at 6 and 12 months?
- Is a novel blended model (digital and physical) of care cost-effective in the rehabilitation of those recovering from COVID-19?
- Is a novel blended rehabilitation programme acceptable to both patients and rehabilitation specialists?

We hypothesise that: (a) the 12-week rehabilitation programme will be effective in improving HR-QoL and reducing symptoms related to COVID-19 at 6 and 12 weeks and those benefits will be retained at 6 and 12 months; (b) the blended model will be cost effective when compared to previously described rehabilitation methodologies, specifically outpatient multidisciplinary pulmonary rehabilitation; (c) we expect the programme to be acceptable to both patients and specialists.

METHODS AND ANALYSIS

Trial Design

The protocol presented herein reflects Protocol Number 01. Any amends to this protocol will be detailed in full within the ISRCTN registry. This observational cohort study will be conducted following the STROBE statement (25) for observational studies with the protocol reported in line with SPIRIT Statement (26). We will examine the effectiveness of a 12-week blended community rehabilitation programme on improvements in HR-QoL and reductions in symptoms of COVID-19, in individuals recovering from the disease.

This evaluation will be conducted in concert with the NHS. Initially the programme will be deployed across 4 NHS locations, namely; University Hospitals of North Midlands Trust, Newcastle upon Tyne Hospitals NHS Foundation Trust; Birmingham and Solihull Community Care Group and Central Manchester University Hospitals Trust. Whilst these locations are clustered in the North and Midlands they are

TABLE 1 | Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
Previous diagnosis of COVID-19	Active COVID-19 symptoms
Able to walk independently for a minimum of 20 m	Are already receiving community rehabilitation
Must have access to the internet and smartphone/tablet/personal computer (with adequate technological literacy)	Have un-managed medical conditions that contraindicate unsupervised exercise
18 years of age and over	Have a formal diagnosis of post-traumatic stress syndrome, clinically significant anxiety or depression where low intensity mental health intervention will not assist
Access to transport for phase 2 attendance	Have been diagnosed with Chronic Fatigue Syndrome

demographically and economically diverse. The NHS sites will be serviced by 8 surrounding Nuffield Health Fitness and Well-being Centres, all of which are registered with the Care Quality Commission and are located within a 20-mile radius of a participating NHS site. Each trust will be assigned 2 rehabilitation specialists from Nuffield Health to support the programme. As this intervention will be offered as a National service, recruitment is open ended beginning in September 2020. Nuffield Health intend to expand provision by operationalising all 112 of its fitness and well-being centres in 2021. These locations cover all 7 geographical regions as defined by NHS criteria. An initial evaluation cohort will not exceed 160 participants ensuring that a participant practitioner ratio of 1:10 is not exceeded.

Those wishing to access the programme can do so via NHS referral, this can be through doctor, nurse or other allied health professional such as a physiotherapist. Patients will only be referred if they meet the qualifying criteria presented in **Table 1**.

Once referred, the patient will complete an online pre-assessment health questionnaire. Once completed the questionnaire is made available digitally to a specialist trained physiotherapist who will contact the patient to conduct a telephone triage assessment. Following successful triage, the patient is handed on to a rehabilitation specialist who then takes up responsibility of the patients care. All patients will receive the identical 12-week programme structure consisting of two 6-week phases, depicted in **Figure 1** and described in detail below.

Rehabilitation Programme

1. NHS healthcare professionals will utilise inclusion/exclusion criteria at the point of discharge to refer a patient to the programme. Alongside the provision of a patient information document, the patient will be fully informed verbally about the programme, being given the opportunity to join the programme should they so wish. The patient will be made aware that their data will be utilised anonymously for research purposes. The patient may also request that their data is not utilised and will still be able to participate in the programme. Should they choose not to progress they will be sign-posted to alternative community/NHS services where available. If the patient accepts to progress on to the programme the NHS healthcare professional will complete an online referral, sent directly to Nuffield Health using a secure online form. Data sharing agreements have been completed between NHS and Nuffield Health and all processes conform to GDPR and NHS digital requirements.
2. When an online referral is completed, an automated booking process is triggered. Via email or telephone (based on patient preference) the patient will choose an appointment time for an initial triage screening. The patient will also be asked to complete pre-screening questions, designed to support the triage process.
3. The patient next joins a telephone or online video triage consultation utilising this feature. The triage is conducted by specialist physiotherapists trained in remote consultation. The triage is designed to be an additional safety step ensuring that the patient is clinically fit to progress onto the 12-week programme. The 45-min triage will also act to collect additional relevant patient information that may be pertinent when tailoring their exercise programme. Information such as details on additional co-morbidities, emotional well-being and medication will be discussed. Should any contraindications to exercise be identified during the triage the patient will be informed that they are unable to progress on the programme at that time. The patient will be sign-posted back to their General Practitioner, who will also be notified in writing. The original referring clinician will also be notified. At the end of triage, if deemed appropriate, the specialist physiotherapist will refer the patient to the rehabilitation specialist with recommendations for the intensity of entry level exercise and specific needs and goals.
4. Following successful triage, the patient will be automatically sent a welcome pack via post as well as email. This will provide full guidance on how to download, access and register on the digital platforms and will provide links to learning materials. The patients GP will also be made aware that their patient has initiated the programme. The digital application platform utilises the functionality of a platform already used extensively across Nuffield Health (MyTherapy, Nuffield Health, London, UK). All other virtual audio-visual communication will be delivered by a separate digital system (Microsoft Teams, Microsoft, Redmond, USA).
5. Within 72 h of referral, the patient will be contacted by their assigned rehabilitation specialist based at a Nuffield Health site within a 20-mile proximity to the referring hospital. practitioner will provide a welcome to the programme, offer the opportunity to ask any questions and to inform them of the start date of the programme.
6. The patient begins the 12-week programme. This programme phases are as follows:

Phase 1

Weeks 1–6 will consist of 3 exercise sessions per week. Session 1 will be an online live streamed activity conducted by 2 Nuffield Health trained rehabilitation specialists. One practitioner will run the exercise session and the second will answer questions via the

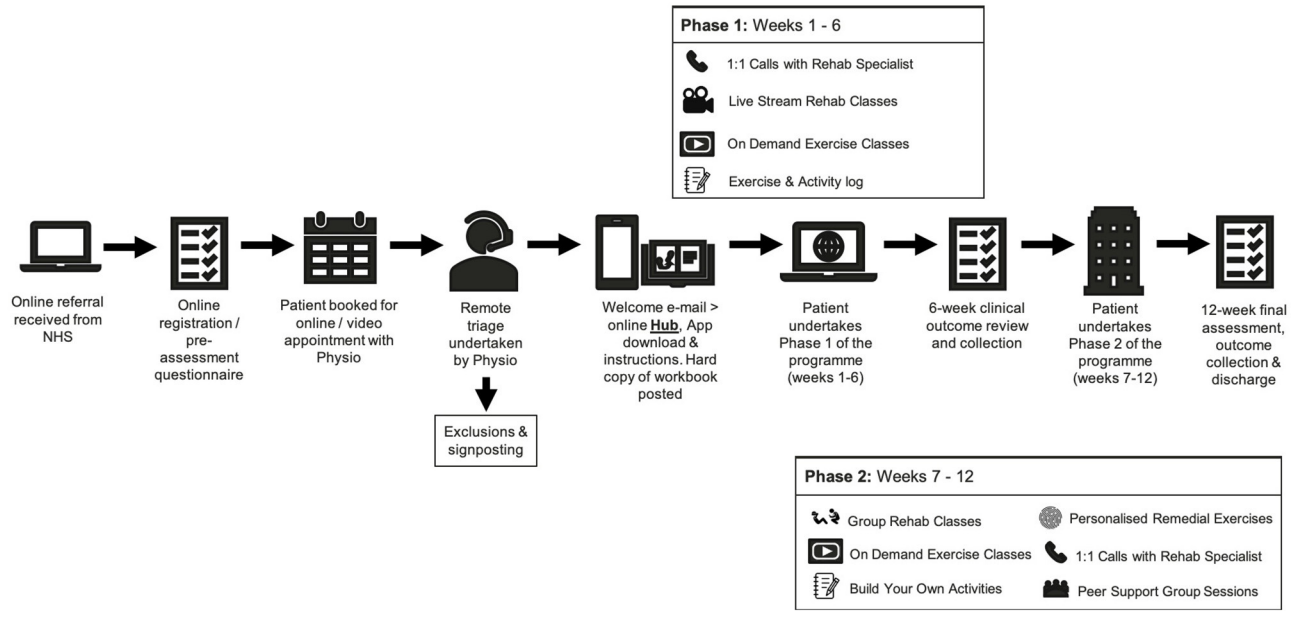

FIGURE 1 | Pathway illustration of the 12-week community rehabilitation programme.

online chat function. The stream will be a 1-way stream, meaning that whilst multiple patients will access the stream at any given time, they will not be recorded/filmed nor will their personal details be visible to the group. A maximum of 10 patients will join a stream at any time. The live stream will last up to 45-min followed by a 15-min period for questions via the secure online chat function or alternatively spoken questions can be provided should the patient have microphone functionality.

The second session of the week during phase 1 will be self-directed. The patient will be directed to a pre-recorded guided exercise session located on a dedicated online platform (Vimeo, New York, USA). This will be a 45-min activity which the patient completes at their leisure. All exercises are designed such that they can be carried out with ease at home.

The third exercise session of the week is described as "build your own." The patient's workbook provides the patient a menu of activities suitable for them, which they may select to populate an exercise session commensurate with their personal threshold.

Each week the patient will be provided a phone call that will last up to 45-min with the rehabilitation specialist. The aim of the phone call is to listen to any patient queries but to also offer support on themes such exercise selection, symptom management and emotional well-being. Nuffield Health specialists have comprehensive training in each of these areas. Prior to progressing to Phase 2 patients will receive a remote mid-point review by the rehabilitation specialist. Progress will be discussed in detail and the patient will be asked if they feel ready and willing to progress to phase 2. If their progress is deemed insufficient, the patient will be recommended to complete another 6-week digital programme in full, before moving into a gym-based setting. Progress will be reviewed weekly and patients will be able to join the face to face component at a later point. All group-based sessions will be offered at two time points across the course of the day, with an AM and PM option. All one to one activity such as the weekly phone calls will be booked according to participant preference on a weekly basis. All above processes intended to maximise participant retention throughout the course of the evaluation.

Phase 2

Following successful completion of phase 1, patients will progress to the phase 2 face to face programme. This phase will be conducted in strictly controlled gym environments conforming to all necessary Government and Public Health England (PHE) guidance. As per phase 1, phase 2 will consist of 3 exercise sessions per week. The first session of the week will be a rehabilitation specialist lead group exercise programme. In appropriately prepared and ventilated spaces, groups of up to 5–10 patients will engage in a 45-min exercise class followed by 15 min for questions and answers. Exercises will consist of aerobic and strength-based exercises as well as stability and mobility. In order to promote continued self-management, the second exercise session of the week will be a remote pre-recorded session that the patient will carry out at home independently, as per phase 1. Similarly, the third session of the week, "build your own" will remain; however, the patient will be encouraged to complete this session within a supervised gym environment. The rehabilitation specialist will be on hand within pre-defined time slots to provide advice and guidance. The patient will again receive a weekly consultation with the rehabilitation specialist following the aforementioned themes. This will culminate between weeks 12 and 13 with a final assessment, summary report and sign-posting to additional services where required.

Practitioner Recruitment and Training

Rehabilitation specialists will be recruited and trained from a pool of exercise professionals working within Nuffield Health. All exercise professionals have a foundation training to a minimum of The Level 3 Personal Training Qualification from an accredited training provider, with preference for professionals trained to level 4. The competencies associated with these qualifications can be found elsewhere. All professionals are registered with The Chartered Institute for the Management of Sport and Physical Activity (CIMSPA). As part of this registration, all professionals are required to engage in continued professional development (CPD) as part of their contract of employment, with a pre-requisite to attain a minimum of 10 CPD points each year. Given the unique structure of Nuffield Health, all exercise professionals have experience working with clinical populations and work closely with clinical professionals on a daily basis within a shared learning environment.

Utilising the Nuffield Health accredited training academy, a multi-disciplinary team of clinical and exercise experts as well as experienced clinical operations specialists will deliver a comprehensive programme of training to up skill exercise professionals to COVID-19 rehabilitation specialists. At present no external standards exist regarding specific competencies in this area. The design of the programme and its content has however been carried out in collaboration with NHS representatives and key authorities from organisations leading rehabilitation nationally.

Training will be delivered via a blended learning approach utilising a mixture of interactive virtual classrooms, online learning, webinars and question and answer sessions. The content to be included covers:

- An overview of the clinical impact of COVID-19, long term effects and the requirement for community rehabilitation and its goals.
- Roles and responsibilities of those involved in delivery of the rehabilitation programme.
- Physiotherapists will be refreshed on initial subjective assessment, screening for inclusion/exclusion and need for onward referral, use of outcome measures and handover process to ensure seamless patient journey.
- Exercise professionals cover week by week roles and responsibilities, systems training, outcome measures, red flags and escalation processes.
- Exercise professionals will further refresh and advance coaching skills, exercise programming, great conversations skills, exercise progression, and regression.
- All will receive mental Health First Aid training—recognising signs and symptoms of emotional distress and understanding how to signpost to appropriate treatment pathway.

All training will be assessed via formal assessment testing theoretical knowledge via online examination and practical skills assessed via role play scenarios and "course-work" tasks.

Patient and Public Involvement

Patients were first involved in this evaluation at their point of clinical referral following a 12-week post discharge follow up. The research questions posed within the protocol paper were constructed with the support of NHS clinicians whom work directly with this clinical population as well-members of NHS Trust management. We believe that the research questions reflect the immediate and on-going needs of the NHS who have a strong insight as to their patient's needs.

Qualitative feedback will be collected from patients throughout their rehabilitation. An initial cohort of 100 patients will be invited to review each milestone of the programme as part of a focus group following their rehabilitation. An evaluation period will then be employed to refine the pathway based on patient feedback, this will include feedback on outcome and recruitment methods.

A patient survey will be provided to all participants that were eligible for the rehabilitation programme gauging views on the dissemination plan and how the intervention may further integrate into community settings.

MEASURES

Outcome data will be collected at 0, 6, and 12 weeks and again at 6- and 12-months post-intervention. Self-report data will be collected via digital application (MyWellbeing, Nuffield Health, London, UK). The patient will be emailed and provided a push-notification prompting them to complete the aforementioned questionnaires. Whilst this evaluation will not utilise any formal comparison group, it is intended that collaboration with trusts will support the analysis of anonymised "usual care" outcome data. This is likely to come from community physiotherapy and/or modified pulmonary rehabilitation.

All data will be securely stored on a dedicated Nuffield Health server and will be retained in line with the organisations publicly available data retention schedule. Modifications to data written to the database will be documented through via an internal inquiry system. Data entered into the database will be retrievable for viewing throughout by those granted secure access privileges associated with an identification code and password. Any data errors will be summarised along with detailed descriptions for each specific problem in a data query reports, which will be sent to the study Outcomes Analyst. The Outcomes Analyst will check any inconsistency, checking other sources to determine required corrections. Any coding changes required within the digital data capture system will then be implemented within 24 h.

Complete back up of the primary database will be performed twice a month. Incremental data back-ups will be performed on a daily basis. In addition to system back-ups, additional measures will be taken to back-up and export the database on a regular basis at site level. The outcomes analyst will send weekly email reports with information on missing data, missing forms, and missing visits to site level co-ordinators who will then rectify immediately as and when required. Data security audits will be completed by the Nuffield Health Information Security group on a quarterly basis. Full details of group membership and details of audit processes can be provided upon request.

Primary Outcome Measures

EuroQoL Five Dimension Five Level Version (EQ-5D-5 L)

This measure is used to assess a person's perception of their general health state and obtain a measure of quality adjusted life years (QALYs). Outcomes can be benchmarked against UK population norms. It covers five dimensions: mobility, self-care, usual activity, pain/discomfort and anxiety/depression, which are rated by the person on five levels of severity: no problems, slight problems, moderate problems, severe problems and extreme problems/unable to function within that domain (27).

Dyspnea-12

Dyspnea-12 consists of 12 descriptor items on a scale of none (0), mild (1), moderate (2), or severe (3). It provides an overall score for breathlessness severity that incorporates seven physical items and five affective items. The time reference period for "these days" captures the current level of breathlessness experienced by patients as opposed to specifically on the day of the test or in response to a specific activity. Total scores range from 0 to 36, with higher scores corresponding to greater severity (28).

Secondary Outcome Measures

Duke Activity Status Index

The Duke Activity Status Index (DASI) is an assessment tool used to evaluate the functional capacity of patients with cardio-pulmonary diseases (29). The activities in the scale are chosen to represent major aspects of physical function, i.e., personal care, ambulation, household tasks, sexual function, and recreational activities. As such, these responses can also be used to assess physical limitations relevant to the patient's HR-QoL. Responses from 12 items are summed up to get a total score, which ranges from 0 to 58.2. Higher scores indicate a higher functional capacity.

30 s Sit to Stand Test

The 30 s Sit to Stand Test is utilised for testing strength and endurance in a variety of cohorts. It is part of the Fullerton Functional Fitness Test Battery (30). This test was developed to overcome the floor effect of the 5 or 10 repetition sit to stand test in older adults. The 30-s chair stand involves recording the number of stands a person can complete in 30 s rather than the amount of time it takes to complete a pre-determined number of repetitions. That way, it is possible to assess a wide variety of ability levels with scores ranging from 0 for those who cannot complete 1 stand to > 20 for more fit individuals.

Generalised Anxiety Disorder-7

GAD-7 comprises 7 items measuring symptoms and severity of anxiety based on the DSM-IV diagnostic criteria for GAD. The GAD-7 has good internal consistency ($\alpha = 0.92$) and good convergent validity with other anxiety scales. Higher scores indicate greater severity of symptoms. The GAD-7 has increasingly been used in large-scale studies as a generic measure of change in anxiety symptomatology, using a cut-off score of 8 (31).

Patient Health Questionnaire-9

The PHQ-9 is a self-report measure of depression that has been widely used in research and is a regular screening measure utilised in primary care and hospital settings. The PHQ-9 items reflect the diagnostic criteria for depression outlined by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition—Text Revision (DSM–IV–TR) (32). Summary scores range from 0 to 27, where larger scores reflect a greater severity of depressive symptoms. The PHQ-9 has been found to discriminate well between depressed and non-depressed individuals using the cut-off total score $\geq$10, with good sensitivity (88.0%), specificity (88.0%) and reliability (33).

Patient Experience and Programme Acceptability

The Patient Experience Questionnaire (PEQ) instrument will be used to assess patient experience and satisfaction. The PEQ contains several quantitative questions and open-ended questions that are used to assess participant's views and satisfaction with service provision (34).

Costs

EQ-5D-5 L utilities will be reported alongside the full evaluation costs of the intervention so as to elicit a "per head" economic evaluation of all participants recruited (35).

Engagement and Usage Measures

The digital systems will collect anonymized descriptive data relating to engagement and usage of the service users with the platforms. Data collected will include the number of sessions attended, time spent in the platform, number of activities completed, number of minutes per log-in, number of resources accessed. A session is defined as an instance where a user logs on to the system. Session time will be always an imperfect calculation, as users may take breaks within a session, without formally log out of the system. To prevent this overestimation, periods of more than 30 min without interaction will be taken as 1 min and periods of inactivity longer than 3 h will start the count on a new session. Use of different program components will be measured.

Statistical Analysis

Participation levels will be monitored throughout the programme and reasons for withdrawal or non-compliance will be recorded. All participants are selected according to clinical criteria alone with no other factors influencing participation so as to limit selection bias. Information biases are limited via the prospective nature of this evaluation and the methodology employed to collect mandatory data at each designated time point. As well as the primary and secondary outcome measures being collected, a detailed clinical history and additional triage will be undertaken for each participant. This will limit confusion bias through the identification of relevant confounding clinical variables. Efficacy of treatments over time will be measured using mixed

effects models. To complement the *post-hoc* comparisons, the magnitude of change on the primary and secondary outcomes measures will be established using Cohen's *d* statistic. Bonferroni corrected *p*-values will be reported for multiple comparisons. Participants with missing data will be removed for analysis with complete-case analysis being utilised.

An interim-analysis will be performed on the primary endpoint when 50% of participants have completed up to the 6-month follow up point. The interim analysis will be performed by an independent statistician. The statistician will report to the principal investigator (BMK) only. The principal investigator will have unblinded access to all data and will discuss the results of the interim analysis with the project team.

Ethics and Dissemination

Manchester Metropolitan University Ethics Committee approved this study on 29/09/2020 (Ref: 25307). Informed consent will be gained from all participants prior to referral onto the rehabilitation programme. Consent will be captured digitally as part of the online referral programme.

Participants will be contacted weekly to ensure that any clinical concerns are addressed and escalated where relevant. Processes are in place to inform the referring clinician and the participants general practitioner should medical intervention be required.

The principal investigators will have access to the cleaned data sets. Project data sets will be housed securely within the project database hosted on a secure Nuffield Health server. Should data sharing be required under reasonable request (e.g., with the NHS) a secure file transfer protocol will be created for the study, and all data sets will be password protected. To ensure confidentiality, data dispersed to project team members will be blinded of any identifying participant information.

Pilot data is expected by December 2021 and will be published in an open access journal. Any intellectual property pertaining to successful delivery of the service will be shared directly with NHS partners.

DISCUSSION

New models of rehabilitation are urgently required to address the immediate gap in provision for those recovering from COVID-19 as well as the escalating back log of rehabilitation cases nationally. Nuffield Health, the UK's largest not-for-profit healthcare charity, have long prioritised exercise as a first line intervention for the treatment and prevention of long-term conditions. This has been successfully achieved via a uniquely structured estate linking hospitals and health and well-being centres as well as the up skilling of exercise professionals to work with clinical populations. Now, by working closely with the NHS, a unique learning partnership will assist in the development of a new rehabilitation pathway, that may later evolve to utilise the expertise of the fitness sector in supporting the NHS and its rehabilitation needs. We must also later review in detail how we create a model that has utility beyond the healthcare system within the United Kingdom, such that patients are able to benefit from this level of support internationally, within varying healthcare structures.

Undertaking the principal aim of this trial will allow for a robust test of the effectiveness of a new 12-week blended rehabilitation programme within a population that has not previously been investigated within a community rehabilitation context. Specifically, this work will provide new insight into changes in HR-QoL and disease specific symptoms related to COVID-19 following 12-weeks of exercise rehabilitation. Positive results of these main outcome measures will allow the programme to consolidate itself as not only a valid treatment option, but as an essential component to the care management pathway of COVID-19 survivors, and indeed those recovering from other serious conditions.

The novel structure of the programme will support further expansion of digital components within the rehabilitation of those recovering from serious illness. This is relevant not just for improving access to information and efficiency of data collection but critically, the remote delivery of care and the ability to individualise programmes of rehabilitation. The relevance of the results will likely have implications for the implementation and success of blended rehabilitation models i.e., digital and physical combined, across health care systems worldwide.

Examining potential mediators and moderators of change will contribute to our understanding of key processes in achieving improvement in services using blended models of rehabilitation. Whilst mediators and moderators of rehabilitation outcomes have been explored, this will be the first exploration of a combined digital/physical intervention. This will inform the tailoring of interventions to best address the needs of the targeted population, ultimately leading to the development of more effective interventions.

An area requiring on-going review and indeed development relates to the provision and impact of rehabilitation across sociodemographic groups. Black, low-income, and immigrant communities are particularly vulnerable and disproportionately impacted by COVID-19 (36, 37). Furthermore, data exists indicating that secondary care-based clinics may be underused by older populations and those in poorer socioeconomic circumstances (37). The proposed evaluation will ensure that sociodemographic variation is considered within analysis and that a representative sample from those that are disproportionately affected are consulted post-programme to understand barriers and facilitators. It is critical that learnings are continuous and that they are built into future re-iterations of the rehabilitation programme.

The proposed economic analysis will add to the current literature in regard to evidence of the cost-effectiveness of home or web-based rehabilitation programmes and will be an innovative analysis of a clinical rehabilitation programme ran independently of allied health professionals within a non-NHS community environment. In a context of health care provision where resources are now especially stretched, cost-effective interventions can only support the delivery of effective health services.

In summary, COVID-19 has proven devastating in its cost of life and long-term impact on survivors. Scalable interventions

must be developed to address what will be a long-term requirement in the rehabilitation of patients having suffered critical illness. To achieve rapid scalability, blended interventions will soon become a recognised viable option across health care. They can be beneficial both in costs and resource management within the NHS and will bring disparate sectors closer together in a combined mission of improving the health of the nation. It is critical that as technology rapidly develops, supporting innovative models of care so too must research in order to rapidly continually and rapidly update on the benefits of providing blended community rehabilitation. This long-term evaluation aspires to drive research and innovation forward, and in doing so support the NHS in its aim of controlling the impacts of COVID-19 and delivering on its long-term plan.

SUMMARY

Strengths and Limitations of This Study

- Evaluates a critical and novel patient cohort.
- This evaluation will review the impact of digital and physical approaches to rehabilitation.
- Evaluates a new model of care delivery and the training of non-clinical staff.
- Demonstrates strong example of NHS/independent sector collaboration.
- A significant proportion of data will be self-reported due to COVID-19 restrictions.

ETHICS STATEMENT

Manchester Metropolitan University Ethics Committee approved this study on 29/09/2020 (Ref: 25307). Informed consent will be gained from all participants prior to referral onto the rehabilitation programme. Consent will be captured digitally as part of the online referral programme. In review Participants will be contacted weekly to ensure that any clinical concerns are addressed and escalated where relevant. Processes are in place to inform the referring clinician and the participants general practitioner should medical intervention be required.

AUTHOR CONTRIBUTIONS

BK, AI, and PD conceived and designed the original protocol. BK, AI, PD, MH, LM, DD, DB, SP, and GW were involved in amending the protocol. BK coordinated the programme throughout and wrote the initial draft of the manuscript with contributions from all authors. All authors further contributed to subsequent drafts and have read and approved the manuscript.

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BRIEF RESEARCH REPORT
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Applying Genomic Epidemiology to Characterize a COVID-19 Outbreak in a Developmentally Disabled Adult Group Home Setting, Arizona

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Individuals living in congregate settings, including those in group homes, have been disproportionately impacted by COVID-19 and may be at increased risk of exposure or infection due to underlying illness. In mid-May 2020, local public health officials responded to an outbreak of COVID-19 among staff and residents associated with a multi-residential group home that provides care for adults with intellectual and developmental disabilities. Samples were collected at 16 of the homes. In four of the homes all the residents tested positive, and in the remaining 12 houses where samples were collected, all residents tested negative. Of the 152 individuals tested, 15/58 (25.9%) residents and 27/94 (28.7%) staff were positive for SARS-CoV-2, including eight hospitalizations and four deaths. Phylogenetic analysis of genomes from this outbreak in the context of genomes from Northern Arizona shows that very few mutations separate the samples from this outbreak. A potential transmission network was developed to illustrate person-place epidemiologic linkages and further demonstrates the dynamic connections between staff and residents with respect to each group home location. Epidemiologic and genomic evidence correlate, and suggest that asymptomatic infected staff likely introduced and spread COVID-19 in this setting. Implementation of public health prevention measures alongside rapid genomic analysis can help guide policy development and guide management efforts to prevent and mitigate future outbreaks.

Keywords: genomic epidemiology, developmental disabilities, public health, COVID-19, outbreak

INTRODUCTION

The COVID-19 pandemic has dramatically impacted individuals in many different congregate settings, including long-term care facilities, homeless shelters, and group homes. Adults with intellectual or developmental disabilities (IDD) are three times more likely to suffer from underlying medical conditions, such as heart disease, diabetes, and respiratory illnesses, that are known COVID-19 risk factors, than those without IDD (1–4). It is also typical for people with IDD to have multiple chronic health conditions, which paired with metabolic and nutritional disorders, elevate the risk of experiencing more severe outcomes of COVID-19. Another analysis showed that COVID-19 patients with IDD, regardless of age, had the highest likelihood of dying from the virus (5). As of June 2020, it is estimated that more than 7,000 IDD congregate-

setting residents have been diagnosed with COVID-19 nationwide, with at least 700 deaths (6). Furthermore, New York health officials have reported infection rates in group homes to be five times higher than the general population (7). Despite these numbers, which are likely an underestimation of the true burden on this population, limited scientific reports have highlighted outbreaks in group homes throughout the United States that care for individuals with IDD.

Arizona has reported COVID-19 cases associated with over 2,000 congregate settings. These cases represent a disproportion of the more than 550,000 cases statewide documented between January and December 2020 (8). While it is recognized that these populations have also experienced disproportionate morbidity and mortality rates, limited reports specifically describe outcomes experienced by individuals with IDD (4, 9, 10). Here, we describe an epidemiologic investigation paired with genomic analysis of a COVID-19 outbreak associated with multiple group home residences in Arizona.

METHODS

Public Health Investigation

On May 15th 2020, public health officials were notified of positive COVID-19 cases associated with a multi-residence group home that provides services for people with IDD. In response, enhanced testing was conducted on May 26th and 27th in resident homes and at an on-site event. This organization has 21 locations throughout Northern Arizona. Each unit houses 2–6 residents that have their own bedrooms, and spend varying amounts of time in shared common areas. Each home is supported by 2–6 medical assistants and caregivers, some of whom work at multiple homes.

Sample collections and testing of residents and staff in the early weeks of the outbreak were performed at healthcare facilities and *via* a community collection site. Extensive contact tracing and collaboration with other public health agencies allowed for identification of all individuals linked with this outbreak. In total, 152 nasopharyngeal swabs collected from 58 residents living in 16 homes and 94 staff were submitted for SARS-CoV-2 PCR testing. Collection dates ranged from April 24 to June 11. Sampling did not occur at five additional locations managed by this organization, as they are used for recreational activities only or are located in another region of Northern Arizona and were not a part of this outbreak.

Public health, working closely with the facility management, collected information on clinical signs, timeline of the outbreak, and exposures of residents and staff working in the homes. The index resident cases in Houses A through D were identified on May 14, 15, 21, and 22, respectively. These houses are located within four to seven miles of each other, experienced a 100% residential infection rate (e.g., all residents in these houses tested positive), and were deemed "positive" houses. Positive resident and staff case samples were identified first in Houses A and B (5/7-5/27), followed by Houses C and D (5/15-6/2). The remaining 12 houses at which samples were collected were classified as "negative" houses (all residents tested negative, although some staff working in these homes (E–G) were positive with collection dates ranging the span of the outbreak, 4/24-6/1). House B initially had two residents; both tested positive and one suffered a severe clinical outcome resulting in death. The surviving resident was transferred to a different home that already had positive residents. None of the other residents were moved between homes throughout this outbreak.

A majority of the staff initially interacted with residents from multiple homes; however, upon identification of additional cases in Houses A and B, staff were assigned to work exclusively at one home. Seven staff that provided care for COVID-19 positive residents were provided alternative housing to avoid exposing their families and close contacts. Strategies to manage COVID-19 in group homes, as well as guidance on isolation, mask efficacy, quarantine, and enhanced personal protective equipment use were provided to the facility on May 22. Daily temperature checks, self-screening for staff, and comprehensive infection prevention procedures were employed to contain the spread once identified in these homes.

Genomic Sequencing and Analysis

RNA was extracted using previously described methods (11, 12) and prepared for whole genome sequencing. SARS-CoV2 cDNA was amplified following the nCoV-2019 sequencing protocol V.124 and using the ARTIC v3 primer set, prepared for sequencing with plexWell384 (SeqWell), and sequenced on a NextSeq 550 with v2 chemistry and 150 X 150 base-pair reads (Illumina). Data were processed and virus genome consensus sequences were built using the Amplicon Sequencing Analysis Pipeline (ASAP) (12). Maximum likelihood phylogenetic trees containing the outbreak genomes and a subset of other Arizona genomes for context were generated using the Wuhan-1 genome as a reference using NextStrain (13, 14). The subset of genomes used was chosen using genome-sampler (15), which selects the most closely related samples from an available dataset collected within the same geographic region and time period.

Epidemiologic Network

Staff and resident cases were loaded into MicrobeTrace (16) as a "Node List" and connections to their respective facilities were loaded as a "Link List" in comma-separated formats. Once loaded in MicrobeTrace: (1) node shapes were mapped to a column distinguishing between persons and places, (2) node labels were mapped to a column populated with a deidentified location ID for all locations, while this column remains empty for all nodes representing persons, (3) node colors were mapped to a column describing the patient outcome, (4) the timeline feature was controlled using the sample collection date as input from the "Node List" file, and finally (5) the graphic was exported as SVG objects at each time interval of interest. The SVG objects exported from MicrobeTrace were further augmented in Inkscape with an additional visualization layer to flag the most interconnected asymptomatic individuals in the network and to customize the figure's legend.

TABLE 1 | Demographic characteristics of COVID-19 positive staff and residents linked to a developmentally disabled adult group home setting.

	Resident ($n = 15$) No. Positive (%)	Staff ($n = 27$) No. Positive^{+} (%)
Age (years)		
<25	0 (0)	4 (14.8)
25–34	0 (0)	13 (48.2)
35–44	2 (13.3)	4 (14.8)
45–54	4 (26.7	3 (11.1)
55–64	6 (40.0)	2 (7.4)
65+	3 (20.0)	1 (3.7)
Sex		
Female	7 (46.7)	22 (81.5)
Male	8 (53.3)	5 (18.5)
Associated Home		
House A	4 (26.7	11 (40.7)
House B	2 (13.3)	6 (22.2)
House C	6 (40.0)	9 (33.3)
House D	3 (20.0)	4 (14.8)
House E	0 (0)	6 (22.2)
House F	0 (0)	5 (18.5)
House G	0 (0)	6 (22.2)
Outcome		
Asymptomatic	8 (53.3)	12 (44.4)
Hospitalized	6 (40.0)	2 (7.4)
Death	4 (26.7)	0 (0)

$^{+}$*Number of positive staff associated with individual houses were not mutually exclusive. Staff were often assigned to work in more than one "positive" home.*

RESULTS

Of the 58 residents sampled, 15 (25.9%) tested positive. Residents ranged in age from 35 to 71 years (mean age = 56 years). None of the residents experienced the hallmark signs of COVID-19 (e.g., fever, cough, shortness of breath); however, staff reported that several infected residents were hypoxic and lethargic. Nine infected residents were confirmed to be asymptomatic at the time of sample collection. Among those who tested positive, 6/15 (40%) were hospitalized, and 4/15 (26.7%) died. The four residents that died ranged in age from 57 to 71 years old and all were reported to be immunocompromised and had extensive co-morbidities prior to becoming infected with COVID-19. These co-morbidities included, but were not limited to, hypothyroidism, seizure disorders, asthma, and previous diagnosis of cancer and tuberculosis in two of the four residents. Twenty-seven of ninety-four staff tested positive (28.7%); two were hospitalized and the remaining were either asymptomatic or developed only mild symptoms (**Table 1**).

Twenty-five of the forty-two positive samples (59.5%) were available for viral genome sequencing with Ct values ranging from 18.0 to 37.5. There was no observable difference in viral load between symptomatic and asymptomatic individuals. 20/25 samples had 90% or greater breadth of coverage across the SARS-CoV-2 genome at ≥10X depth of coverage. Phylogenetic analysis based on single nucleotide polymorphisms (SNPs) comprised a subset of Arizona SARS-CoV-2 genomes, and included five that were previously shown to cluster with the outbreak group. Results show the majority of genomes associated with this outbreak fall into a monophyletic clade defined by 2 distinct SNPs, C13860T and C21575T, the latter of which confers an L5F amino acid substitution in the spike protein gene (**Figure 1**).

Virus genomes from two staff exposed outside the workplace are not closely related to the others, indicating they are not part of the transmission network of this outbreak and did not seed the outbreak, while five community samples are clonal to this group, showing that this outbreak was not confined solely to the group home. Two of the five samples are from healthcare workers, one is a confirmed household contact of a staff member, and two additional samples have no known epidemiological connections. Residents did not have outside interactions other than receiving necessary medical care, including at the same healthcare facility where the above two healthcare workers were employed. Collection dates of the earliest staff cases precede both the resident and community cases; therefore, this transmission network was likely fueled by staff encounters in the group homes and community. Ongoing viral sequencing efforts of positive samples in subsequent months in the region revealed no additional cases associated with this outbreak.

To further characterize and understand the dynamics of this outbreak, a potential transmission network was developed using MicrobeTrace (16), which incorporates person-place linkages of all 42 positive cases ascertained through public health investigations and contact tracing (**Figure 2**). The timeline of the network relies on the earliest collection dates for the positive case samples. Each node is sized according to the number of person-place connections (e.g., more cases are associated with House A than House B). The letters reference the residential homes (A–G) and the hospital (H). Panels A–C illustrate the positive individuals and their associated locations by week of the outbreak, with panel D demonstrating the complete network highlighting the interactions and connections between staff with respect to each group home location and its residents. The network also displays the hypothesized movement of the virus throughout the homes, and indicates that asymptomatic staff connected to multiple homes likely played a significant role in sourcing this outbreak.

DISCUSSION

People with IDD often require a high level of direct care, may be unable to communicate symptoms of illness, and are dependent on close physical contact with support staff; thus, coping with the COVID-19 pandemic has been especially challenging for this demographic. Social distancing in this setting is not always feasible; therefore, despite measures taken to protect their patients and limit potential spread, caregivers can pose risk to residents. In this case study, we highlight an outbreak involving 42 individuals linked by a multi-residential group home environment that cares for adults with IDD.

While many staff were not in frequent close contact with one another in the work setting, several were housemates. Additionally, at least six were exposed through other means (e.g., family gatherings). Several staff also had close connections

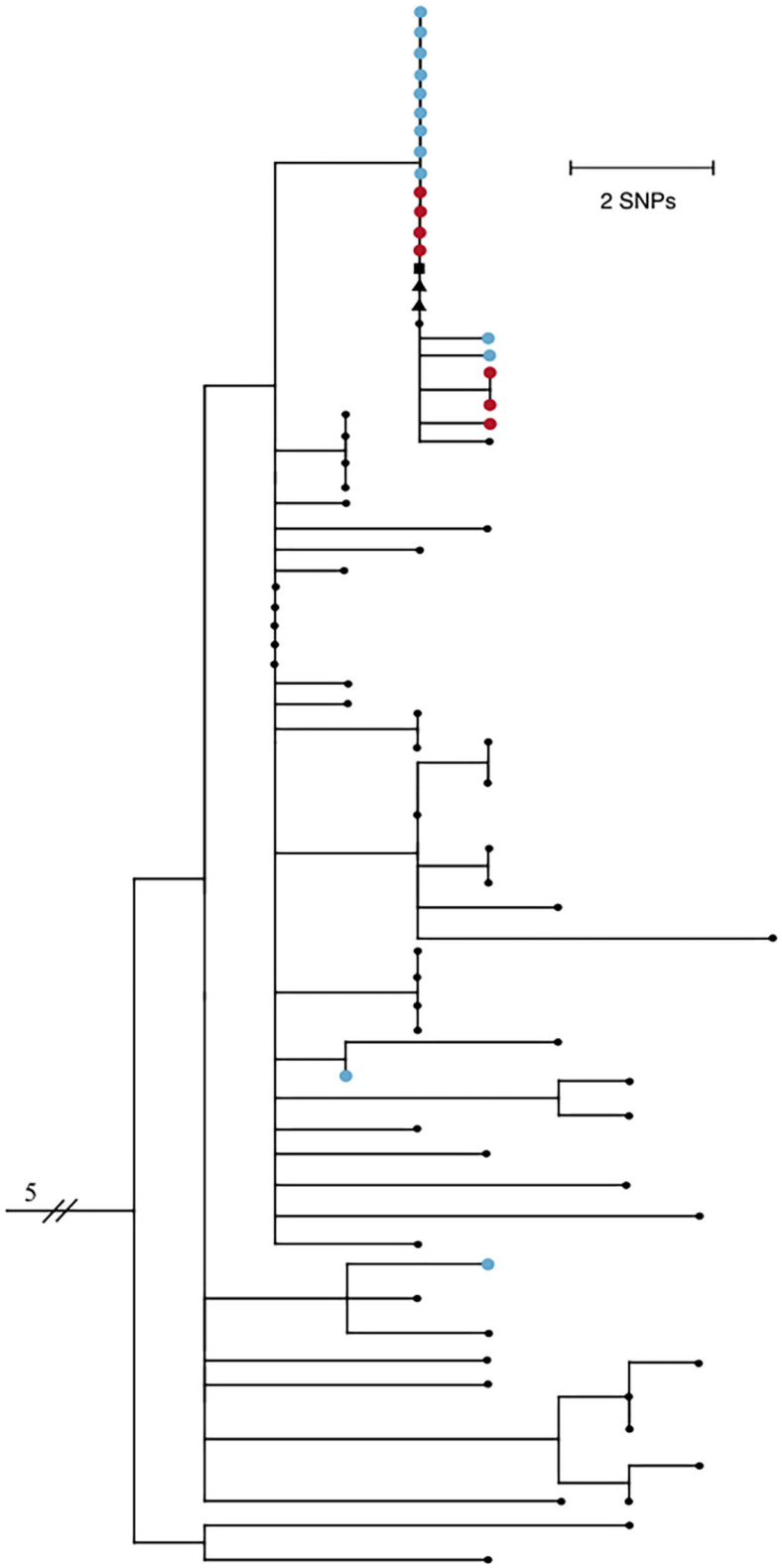

FIGURE 1 | Maximum likelihood phylogenetic tree of 74 SARS-CoV-2 genomes from Northern Arizona, May-June 2020 generated by Nextstrain (13, 14) using the Wuhan1 genome as a reference (EPI_ISL_402125), showing 18/20 samples sequenced from this outbreak form one tight clade. Blue nodes represent sequences

(Continued)

FIGURE 1 | from staff and red nodes represent sequences from residents. Square shaped nodes represent the household contact of an infected staff and triangular shaped nodes represent healthcare workers. Genomes have been published to GISAID. EPI_ISL_694009-023, 025–040, 228, 231–235, 237–239, 241–242, 244, 318, 320, 322, 324, 328, 330, 335.341, 342, 345, 350, 351, 355, 378, 380, 381, 387, 389, 391, 398, 399, 434, 437, 442, 451, 455, 601, 607, 914212, 299. All Arizona samples in the tree have the D614G mutation.

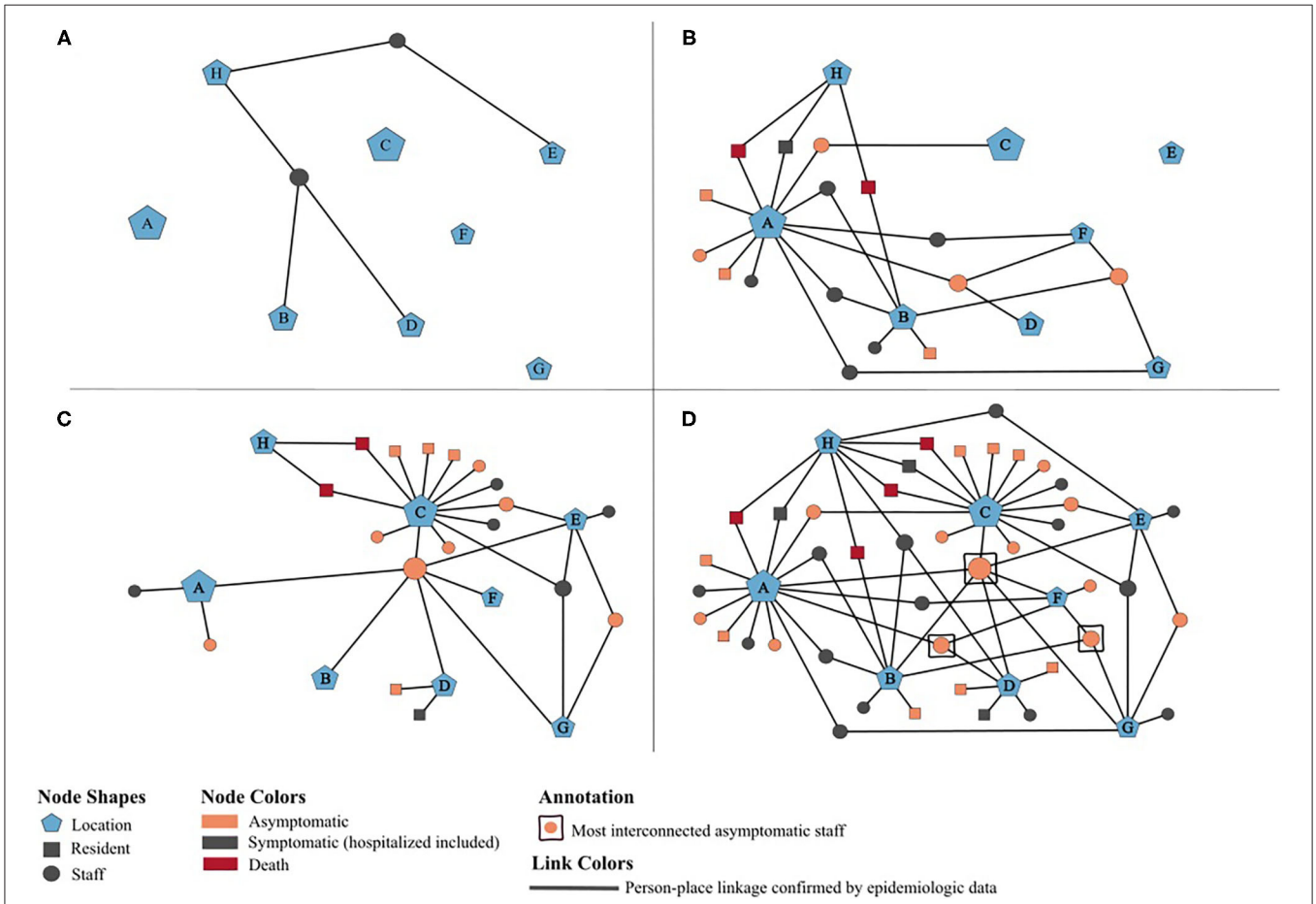

FIGURE 2 | Potential transmission network of 42 SARS-CoV-2 positives cases associated with an outbreak at an adult group home setting, May-June 2020. **(A–D)** illustrate person-place linkages of staff and residents throughout the weeks of the outbreak. **(A)** Two initial positive staff pre-outbreak, May 1–10. **(B)** Staff associated with additional homes and residents in houses A and B test positive, May 11–20. Public health notified on May 15. **(C)** Residents in houses C and D test positive, May 21–30. Enhanced testing by public health on May 26–27. **(D)** Complete network of all positive cases at the end of the outbreak and associated houses, May 31–June 30.

with Native American communities experiencing high COVID-19 attack rates during this timeframe. Given the continual risk of exposure both in and outside of the workplace and common practice for staff to work in multiple houses, it was difficult for public health officials to determine the most appropriate timeframe for quarantining and testing of staff. Furthermore, since many of the early cases were asymptomatic, our understanding of the variation of viral spread before and after implementation of distancing, isolation, and prevention measures relies on dates of collection (as mentioned above for the network) versus dates of symptom onset for positive case samples.

Sequencing data were not available for every positive case, a well-understood limitation when conducting genomic epidemiologic analyses, making it difficult to infer informative transmission maps. However, while a clear transmission pattern could not necessarily be ascertained through the genomics alone, the phylogeny of the outbreak shows a highly connected genomic network, and heightens the importance of using epidemiologic information in the context of the sequencing data when interpreting findings. Furthermore, public health was able to gather evidence. Overall, genomic and epidemiologic evidence supports our hypothesis and suggests that infected staff introduced COVID-19 into this setting, played a role in spreading the virus among the multiple homes, and contributed to limited community transmission.

Despite these challenges, enhanced precautions required of staff and timely interventions by the facility and public health

curbed this outbreak. After the implementation of these measures on May 22 and widespread testing on May 26–27, only a small number of individuals tested positive (6/42; 14%). Given the vulnerable nature of people living in congregate settings, it is critical to have policies and procedures in place to manage disease outbreaks. Since the May outbreak, a number of prevention measures continue to be implemented by the organization, and are proving to be successful at mitigating the spark of new clusters or outbreaks, as there has only been a few sporadic cases in staff members. These measures specifically include oxygen and temperature checks on every resident multiple times throughout the day, daily temperature checks on staff, enhanced monitoring of staff exposures outside of work followed by at home isolation, limitation of visitors, and thorough cleaning of homes. Staff are now assigned to working at no more than two houses, and any staff that work at a higher risk home only provide care for residents in that home. Ongoing widespread screening of staff and residents is also occurring in partnership with public health to ensure early identification of potential asymptomatic infected individuals. Early interventions, paired with rapid genomic epidemiologic analyses, can provide a better understanding of transmission patterns and further guide public health efforts.

DATA AVAILABILITY STATEMENT

The genomic data supporting the conclusions of this article are publicly available from GISAID. Additional data will be made available by the authors without undue reservation, upon request.

ETHICS STATEMENT

Ethical approval for this study and written informed consent from the participants of the study were not required in accordance with local legislation and national guidelines. This work was conducted in collaboration with a local public health agency as a public health surveillance activity, involving genomic sequencing and analysis of de-identified remnant biospecimens, and therefore is exempt from needing human subjects research board approval.

AUTHOR CONTRIBUTIONS

HY coordinated the work between the two agencies and prepared the manuscript alongside JB. AP and DJ-S assisted with development of the figures. MG, MO, and MM conducted the investigations and provided epidemiologic data. AP, DJ-S, MF, and DL performed genomic sequencing and data analysis. JB and DE oversaw the genomic response efforts and provided critical revisions of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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REVIEW
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Challenges and Issues of Anti-SARS-CoV-2 Vaccines

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At the beginning of 2021, anti-SARS-CoV-2 vaccination campaigns had been launched in almost 60 countries with more than 500 million doses having been distributed. In addition to the few vaccines already in use, many other candidates are in preclinical phases or experimental stages in humans. Despite the fact that the availability of anti-SARS-CoV-2 vaccine constitutes a major advance and appear to be the only way to control the pandemic, some investigation remains to be carried out, and this is notably concerning the impact on transmissibility, the duration of the conferred protection in the mid- and long term, the effectiveness against present and future viral mutants, or the ideal schedule that should be applied. In this paper, we review the circumstances that facilitated such a rapid development of anti-SARS-CoV-2 vaccines and summarize the different vaccine platforms under investigation as well as their present results and perspectives in different settings. We also discuss the indications of vaccination under special conditions, such as a history of previous COVID-19 infection or belonging to extreme age categories like children and elderly. Overall, this review highlights the multiple challenges to face if aiming to find a global solution to the pandemic through high vaccination coverage all over the world.

Keywords: vaccination, COVID-19, SARS-CoV-2, prevention strategy, vaccine formulation

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INTRODUCTION

For more than 1 year, SARS-CoV-2 has beenspreading all over the world creating a huge burden of disease with millions of cases of infection and thousands of deaths recorded every day (1).

Even though significant advances have been made in patient management, notably thanks to better understanding and treatment of pulmonary and thrombo-embolic lesions, there is currently no universally approved viral treatment, making until recently from physical distancing and hygiene measures the only means of slowing down the pandemic but at heavy psychosocial, educational, medical and economical costs. While the third wave is ongoing in Europe and an upsurge of cases is observed due to new variants issued from neighboring countries, there is rising hope to control the pandemic thanks to the arrival of the awaited vaccines (2, 3). As of early April 2021, SARS-CoV-2 vaccination campaigns have been launched in roughly 60 countries with more than 500 million doses having been administered globally.

Worldwide, outstanding resources have been deployed to support vaccine development by recruiting thousands of researchers, using high technology, and calling for important financial subsides. Though the availability of vaccines is unanimously considered to be a dramatic progress among scientists, much uncertainty and questions remain inside the general population; these are easily understandable regarding the innovative techniques applied, the uncommon rapidity of commercialization, and the daily flow of conflicting information delivered by the media.

In this setting, we aim to clarify the scientific background that allowed for such a rapid development of a vaccine, to provide a summary of the different formulations available, to discuss the perspectives of vaccination campaigns, and to highlight how challenging such a vaccination program could be in the setting of a pandemic due to a new pathogen.

Our literature review was mainly based on peer-reviewed articles listed on a platform developed by the French Agency for Research on AIDS, Hepatitis, and Emerging Infectious Diseases that selects on a weekly basis the most relevant papers published on COVID-19 vaccines and therapeutics in high-ranked journals of choice. Moreover, we gave much consideration to all scientific information provided by the European Centre of Disease Control and Prevention (ECDC) as well as the World Health Organization (WHO) from which we consulted the website sections dedicated to professionals on weekly basis. Based on these two major sources, preprints papers that were judged to be reliable and highly relevant in context were also included.

BACKGROUND AND OPPORTUNITIES

According to the WHO (2), as of April 1, 2021, there were no <84 vaccine candidates in clinical evaluation, 184 candidates in preclinical evaluation, and more than 100 vaccine studies. If so many vaccine candidates are close to the marketing stage only 15 months after the first manifestations of COVID-19, this high-speed development has been facilitated by numerous circumstances and opportunities that are detailed below.

Background From Previous Studies on Other Coronaviridae

Until recent work against SARS-CoV-2, there was no vaccine approved for human use against coronaviruses. The low pathogenicity of alpha and beta coronaviridae (mainly responsible for common colds) did not make them a priority for vaccine research. When SARS-CoV-1 emerged in 2003, vaccines against this virus were tested in the preclinical phase and phase I in humans, but their industrial development was stopped with the spontaneous resolution of the epidemic (4). Vaccines against MERS-COV were tested for several years, but none reached the marketing stage (4, 5). While all this work did not result in vaccines used in humans, it allowed for the identification of the antigens of the coronaviruses targeted by our immune responses. Neutralizing human antibodies are directed against the Spike (S) protein (responsible for the particular crown aspect observed in structural studies of coronaviruses), and especially against one of its sequences called Receptor Binding Domain (RBD) (6). The S-protein is responsible for the invasion of human cells through interaction between its RBD region and, in the case of SARS-CoV-2, a specific receptor for the angiotensin 2 converting enzyme (ACE2) expressed by many human cell types, in particular in the pulmonary and vascular tissues. The S-protein was therefore selected as the main target against which an immunization by vaccine should be generated in order to obtain a protective immune response capable to hamper attachment and invasion by the virus the way natural antibodies do. Prior knowledge of these elements from related-coronaviruses studies largely contributed to accelerating the identification of SARS-CoV-2 vaccine targets and the determination of their corresponding genomic sequences (4, 7).

Research on Immunological Responses Elicited by SARS-CoV-2 Infection in Humans and Other Primates

Although vaccine does not have to exactly reproduce the natural immunity, immunological studies conducted *in vivo* during infection by SARS-CoV-2 were also of great help to presume what should be ideally induced by vaccination.

Irrespective of the presence of symptoms, the virus induces production of specific antibodies, following a pattern similar to that observed in most viral infections: rapid production of IgM-antibodies (peak at 10 days) then rising of IgG with a peak around 20 days to decline onwards (8, 9). It is estimated that within 1 month of infection, over 90% of patients will have produced specific IgG (10). In asymptomatic patients—who initially produce fewer antibodies—specific IgG may be no longer detectable as early as 2 months after the infective contact (8), whereas, in some other people who generated a stronger immune response (often but not always associated with disease severity), the IgG could still be detectable up to 8 months later (11). How long would last the protection remains nevertheless unpredictable yet given the slight decline over time. Of note in the case of SARS-CoV-1, IgG antibodies were measured even more than 2 years after infection (12). The neutralizing antibodies are very specific and do not cross-protect against other coronaviruses. Besides the production of IgG antibodies, there is a production (then a decay) of IgA antibodies in the respiratory mucous membranes. These have been shown of major importance to prevent asymptomatic carriage and transmission of infection (13). Moreover, the Spike-protein stimulates the genesis of CD4 + lymphocytes, with a weaker effect on CD8+ lymphocytes. In addition to the Spike-protein, structural and non-structural regions of the nucleocapsid contribute to the stimulation of T cell responses and might be considered as additional targets for future vaccines especially to prevent escaping mutants (14). Unlike antibodies, there may be cross-reactivity on CD8+ lymphocytes between other epitopes from SARS-CoV-2 and from previously met coronaviruses, suggesting why some individuals could benefit from prior protective immunization (14). The development of a coordinated, specific adaptive immune response involving genesis of CD4+, CD8+, and neutralizing antibodies has been statistically associated with a milder pattern of infection while a suboptimal cellular immune response has been correlated with advanced age and worse outcome (15). In some individuals, however, the host immune responses can be amplified in such an uncontrolled manner that an inappropriate secretion of inflammatory cytokines will be triggered, which is responsible for major tissue damages (16).

In addition to human studies, experiments in other primates were of great use, especially at the beginning of the pandemic when the production of neutralizing antibodies against SARS-CoV-2 was demonstrated as well as their contribution to the

resolution of infection in a macaque model (17). The observation that in primates a primary infection protects against reinfection (18) gave additional arguments to assume the efficacy of a vaccine, as did the evidence from laboratory assays of a human functional immune memory persisting months after infection (11, 19). However, cases of re-infections (symptomatic and asymptomatic) have been reported for SARS-CoV-2 in humans (19) and also for MERS-CoV in animals (20), irrespective of the circulation of mutant strains. Only a few reinfection cases were well-documented on the immunological side by investigating the type and function of immune memory responses. In addition to the issue of escaping variants (21), the existence of reinfection questions the possibility of waning immunity as well as the role of memory cells and the way to efficiently induce them by vaccines. So far, there is no surrogate of protection allowing for identification of previously-exposed individuals at risk for re-infection, nor to quantify the duration of protection provided by the various vaccines. Comprehensive immunological studies allowing for the definition of standardized correlates of protection are importantly needed. Such studies will also be helpful to clarify concerns about the hypothesis of Antibody-dependent Enhancement of Disease (ADE) during which an aggravation of the disease linked to the production of facilitating antibodies induced after infection or by vaccination is observed (22). The ADE phenomenon has been well-documented for Flaviviridae like Dengue fever and mainly occurs when low antibody titers or low-affinity antibodies are produced. The reports of ADE in some animal models during trials of SARS-CoV-1 and MERS-CoV vaccines (23), as well as the observation that high antibodies rates correlated with the severity of outcome in COVD-19 patients (24) have raised concerns on safety and efficacy on futures anti-SARS-CoV-2 vaccines at the early stage of their development. Fortunately, this hypothesis is rendered unlikely for the moment considering the results of most clinical trials that did not demonstrate any case of ADE, neither after natural infection nor after vaccination of previously infected people. Nevertheless, until now, we do not benefit from any immunological assay or biomarker that is able to distinguish between a severe viral infection from an immune-enhanced disease (whatever this would be enhanced by antibodies, T cells, or innate-immunity pathways). Further in-depth investigation assessing the host immune responses and evaluating the risk of immunopathology after natural infection or vaccines will be of utmost importance to improve future prevention strategies, even now that vaccination campaigns have been globally rolled out.

Prior Existing Vaccine Platforms and Regulatory Facilities Adapted to Emerging Virus

All the above information could not have been exploited in such an efficient way without the experience drawn from previous epidemics, which had already led to the creation of vaccine platforms, international collaborations and regulatory facilities (like emergency use authorization procedure) adapted to emerging viruses[1]. In common circumstances, the production and marketing of a new vaccine take more than 10 years. However, an epidemic setting requires shortening the duration of vaccine development stages by overlapping the phases by starting from the outset with a phase "1/2" followed by the launch of phase 3 if intermediate results appear favorable. Such a fastened procedure was implemented to develop the pioneering vaccine against the Ebola virus (25), for which a vaccination campaign could be started after 5 years only. Given the state of emergency triggered by the COVID-19 pandemic, the American [the Food and Drug Administration-(FDA)] and European [the European Medicines Agency-(EMA)] regulatory agencies and the WHO were immediately solicited to define the level of performance required to allow marketing of a SARS-CoV-2 vaccine: clinical efficacy of 50% (with a lower limit of confidence interval $\geq$ 30%) was set as a sine-qua-non condition for a vaccine to be considered beneficial to public health (26). For the most promising vaccine candidates, commercial production started well before the results of phase 3 were obtained. To support research, extraordinary funding has been granted by various governments and international associations allowing for the precious gain of time. The accelerator COVAX platform was built by the Global Vaccine Alliance (GAVI), the Coalition for Epidemic Preparedness Innovations (CEPI), and the WHO to promote research, development, and manufacture of many SARS-CoV-2 vaccine candidates at an affordable price; the aim is to offer equitable access to vaccination all over the world and thereby to provide a global solution to the pandemic (Fair Allocation Framework)[2].

TYPES OF VACCINE AND CURRENT RESULTS

It is common wisdom that having a safe and efficient vaccine remains the best way to control the COVID-19 pandemic. Among all the candidates in development (2, 3), some of them use traditional approaches like virus-inactivated or virus-live attenuated vaccines while others are based on more recent technologies like vectored-vaccines or mRNA vaccines, two innovations developed throughout this last decade. **Table 1** displays the main platforms used for COVID-19 vaccine development with their respective specificities and inconveniences.

In total, 15 vaccines are now evaluated in phase 3, whereas five have already achieved phase 4 (2). As of early December 2020, two vector vaccines and four inactivated vaccines were already approved by Chinese and Russian authorities and are now being distributed in these countries and partner ones. Out of these six candidates, only the Gamaleya National Research Centre published until now interim data of phase 3 clinical trial for its AdV5/AdV26 not-replicating-vectored-vaccine (Gam-COVID-Vac) (27). With the United Kingdom starting first, mass vaccination campaigns have been launched in many European

[1]https://www.fda.gov/vaccines-blood-biologics/vaccines/emergency-use-authorization-vaccines-explained

[2]https://www.who.int/initiatives/act-accelerator/covax

TABLE 1 | Vaccine platforms used for COVID-19 vaccine.

Vaccine platform	Subtype of candidate	Principles	Advantages	Inconveniences
1) Modified virus-containing vaccines		• Well-known technology, used in many other vaccines • Injection of the virus itself after it has been rendered unharmful by various processes.		
	1.1 Weakened	• Attenuation of the replicative capacities of the virus by culture methods or genes deletion	1) Induction of a robust immune response against various viral antigens (not only the S protein) 2) Generate humoral and cellular specific immunity. 3) Intranasal formulations possible allowing for IgA formation and prevention of asymptomatic carriage	1) Containing weakened but live virus, posing risks of disease in immunocompromised individuals 2) Heavy manufacturing conditions due to use of live virus
	1.2 Inactivated	• Killing of the virus by heat.	1) No live virus avoiding the risk of disease 2) Induction of immune response against various viral antigens (not only the S protein)	1) Need of an adjuvant to generate sufficient immune stimulation 2) Need for highly secured manufacture conditions due to manipulations on the virus 3) Generate only humoral specific immunity.
2) Protein subunits vaccines		• Well-known technology, used for many other vaccines • Injection of viral surface proteins that have been prior recognized as immunogenic. Formulations differ by the parts of proteins used (i.e., the entire protein S or only its receptor-binding domain)	1) Very safe. No pathogen agent used so no risk of disease and a well-known procedure 2) Easier manufactures (recombinant proteins produced by bacteria, yeasts or cell culture	1) Need of an adjuvant to generate sufficient immune stimulation 2) Generate mostly humoral specific immunity.
3) Vectored vaccines		• Innovative technology applied for a decade to fight against other epidemic viruses (like Ebola) (19). • Sars-Cov2 gene(s) introduced in a different unharmful virus used as a vector to infect humans' cells. Host cells will produce the Sars-Cov2 antigens selected for immunization +/- new vector viruses.		
	3.1 Replicating vector	• The vector virus has been attenuated to lose its pathogenic capacity and modified to carry Sars-COV2 genes, but it remains able to replicate in infected cells. • Example of viruses used are Measles, VSV, New Castle virus…	1) Highly immunogenic 2) Generate humoral and cellular-specific immunity. 3) Intranasal formulations possible allowing for IgA formation and prevention of asymptomatic carriage	1) Containing weakened but live virus, so there is a risk of disease in immunocompromised individuals
	3.2 Non-replicating vector	• Deletion of some genes of the vector renders it unable to replicate in host cells. Most commonly used viruses are modified adenovirus (AdV5/AdV26, AAV) or animals' viruses (ChAdOx1…). Vectors are selected to minimize previous natural immunity. Some formulations contain also antigen-presenting cells.	1) Generate humoral and cellular-specific immunity. 2) Some schedule involving one single dose	1) Possible immunization against the vector virus leading to loss of efficacy (because of previous contact with related viruses or immunization between both doses). 2) No intranasal administration
4) Nucleic acid-based vaccines		• Innovative technology based on the delivery to human cells of the genetic information necessary to produce SARS-COV2 proteins selected as a target for immunization.	1) Generate humoral and cellular-specific immunity. 2) Easy manufacture (*in vitro*, without live viruses)	1) No intranasal administration

(Continued)

TABLE 1 | Continued

Vaccine platform	Subtype of candidate	Principles	Advantages	Inconveniences
	4.1 DNA vaccine	• Selected viral genes are introduced into bacterial plasmids easy to reproduce in a sufficient amount. The vaccine contains plasmids that will enter thanks to a small electric shock (transfection) inside the human cell nucleus where they will be translate and lead to viral protein synthesis.	1) Very stable and easy to store	1) Necessity of material for electroporation 2) Less immunogenic than RNA vaccine
	4.2 mRNA vaccine	The genetic sequence corresponding to the viral protein is already translated into mRNA, which is immediately readable by the human ribosomes bypassing the nucleus steps. The mRNA is delivered inside human cells through lipid shells. This pioneer technology has been already studied for other viral vaccines (against ZIKA virus, HIV-1) in animal and human phase 1/2 trials and appears promising for therapy against metastatic cancers (27)	1) Highly immunogenic 2) No live virus, so no risk of disease even in immunocompromised people 3) No modification of the human genetic pool (no entry in the nucleus)	1) Very unstable product (storage at ≤20–70°C for a maximum of 5 days) 2) Limited data in humans (pioneer technology used for only a decade)[1]

countries, starting at the end of December 2020, using first mRNA vaccines (the Pfizer/BioNTech BNT162b2 mRNA vaccine and the Moderna mRNA-1273 vaccine) then also the Astra Zeneca/Oxford AZD1222 vectored vaccine—all three approved for use by the EMA [3]. The candidate from Johnson and Johnson, which is part of the COVAX program, has now also been authorized for use in Europe, while the Gam-COVID-Vac, the Novavax, and the Curevac candidates are under EMA review. As detailed in **Table 1**, compared to the mRNA formulations, the vectored vaccines or protein recombinant vaccines require less stringent storage conditions (and a single dose schedule for the candidate of Johnson and Johnson), whereas the Gam-COVID-Vac applies a heterologous prime-boost strategy (see below).

Many publications assessing candidates at various stages are available but a comparison between performances of each vaccine is rendered complicated by the variability of design and methodologies applied. For example, in immunogenicity studies, the minimal inhibitory concentration used to estimate the capacity of antibody neutralization ranges from 50 to 100%. Since COVID-19 is a new disease, we do not yet benefit from validated immunological surrogates of protection (i.e., a threshold level of antibodies or neutralization functional testing) that will allow for standardized evaluation of vaccine effectiveness. The same problem arises when willing to compare clinical efficacy since most phase 3 studies only recorded symptomatic cases whose definitions are eminently variable.

At the time of writing this review, four clinical phase 3 trials have been published, enrolling each 20–40,000 healthy adult volunteers (plus 100 12–16-year-old adolescents in the Pfizer study). Pfizer/BioNTech study showed 95% efficacy (95% CI, 90.3–97.6), as assessed 7 days after the second dose (28) and recent data under review are reassuring about the protection conferred against two new variants (29). Along the same line, the trial from Moderna reported 94.1% efficacy (95% CI, 89.3–96.8%;) after two doses (30). The publication from the Astra Zeneca/Oxford team demonstrated a mean efficacy of 70% for its ChAdOx1-S not-replicating vectored-vaccine (efficacy of 90% for patients having been given half dose first then a full second after 1 month; the efficacy was 62% for those having received two full doses 1 month apart) (31). However, these results were obtained in people 18–55 years old so that restricted use was firstly recommended by National Immunization Technical Advisory Groups (NITAGs) of some countries. More data are thus warranted to evaluate efficacy in older individuals though this is expected by observation from the prior immunological study (32). Last published was the interim analysis of the phase 3 trial of the Gam-COVID-Vac that showed 91.1% efficacy (95% CI 83.8–95.1) against documented COVID-19 after two doses (27). The firm Johnson and Johnson has already announced its candidate provided 66% efficacy (72% in the US cohort) in preventing symptomatic, laboratory-confirmed COVID-19 from 28 days after injection with even higher efficacy against severe forms of infection and including against the south African variant from the B.1.351 Lineage (33).

Concerning safety data, all phase 3 trials enrolled thousands of participants, allowing for a good assessment of short-term adverse reactions, which are known to occur within 6 weeks after injection (34). No trial reported major adverse events. As for minor to moderate reactions, they appear more frequent in young people and after either the second dose for the mRNA vaccine or the first one for vectored vaccines. Rapidly, some

[3]https://www.ema.europa.eu/en/human-regulatory/overview/public-health-threats/coronavirus-disease-covid-19/treatments-vaccines/covid-19-vaccines

concerns arose about allergic reactions following administration of the Pfizer vaccine, mainly due to the lipid envelop necessary to transport the nucleic acid. Despite the media impact, the rate of anaphylaxis observed so far was not estimated to be a major issue or cause of contraindication by the competent safety authorities and WHO, but caution is still advised (medical monitoring 15–30 min after injection) especially when administrating this vaccine to individuals with a history of a previous severe allergic reaction[4] (35). Some warnings were also published about facial palsy after the Pfizer vaccine but a causal relationship could not be retained so far. As for the candidate from Astra Zeneca, concerns were raised after three cases of transverse myelitis occurred in the post-vaccine period, but any relationship with the vaccine administration was discarded for two of the three cases (31). However, for all candidates, the period of follow-up before approval was a fortiori very short (3 months maximum after the second dose and 6 months in total) due to the emergency state. If safety concerns seem low for the moment and far away from outweighing the benefits, awareness will be of major importance during the universal mass vaccination campaign. As for all previously licensed vaccines, enlarging the vaccinated population and the follow-up period will likely unmask the occurrence of very rare events ($<1/10^5$-10^6), as serious anaphylaxis reactions or neurological/auto-immune disorders. A much longer time is therefore needed to identify a true causal relationship in vaccine recipients. Implementation of an international surveillance system recording all secondary reactions is now of utmost importance to guide vaccination policies and has been launched by the WHO. The fundamental role of pharmacovigilance reporting systems has been recently emphasized by the warning raised by some European countries about serious blood clots events occurring in individuals shortly after reception of the AstraZeneca vaccine. Although rare, the incidence of this disorder has been found higher than expected in unvaccinated populations, in particular among young vaccinated women. At the time of writing this paper, the causality could not be formally established, but the problem is under thorough investigation by EMA experts and international surveillance is ongoing[5]. This concern should be all the more seriously considered that COVID-19 is associated with a high prevalence of thromboembolic complications for which an immunological origin through the formation of anti-platelets antibodies has already been hypothesized (36).

DISCUSSION AND KEY QUESTIONS

Vaccination has started in many countries, using various types of vaccines and schedules. However, important questions remain, and these should be addressed in the near future to ensure the success of the vaccination campaigns.

What Could We Presently Expect in Terms of Effectiveness?

Until now, whatever was the studied candidate, the rate of efficacy published only reflected the individual rate of protection against disease (decrease in the number of patients getting symptomatic infections with a variable degree of severity, as compared to the placebo group). No data currently allow us to assess the impact on viral transmission, although expected according to mathematical modeling (37). Animal studies showed that neutralizing IgG reduces viral shedding in upper airways without however abrogating it (38). All phase 3 candidates induce circulating neutralizing IgG antibodies, but none of them have been proven to generate IgA antibodies that favor sterilization of the upper respiratory tract and therefore hinder the asymptomatic carriage of the virus (13). Such antibodies are preferentially generated when antigens are delivered intranasally, but only a few vaccines that are suited for intranasal administration have been developed, and even fewer have already entered in clinical trials.

Another key point is the duration of the induced protection, especially considering the lack of knowledge about anti-SARS-Cov-2 natural immune memory responses and the existence of reinfection with the same strain. The period of follow-up in the first published vaccine studies did not exceed 3 months after the second dose; hence we can wonder about the persistence of the induced immune responses (both cellular and humoral) in the mid- and long term and the need for additional booster doses. Whether the number of doses administered during the primary vaccination series could influence the robustness and duration of protection, remains another poorly documented issue.

On the same line, a discussion ensued about the maximum time interval between the two requested injections, originally designed to be 21 days for the Pfizer/BioNtech mRNA vaccine. This was based on the observation that specific immunity starts to be detectable 12 days after the first dose. Since numerous countries are facing a resurgence in the epidemic, notably due to the raising of more transmissible variants, the WHO and EMA have authorized to extend the interval between the two doses up to 42 days[6]. While delaying the second injection would not reduce overall efficacy after complete vaccination, the extended window period between the two doses could prolongate a suboptimal immunization status, insufficient to fully protect the recipients and perhaps favorable to the selection of escape mutants. It thus seems important to follow at best the originally recommended vaccination schedule and to postpone the second injection only if the circumstances absolutely require it. Individuals should be aware that they are not fully protected after a single dose and that control measures should absolutely not be relaxed. Creating an extended window period during which the immune response is suboptimal could furthermore constitute a theoretical risk factor for the development of ADE, which could mainly occur when low antibody titers or low-affinity antibodies are produced.

[4]https://www.gov.uk/government/publications/regulatory-approval-of-pfizer-biontech-vaccine-for-covid-19/information-for-healthcare-professionals-on-pfizerbiontech-covid-19-vaccine

[5]https://www.ema.europa.eu/en/news/covid-19-vaccine-astrazeneca-benefits-still-outweigh-risks-despite-possible-link-rare-blood-clots

[6]https://www.who.int/news-room/events/detail/2021/01/05/default-calendar/extraordinary-meeting-of-the-strategic-advisory-group-of-experts-on-immunization-(sage)-$-$5-january-2021

Who Should Be Vaccinated?

Another major issue is to define the population to vaccinate. It is commonly admitted that vaccination should not be kept for all risk groups, this includes low, moderate and high risk groups [who obviously should be given priority (39)] but should be distributed to the highest number of people in order to slow down or even eradicate the circulation of the virus. Human history overflows with examples showing that controlling viral pandemic through vaccination is achievable, like for smallpox, poliomyelitis, and measles. However, it demonstrates also that as soon as the vaccine coverage becomes insufficient, outbreaks are observed (40, 41). The minimum rate of vaccine coverage requested to achieve suppression of community transmission and herd immunity is the function of each pathogen characteristic (way of transmission, incubation period, and fatality rate, which are all involved in the calculation of the basic and effective reproductive numbers) (42). This vaccination coverage rate was estimated around 60–70% for SARS-CoV-2, far from the >95% required to control measles. However, this estimation is susceptible to change over time since more transmissible variants are unfortunately emerging, the calculation also depends on social behavior and population heterogeneity, and as the first estimation implies that all infected (or vaccinated) individuals remain fully immunized for several months, which is uncertain especially regarding the possibility of asymptomatic carriage. Moreover, although host risk factors for severe COVID-19 are progressively identified (43, 44), it is basically impossible to predict who will develop serious forms of COVID-19 or its post-infective complications. Neither age nor the absence of comorbidity can guarantee a benign evolution of disease. The rise in incidence among children and young adults of a post-infectious multiple inflammatory syndrome (MIS-C) well illustrates this concern and sustains the universal mass vaccination policy (45). This way, fragile people who could either not quickly access the vaccine or who might not be eligible for vaccination because of their medical status will also benefit from protection thanks to the indirect effect and as will those ones who will only develop a suboptimal immune response.

At first glance, the solution seems straightforward: everyone without formal contraindication should be vaccinated to eradicate at most the human reservoir and hamper the circulation of the virus.

However, other questions arise. Firstly, should we somewhat adapt the schedule to subjects who have had a documented resolved infection or had been identified as a carrier? To date, no one can guarantee the duration and intensity of the protection conferred by the natural infection, although *in-vitro* indicators of immune memory have been found 6–8 months after infection (11, 46). Cases of re-infections (symptomatic and asymptomatic) have been clearly reported (19, 20), and the reinfection rate (defined as 2 positive PCR > 90 days apart with 7 days minimum without symptoms before the second sample) is currently estimated around 0.7–3.9% (47, 48). At the individual level, the decision to vaccinate could partly be guided by the serological status, pending more thorough testing assessing also cellular immunity will become available. If a large amount of antibody persists, the vacciney appears useless but follow-up testing could be advised. Vaccination could be indicated when the level of suspected neutralizing antibodies declines significantly. However, no cut-off has been validated, and assessment of the immune status of all vaccine recipients constitutes an unrealistic scenario implying carrying out serological testing on a large scale and spending considerable logistical and financial resources. Since the ADE hypothesis is not supported to date by clinical trials results (including previously infected people), and since series of data seem to indicate that most individuals are protected at least until 3–6 months after a documented infection (49), providing the vaccine after this delay appears a wise option. The vaccine is then expected to act as a booster, helping to mount a faster immune response in case of further contact and reinforcing immune memory. As supported by some recent immunological studies (50, 51), a single dose schedule might be sufficient in previously infected people and is now proposed by some regulatory agencies[7]. Of note, according to our opinion, the benefits from vaccination remain a matter of debate in subjects who presented with a severe form of COVID-19 with cytokines storm, for which the greatest precautions should probably be taken before reintroducing any SARS-CoV-2 antigen. Individuals with a history of severe COVID-19 were actually excluded from phase 3 trials, and much more data are needed to guide this decision. As well, knowledge of the serological status could be helpful in particular subgroups of more fragile individuals such as the elderly, more prone to develop ADE, to tailor the number of doses in case of prior infection. Again, data from phase 3 trials concerning extreme age groups are still awaited. These groups obviously deserve specific attention considering particular features of their immune systems, like the well-documented immunosenescence phenomenon characterized by lower immune responses to several vaccines in the elderly. Moreover, it has been found that anti-SARS-CoV-2 T cell responses are disrupted after the age of 65 years (15). Since the elderly are at the highest risk for life-threatening COVID-19, almost all countries have decided to launch their vaccination campaign by giving them absolute priority, especially for those living in care homes. Further assessment of efficacy and safety is still ongoing in this cohort and will be important in order to tailor the vaccine schedule if necessary (interval and number of doses or amount of antigens). Moreover, a deeper investigation into the scarcity of cellular immune responses observed in elderly people exposed to SARS-CoV-2 could have important implications to guide the design of future new vaccines against this virus and other related ones.

What about the other extreme age group: children? Unlike other respiratory viruses, children are less susceptible to COVID-19 than adults are. Not only do they present with milder forms of infections (52), but they seem less likely to become infected after exposure (especially for the youngest) (53, 54). Adolescents, however, show the same features of transmission and disease as adults. Many studies are ongoing to assess to which extent children contribute to the spread of the SARS-CoV-2 pandemic and the reasons why they are less susceptible. If it is generally

[7]https://www.has-sante.fr/jcms/p_3237271/fr/strategie-de-vaccination-contre-le-sars-cov-2-vaccination-des-personnes-ayant-un-antecedent-de-covid-19

admitted that the children (especially until primary school age) are not the motor of transmission, they can still transmit the disease once infected, irrespective of their age (52, 55) and this is all the more difficult to estimate that they are often asymptomatic carriers. A reflection should therefore be carried out on whether, once vaccination of priority groups is be completed, children should also be considered for vaccination and if so, for which age group. Regarding features of infection and transmission, consideration should probably be given to adolescents first and then to school-age children as well as those with comorbidities irrespective of their age. The main goal would be to decrease the circulation and reservoir of the virus inside the community, especially if willing to achieve an optimal vaccine coverage, provide herd immunity and prevent the rapid spread of more transmissible new variants, which showed increased infectivity also among children (56). Although it should be stressed out that children represent only 17.4% percent of the EU population and <2% of hospitalized COVID-19 cases, they constitute a very dynamic part of the population, even beyond their school and household, by traveling and gathering during collective activities and have regular contacts with their grandparents. Compliance with social distancing measures is also more difficult to achieve in young individuals. Some popular waves are pushing now to vaccinate in priority young adults and adolescents, whom the psychosocial burden of the pandemic is estimated to be among the highest after health care workers and elderly (57). The increased incidence of MIS-C in the pediatric population this summer as well as the existence of severe cases (though rare) within the youngest is an additional argument to consider for vaccination in the mid- or long term if high epidemic circulation is still ongoing. Moreover, co-infections with SARS-CoV-2 and other respiratory viruses like Influenza or RSV have been described to lead to severe pneumonia (58). Even though winter epidemic viruses were almost absent from the landscape this year, we can hypothesize that a problem could arise once others respiratory viruses will come back and affect the youngest population again. RSV and influenza are major causes of morbidity and hospitalization every year in pediatrics, and no one can predict what could give co-infection with SARS-CoV-2, especially for infants and children with comorbidities.

Besides the encouraging results of the mRNA Pfizer vaccine in hundreds of adolescents, data on vaccine efficacy and safety are awaited in children who usually presented with higher immune responses. If considering vaccination in pediatric groups in the future, the number of doses and the optimal amount of antigen should be determined for each age category in order to maximize efficacy but also to minimize the risk of reactions (like fever, pain, rash, etc.). It should also be determined to which extent the history of atopia (a frequent problem in pediatrics) requires more caution or constitute a contraindication. Last but not least, a place should be found in the already tight vaccine schedule, without hampering compliance to other vaccinations and in respecting intervals with other injections to minimize adverse events.

Choosing the vaccine candidates that are the most adapted for children might be a crucial point in this debate and could differ from these for adults. The ideal vaccine for pediatric setting should, besides offering optimal protection and long-term immune memory, be not too immunogenic, be administered following a single dose schedule, be suitable for intranasal delivery (no needle and prevention of carriage frequent in the youngest), require no strict storage conditions, and, if possible, provide simultaneous protection against other viruses whose others vaccines could then be avoided.

Finally, the question of pregnant women and immunocompromised patients deserves specific attention. Whereas, formulations containing live replicating viruses have formally to be avoided, no data are available for mRNA vaccines in these cohorts. As for not replicating vectored vaccines, the precaution principle should prevail while waiting for further recommendations. Risk assessments of COVID-19 in pregnant women have given conflicting results considering the rate of serious infections, hospitalization, and complications like preterm delivery (59, 60). Pregnant women are however considered as a risk group by the CDC[8]. Even if no specific physio-pathological argument or animal study raises concerns regarding mRNA vaccination in this cohort, the WHO and the EMA do not recommend systematic vaccination given the absence of specific data but rather a case-by-case approach with cost-benefit assessment, especially for women belonging to other risk groups[1,6,9,10]. It should be highlighted that vaccine studies including pregnant women are definitely needed if willing to provide reliable recommendations in the near future. For women who are breastfeeding, a recent EMA report indicated that no particular risk should be considered for the mRNA vaccine, due to quick degradation of the product that is not suspected to be armful once entering the digestive tract of the newborns. For persons living with HIV or other immunocompromising comorbidities, as long as they are treated and stable, and given they are at higher risk of severe COVID-19, vaccination is recommended after medical advice[1,6,9]. However, not all types of vaccine would be acceptable in this cohort since no live virus could be administered. Protein subunit or mRNA vaccines would therefore be preferred.

How Do We Choose Between the Different Vaccines?

Table 1 displays the different types of vaccines, each offering various advantages and inconveniences. Until now, their use depends on the performances achieved as well as on marketing authorization earned from regulatory agencies and commercial agreements. Some formulations may better suit some settings than others depending on their conditions of their supply, storage, and schedule of administration. However, equity and accessibility for all must be protected, and research is encouraged to provide the best candidate vaccine for each socioeconomic and geographical situation.

[8]https://www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/pregnancy-breastfeeding.html

[9]https://extranet.who.int/pqweb/vaccines/who-recommendation-covid-19-mrna-vaccine-nucleoside-modified-comirnaty%C2%AE

[10]Available online at: https://www.ecdc.europa.eu/en/covid-19-pandemic

All vaccines are directed at least against epitopes of the S protein or its RBD sequence that either vectored or presented in different ways, could be theoretically used in a "heterologous prime boost strategy." This strategy consists of giving two doses of vaccine where each belongs to a different formula and therefore presenting the antigens differently. This process seems to induce a higher immune response than using the same formulation twice (61). The heterologous prime boost has already shown promising results in vaccination against HCV and HBV. Such a strategy could be of great interest against SARS-CoV-2, but further studies are needed to investigate its superiority and harmlessness in humans and animals.

Finally, the choice of vaccine type should be continuously evaluated in the future to fit at most the host and the pathogen. If necessary, adaptations should be envisaged for subgroups of individuals according to their age, immune and medical status, and history of allergy or pregnancy. Private–public partnerships would facilitate the establishment of broad international cohorts, which is mandatory to monitor vaccine effectiveness and safety among individuals suffering from rare conditions.

Adaptation of Vaccine to Viral Evolution

Last but not least, the success of universal mass vaccination also relies on the implementation of continuous surveillance of circulating viral strains as well as of an active reporting system of cases to identify vaccine failure. Like all RNA viruses, the SARS-CoV-2 genome undergoes frequent spontaneous mutations or deletions that are fortunately less frequent than other RNA viruses due to the presence of a corrective enzyme (62).

Whereas, not every mutation leads to consequences on pathogenicity, some could be the source of trouble, either through increasing virulence or transmissibility or by impairing the protection achieved by prior infection or vaccination (62). Such events could happen when significant mutations occur in genes encoding the S-protein: the main target of the majority of vaccines. As seen with many other pathogens, new variants can outcompete the local dominant clone (s) because the acquired mutations confer selective advantages for survival and dissemination.

From the beginning of the pandemic, numerous SARS-CoV-2 variants characterized by mutations on surface proteins compared to the original strain isolated in Wuhan in December 2019 have been identified within the forefront the D614G mutated strain that early became dominant in Europe and the Americas (63). Further new variants have recently been identified spreading all over the world (62, 64). At the time of writing this paper, the most harmful variants in Europe either belong to the lineage B.1.1.7 (UK variant VOC 202012/01) or to the lineage B.1.351 that originated from South Africa (48, 61). Both of them harbor mutations affecting the sequence of the S-protein, of which one (N501Y) affects its RBD. These mutations are hypothesized to increase viral affinity for human cell receptors and facilitate replication, leading to higher transmissibility (64). Fast recrudescence of cases has actually been observed with these strains (47, 56), requiring the implementation of more stringent lockdown measures in some regions. Though data are conflicting, results from Britain epidemiological reports tend to indicate increased severity of infection with the B.1.1.7 mutant (48, 56). Fortunately, according to preliminary immunological studies, the genetic changes found in this variant seem only to marginally affect the efficacy conferred by currently available vaccines (29, 65). However, real concerns exist about the protection against the South-African and Brazilian variants that both carrying the mutation E484K believed to impair the neutralizing capacity of vaccine-induced antibodies (64, 65). Strikingly, this mutation has been identified additionally in some B.1.1.7 UK strains that will now deserve particular attention and monitoring of cases. The Brazilian variant (P.1 lineage) has been first reported in the city of Manaus (as well as in some travelers in Japan and South Korea), creating an important upsurge of cases in this city thought to have, however, reached a high level of community immunization. Only a minority of cases have been reported to date in Europe and are mostly associated with travel history, but further monitoring is required.

Since mutations belong to the natural dynamic evolution of RNA viruses, it seems likely that several other SARS-CoV-2 variants will emerge over time, with more or fewer implications on pathogenicity and transmissibility but requiring constant assessment of vaccines effectiveness and perhaps adaptation of the presented antigens to enlarge protection. A similar model is -already applied with the Flu vaccine in which vaccination must be repeated yearly and vaccine production adapted anticipatively according to the most likely antigenic drifts for the four dominant influenza A/B strains. International collaboration and elaboration of a reference database are crucial to identify new lineages and understand the implications of mutations on pathogenesis and on protection confer by the available vaccines. Whereas, effects of new mutations on disease severity remain uncertain to date, we can wonder whether future genetic variations in SARS-CoV-2 associated with host immune adaptations will result in persisting seasonal epidemics with, however, a less serious pattern of infection, like observed for H1N1 for almost a century (66).

CONCLUSION AND PERSPECTIVES

In nearly 15 months, SARS-CoV-2 has been responsible for a dramatic burden of disease and a global economic recession. To date, the collective immunity achieved is largely insufficient, as evidenced by the persistence of the pandemic, and the physical distancing and hygiene measures, while mandatory to avoid overflow of the healthcare system, are not enough on their own to control the spread of the disease especially in a long-term perspective. The emergency state generated by COVID-19 sparked important rallying all over the world, which, in addition to the experience drawn from prior viral epidemics, allowed for faster development of a vaccine.

Broadly vaccinating the population remains the best way to fight COVID-19 even if additional data are needed to better tailor vaccine schedules (notably for particular subgroups and previously ill people) and identify long-term side effects. Many

promising options, like new vaccine candidates and prime boost strategies, are still under investigation.

Continuous monitoring of the circulating viral strains, associated with the international post-vaccination surveillance system reporting host infections and reactions, will be the cornerstones to ensure effectiveness and safety for everyone. A judicious choice of the best formulation, based on economic and logistical constraints but also on scientific and medical arguments, could help to optimize the success of vaccination campaigns worldwide in addition to constant evaluation of vaccine effectiveness on new variants to avoid breakthrough infections.

Control of the pandemic will, however, only be achieved through international coordination on preventive strategies and vaccination policies and if social distancing and hygiene measures are kept long enough while reaching sufficient vaccine coverage to interrupt viral epidemic circulation.

AUTHOR CONTRIBUTIONS

SB and PD contributed equally to this article in reviewing the literature and writing the manuscript. All authors approved the submitted version.

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frontiers in Public Health

REVIEW
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One-Year Update on Salivary Diagnostic of COVID-19

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Background: Coronavirus disease 2019 (COVID-19) is a global health problem, which is challenging healthcare worldwide. In this critical review, we discussed the advantages and limitations in the implementation of salivary diagnostic platforms of COVID-19. The diagnostic test of COVID-19 by invasive nasopharyngeal collection is uncomfortable for patients and requires specialized training of healthcare professionals in order to obtain an appropriate collection of samples. Additionally, these professionals are in close contact with infected patients or suspected cases of COVID-19, leading to an increased contamination risk for frontline healthcare workers. Although there is a colossal demand for novel diagnostic platforms with non-invasive and self-collection samples of COVID-19, the implementation of the salivary platforms has not been implemented for extensive scale testing. Up to date, several cross-section and clinical trial studies published in the last 12 months support the potential of detecting SARS-CoV-2 RNA in saliva as a biomarker for COVID-19, providing a self-collection, non-invasive, safe, and comfortable procedure. Therefore, the salivary diagnosis is suitable to protect healthcare professionals and other frontline workers and may encourage patients to get tested due to its advantages over the current invasive methods. The detection of SARS-CoV-2 in saliva was substantial also in patients with a negative nasopharyngeal swab, indicating the presence of false negative results. Furthermore, we expect that salivary diagnostic devices for COVID-19 will continue to be used with austerity without excluding traditional gold standard specimens to detect SARS-CoV-2.

Keywords: nasopharyngeal swabs, saliva, diagnostic test, salivary diagnostic, SARS-CoV-2, COVID-19

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INTRODUCTION

The World Health Organization (WHO) indicates the pivotal importance of mass testing in the find–test–trace–isolate–support strategy to contain COVID-19 transmission (1). The increase in massive testing capacity coupled with artificial intelligence multidisciplinary data should be used to prevent and combat the negative effects of COVID-19 and strengthen global health public systems to improve COVID-19 response (2). The National Institute of Health supports a rapid scaling up of SARS-CoV-2-detecting tests in the United States (3); however, the need for better diagnostic tests with high sensitivity has been considered critical to mitigate and suppress the spread of COVID-19 (4). Novel continued efforts need to be performed to reduce the presence of false-negative molecular testing in presymptomatic and asymptomatic subjects, as well as the presence of hospitalized patients with initial false-negative testing and clinical signs and symptoms consistent with SARS-CoV-2 infection (4).

In this critical review, at the salivary SARS-CoV-2 detection's 1-year mark, we discussed the advantages and limitations in the implementation of salivary diagnosis of COVID-19 and point out some recommendations to this potential application to provide a comprehensive summary on the scientific advances performed in the last 12 months. The diagnostic test for COVID-19 by invasive nasopharyngeal collection is uncomfortable for the patients and requires specialized training of the frontline workers in order to perform an appropriate collection of samples. Additionally, these frontline professionals are in close contact with infected patients or suspected cases of COVID-19, leading to increased morbimortality of healthcare workers. It imposes the development of new strategies for COVID-19 diagnosis; however, despite the colossal demand for novel diagnostic platforms with non-invasive and self-collection samples of COVID-19, the accuracy of salivary SARS-CoV-2 platforms are still not well-elucidated. The pivotal impact on social, health, economic, and educational fields in a global emergency due to COVID-19 makes it more challenging to compare the advantages and limitations in implementing novel potential salivary platforms (1, 2, 4).

BACKGROUND

The coronavirus disease 2019 (COVID-19) is an international public health emergency, which also impacts social, economic, and educational aspects worldwide. The outbreak of COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which has been spread across all continents with more than 117 million cases and ∼2.5 million deaths (5). The centers for disease control and prevention around the world have recommended testing for SARS-CoV-2 in upper respiratory specimens.

The COVID-19 diagnostic is mainly based on the detection of SARS-CoV-2 by real-time polymerase chain reaction (RT-PCR). The sensitivity of this gold standard test is higher in symptomatic than in asymptomatic COVID-19 subjects (6). Besides, the false-negative results have uncertain frequency especially in the incubation period of the disease. Although SARS-CoV-2 RNA detection in the nasopharyngeal swab was reported as the gold standard method for COVID-19 diagnosis, sample collection by this method requires that healthcare frontline workers are in close contact with infected patients or suspected cases of COVID-19. Besides, this specimen-collecting procedure is invasive and inconvenient for patients, and it requires specialized training for healthcare workers (7).

Salivary biomarkers are an alternative method to other invasive procedures in the early diagnostic of systemic diseases (8). The collection of saliva samples represents a non-invasive, convenient, and easy self-collection method, with no direct contact between healthcare workers and patients. Saliva contains more than 3,000 proteins, 3,000 mRNA, ∼50 microRNAs, hundreds of metabolites, and more than 700 species of microorganisms such as viruses (9). The 198 extracellular RNAs (ExRNAs) detected in saliva were also considered potential biomarkers for systemic and oral diseases. In this context, salivary exRNA related to SARS-CoV-2 infection could be used to develop novel salivary platforms of COVID-19 (10, 11). Previously, we detailed the potential of salivary diagnosis for COVID-19 (12), which was confirmed in several studies by detecting SARS-CoV-2 in human saliva (13–37) and in saliva associated with oropharyngeal fluid (38, 39). SARS-CoV-2 was also detected in animal models of COVID-19. The viral RNA was detected in saliva and nasal washes from 2 to 8 days post-infection of infected ferrets as an animal model of COVID-19 (40). In this context, analysis of SARS-CoV-2 RNA from saliva can provide clues in the early diagnosis of COVID-19. Higher viral loads of SARS-CoV-2 in oropharyngeal fluid mixed with saliva were detected on symptom onset, which then gradually declined toward the detection limit until 25 days after symptoms started (39, 41).

FUTURE DIRECTIONS

As the landscape of SARS-CoV-2 diagnosis comprises limitations for the current gold standard diagnosis methods and potential benefits for novel applications in COVID-19 diagnosis, a critical evaluation on the advantages and limitations of concurrent emerging salivary diagnosis is mandatory.

Search Strategy and Study Selection

We searched three electronic databases, PubMed, LILACS, and Google Scholar, from February 2020 when the SARS-CoV-2 was first indicated in saliva (12, 38) until February 2021. The selected keywords were COVID-19, coronavirus, SARS-CoV-2, saliva, and nasopharyngeal swabs. We selected studies that analyzed the accuracy and sensitivity of saliva compared with nasopharyngeal swabs for SARS-CoV-2 detection by RT-PCR. Positive detection of SARS-CoV-2 in saliva or nasopharyngeal fluid was considered as a reference standard.

Table 1 compares the sensitivity of nasopharyngeal and salivary samples in SARS-CoV-2 RNA detection using RT-PCR and points out the presence of samples negative in nasopharyngeal swab and positive in saliva, which indicates the presence of false-negative results.

TABLE 1 | Sensitivity of COVID-19 salivary diagnosis comparing to nasopharyngeal swab (NPS) specimens.

References	Total samples	Positive (NPS+Saliva)	Negative	Sensitivity Saliva	Sensitivity NPS	Percentage of Saliva + and NPS -
Rao et al. (30)	217	160	57	93.1% (149/160)	52.5% (84/160)	47.5% (76/160)
Caulley et al. (17)	272	13	259	84.6% (11/13)	61.5% (8/13)	38.5% (5/13)
Senok et al. (32)	401	35	366	80% (28/35)	74.3% (26/28)	25.7% (9/35)
Uwamino et al. (35)	196	58	138	74.1% (43/58)	81% (47/58)	19% (11/58)
Moreno-Contreras et al. (27)	71	34	37	73.5% (25/34)	82.3 (28/34)	17.6% (6/34)
Yokota et al. (37)	1,924	52	1,872	92.3% (48/52)	88.5% (46/52)	11.5% (6/52)
Jamal et al. (21)	91	72	19	72.2% (52/72)	88.9% (64/72)	11.1% (8/72)
Iwasaki et al. (20)	76	10	66	90% (9/10)	90% (9/10)	10% (1/10)
Pasomsub et al. (28)	200	21	179	85.7% (18/21)	90.5% (19/21)	9.5% (2/21)
Torres et al. (34)	943	108	835	42.6% (46/108)	92.6% (100/108)	7.4% (8/108)
Hanson et al. (19)	354	86	268	94.2% (81/86)	93% (80/86)	7% (6/86)
Kandel et al. (22)	429	46	383	91.3% (42/46)	93.5% (43/46)	6.5% (3/46)
Landry et al. (23)	124	35	89	85.7% (30/35)	94.3% (33/35)	5.7% (2/35)
Babady et al. (14)	87	18	69	94.4% (17/18)	94.4% (17/18)	5.5% (1/18)
Miller et al. (26)*	91	36	55	97.2% (35/36)	94.4% (34/36)	5.5% (2/36)
Skolimowska et al. (33)	131	19	112	84.2% (16/19)	94.7% (18/19)	5.3% (1/19)
Altawalah et al. (13)	848	361	487	84.2% (304/361)	95.3% (344/361)	4.7% (17/361)
Matic et al. (24)	74	22	52	72.7% (16/22)	95.4% (21/22)	4.5% (1/22)
Chau et al. (18)	27	28	NA	75% (21/28)	96.4% (27/28)	3.6% (1/28)
Vaz et al. (36)	155	73	82	94.5% (69/73)	97.3% (71/73)	2.7% (2/73)
Barat et al. (15)	459	38	421	81.6% (31/38)	97.4% (37/38)	2.6% (1/38)
McCormick-Baw et al. (25)	156	50	106	96% (48/50)	98% (49/50)	2% (1/50)
Bhattacharya et al. (16)	74	58	16	91.4% (53/58)	100% (58/58)	0% (0/58)
Ranoa et al. (29)	100	9	91	100% (9/9)	100% (9/9)	0% (0/9)
Rutgers (31)	53	26	27	100% (26/26)	100% (26/26)	0% (0/26)
Overall	7,553	1,468	6,086	83.6% (1,227/1,468)	88.4% (1,298/1,468)	11.6% (171/1,468)

** The specificity of both salivary and nasopharyngeal (NPS) swabs were 100%. Considering the presence of false-negative results with NPS, a positive detection of SARS-CoV-2 in saliva or nasopharyngeal fluid was considered as a positive standard reference.*

Limitations of SARS-CoV-2 Detection in Nasopharyngeal Specimens

Although the nasopharyngeal swab tests have been considered as gold standard specimens, multisite assessment or other specimens for SARS-CoV-2 diagnosis have been suggested to reduce false-negative test results and to increase testing capacity (42), especially due to limitations in low–middle income countries (LMICs) (43). Nasopharyngeal collection is performed using a flexible plastic swab with a nylon tip, which is inserted into the nostrils until the healthcare worker observes resistance. Subsequently, the swab is rotated three times in the nasopharynx and removed after 5 s, a procedure which is considered invasive and uncomfortable (44, 45). However, the swab collection protocol can be different in each country. Appropriate nasopharyngeal swab collection is more difficult in children and patients with a deviated nasal septum or coagulopathy (46). The sputum is another respiratory specimen tested to be used in COVID-19 diagnosis. Due to the limitations of sampling, sputum collection was used in possibly only one third of COVID-19 patients, which reveals a robust restriction of this diagnostic method (12, 38). Self-collection of samples from suspected cases of COVID-19 or infected patients is still limited and the direct contact between healthcare workers and patients during the standard collection procedures resulted in about 20% of the healthcare workforce becoming infected, and some deaths were reported (47). Frontline workers may experience intense anxiety and additional adverse emotion due to the risk of contamination during the collection procedure (48). Although most studies showed higher levels of the virus in nasopharyngeal specimens compared with saliva, lower levels of SARS-CoV-2 in nasopharyngeal swabs can result in false-negative outcomes due to inaccurate collection (48). The personal protective equipment and creation of exclusive sampling rooms have been reported as tools capable of enhancing the protection of frontline workers (47, 48). Currently, COVID-19 cases have been significantly increasing worldwide, overloading national health systems. Furthermore, the situation might be even worse in LMICs, since there is a scarcity of trained healthcare and other frontline workers to face the COVID-19 pandemic (49). Taken together, these issues demonstrate the critical demand for new approaches for COVID-19 diagnosis.

The Potential of Salivary Diagnosis of COVID-19

The enthusiasm in developing new salivary platforms for COVID-19 diagnosis and monitoring is comprehensible; however, the true accuracy of these new protocols to detect SARS-CoV-2 in saliva has been discussed in several scenarios such as during the incubation period, the viral response phase, and the host inflammatory phase of symptomatic patients. Besides, the diagnostic sensitivity levels in COVID-19 asymptomatic patients also remain unclear in both salivary and nasopharyngeal specimens. It is important to emphasize that the implementation of salivary diagnosis for COVID-19 before a comprehensive knowledge of its limitation could promote future issues about the application of salivary diagnostic tests to other systemic diseases. However, the colossal demand for novel diagnostic platforms for COVID-19 with non-invasive and self-collection samples could be used after the creation of a well-designed strategic plan for its implementation until this true efficacy will be completely investigated.

Preponderance of Reviews and Letters Over Primary Clinical Trials

The most remarkable data on COVID-19 salivary diagnosis implementation is the unbalanced number of published clinical trials or reviews and letters. PubMed reveals 20 cross-sectional and case-control designed studies, five cross-sectional studies with no control subjects, and more than 200 reviews and letters published from February 2020 up to February 2021. Additionally, there are 14 additional cross-sectional studies that evaluated the oropharyngeal fluid mixed with saliva as a diagnostic fluid for COVID-19. It suggests that opinions concerning salivary diagnostic platforms have been consolidated primarily from letters and reviews. On the other hand, it is important to emphasize that cross-sectional studies with salivary diagnostics indicated a higher correlation of sensitivity compared with the gold standard nasopharyngeal samples in COVID-19 diagnosis. We performed this critical review due to the limitations concerning current reviews focusing on the counterbalance between the inevitable obstacles and encouraging results of COVID-19 salivary diagnosis.

Sample Size of Studies

In order to obtain a more robust comparison of saliva with gold standard specimens, a limited number of comparative studies and lower sample sizes have been overcome in these 12 months after SARS-CoV-2 detection in saliva (38) Altogether, the total number of salivary samples (non-infected subjects and COVID-19 patients), which was compared with gold standard nasopharyngeal respiratory specimens was 7,553. In this context, 5,172 subjects were from published studies/original articles, 53 subjects from the FDA emergence-approved study, 191 subjects from preprint articles, 559 subjects from short communications/brief reports, and 1,578 subjects from letters to the editor. To detect the salivary sensitivity 1,468 salivary and/or NPS-positive samples from COVID-19-infected patients were used and evaluated in this review (**Table 1**).

The Relevance of Specificity in SARS-CoV-2 Detection

In general, the absence of analysis in control subjects can be considered a negative condition; however, the main limitation in the use of RT-PCR tests is the detection of RNA in levels near the sensitivity limits. The detection of unspecific RNA is not a classical limitation of RT-PCR tests (50), which is considered 100% specific due to the intrinsic characteristics of this platform (51). It must be considered that the presence of SARS-CoV-2 RNA in saliva and negative results in nasopharyngeal samples analyzed by RT-PCR cannot be classified as false positive, but a misclassification of nasopharyngeal samples. This pivotal view is well-documented in a previous study that showed 71% of matched detection of SARS-CoV-2 RNA in saliva and

nasopharyngeal swabs, 21% only in saliva, and 8% only in nasopharyngeal swabs (52). It can be related with the limitations in nasopharyngeal swab procedure and/or with low produced nasopharyngeal mucous secretion in COVID-19 patients. In this new pandemic era, the centers for disease control and prevention worldwide took maximal efforts to establish reference standards for COVID-19 diagnosis in a fast and efficient way, based on the outbreak of severe acute respiratory syndrome (SARS) in 2003 (53). It is well-recognized that updates in COVID-19 diagnosis protocols are crucial, and the reference standards are not perfect, especially in samples collected in the 1st days after infection (54). The procedures related to sample preservation and RNA extraction were reported in all included studies, and it seems suitable, and presumably these factors did not influence the results. In this context, it is important to emphasize that the absence of a control group in the studies with oropharyngeal fluid mixed with saliva is not a significant limitation (38, 39, 55–57). The reference standard using nasopharyngeal specimens was considered as an unsolved issue that needs imperative debate to increase confidence in COVID-19 tests (54).

Saliva Collection and Its Correlation With Sensitivity

The pioneer study that detected viable SARS-CoV-2 in oral fluid promoted a paradigm shift in diagnosis, monitoring, and infection control for COVID-19 (38). However, the sensitivity of salivary SARS-CoV-2 RNA to diagnose COVID-19 needs to be carefully checked because some data are based on trials designed to evaluate oropharyngeal fluid mixed with saliva (38, 39, 57–59). In classical studies with salivary collection, the patient is not required to cough out fluid from their throat. Frequently, total saliva is collected from the mouth under an unstimulated or stimulated flow rate (9). Some collection devices were also developed to collect saliva specifically from parotid, submandibular/sublingual, and minor and palatine glands (9). Here, this review considered studies that collected saliva by the traditional drooling technique and without coughing into a container. **Table 1** shows a similar sensitivity to detect SARS-CoV-2 RNA undergoing paired collection of saliva and NPS. SARS-CoV-2 RNA was detected overall in 83.6% (1,227/1,468) of saliva samples and also in 88.4% (1,298/1,468) of samples of NPS, which supports the potential of salivary SARS-CoV-2 RNA as a biomarker for COVID-19 in a preliminary analysis. We also highlight a substantial salivary detection of SARS-CoV-2 in patients with a negative nasopharyngeal swab (11.6%, 171/1,468), indicating an expressive indication of false-negative results with gold standard specimens. Thus, based on these data, we suggest that saliva is an accurate sample to be used for a mass screening test, and this biofluid could be used to reduce the rate of false negatives in the clinical performance of COVID-19 diagnostic tests. We also observed that the majority of articles analyzed unstimulated saliva, which avoids a potential dilution of the SARS-CoV-2, which could occur in mouth rinsing or stimulated saliva collection (60).

The Importance of Home- and Self-Sample Collection

It was indicated that the primary choice for sampling during illness experience is home-based tests compared with clinic-based strategies. The higher compliance to test for SARS-CoV-2 was verified when a lower degree of contact with frontline healthcare workers was required to collect samples: as expected, home testing was the most preferred, followed by tests in drive-through sets and, subsequently, hospital-based testing. It is crucial to provide self-saliva collection and home-based tests to suspected cases of COVID-19 as profitable strategies in order to guarantee the social distance of the population. It also contributes to reduce direct contact with frontline workers, which offers a potential for early diagnosis due to the hierarchy of willingness to test for COVID-19. The self-sample collection and home-based tests should be validated as soon as possible to be applied in public and private healthcare systems (61).

Spectrum of Patients

In order to provide a suitable spectrum of COVID-19 patients with distinct severity of diseases, it is important to envisage patients searching for a diagnostic test in the onset of symptoms and in the late stage of the disease. Bearing in mind that the higher salivary SARS-CoV-2 levels occur during the acute phase of disease with gradual decline after symptom onset (39), it is important to point out the limitations of longitudinal analysis with SARS-CoV-2 level in asymptomatic COVID-19 subjects. In this context, the temporal analysis of SARS-CoV-2 viral load in saliva should receive more attention among asymptomatic and non-hospitalized COVID-19 patients, which could be pivotal for the translation of salivary tests in the clinic. However, the current gold standard protocols are also unable to raise this query (54). The comparison between sensitivity shown in **Table 1**, in different studies, reported a limited heterogeneity, which should not be ignored to improve this new potential gold standard protocol.

Obstacles to SARS-CoV-2 RNA Extraction From Saliva and New Applications

A critical hurdle for salivary diagnosis may be the broad-spectrum validation in COVID-19 patients during the incubation period, the viral response phase, and the host inflammatory phase in asymptomatic and symptomatic patients. It has been proposed that patients can be infected and after 24 to 72 hours the onset of symptoms could occur. About 50% of the transmission of cases is from asymptomatic COVID-19 individuals. The viral levels of SARS-CoV-2 RNA are presumably detected in nasopharyngeal swabs before or sooner than symptom onset, which is a leading challenge in the diagnosis, spread, and containment of COVID-19 (6).

Some critical issues in the isolation of RNA methods to process saliva are unique to this biofluid. The know-how and practice to pipette a biofluid with higher density could explain the discrepancy between the overall sensitivity in different studies. Some protocols indicated the dilution of saliva in a standard liquid as that occurring in nasopharyngeal swabs. This action can

change the SARS-CoV-2 concentration and reduce the sensitivity of tests. In this context, the higher saliva density that makes pipetting difficult, tooth-brushing contaminants, and changes in volume are parameters that could interfere with the result (62). In general, the use of the magnetic bead methodology showed good results for saliva sensitivity, 97.2% (26), possibly due to the RNA extraction insulation kit used. In addition, the enzymes present in saliva also makes RNA naturally degrade, so choosing a more robust methodology is important for the sensitivity and specificity of the experiment (26).

Various methods are available to extract RNA from saliva, such as methods using phenol and guanidinium isothiocyanate, or commercially available silica membrane spin columns or magnetic bead-based RNA isolation kits (63). Other molecular diagnostic methods, such as reverse transcription–loop-mediated isothermal amplification (RT-LAMP), have also been reported as useful for diagnosing COVID-19 in settings of point-of care testing (64–66). Rapid and extraction-free detection of SARS-CoV-2 from saliva by colorimetric RT-LAMP is a simple, sensitive, rapid, and cost-effective approach with broad potential to expand diagnostic testing for the virus causing COVID-19 (67, 68). Although full validation on additional clinical samples is necessary before such an assay can be widely used, a few studies have evaluated this technique. These preliminary results demonstrate a promising approach to overcome the current bottlenecks that limit widespread testing.

Furthermore, the sequencing of the genome using salivary samples from COVID-19 patients could contribute in the incorporation of new targets (69), identification of the new SARS-CoV-2 variants of concern (as B.1.1.7 emerged in the United Kingdom, B.1.351 was first identified in South Africa, and P.1 emerged in Brazil) (70, 71), or even in the identification of critical mutations (72). In addition, a nanopore sequencing analysis of saliva suggests that host factors play a more important role in the clinical outcome than viral genetic variation (69), as demonstrated by emerging clinical studies (73).

FINAL REMARKS

These tests seem to be in agreement with FDA emergence approval, which includes a home collection of saliva to diagnose COVID-19 when indicated by a healthcare provider. Up until now, the FDA had authorized at least five salivary tests for COVID-19 diagnosis. The patients are also informed that a negative result is not a guarantee of the absence of COVID-19 infection. However, due to the high specificity of RT-PCR analysis, the detection of SARS-CoV-2 in saliva can be acceptable when the diagnostic test for COVID-19 is positive. We also highlight a substantial salivary detection of SARS-CoV-2 in patients with a negative nasopharyngeal swab (11.6%), indicating an expressive indication of false-negative results with gold standard specimens. Besides, the higher compliance to test for SARS-CoV-2 under reduced direct contact, requiring the collection of saliva, may contribute to an early diagnosis of COVID-19, resulting in optimal clinical care, encouraging isolation and reducing the spread of the disease. In this regard, the potential implementation of salivary SARS-CoV-2 diagnosis under a pandemic situation and social, health, economic, and educational issues due to COVID-19 is an additional challenge. These results support the potential of SARS-CoV-2 RNA as a biomarker for COVID-19, providing a self-collection, non-invasive, safe, and comfortable analysis, suitable to protect dentists, dental assistants, dental hygienists, and other frontline workers with self-collection and/or home collection saliva samples. Furthermore, we expect that salivary diagnostic devices for COVID-19 will continue to be used with austerity without excluding traditional gold standard specimens to detect SARS-CoV-2.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: RS-S, TC, LG, LC-S, and MM are inventors of patents related to the use of photonic devices for COVID-19 diagnosis using saliva and nasopharyngeal samples titled, Spectral profile for COVID-19 diagnostic, use of the same, method, system, and platform to COVID-19 diagnosis—BR 10 2020 010992 8.

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BRIEF RESEARCH REPORT
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SARS-CoV-2-Specific Antibody Prevalence and Symptoms in a Local Austrian Population

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Background: Since December 2019 the novel coronavirus (SARS-CoV-2) is the center of global attention due to its rapid transmission and toll on health care systems and global economy. Population-based serosurveys measuring antibodies for SARS-CoV-2 provide one method for estimating previous infection rates including the symptom-free courses of the disease and monitoring the progression of the epidemic.

Methods: In June 2020 we succeeded in testing almost half of the population of an Austrian township (1,359 inhabitants) with a reported higher incidence for COVID-19 infections (17 PCR positive cases have been officially reported until the date of sample collection, i.e., 1.2% of the total population). We determined the prevalence of SARS-CoV-2-specific antibodies in this population, factors affecting, and symptoms correlated with prior infection. Antibodies were determined using a CE-certified quality-controlled ELISA test for SARS-CoV-2-specific IgG and IgA antibodies.

Results: We found a high prevalence of 9% positive antibodies among the town population in comparison to 6% of the neighboring villages. This was considerably higher than the officially known RT-PCR-approved COVID-19 cases (1.2%) in the town population. Twenty percent of SARS-CoV-2-antibody positive cases declared being asymptomatic in a questionnaire. On the other hand, we identified six single major symptoms, including anosmia/ageusia, weight loss, anorexia, general debility, dyspnea, and fever, and especially their combination to be of high prognostic value for predicting SARS-CoV-2 infection in a patient.

Conclusions: This population study demonstrated a high prevalence of antibodies to SARS-CoV-2 as a marker of past infections in an Austrian township. Several symptoms revealed a diagnostic value especially in combination.

Keywords: SARS-CoV-2, COVID-19, immunology & infectious diseases, antibody prevalence, disease symptom assessment

The world is still in the midst of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection pandemic, with Austrian towns, such as Ischgl, acting as local epicenters. In June 2020, we succeeded in testing approximately half of the population (47%) of an Austrian township with a reported high incidence of coronavirus disease (COVID-19) infections. We determined the prevalence of SARS-CoV-2 infections in this population, factors affecting it, and the symptoms associated with prior infection. The study's design and execution were in accordance with the local ethics committee and were approved by the local and national authorities.

The township of Weißenkirchen/Wachau (1,359 inhabitants) comprises the town Weißenkirchen (926) and the communities Wösendorf (296), Joching (150), and St. Michael (23). Participants were recruited with a public call that was supported by local authorities as well as the Austrian red cross. A group of 835 participants comprising people of all ages (ranging from 7 to 89 years) with a uniform distribution of sex (48% male) was tested for SARS-CoV-2-specific immunoglobin G (IgG) and immunoglobulin A (IgA) antibodies. The participants completed a questionnaire on personal data as well as disease symptoms, their onset, and duration.

Blood samples from the study group were tested in a certified diagnostic laboratory (Bioscientia, Ingelheim, Germany) using an EC-certified semiquantitative enzyme-linked immunosorbent assay (ELISA) (Euroimmun Anti-SARS-CoV-2-ELISA IgG and IgA). Although, the reference method for screening and diagnosis of acute COVID-19 infections is reverse transcription polymerase chain reaction (RT-PCR), the detection of antibodies against SARS-CoV-2 (IgG, IgA) plays a complementary role. It is particularly important for providing epidemiological information about previous infections, especially in the early times of the pandemic, when information about the dark figure, the number of unreported cases was an unknown factor (1). Seroprevalence has been observed in patients with COVID-19 confirmed by RT-PCR, as recently reviewed (2). So far, only a few studies have assessed seroprevalence in primarily asymptomatic individuals. The numbers during the early phase of the pandemic were overall low (1.6%) even among high-risk groups of healthcare workers having frequent contact with patients with COVID-19 (3). Additionally, only up to 5% seroprevalence was discovered in smaller studies in the general population (4).

Using the sensitive and reliable laboratory-based ELISA assay, 8.5% (71/835) and 9.0% (75/835) of the participants tested in our study showed SARS-CoV-2-specific IgG and IgA antibodies, respectively (**Figure 1**). Both classes of antibodies were found in 5.7% (48/835) of the participants. The high number of participants with SARS-CoV-2-specific IgA antibodies could be a hint of more recent infections (5). Due to their stickiness the detection of IgA antibodies is inherently less reliable than that of IgG. Thus, these data must be treated with caution. The day of sample collection was clearly after the first pandemic peak in Austria with very low infection rates at that time. Furthermore, we excluded cases with acute disease symptoms from our study. Therefore, no acute symptomatic COVID-19 cases should be included. Consequently, we considered only participants with

FIGURE 1 | Venn diagram showing the number of cases with SARS-CoV-2-specific antibodies.

SARS-CoV-2-specific IgG antibodies as cases having previous contact with SARS-CoV-2.

Individuals who showed SARS-CoV-2-specific IgG antibodies stated significantly more often that they either stayed abroad or in the Austrian state of Tyrol (42%, 30/71) as compared to the total tested population (26%, 206/806). Notably, the national hot-spot, Tyrol, was not the source of the virus, but other countries, mainly Israel, were sources. From those who visited Israel in early 2020, 53% (10/19) developed SARS-CoV-2-specific IgG antibodies. Thus, the virus most likely was introduced from external hot-spots into the local population, where it proliferated.

Nine percent (61/695) of the tested individuals in the township of Weißenkirchen developed SARS-CoV-2-specific IgG antibodies in contrast to 6% (10/167) in the control group of tested individuals from neighboring municipalities. Within the township of Weißenkirchen, 10% (45/458) of the participants from the town of Weißenkirchen, 38% (6/16) from St. Michael, and only 5% (6/114) from Wösendorf and 6% (4/71) of Joching showed virus-specific antibodies. Thus, as expected, the township of Weißenkirchen was more affected by COVID-19 than were the neighboring municipalities. Moreover, within the township, the infection rates could be mainly localized to the town of Weißenkirchen and the community of St. Michael. The official number of known RT-PCR-approved COVID-19 cases in the town population was 1.2% (17/1359) until the time point of sample collection. Thus, the dark figure of unknown infections in Weißenkirchen can be estimated to 7%.

Fifty-four percent (38/71) of participants with SARS-CoV-2-specific IgG antibodies were male, as compared to 48% (404/834) in the total tested population. From our data, a higher vulnerability of the male population, as has been indicated by some studies, is not evident. However, these studies were based on recent epidemiological data from Asia and an especially large population analysis in China (6). Similarly, we could not find significant influences of age, body mass index, and alcohol intake on the level of infection within the tested population.

Smokers turned out to be underrepresented among the participants with SARS-CoV-2-specific antibodies. Eight percent of participants with SARS-CoV-2-specific IgG antibodies identified themselves as smokers as compared to the 17% in the

total population. Since this observation did not reach statistical significance, it remains unclear whether smoking may reduce the risk of SARS-CoV-2 infection. The current data suggest that smokers are more vulnerable and that smoking is a predictor of negative outcomes, but not necessarily for a higher susceptibility to (asymptomatic) infection (7).

Twenty percent (14/71) of participants who developed SARS-CoV-2-specific IgG antibodies self- declared not to have noticed any of the 19 different disease symptoms listed in the questionnaire (**Table 1**) whereas 80% (57/71) self-declared one or more disease symptoms. Although some of these symptoms may have been related to other diseases during the evaluation period, our data suggest that asymptomatic SARS-CoV-2 infections are rather uncharacteristic for the tested population. In fact, participants with SARS-CoV-2-specific IgG antibodies self-declared to have significantly more disease symptoms during the evaluation period than the total population tested in our study (**Table 1**). Furthermore, participants having contact with SARS-CoV-2 demonstrated anosmia/ageusia, weight loss, anorexia, general debility, dyspnea, and fever more significantly than the total tested population. The enrichment of disease symptoms becomes more distinct when comparing participants with SARS-CoV-2-specific IgG antibodies with participants lacking both virus-specific IgG and IgA antibodies (**Table 1**). Participants of the IgG and IgA negative group had most likely no contact to the virus before. More and larger samples will be required to confirm the prognostic values of symptoms found in other local studies (8).

In this community-based SARS-CoV-2 population study in Austria, we found a higher seroprevalence (9%) in the town population than in the neighboring villages (6%). The seroprevalence exceeded the number of officially documented COVID-19 cases (1.2%). Considering this large sample comprising approximately half of the town population, we identified six single major symptoms, especially in combination, to be of a high prognostic value for predicting SARS-CoV-2 infection in a patient. This study has limitations; selection bias cannot be ruled out due to the voluntary nature of the study. Therefore, the estimated prevalence may be biased due to non-response or because previously symptomatic persons may have been more likely to participate. Ongoing additional

TABLE 1 | Disease symptoms in the tested population.

Disease symptoms in the evaluation period (January to June 2020)	Number of total cases with disease symptoms	Number of cases lacking both SARS-CoV-2-specific IgG- and IgA antibodies but describing disease symptoms	Number of cases with SARS-CoV-2-specific IgG antibodies and with disease symptoms
Cases with symptoms	532 (63.7%)	456 (61.9%)	57 (80.3%)
Cases without any symptoms	296 (35.4%)	274 (37.2%)	14 (19.7%)
Cases without data	7 (0.8%)	7 (1.0%)	0 (0.0%)
*Anosmia/ageusia	63 (7.5%)	35 (4.8%)	26 (36.6%)
*Weight loss	33 (4.0%)	21 (2.9%)	11 (15.5%)
Apathy	9 (1.1%)	5 (0.7%)	3 (4.2%)
*Anorexia	49 (5.9%)	33 (4.5%)	15 (21.1%)
Pneumonia	4 (0.5%)	3 (0.4%)	1 (1.4%)
*General debility	147 (17.6%)	119 (16.2%)	24 (33.8%)
*Dyspnea	51 (6.1%)	43 (5.8%)	8 (11.3%)
*Fever	133 (15.9%)	109 (14.8%)	20 (28.2%)
Diarrhea	105 (12.6%)	88 (11.9%)	14 (19.7%)
Stomach ache	60 (7.2%)	51 (6.9%)	7 (9.9%)
Headache / Pain in the limbs	224 (26.8%)	190 (25.8%)	26 (36.6%)
Eczema	21 (2.5%)	18 (2.4%)	2 (2.8%)
Tussis	278 (33.3%)	247 (33.5%)	25 (35.2%)
Rhinitis	301 (36.0%)	261 (35.4%)	27 (38.0%)
Somnolence	12 (1.4%)	11 (1.5%)	1 (1.4%)
Sore throat	222 (26.6%)	200 (27.1%)	16 (22.5%)
Swelling of the lymph node	45 (5.4%)	41 (5.6%)	3 (4.2%)
Nausea/vomiting	49 (5.9%)	44 (6.0%)	3 (4.2%)
Conjunctivitis	28 (3.4%)	24 (3.3%)	1 (1.4%)

Data are based on self-declarations of tested cases. Disease symptoms are ordered according to their enrichment in cases with SARS-CoV-2-specific IgG antibodies as compared to total cases (: significant enrichments).*

population tests and follow-up tests investigating the prevalence in the town will provide further insights into the still developing and currently dynamic pandemic situation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RJB, YH, and IY collected the data. DeL, RJB, DoL, and YH analyzed the data. RJB and DeL wrote the manuscript. OH markedly contributed to the concept of the manuscript. All authors agreed with the content of the manuscript.

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Determinants of Social Distancing Among South Africans From 12 Days Into the COVID-19 Lockdown: A Cross Sectional Study

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Introduction: Social or physical distancing has been an effective measure for reducing the spread of COVID-19 infections. Investigating the determinants of adherence to social distancing can inform public health strategies to improve the behaviour. However, there is a lack of data in various populations. This study investigates the degree to which South Africans complied with social distancing during the country's COVID-19 lockdown and identifies the determinants associated with being in close contact with large numbers of people.

Materials and Methods: Data was collected from a South African national online survey on a data free platform, supplemented with telephone interviews. The survey was conducted from 8 to 29 April 2020. The primary outcome was the number of people that participants came into close contact with (within a 2-metre distance) the last time they were outside their home during the COVID-19 lockdown. Multivariate multinomial regression investigated the socio-demographic, psychosocial and household environmental determinants associated with being in contact with 1–10, 11–50 and more than 50 people.

Results: Of the 17,563 adult participants, 20.3% reported having not left home, 50.6% were in close physical distance with 1–10 people, 21.1% with 11–50 people, and 8.0% with >50 people. Larger household size and incorrect knowledge about the importance of social distancing were associated with being in contact with >50 people. Male gender, younger age and being in the White and Coloured population groups were significantly associated with being in contact with 1–10 people but not with larger numbers of people. Employment, at least secondary school education, lack of self-efficacy in being able to protect oneself from infection, and moderate or high risk perception of becoming infected, were all associated with increased odds of close contact with 1–10, 11–50, and >50 people relative to remaining at home.

Conclusion: The findings identify subgroups of individuals that are less likely to comply with social distancing regulations. Public health communication, interventions and policy can be tailored to address these determinants of social distancing.

Keywords: COVID-19, South Africa, lockdown, social determinants of health (MeSH), stay at home directive, physical distancing and research, social distancing behaviour

INTRODUCTION

The coronavirus, COVID-19, that was first discovered in China in December 2019, continues to pose a significant global public health threat. At the end of October 2020, there were over 43 million confirmed cases and 1.1 million COVID-19 related deaths globally (1). SARS-CoV-2, the strain of coronavirus that causes COVID-19, can be spread by respiratory droplets from person to person when in close contact (2) while airborne transmission is also plausible (3). In the absence of pharmaceutical interventions, governments have promoted behavioural change measures such as social or physical distancing, wearing of face masks, and frequent hand washing or sanitising, to reduce viral transmission (4–6).

Social distancing refers to maintaining at least a metre distance between individuals and the avoidance of crowded gatherings with the potential for close contact (4). It has been demonstrated that social distancing resulted in reduced COVID-19 infections and transmissions (5, 7–9). In the United States, a 50% decrease in non-essential business visits was associated with a 45% decrease in transmissibility (9). In order to reduce the number of social contacts and thereby slow the viral spread, countries have introduced regulations such as closing of shops, educational institutions, and restaurants, prohibition of mass gatherings and public events, and work from home directives (10). Data from modelling and observational studies have shown that social distancing interventions, such as bans on mass gatherings, school and workplace closures and movement restrictions, are associated with lower incidence of COVID-19 infections and reduced mortality (11–15). Longitudinal analysis of outbreak epicentres in 37 OECD countries during the first pandemic wave found that a 1-day delay in the mass gatherings ban and a 1-day delay in school closures were associated with increases in COVID-19 cumulative mortality of 6.9% and 4.4%, respectively (15).

In South Africa, the first COVID-19 cases were discovered in early March 2020. To promote social distancing and minimise COVID-19 spread, measures to reduce interpersonal interactions were introduced in mid-March and a national 21-day lockdown was imposed to begin on 27 March 2020 (16). South Africans were required to remain at home and were only allowed out during strictly controlled conditions such as purchasing of food, medicines and other essentials; to seek medical care or to collect a social grant. The lockdown created an opportunity to break COVID-19 transmission, as a 14-day incubation period exists during which the infection symptoms can become distinct. The regulations were accompanied by public health advice on hygiene and keeping a 1–2 metre distance from others when outside of the home. Under a risk adjusted strategy, the lockdown was extended by 2 weeks to 30th April 2020, allowing for the economy to reopen partially. The behavioural practises of wearing a face mask and disinfecting surfaces were made mandatory. From 1 to 31 May the country transitioned to an "Alert level 4" lockdown, and then from 1 June to 17 August to "Alert level 3," with more economic sectors reopening at each stage. Following a second wave of infections during November 2020 to January 2021, the country is at level 1 lockdown with economic sectors having reopened, and therefore with the need for strict social distancing measures to be heeded (17).

Despite governmental regulations to combat the outbreak, the degree of success in mitigating the spread of COVID-19 is largely dependent on public adherence to social distancing and other behavioural interventions that were regulated. It was estimated that if social distancing among South Africans declined by 2% there could be a 23% increase in cases (18). Further, the South African COVID-19 Modelling Consortium (19) indicated that adherence to social distancing and other public health regulations during the lockdown in the first wave could result in delaying the epidemic peak by 2–3 months, allowing time for the health system to adequately prepare. It is unclear for how long social distancing measures will need to be practised as subsequent waves of COVID-19 may emerge.

Understanding the determinants of adherence to social distancing will inform targeted and tailored public health interventions to address the behaviour. Studies have shown that transmission reducing behaviours like social distancing during infectious disease outbreaks were influenced by increased risk perceptions (6, 20, 21), self-efficacy to implement these behaviours (21, 22) and high knowledge about prevention and transmission (23–26). In addition, gender, older age, income and education were also associated with adherence to social distancing (22, 25, 27–29). However, evidence from low- and middle-income countries (LMICs) is scarce. COVID-19 research priorities need to factor in social determinants of health, as compliance with distancing behaviours are challenging for individuals with adverse social determinants such as crowded living conditions (30). Notably, a large proportion of South Africans live in crowded settlements where large numbers of people live in small homes and families share water and sanitation services.

Using data from a nationwide population-based survey, this study investigates the degree to which South Africans complied with social distancing during the country's COVID-19 lockdown, as measured by the number of people that they came into close contact with the last time they were outside their homes. It further identifies the socio-demographic, psychosocial and

household environmental determinants associated with being in contact with 1–10, 11–50 and more than 50 people, where contact with more than 50 people is indicative of crowding. We hypothesised that participants from lower socioeconomic environments, from households with large numbers of people and who use public transport would report being in contact with larger numbers of people. At the time of writing this paper, the role of psychosocial and environmental factors on the number of close contacts had not been previously assessed in South Africa. The findings will allow us to confirm if the patterns of association with knowledge, self-efficacy, risk perception and demographic factors found in studies from high income countries persist in South Africa. The findings can inform public health and policy directives to improve adherence to social distancing.

MATERIALS AND METHODS

A rapid online survey, supplemented with telephone facilitated interviews, was conducted during the COVID-19 lockdown in South Africa. South African adults in all nine provinces were eligible to participate. The invitation links to participate in the survey were widely distributed on a data-free mobile messaging platform and via numerous communication and media channels, including social media, national and local radio, national television news, email, local websites and a wide network of strategic partners in government, education, faith-based and community organisations, non-profit organisations and the private sector. All participants were encouraged to share the survey link. The data-free mobile messaging platform allowed participants to complete the survey without incurring data costs. The platform was chosen because it has a large user-base of more than four million South Africans and can be downloaded from all application stores. The online questionnaire and telephonic interviews were available in English, Afrikaans, Sepedi, isiZulu, and isiXhosa.

Telephone interviews supplemented the online survey approach, to include participants that may not have responded to an online survey. Interviewers were trained by the research team in obtaining informed consent and telephone interview procedures. The team of interviewers were collectively fluent in the five languages in which the survey could be completed. A list of telephone numbers of over one million people in predominantly densely populated areas such as informal settlements and townships (urban residential settlements) was used to recruit participants in the telephone survey, where 3,602 people from the list were telephonically contacted and 2,682 participated.

The questionnaire was developed in consultation with epidemiologists, behavioural and public health scientists. Discussions were held to identify key thematic areas that would provide insight into the attitudes and behaviours among the general South African population. Questionnaire development occurred as South Africa begun its lockdown and when very little was known about COVID-19 local transmission in the country. Questionnaire development was informed by previous work on public reactions to the pandemic (31, 32) and from a South African survey conducted a few weeks prior (33). Consultations with stakeholders in scientific and civil society networks were used to further refine the questionnaire. The thematic areas identified and included in the questionnaire were demographic and household characteristics, knowledge about COVID-19 and preventative measures, public concerns about the pandemic, self-efficacy about the ability to protect oneself from infection, risk perception, personal experience with testing and screening, attitudes toward lockdown measures, travel behaviour, physical distancing, access to essentials like food, water, sanitation, healthcare and chronic medicines, and the socio-economic impact of the lockdown measures. Five-point Likert scales were used for the questions on self-efficacy, risk perception and socio-economic impact. The questionnaire comprised 55 items of which 54 were close ended. The survey was conducted during 8–29 April 2020. The online survey was available to complete during 8–24 April and the telephone interviews were conducted during 8–29 April, as telephone data collections required a longer time to complete. The survey period corresponded to the 2nd−4th week (12th−33rd day) of the state-implemented "Alert level 5" lockdown period.

Ethical Procedures

Ethical approval to conduct the study was received from the Human Sciences Research Council Research Ethics Committee (HSRC REC) (Protocol number: REC 5/03/20), which is aligned with the principles expressed in the Declaration of Helsinki. Informed consent was obtained from participants before they were directed to the survey questions. Participants were informed of voluntary participation, the anonymity of their responses, and the option to withdraw from the survey at any time.

Measures

The dependent variable was derived from the question "The last time you were away from home, how many people did you come into close contact with? (within 2 metres)," and the response had 6 options (have not left home, 1–3, 4–10, 11–20, 21–50, and more than 50 people).

The independent variables were classified into socio-demographic variables, psychosocial determinants of behaviour, household environmental and living conditions and economic capability. The questions analysed in the current paper are presented in **Supplementary Table 1**. The socio-demographic variables considered were gender, age, population group, residential community type, education and employment, where population group was reported in consistency with Statistics South Africa's standard classification categories (34). Variables measuring psychosocial determinants of behaviour were knowledge about behaviours to prevent COVID-19 transmission, feeling that the lockdown was unnecessary and being angered by it, self-efficacy in protecting oneself from infection, and risk perception of becoming infected. Self-efficacy in protecting oneself from infection was evaluated by participants' agreement or disagreement with the statement "I am confident that I can prevent myself from getting COVID-19." Risk perception was assessed by a single item asking participants to rate their level of personal risk of becoming infected with the virus, with

five response options ranging from very high risk to very low risk. The two knowledge items were assessed by participants affirmative responses to the statements "I can prevent myself from becoming infected with the Coronavirus (COVID-19) by staying away from people who are infected" and "I can prevent myself from becoming infected with the Coronavirus (COVID-19) by staying 2 metres away from another person," with "Yes" or "No" response options. Agreement with the statement "The lockdown was unnecessary and has made me angry" assessed feelings about the lockdown. The variables related to household environmental conditions were whether participants lived in a household that shared water facilities with other households, the number of household members, and access to food during the lockdown. Economic capability referred to the perceived financial difficulty as a result of the COVID-19 lockdown. It was calculated as a sum score of four items, related to feeling that the lockdown was making it difficult to earn their income; to keep their job; would make it difficult to feed their family; and to pay their bills or debts. Each item was measured on a 5-point Likert scale, with options ranging from 1 (strongly disagree) to 5 (strongly agree). The Cronbach's alpha for the four items was 0.91, demonstrating high inter-item reliability. Lower values of the composite score indicated higher perceived financial difficulty. The sum score was grouped into high, moderate and low using the 25th and 75th percentiles. The selection of independent variables was informed by the literature, the Health Belief Model (35) and the Social Determinants of Health (36).

Statistical Analysis

The data were benchmarked using the South African adult mid-year population estimates by age, race, sex, and province (34) to increase generalizability of the estimates to a national level. All analyses were conducted in Stata 15.0 (Stata Corporation, College Station, Texas, USA). The "svy" command was used to incorporate benchmarking weights into the analysis. Summary statistics were used to describe the characteristics of the sample. Pearson Chi-square tests were used to detect significant differences in estimates of categorical variables.

Preliminary cross-tabulations and multinomial regressions showed that for the majority of independent variables, the patterns of association (the relative risk ratios) for coming into contact with 1–3 people were similar to those of coming into contact with 4–10 people. A similar finding was observed between the categories of 11–20 and 21–50 people. The response options for close contacts were therefore recoded into four categories; 1–10 people, 11–50 and >50 and did not leave home, in order to facilitate meaningful comparisons across a smaller number of categories.

A multivariate multinomial logistic regression analysis was used to determine factors associated with the numbers of people that participants came into close contact with, where "None/did not leave home" was used as the reference category. All independent variables that had a significant univariate association with the outcome variable, as measured by the Chi-square tests, were used in the multivariate multinomial model. Odds ratios with 95% confidence intervals (CIs) were used to assess the strength and direction of the associations. All statistical tests were considered significant at a p-value < 0.05.

RESULTS

Characteristics of the Weighted Sample

Table 1 shows that of the sample of 17 563 individuals, more than half were female (53%); 70.1% were 25–59 years old; the majority were African (77.9%) and had matric (grade 12) or higher-level education (79.6%); 36.6% had full time employment, 37.2% were unemployed, 33.9% resided in townships and 21% lived in rural traditional tribal areas.

More than 95% reported correct knowledge that staying away from infected people as well as maintaining a 2-metre distance between other people can prevent COVID-19 (**Table 1**). A quarter (25.6%) perceived themselves at high risk of infection, 82.6% reported self-efficacy in protecting themselves from COVID-19 infection, and 11.7% felt that the lockdown was unnecessary and had made them angry. Just under one quarter (23.2%) did not have enough money to buy food during lockdown and over one quarter (26.8%) had perceived financial difficulty during lockdown. The average household size was 4.8 people and 26.6% lived in households that shared their water sources with other households.

Numbers of Close Contacts

Table 2 shows that a fifth of participants (20.3%, 95% CI: 19.4–21.2) reported not being within a 2-metre distance from anyone because they had not left home, 50.6% (49.5–51.7) had come into close physical distance with 1–10 people on the last occasion that they were away from home, 21.1% (20.2–22.0) with 11–50 people, and 8.0% (7.4–8.6) with more than 50 people.

The number of people that participants were in close distance with varied significantly with all the independent variables. The percentage who stayed home and did not come into close distance with others was higher among residents of rural traditional communities (31.3%), Africans (22.5%), participants who did not complete secondary school (45.4%), unemployed participants (28.1%), those with low risk perceptions (25.9%) and those who reported being unable to afford food during the lockdown (28.4%). Having been in close proximity with >50 people was highest among residents of informal settlements, rural traditional areas, and townships; among those with incorrect knowledge about social distancing as a preventive measure, and among those with low self-efficacy to protect themselves from infection.

Factors Associated With Close Contacts

In **Figure 1** we summarise the key findings presented in **Table 3**. The analyses highlight 8 broad indicators within demographic, psychosocial, household living conditions and economic capabilities as being associated with numbers of close contacts.

Characteristics of Those Who Came Into Close Proximity With 1–10 People

The odds of coming into close proximity with 1–10 people outside the home compared to having not come into contact

TABLE 1 | Characteristics of the study sample.

Variables	Total	%	95% CI
Socio-demographic			
Gender			
Female	10,769	53.0	51.9–54.1
Male	6,614	47.0	45.9–48.1
Other	180	<0.1	0.0–0.0
Age (years) (Mean, S.E.)		40.3	0.21
18–24	2,850	15.2	14.5–15.8
25–59	13,160	70.1	69.0–71.2
60–69	1,107	8.7	8.0–9.5
≥70	341	6.0	5.2–7.0
Population group			
African	8,689	77.9	77.2–78.5
White	6,018	10.4	10.1–10.8
Coloured	1,877	8.6	8.2–9.1
Indian/Asian	979	3.1	2.8–3.3
Residential community			
City	2,433	10.2	9.6–10.8
Suburb	7,263	28.5	27.5–29.4
Township	4,072	33.9	32.9–34.9
Informal settlement	622	4.3	4.0–4.8
Rural (Traditional tribal area)	2,548	21.0	20.2–22.0
Farm	625	2.1	1.8–2.3
Highest education level			
Less than secondary	557	6.0	5.4–6.6
Secondary	2,301	14.4	13.6–15.1
Matric or higher	14,705	79.6	78.7–80.5
Employment			
Employed full time	7,152	36.6	35.6–37.6
Employed informally/part time	1,674	9.8	9.2–10.4
Student	1,418	7.9	7.4–8.4
Unemployed	5,387	37.2	36.1–38.3
Self employed	1,932	8.6	8.0–9.2
Psychosocial determinants of behaviour			
Correct knowledge that staying away from people who are infected can prevent COVID-19 infection	16,752	96.8	96.4–97.2
Correct knowledge that staying 2 metres away from another person can prevent COVID-19 infection	16,521	95.6	95.1–96.0
The lockdown was unnecessary and has made me angry	1,619	11.7	11.0–12.5
Self-efficacy - I am confident in preventing myself from getting COVID-19			
Agree	13,626	82.6	81.8–83.4
Neutral	2,901	13.7	13.0–14.5
Disagree	897	3.6	3.3–4.0
Risk perception of becoming infected			
Low	8,127	45.0	44.0–46.1
Moderate	5,570	29.3	28.4–30.3
High	3,866	25.6	24.7–26.6
Household environment and living conditions			
Ability to get food to your household during the lockdown			
We can buy from a shop within walking distance from my house	4,148	25.7	24.8–26.6
We can buy from a shop, which I reach using a taxi/bus	1,949	15.3	14.5–16.1
We can buy from a shop, which I reach using my car	7,922	35.8	34.8–36.9
Do not have enough money to buy food during the lockdown	3,508	23.2	22.4–24.1

(Continued)

TABLE 1 | Continued

Variables	Total	%	95% CI
Share water facilities with other households	4,074	26.6	25.6–27.5
Household size (Mean, S.E., range)	4.8	0.03	1–20
Economic capability			
Perceived COVID-19 related financial difficulty			
High	4,006	26.8	25.8–27.8
Moderate	7,414	49.0	47.8–50.2
Low	3,382	24.2	23.1–25.3

with anyone were significantly higher among males than females (AOR = 1.37, 95% CI: 1.18–1.58); White [AOR = 1.86 (1.41–2.47)] and Coloured [AOR = 1.69 (1.33–2.15)] than African population groups; participants with secondary school education and who completed secondary school [AOR = 1.51 (1.03–2.21) and AOR = 2.35 (1.64–3.37)] than those with less than secondary school education; full-time employees, self-employed participants and those employed informally or part-time than unemployed participants [AOR = 1.53 (1.27–1.84), AOR = 1.32 (1.01–1.71) and AOR = 1.44 (1.14–1.82), respectively]; participants who were unsure about their self-efficacy in protecting themselves from infection than those with high self-efficacy [AOR = 1.78 (1.39–2.28)]; participants with moderate and high risk perception than low risk perception [AOR = 1.55 (1.28–1.88) and AOR = 1.21 (1.01–1.45)]; and participants who travelled to shops by their own car [AOR = 1.27 (1.04–1.54)] than by walking there.

The odds of coming into close proximity with 1–10 people compared to having not come into contact with anyone were significantly lower for 25–59 year olds [AOR = 0.68 (0.56–0.83)] and 60–69 year olds [AOR = 0.58 (0.40–0.84)] than younger people aged 18–24 years; residents of rural traditional areas [AOR = 0.57 (0.42–0.76)] than city dwellers; and participants who were unable to afford food during the lockdown than those who walked to shops to buy food [AOR = 0.78 (0.64–0.95)].

Characteristics of Those Who Came Into Close Proximity With 11–50 People

The odds of coming into close contact with 11–50 people was significantly higher for participants with secondary school education [AOR = 1.63 (1.04–2.56)] and who completed secondary school [AOR = 2.37 (1.52–3.70)] than those with less than secondary school education; full-time employees, self-employed participants and those employed informally or part-time [AOR = 1.84 (1.47–2.29), AOR = 1.48 (1.08–2.04), and AOR = 1.54 (1.18–2.02), respectively]; participants who were unsure or disagreed about having self-efficacy to protect themselves from infection than those with high self-efficacy [AOR = 2.50 (1.92–3.26) and AOR = 1.83 (1.17–2.85)]; participants with moderate and high risk perception than low risk perception [AOR = 2.02 (1.63–2.52) and AOR = 1.58 (1.28–1.94), respectively]; participants who travelled to shops by public transport [AOR = 1.33 (1.03–1.72)] or by their own car (AOR = 1.51 (1.20–1.90)] than those who travelled to shops by walking; and participants who lived in households that share water facilities with other households [AOR = 1.22 (1.00–1.47)]. The odds of coming into close contact with 11–50 people was significantly lower for participants who were unable to afford food during the lockdown [AOR = 0.73 (0.58–0.92)] than those who travelled to shops by walking.

Characteristics of Those Who Came Into Close Proximity With >50 People

The odds of coming into close contact with >50 people compared with having not come into contact with anyone was significantly higher for participants who completed secondary school than those with less than secondary school education [AOR = 1.68 (1.00–2.81)]; full-time employees, self-employed participants and those employed informally or part-time than unemployed participants [AOR = 2.59 (1.94–3.47), AOR = 2.14 (1.38–3.32), and AOR = 1.83 (1.28–2.61), respectively]; those with incorrect knowledge that staying away from infected people was a preventive measure [AOR = 2.11 (1.12–3.97)]; those with incorrect knowledge that staying 2 metres away from other people was a preventive measure [AOR = 1.91 (1.13–3.25)]; participants who were unsure or disagreed about having self-efficacy to protect themselves from becoming infected than those with high self-efficacy [AOR = 2.4 (1.74–3.31) and AOR = 2.39 (1.47–3.9)]; participants with moderate and high risk perception than low risk perception [AOR = 1.87 (1.41–2.48) and AOR = 2.72 (2.10–3.52)]; participants who travelled to shops by public transport [AOR = 1.83 (1.33–2.53)] or by their own car [AOR = 1.42 (1.04–1.95)] instead of walking; and those with higher household sizes [AOR = 1.04 (1–1.08)]. The odds of coming into close contact with >50 people were significantly lower for participants with high perceived financial difficulty as a result of the lockdown than those with low financial difficulty [AOR = 0.71 (0.51–0.98)].

DISCUSSION

The study provides evidence on the extent of social distancing, assessed by the number of people that participants came within close proximity to, on the last occasion that they were outside their homes, during the early days of the COVID-19 lockdown in South Africa. It provides the first evidence of psychosocial and environmental determinants associated with social distancing in the country. A fifth of South Africans reported that they were

TABLE 2 | The number of close contacts when outside the home by socio-demographic characteristics and behavioural determinants.

	Number of people in close contact with (within a 2-metre distance) the last time the participant was away from home								
	Did not leave home		1–10 people		11–50 people		>50 people		
	%	95% CI	%	95% CI	%	95% CI	%	95% CI	*p*-value
Total	20.3	(19.4–21.2)	50.6	(49.5–51.7)	21.1	(20.2–22.0)	8.0	(7.4–8.6)	
Socio-demographic									
Gender									
Female	21.5	(20.3–22.7)	48.8	(47.4–50.1)	21.3	(20.2–22.5)	8.4	(7.6–9.3)	0.004
Male	19.0	(17.6–20.4)	52.7	(51.0–54.4)	20.7	(19.3–22.2)	7.6	(6.8–8.5)	
Other	17.8	(12.9–24.1)	55.6	(48.2–62.6)	21.1	(15.8–27.7)	5.6	(3.0–10.0)	
Age group (years)									
18–24	20.6	(18.9–22.5)	53.7	(51.5–56.0)	18.3	(16.6–20.1)	7.3	(6.3–8.6)	0.021
25–59	19.2	(18.3–20.2)	50.5	(49.4–51.7)	21.7	(20.8–22.7)	8.5	(7.9–9.2)	
60–69	22.6	(18.9–26.9)	48.7	(44.0–53.4)	22.3	(18.4–26.7)	6.4	(4.5–9.1)	
≥70	28.7	(22.2–36.3)	46.2	(38.6–54.1)	18.6	(12.5–26.7)	6.5	(3.4–11.9)	
Population group									
African	22.5	(21.4–23.6)	46.8	(45.5–48.1)	21.6	(20.5–22.8)	9.1	(8.3–9.8)	<0.001
White	10.8	(9.7–12.0)	68.1	(66.4–69.7)	18.2	(16.9–19.5)	3.0	(2.5–3.6)	
Coloured	14.2	(12.6–16.1)	62.3	(59.7–64.9)	17.9	(15.9–20.0)	5.6	(4.5–6.8)	
Indian/Asian	13.8	(11.4–16.7)	55.1	(51.3–58.9)	25.3	(22.1–28.8)	5.7	(4.4–7.4)	
Community of residence									
City	16.1	(13.8–18.8)	60.4	(57.3–63.4)	17.7	(15.5–20.2)	5.7	(4.4–7.4)	<0.001
Suburb	13.0	(11.6–14.5)	60.4	(58.4–62.3)	21.5	(19.9–23.1)	5.2	(4.4–6.1)	
Township	21.1	(19.6–22.6)	47.2	(45.4–49.0)	22.1	(20.6–23.8)	9.6	(8.5–10.8)	
Informal settlement	19.8	(16.4–23.8)	49.0	(44.4–53.6)	20.1	(16.8–23.8)	11.1	(7.9–15.3)	
Rural (Traditional tribal area)	31.3	(28.9–33.8)	37.7	(35.1–40.3)	20.8	(18.6–23.2)	10.2	(8.9–11.7)	
Farm	17.6	(13.4–22.7)	59.7	(53.4–65.7)	18.6	(14.5–23.6)	4.1	(2.0–8.1)	
Highest educational level									
Less than secondary	45.4	(40.0–50.9)	31.1	(26.0–36.8)	15.2	(11.9–19.2)	8.3	(5.9–11.6)	<0.001
Secondary	27.8	(25.3–30.5)	43.8	(41.1–46.6)	18.3	(16.3–20.6)	10.0	(8.2–12.2)	
Matric or higher	17.1	(16.2–18.0)	53.3	(52.1–54.5)	22.0	(21.0–23.0)	7.6	(7.0–8.3)	
Employment									
Employed full time	13.0	(11.9–14.2)	52.7	(51.1–54.3)	24.6	(23.2–26.1)	9.7	(8.7–10.8)	<0.001
Employed informal/part time	19.1	(16.7–21.7)	50.1	(47.0–53.3)	22.4	(19.9–25.1)	8.4	(6.8–10.2)	
Student	21.6	(19.1–24.4)	49.3	(46.1–52.6)	19.3	(16.9–22.0)	9.7	(7.7–12.2)	
Unemployed	28.1	(26.3–29.9)	47.5	(45.6–49.5)	18.1	(16.5–19.9)	6.3	(5.4–7.3)	
Self employed	18.0	(15.4–20.9)	56.8	(53.2–60.3)	18.5	(15.8–21.6)	6.7	(5.0–8.8)	
Psychosocial factors									
Correct knowledge that staying away from people who are infected can prevent COVID-19 infection									
No	18.1	(13.4–24.1)	40.1	(34.2–46.4)	25.4	(20.4–31.2)	16.3	(11.8–22.2)	<0.001
Yes	20.2	(19.3–21.1)	51.0	(49.9–52.1)	21.1	(20.2–22.0)	7.8	(7.2–8.4)	
Correct knowledge that staying 2 metres away from another person can prevent COVID-19 infection									
No	16.4	(12.9–20.7)	47.7	(42.5–52.9)	22.7	(18.9–26.9)	13.2	(10.2–17.0)	0.001
Yes	20.1	(19.2–21.1)	50.9	(49.8–52.0)	21.2	(20.2–22.1)	7.8	(7.2–8.4)	
Thought that the lockdown was unnecessary and were angered by it									
No	18.9	(17.9–19.9)	51.5	(50.2–52.7)	22.1	(21.0–23.2)	7.6	(7.0–8.3)	<0.001
Yes	28.8	(25.7–32.1)	42.6	(39.4–46.0)	17.2	(15.0–19.7)	11.4	(9.5–13.6)	
Self-efficacy in preventing one's self from COVID-19 infection									
Agree	22.4	(21.4–23.5)	50.9	(49.7–52.1)	19.4	(18.4–20.4)	7.3	(6.7–8.0)	<0.001

(Continued)

TABLE 2 | Continued

	Number of people in close contact with (within a 2-metre distance) the last time the participant was away from home								
	Did not leave home		1–10 people		11–50 people		>50 people		
	%	95% CI	%	95% CI	%	95% CI	%	95% CI	*p*-value
Unsure	9.8	(8.3–11.5)	50.2	(47.2–53.1)	29.6	(26.8–32.5)	10.4	(8.8–12.3)	
Disagree	11.2	(8.3–14.8)	46.9	(42.0–51.9)	27.2	(23.0–32.0)	14.7	(11.4–18.7)	
Risk perception									
Low	25.9	(24.5–27.3)	50.8	(49.2–52.4)	17.9	(16.6–19.3)	5.4	(4.8–6.1)	<0.001
Moderate	13.6	(12.1–15.2)	54.1	(52.2–56.1)	25.2	(23.5–27.0)	7.1	(6.1–8.2)	
High	18.2	(16.6–20.0)	46.3	(44.1–48.5)	21.8	(20.1–23.5)	13.8	(12.2–15.4)	
Environmental /household living conditions									
Ability to get food to your household during the lockdown									
Can buy from a shop, which I reach using my car	13.6	(12.2–15.0)	55.5	(53.6–57.4)	24.1	(22.4–25.9)	6.8	(5.9–7.8)	
Can buy from a shop within walking distance from my house	21.1	(19.3–22.9)	52.5	(50.4–54.5)	19.3	(17.8–21.0)	7.2	(6.1–8.4)	<0.001
Can buy from a shop, which I reach using a taxi/bus (public transport)	22.1	(19.8–24.5)	41.9	(39.2–44.7)	22.9	(20.6–25.3)	13.1	(11.3–15.2)	
Do not have enough money to buy food during the lockdown	28.4	(26.6–30.3)	47.1	(45.0–49.1)	16.9	(15.5–18.5)	7.6	(6.6–8.7)	
Do you share water facilities with other households									
Yes	22.2	(20.5–23.9)	47.6	(45.6–49.7)	21.0	(19.4–22.7)	9.2	(8.1–10.5)	0.001
No	19.6	(18.6–20.7)	51.7	(50.5–53.0)	21.1	(20.0–22.2)	7.6	(6.9–8.3)	
Household size									
1–5 people	19.5	(18.5–20.5)	52.3	(51.0–53.6)	20.9	(19.8–22.0)	7.4	(6.7–8.1)	<0.001
6–10 people	21.9	(20.0–24.0)	47.6	(45.5–49.8)	21.7	(19.9–23.6)	8.8	(7.6–10.1)	
≥10 people	22.3	(18.6–26.5)	42.7	(38.2–47.3)	21.8	(18.3–25.8)	13.2	(9.9–17.3)	
Economic capability									
Perceived COVID-19 related financial difficulty									
High	22.2	(20.5–24.1)	51.2	(49.1–53.3)	18.9	(17.4–20.5)	7.7	(6.7–8.9)	<0.001
Moderate	20.0	(18.6–21.5)	51.2	(49.5–52.9)	21.1	(19.6–22.6)	7.7	(6.9–8.7)	
Low	16.6	(14.6–18.8)	47.8	(45.1–50.4)	26.2	(23.9–28.7)	9.4	(8.0–11.1)	

not in close proximity (within 2-metres) to others outside their homes because they had not left home. More than half were in close proximity to up to 10 people and 29% said they were in close proximity to more than 10 people. The findings provide perspective on the effectiveness of state implemented lockdown orders, which have thus far been dominated by studies in high income countries (9, 37). Abrupt social distancing regulations caught many people off guard, as there were just under 5,000 cases reported between 4th March and 27th April 2020, at the time of the lockdown enforcement (38).

The findings must be interpreted within the context of the South African lockdown where all citizens were required to stay at home, unless they needed to leave home to access essential services (39). Essential service providers and public transport were mandated to implement protocols that ensured that their patrons kept at least one square metre apart and reduce 50% of their capacity, respectively. The survey did not ask about the types of activities the participants were engaged in or request the reasons for coming into close contact with people outside their homes.

However, a review of Google's COVID-19 Community Mobility Reports, which tracked how visits and duration of time spent at various locations changed over time among users of Google Maps, provides some insight. These reports indicated that during the lockdown there was over 70% reduction in mobility in retail and recreation locations and in transit stations, over 60% reduction in workplace mobility but only a reduction of 40% in grocery and pharmacy locations (40). Larger reductions in mobility were observed in the Western Cape, Gauteng and Kwa-Zulu Natal provinces, which have large industrial urban areas, larger economies and had greater numbers of cases (41). Similarly, another national study in July 2020 found that while many South Africans reported avoiding large groups, only 25% practised physical distancing (42).

At the time of the survey, face masks were newly introduced and only became compulsory to be worn in public on 29 April 2020 (43). Our study did not ask participants if they had worn a mask when outside their homes. Visits to essential service providers such as grocery stores and pharmacies increase the chances of close proximity to people, depending on the environments in which these activities occur. For example, it is likely that travelling to, queuing outside and shopping in a crowded township mall would result in contact with more people than at a quieter suburban shopping centre. However, participants who were in close proximity of over 50 people can be seen as having not avoided, or been able to avoid, crowds.

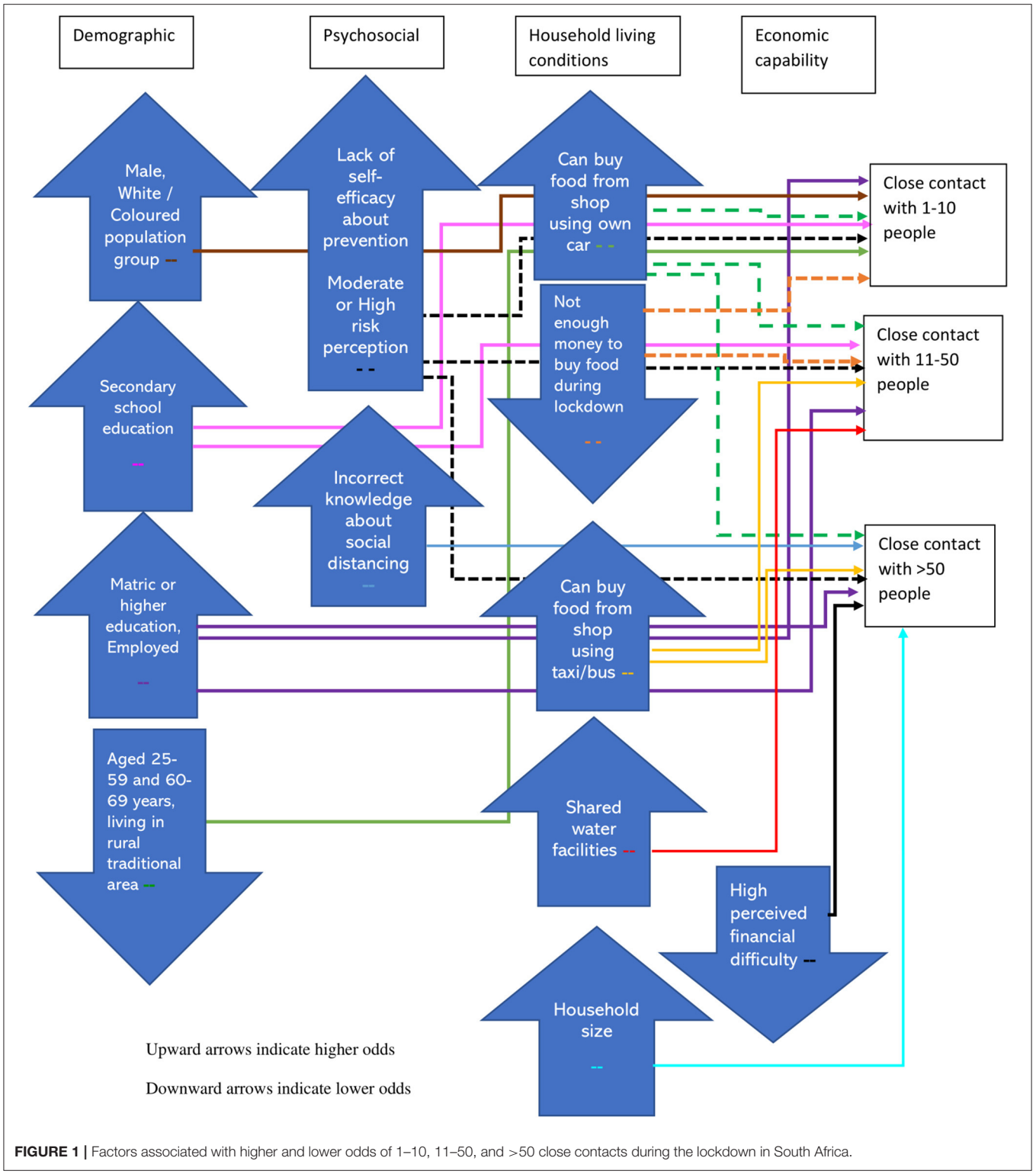

FIGURE 1 | Factors associated with higher and lower odds of 1–10, 11–50, and >50 close contacts during the lockdown in South Africa.

These individuals were least compliant with social distancing regulations, and could have contributed to the rapid community transmission rates.

It is, therefore, within this context, that differing patterns and strengths of association with the determinant variables were observed for those who came into contact with smaller numbers of people vs. those who were in contact were in contact with large numbers of people. Murphy et al. (44) show that various factors are linked to compliance with laws affecting freedom of movement. However, Bish and Michie (45) found few studies

TABLE 3 | Multiple multinomial regression showing factors associated with being in contact with 1–10, 11–50 and >50 people when outside the home.

	1–10 people		11–50 people		>50 people	
	OR	**95% CI(OR)**	**OR**	**95% CI(OR)**	**OR**	**95% CI(OR)**
Did not leave home (base category)						
Sociodemographic variables						
Gender						
Female	ref	–	ref	–	ref	–
Male	1.37**	(1.18–1.58)	1.15	(0.96–1.37)	0.99	(0.8–1.24)
Other	1.01	(0.61–1.67)	1.18	(0.66–2.09)	1.04	(0.46–2.32)
Age group (years)						
18–24	ref	–	ref	–	ref	–
25–59	0.68**	(0.56–0.83)	0.82	(0.65–1.03)	0.79	(0.58–1.08)
60–69	0.58*	(0.4–0.84)	0.89	(0.58–1.36)	0.77	(0.43–1.37)
≥70	0.56	(0.32–1.01)	0.79	(0.38–1.65)	0.61	(0.23–1.63)
Population group						
African	ref	–	ref	–	ref	–
White	1.86**	(1.41–2.47)	1.13	(0.82–1.55)	0.74	(0.5–1.09)
Coloured	1.69**	(1.33–2.15)	1.2	(0.91–1.59)	0.88	(0.6–1.29)
Indian/Asian	0.99	(0.72–1.36)	1.08	(0.76–1.54)	0.91	(0.59–1.42)
Community of residence						
City	ref	–	ref	–	ref	–
Suburb	1.15	(0.89–1.49)	1.33	(0.98–1.8)	0.99	(0.65–1.5)
Township	0.8	(0.62–1.04)	1.3	(0.95–1.79)	1.29	(0.85–1.95)
Informal settlement	0.87	(0.6–1.26)	1.18	(0.75–1.84)	1.54	(0.82–2.88)
Rural (Traditional tribal area)	0.57**	(0.42–0.76)	1.06	(0.74–1.53)	1.22	(0.78–1.91)
Farm	0.99	(0.59–1.67)	1.25	(0.71–2.2)	0.85	(0.31–2.35)
Highest educational level						
Less than secondary	ref	–	ref	–	ref	–
Secondary	1.51*	(1.03–2.21)	1.63*	(1.04–2.56)	1.56	(0.86–2.82)
Matric or higher	2.35**	(1.64–3.37)	2.37**	(1.52–3.7)	1.68*	(1–2.81)
Employment						
Unemployed	ref	–	ref	–	ref	–
Employed full time	1.53**	(1.27–1.84)	1.84**	(1.47–2.29)	2.59**	(1.94–3.47)
Employed informal/part time	1.44*	(1.14–1.82)	1.54*	(1.18–2.02)	1.83*	(1.28–2.61)
Student	0.89	(0.69–1.15)	1.03	(0.76–1.39)	1.49	(0.98–2.28)
Self employed	1.32*	(1.01–1.71)	1.48*	(1.08–2.04)	2.14*	(1.38–3.32)
Psychosocial factors						
Incorrect knowledge that staying away from people who are infected can prevent COVID-19 infection	1.15	(0.72–1.84)	1.31	(0.76–2.27)	2.11*	(1.12–3.97)
Incorrect knowledge that staying 2 metres away from another person can prevent COVID-19 infection	1.35	(0.89–2.04)	1.4	(0.92–2.14)	1.91*	(1.13–3.25)
Thought that the lockdown was unnecessary and were angered by it	0.85	(0.69–1.05)	0.78	(0.61–1.01)	1.23	(0.91–1.65)
Self-efficacy in preventing one's self from COVID-19 infection						
Agree	ref	–	ref	–	ref	–
Unsure	1.78**	(1.39–2.28)	2.5**	(1.92–3.26)	2.40**	(1.74–3.31)
Disagree	1.18	(0.78–1.79)	1.83*	(1.17–2.85)	2.39**	(1.47–3.9)
Risk perception						
Low	ref	–	ref	–	ref	–
Moderate	1.55**	(1.28–1.88)	2.02**	(1.63–2.52)	1.87**	(1.41–2.48)
High	1.21*	(1.01–1.45)	1.58**	(1.28–1.94)	2.72**	(2.1–3.52)
Household living conditions						
Ability to easily get food to one's household during the lockdown						
Can buy from a shop within walking distance from my house	ref	–	ref	–	ref	–
Can buy from a shop, which I reach using a taxi/bus (public transport)	1.08	(0.86–1.36)	1.33*	(1.03–1.72)	1.83**	(1.33–2.53)

(Continued)

TABLE 3 | Continued

	1–10 people		11–50 people		>50 people	
	OR	**95% CI(OR)**	**OR**	**95% CI(OR)**	**OR**	**95% CI(OR)**
Can buy from a shop, which I reach using my car	1.27*	(1.04–1.54)	1.51**	(1.2–1.9)	1.42*	(1.04–1.95)
Do not have enough money to buy food during the lockdown	0.78*	(0.64–0.95)	0.73*	(0.58–0.92)	0.91	(0.66–1.25)
Share water facilities with other households	1.12	(0.95–1.32)	1.22*	(1–1.47)	1.11	(0.87–1.42)
Household size	0.99	(0.96–1.02)	1.02	(0.99–1.05)	1.04*	(1–1.08)
Economic capability						
Perceived COVID-19 related financial difficulty						
Low	ref	–	ref	–	ref	–
Moderate	1.1	(0.89–1.37)	0.82	(0.64–1.05)	0.78	(0.58–1.05)
High	1.08	(0.86–1.38)	0.79	(0.61–1.03)	0.71*	(0.51–0.98)

*$p < 0.05$, **$p < 0.001$.

aimed at understanding avoidant behaviours, in their review of demographic and attitudinal determinants of behaviours during a pandemic.

Male gender, younger age and being in the White and Coloured population groups were associated with being in contact with 1–10 people and not with larger numbers of contacts. Notably, larger household size and a lack of knowledge about the importance of social distancing were associated with being in contact with more than 50 people. Employment, having at least secondary school education, lack of self-efficacy in being able to protect oneself from infection, and moderate or high risk perception of becoming infected, were all linked to increased odds of close contact with other people, where there were signs of a dose response relationship, in that the strengths of associations were higher for having >50 and 11–50 close contacts than with having 1–10 contacts.

Additionally, people who could travel to shops using their own vehicles were more likely to be in contact with others relative to remaining at home, and those who travelled by minibus taxis or other public transport were more likely to be in contact with over 10 people. Individuals who shared water facilities with other households, such as communal taps and water tanks, came into contact with 11–50 people more often than those with their own water facilities.

In agreement with the finding that males came into contact with 1–10 people more often than females, other studies have shown that men were less likely to comply with public health precautions, including hand washing and social distancing (46–48). The lower adherence to preventive measures among men may be explained by socially constructed behaviours relating to masculinity, such as masking of fear and the tendency to downplay risk (45, 49). Gender differences in labour market participation, work arrangements and household roles also determine the extent of being able to stay at home during lockdowns (50). In congruence with current findings on age effects, studies in the United States and Germany also reported less social distancing among young people (27, 51). Youth tend to have more social contacts than older people (52, 53) and in sub-Saharan African countries large multigenerational households can increase risk transmission between young and old (29). Understanding young people's motivating factors for engaging in social distancing, such as increased social responsibility (54), will inform strategies to increase social distancing among youth.

Other studies also found lower engagement in COVID-19 preventive behaviours, including social distancing, among individuals with low self-efficacy and low knowledge about preventive behaviours (26, 55, 56). Individuals with poor knowledge about the role of distancing in preventing infection were twice as likely to have over 50 close contacts, which suggests the need for public health communication to explain the mechanisms of viral transmission and thereby provide a clear rationale for distancing behaviours. Public health communication should enhance self-efficacy by providing practical solutions to perceived barriers of distancing behaviour.

While increased risk perception generally increases protective behaviours (57), in this study, individuals with high risk perceptions had more close contacts. Further research is required to understand this association. Perceived fatalism of COVID-19 has been shown to be associated with lower intention to practise protective behaviours such as social distancing (58). Additionally, coming into close contact with others is not always autonomous but could be dependent on the circumstances that allow for social distancing. Other studies argue that the perceived risk and behaviour relationship cannot be fully examined in cross sectional studies because one's current risk perception can be reflective of their risk behaviours over time (59).

Individuals with secondary or higher levels of education and those who were employed either full time, informally, part-time or self-employed were more likely to have close contact with others, because those with less than secondary school education and the unemployed reported higher rates of staying at home. Individuals with low education have previously been shown to be more compliant with preventive measures during disease outbreaks (60). Employed individuals are more often the breadwinners of the family and would have been expected to go out during lockdown for activities such as grocery shopping or to work in essential services. Self-employed and informal sector employees such as market sellers or maintenance services may have been informally seeking work during lockdown, which increased their likelihood of contact with large numbers

of people. Bish and Michie (45) found the relationship of educational level and avoidant behaviour, such as avoiding large crowds, to be unclear but there was evidence of more educated people complying with avoidant behaviour.

Factors indicative of lower socio-economic living conditions, that is communal water sources, large households, and using public transport to shops were all associated with high numbers of close contacts. Residents of densely populated neighbourhoods and those who use minibus taxis or buses have a higher frequency of close contacts (52) because social distance is constrained in these environments (61–63). It is critical that preventive measures for public transport are adhered to, including disinfecting surfaces in public transport and maintaining safe distances between commuters in minibus taxi rank queues. Contrastingly, individuals who travelled to shops with their own vehicle, which is usually indicative of affluence, were also more likely to have close contacts. Having a private vehicle provides the opportunity for increased mobility, which is in turn leads to increased probability of contacts.

The determinants associated with non-compliance with social distancing can inform the development of tailored health promotion and communication strategies. As differential risks of exposure are considered for preferentially vaccinating individuals, so too should heterogeneity of group risks be considered when designing interventions. Using an intervention mapping approach, health educators can tailor and target health education messages for subgroups of individuals that were less likely to comply with social distancing regulations, such as males, young people, individuals in densely populated areas with shared water sources, the employed, and taxi commuters. Information campaigns need to improve individuals' knowledge of social distancing as a preventive measure, thereby enhancing cooperation to comply with public health advice. Campaigns should reiterate the combined effect of mask wearing, reducing gatherings, distancing and hand hygiene, particularly as fatigue in practising these behaviours sets in. Intervention development efforts need to recognise that distancing behaviours are due to willingness and perceived control but are also dependent on circumstantial feasibility of distancing. From a policy perspective, enabling environments therefore need to be created to enhance individuals' self-efficacy to protect themselves from infection and promote social distancing. Measures being implemented in several countries include home delivery of essential services, chronic medication, food parcels and social grants; temporary sites for people unable to quarantine at home (29); and enforcement of 1–2 metre distance marks in queues, shops and transit stations. This is particularly relevant because many South Africans, particularly pensioners, waited in long queues to collect social grants or food parcels during lockdown. Other policy directives include investing in better infrastructure such as sanitation, water, housing, and ventilation, as well as better infrastructure for public transport. Regulations for public transport need to be reviewed and enforced, including disinfecting surfaces and distancing protocols for commuters. Distancing and infection control protocols need to be strengthened and enforced in workplaces and areas where informal sector work is common, like streets with street vendors. Given that South Africa's second epidemic wave emerged after public events and mass gatherings during the festive season in December 2020, the regulations for gatherings needs to be reviewed and enforced.

Health communication needs to include simplified and language-appropriate targeted messages to change health behaviour and social norms, increase public accountability, and guide individuals in crowded living conditions on how to effectively social distance when outside their homes. Interventions that are community-led are more likely to increase public support for and adoption of social distancing. Notably, ways to maintain social connexion should be considered when promoting social distancing, because distancing behaviours during the pandemic have led to a decline in social connexions that are linked to poor mental health and increased desire for material wealth (64).

Finally, the unemployed, those who were unable to access or afford food and those with highest perceived financial difficulty had the highest prevalence of staying at home. Although these very poor communities adhered to lockdown regulations, they experienced severe economic impacts of the lockdown including loss of income and hunger (65). The unemployed, elderly and uneducated often rely on the economic activity of others in the household, who cannot afford to stay home and waive their means to earn an income. The South African COVID-19 lockdown is viewed as having intensified the country's pre-existing inequalities and inequities (29, 41). Lockdowns should assist those living in financial hardship in terms of service provision, economic enablement, mental health support services, infrastructure to increase living spaces, and health education so that their time spent under lockdown is more manageable.

A limitation of this study is that adherence to social distancing and other behaviours were self-reported, which is subject to recall and social desirability bias. Secondly, online surveys introduce selection bias as individuals who utilise the internet and smart phones are more likely to complete online surveys. To minimise the impact of this limitation, the online surveys were supplemented with telephonic interviews that purposely targeted individuals in poorer and high-density areas, and the data was benchmarked to the general population to increase generalisability of the findings. Thirdly, risk perception and self-efficacy were measured by single items instead of scales. Fourth, other potential mediating psychosocial variables such as social norms, perceived loss of control and perceived safety climate were not measured in this study. Strengths of the study include the rapid online survey method that provides real-time results as the COVID-19 pandemic continues. The study reports on a wide range of determinant variables from a large population-based sample. It highlights the important role of the social determinants of health in social distancing compliance in South Africa.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article can be made available from the lead author upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Sciences Research Council Research Ethics Committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

RS conceptualised the paper, conducted the analysis, and led the writing. MM, IN, and TM contributed to the analysis. SPR conceived the study. ND, NV, MM, SPR, and ASD contributed to interpreting results and drafting the manuscript. All authors provided critical review of the manuscripts draughts and approved the final manuscript before submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.632619/full#supplementary-material

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Clinical Significance of Plasma D-Dimer in COVID-19 Mortality

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It is not clear whether D-dimer can be an independent predictor of coronavirus disease 2019 (COVID-19) mortality, and the cut-off of D-dimer for clinical use remains to be determined. Therefore, a comprehensive analysis is still necessary to illuminate the clinical significance of plasma D-dimer in COVID-19 mortality. We searched PubMed, Embase, Cochrane Library, and Scopus databases until November 2020. STATA software was used for all the statistical analyses. The identifier of systematic review registration was PROSPERO CRD42020220927. A total of 66 studies involving 40,614 COVID-19 patients were included in our meta-analysis. Pooled data showed that patients in high D-dimer group had poor prognosis than those in low D-dimer group [OR = 4.52, 95% CI = (3.61, 5.67), $P < 0.001$; HR = 2.81, 95% CI = (1.85, 4.27), $P < 0.001$]. Sensitivity analysis, pooled data based on different effect models and the Duval and Tweedie trim-and-fill method did not change the conclusions. Subgroup analyses stratified by different countries, cutoffs, sample size, study design, and analysis of OR/HR still keep consistent conclusions. D-dimer was identified as an independent predictor for COVID-19 mortality. A series of values including 0.5 μg/ml, 1 μg/ml, and 2 μg/ml could be determined as cutoff of D-dimer for clinic use. Measurement and monitoring of D-dimer might assist clinicians to take immediate medical actions and predict the prognosis of COVID-19.

Keywords: COVID-19, SARS-CoV-2, D-dimer, independent, cutoff, meta-analysis

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INTRODUCTION

The outbreak and spread of Coronavirus Disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), had caused a pandemic around the world (1). Though most of patients had mild symptoms, a small minority of cases suffered from acute respiratory distress syndrome (ARDS) and even death (2). As of November 28, 2020, about 60 million cases have been reported by world health organization (WHO) and included around 1.5 million deaths globally (1). Worse still, the numbers of death are persistently increasing especially in the United States, the epicenter of COVID-19 (3). Therefore, identification of the independent predictors for COVID-19 mortality is still urgent and necessary to reduce the poor outcomes.

D-dimer, a fibrinogen degradation product, consists of two covalently bound fibrin D domains, which reflect the high coagulation and enhancement of secondary fibrinolytic activity *in vivo* (4, 5). Previous studies demonstrated that D-dimer was associated with the severity of COVID-19 (6–8).

Hyperinflammation and hypoxia-induced injury caused by SARS-CoV-2 infection could cause the dysfunction of endothelial cells and stimulate thrombosis and elevation of D-dimer (9). Elevated D-dimer could cause the formation of pulmonary microthrombus, deep venous thrombosis, and disseminated intravascular coagulopathy, which were associated with the poor prognosis (10–12). Nowadays, increasing studies showed that D-dimer could be used as a predictor for COVID-19 mortality (9, 13). Moreover, numerous review and meta-analyses highlighted the prognostic value of D-dimer in COVID-19 mortality (14–16). However, one of the drawbacks of these analyses was that more attention was paid to D-dimer levels between survivors and non-survivors (17, 18). Actually, the abnormal elevation of D-dimer was more valuable to reflect hemodynamic changes in clinic. In addition, these meta-analyses were based primarily on the studies using univariate analysis, and it was not clear whether D-dimers play an independent role in predicting COVID-19 mortality on admission (14, 19). Another challenge is that the cutoff of D-dimer for clinical use remains to be determined (8). From the above, a comprehensive analysis of all the published studies is

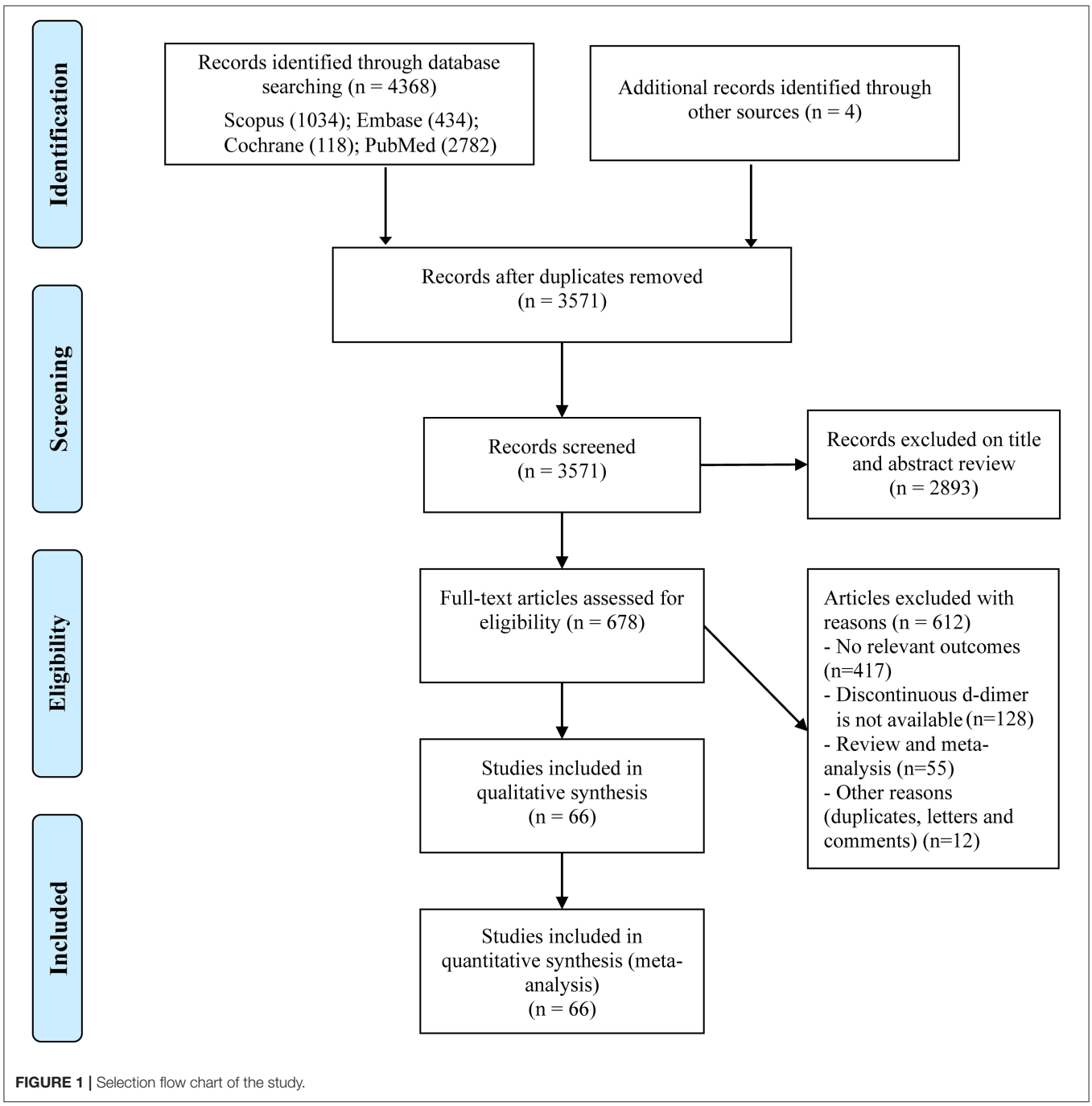

FIGURE 1 | Selection flow chart of the study.

TABLE 1 | Characteristics of eligible studies.

References	Country	Study design	Cases	Age (years)	Sex (male %)	Cutoff (μg/ml)	Variables	NOS scores
Aloisio et al. (20)	Italy	Single-center	427	61.4 ± 17.1	293 (68.6%)	16.28	OR	7
Ayanian et al. (27)	USA	Single-center	299	–	161 (53.8%)	3	OR	7
Bahl et al. (28)	USA	Multi-center	1461	62.0 ± 17.8	770 (52.7)	0.5 1	OR	7
Barman et al. (29)	Turkey	Multi-center	607	59.5 ± 15.6	334 (55.0%)	0.5	OR*	8
Bazzan et al. (30)	Italy	–	88	60.7 ± 12.8	60 (68.1%)	3	OR	6
Berenguer et al. (31)	Spain	Multi-center	4035	68.6 ± 17.8	2433 (61.0%)	0.5	OR	7
Berger et al. (32)	USA	Multi-center	2377	63.3 ± 16.3	1495 (62.8%)	0.23 2	OR*	9
Bhargava et al. (33)	USA	Single-center	419	–	211 (50.4%)	1.5	OR	7
Cao et al. (34)	China	Single-center	102	52.6 ± 22.6	53 (52.0%)	0.5	OR	7
Chen et al. (35)	China	Multi-center	1590	48.3 ± 68.2	904 (57.3%)	0.5	OR	7
Chen et al. (36)	China	Single-center	203	55.1 ± 53	108 (53.2%)	0.5	OR	7
Chen et al. (37)	China	Single-center	274	58.5±19.4	171 (62.4%)	21	OR	7
Chen et al. (38)	China	Single-center	73	65.8 ± 10.1	42 (57.5%)	2.35	OR	7
Chen et al. (39)	China	Multi-center	635	59.9±14.1	318 (50.0%)	0.5	HR	7
Cheng et al. (40)	China	Single-center	305	62.5 ± 14.2	184 (60.3%)	0.845	HR	7
Chilimuri et al. (41)	USA	Single-center	375	62.3 ± 14.9	236 (62.9%)	1	OR*	9
Cortés-Tellés et al. (42)	Mexico	Single-center	200	53.6 ± 17.9	138 (69.0%)	0.7	OR	7
Du et al. (43)	China	Single-center	179	57.6 ± 13.7	97 (54.2%)	0.5	OR	7
Feng et al. (44)	China	Multi-center	476	52.3 ± 17.8	271 (56.9%)	1	HR*	8
Giacomelli et al. (45)	Italy	Single-center	233	–	72 (30.9%)	0.5 1	OR HR*	8
Guisado-Vasco et al. (46)	Spain	Single-center	607	69.0 ± 16.3	394 (65.0%)	2.5	OR*	9
Huang et al. (47)	China	Multi-center	676	54.2 ± 21.5	314 (46.4%)	0.5	OR HR*	8
Laguna-Goya et al. (48)	Spain	Single-center	501	52.0 ± 11.9	317 (63.3%)	1.368	OR	7
Li et al. (50)	China	Multi-center	523	53.4 ± 15.3	275 (52.6%)	1.09	HR*	8
Li et al. (49)	China	Single-center	2068	61.2 ± 14.1	1005 (48.6%)	0.5	OR	7
Li et al. (51)	China	Single-center	102	57.4 ± 18.8	59 (57.8%)	0.5 1	OR	7
Li et al. (52)	China	Single-center	113	67.3 ± 14.1	68(60.2%)	20	OR	7
Li et al. (53)	China	Multi-center	245	51.5 ± 20.1	118 (48.2%)	1	HR*	8
Li et al. (54)	China	Multi-center	132	64.3 ± 10.5	70 (53.0%)	1.5	OR*	8
Liao et al. (55)	China	Multi-center	380	63.3 ± 14.9	206 (54.2%)	2	OR*	9
Liu et al. (56)	China	Single-center	214	67.6 ± 12.7	119 (55.6%)	1	HR*	8
Liu et al. (57)	China	Single-center	1190	57.0 ± 14.8	635 (53.4%)	0.5 1	OR	7
Lu et al. (58)	China	Single-center	20	69.8 ± 12.0	8 (40.0%)	1	OR	7
Luo et al. (59)	China	Single-center	403	54.2 ± 21.6	193 (47.9%)	0.55 5	OR	7
Ma et al. (60)	China	Multi-center	523	43.3 ± 16.4	289 (55.3%)	0.5 1	OR	7
Manocha et al. (61)	USA	Multi-center	446	64.9 ± 15.2	291 (65.2)	6.106 6.99	OR	7
Mikami et al. (62)	USA	Multi-center	2820	65.3 ± 18.1	1611 (57.1%)	2	OR HR*	8
Musoke et al. (63)	USA	Single-center	355	66.2 ± 14.2	181 (51.0%)	1.5	OR*	9
Pan et al. (64)	China	Single-center	124	68.0 ± 10.5	85 (68.5%)	3.06	OR	7
Paranjpe et al. (65)	USA	Multi-center	1078	74.7 ± 58.7	627 (58.1%)	2	OR	7
Peng et al. (66)	China	Multi-center	49	63.0 ± 15.3	32 (65.3%)	0.5	OR	7
Petrilli et al. (67)	USA	Multi-center	2741	62.6 ± 17.1	1678 (61.2%)	2.5	HR	7
Piñana et al. (68)	Spanish	Multi-center	244	56.3 ± 64.1	132 (54.1%)	0.5	OR	7
Qin et al. (69)	China	Single-center	118	63.1 ± 15.7	49 (41.5%)	0.5 1	OR	7
Quintana-Díaz et al. (70)	Spanish	Single-center	3373	62.4 ± 23.0	1725 (48.9%)	0.5	OR*	8
Singh et al. (71)	USA	Single-center	276	61.6 ± 17.1	130 (47.1%)	1.18	OR	7
Song et al. (72)	China	Multi-center	248	63.4 ± 9.7	128 (51.6%)	0.5	OR	7
Tu et al. (73)	China	Single-center	174	53.0 ± 19.5	69 (39.7%)	0.5	OR	7
Volo et al. (74)	Italy	Single-center	23	64.7 ± 33.2	21 (91.3%)	4	OR	7
Wang et al. (75)	China	Single-center	548	58.7 ± 15.7	279 (50.9%)	1	OR	7

(Continued)

TABLE 1 | Continued

References	Country	Study design	Cases	Age (years)	Sex (male %)	Cutoff (μg/ml)	Variables	NOS scores
Wang et al. (76)	China	Single-center	213	60.6 ± 13.4	95 (44.6%)	0.55	OR	7
Wendel Garcia et al. (77)	Switzerland	Multi-center	639	62.3 ± 13.4	447 (75·1%)	1.5	HR*	8
Xia et al. (78)	China	Single-center	81	66.7 ± 11.4	54 (66.7%)	5 21	OR	7
Xie et al. (79)	China	Single-center	140	58.2 ± 15.7	72 (51.4%)	0.45	HR*	8
Xu et al. (80)	China	Multi-center	703	46.1 ± 15.2	382 (54.3%)	0.5	OR	7
Yang et al. (81)	China	Multi-center	203	59.9 ± 14.9	115 (56.7%)	1	OR*	8
Yang et al. (82)	China	Multi-center	205	63.0 ± 10.5	96 (46.8%)	0.5	OR	7
Yao et al. (83)	China	Single-center	108	48.8 ± 15.8	43 (39.8%)	1	OR	7
Yao et al. (84)	China	Single-center	248	63.0 ± 13.4	135 (54.4%)	2	OR*	8
Yu et al. (85)	China	Single-center	1464	61.9 ± 14.8	736 (50.3%)	0.5	OR*	9
Zhang et al. (86)	China	Multi-center	289	55.6 ± 49.2	154 (53.3%)	0.5	OR	7
Zhang et al. (9)	China	Single-center	343	59.5 ± 15.6	169 (49.3%)	0.5 2	OR HR	7
Zhang et al. (87)	China	Multi-center	828	60.6 ± 13.4	447 (53.99%)	1	HR	7
Zhou et al. (13)	China	Multi-center	191	56.4 ± 15.7	119 (62.3%)	0.5 1	OR*	8
Zhou et al. (88)	China	Single-center	67	70.6 ± 6.9	22 (32.8%)	median high	OR*	9
Zhou et al. (89)	China	Single-center	220	58.4 ± 16.4	104 (47.3%)	0.43 1	OR	7

*OR, odds ratio; HR, hazard ratio; * Variables are calculated by multivariable analysis.*

still necessary to illuminate the clinical significance of plasma D-dimer in COVID-19 mortality.

To our knowledge, this is the largest meta-analysis about the association between D-dimer with COVID-19 mortality. Our study will determine its cutoff and highlight the independent prognostic value of D-dimer in COVID-19 mortality to assist clinicians to take immediate medical actions and evaluate the prognosis of COVID-19.

MATERIALS AND METHODS

Search Strategy

Our meta-analysis was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement. The following databases were searched: PubMed, Embase, Cochrane Library, and Scopus databases, from their inception to November 2020. No language restrictions were applied. The search terms were as follows: ("Coronavirus disease 2019" OR "Coronavirus 2019" OR "COVID-19" OR "COVID19" OR "Severe acute respiratory syndrome coronavirus 2" OR "SARS-CoV-2" OR "nCoV-2019" OR "2019-nCoV" OR "Novel coronavirus") AND ("Mortality" OR "Death" OR "Dead" OR "Fatality" OR "Non-survival" OR "Non-survivors" OR "Non-survivor" OR "Prognosis" OR "Deceased") AND ("D-dimer" OR "Laboratory"). Three of the authors (GD, FZ, and YL) independently screened initial records, titles, abstracts, and full text articles. Disagreements were resolved by discussion. In order to avoid missing relevant articles, we also manually reviewed the reference lists of selected retrieved papers as well as the major reviews and meta-analyses. The identifier of systematic review registration was PROSPERO CRD42020220927.

Inclusion and Exclusion Criteria

Any study reporting the relationship between D-dimer and COVID-19 mortality should be included if they met the following criteria: (1) patients were diagnosed as COVID-19; (2) dichotomous D-dimer was available to evaluate the risk of COVID-19 mortality; or (3) odds ratio (OR) or hazard ratio (HR) of the D-dimer was accessible or estimated by the provided data or Kaplan-Meier curves based on the method previously described (20, 21). Exclusion criteria were as follows: (1) patients were asymptomatic carriers of SARS-CoV-2; (2) studies with smaller sample size from the same authors or institutions; and (3) patients or studies did not fulfill the inclusion criteria.

Data Extraction and Quality Assessment

We used Endnote X9 to exclude any duplicate and irrelevant studies in our initial search. We extracted the following basic information: first authors, publication date, country of origin, study design, cases, age, sex, cutoff of D-dimer, OR, HR, and its associated 95% confidence intervals (CI). OR and HR were extracted preferentially from multivariable analysis based on lower cutoff of D-dimer. Stratified data or interquartile range such as age were converted to mean (standard deviation) based on the mathematical formulas for meta-analysis (22, 23). We used Newcastle-Ottawa Scale (NOS) for quality assessments. Two authors (GD and FZ) independently selected and evaluated the included articles. When a consensus was lacking, a third reviewer (LY) was consulted to solve the disagreements.

Statistical Analysis

STATA (Version 12.0; STATA Corporation, College Station, TX, USA) and TSA (Copenhagen trial unit) software were used for all the statistical analyses. OR with 95% CI was calculated for binary outcomes, and HR for time-to-event outcomes (24). Random-effect and fixed-effect models were both adopted in all analyses to assess the stability of results. Additionally, sensitivity analyses were performed by omitting one study each time; meta-regression and subgroup analyses

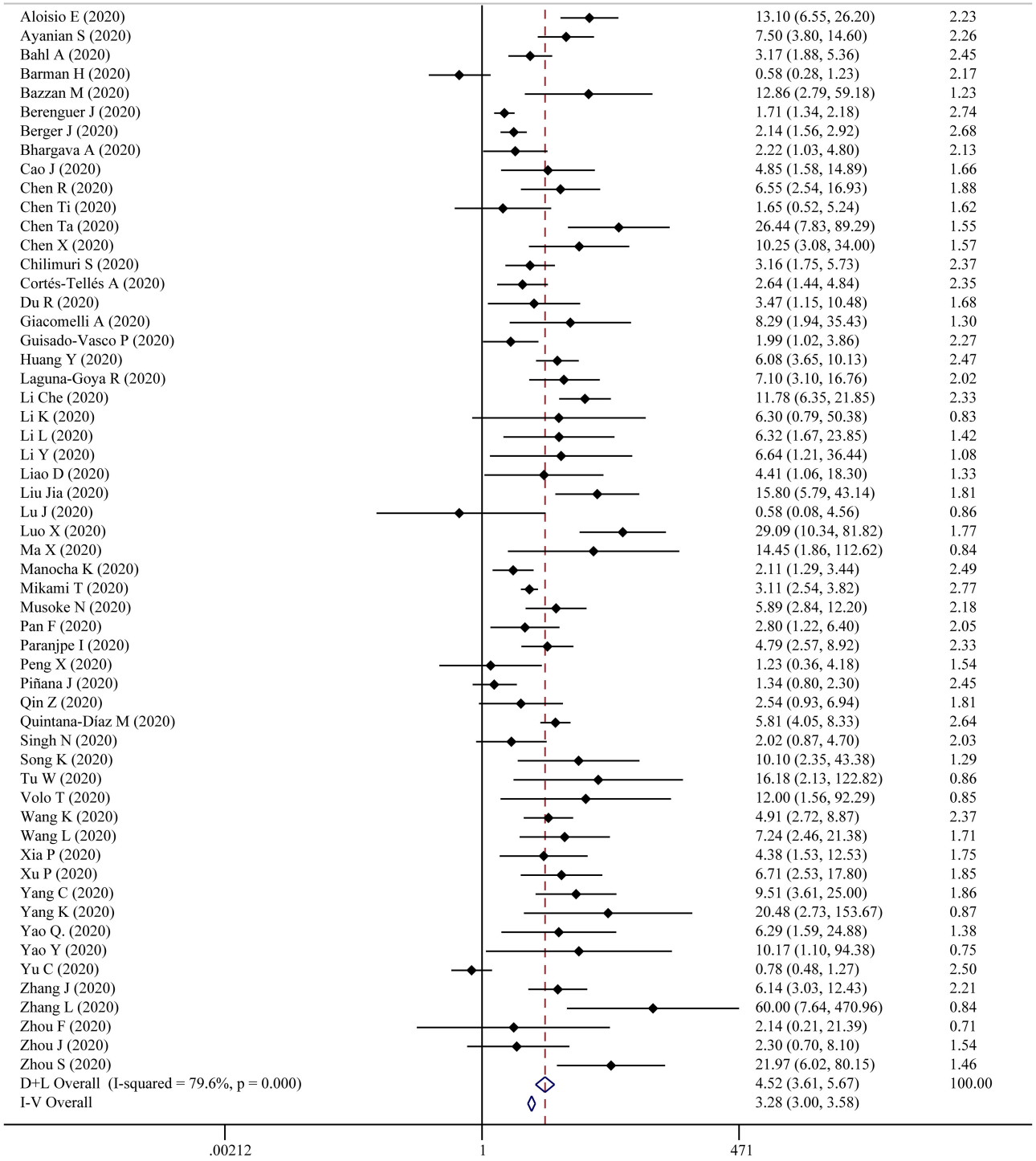

FIGURE 2 | Forest plot to assess odds ratio (OR) of COVID-19 mortality for D-dimer.

were conducted based on different countries, cutoffs, sample size, study design, and analysis of OR/HR to further evaluate the consistency of our conclusions. The funnel plot and Egger test was used to evaluate publication bias, and the Duval and Tweedie trim-and-fill method was performed to adjust for this bias (25). Trial sequential analysis was used to eliminate early false positive findings. $P < 0.05$ was considered statistically significant.

RESULTS

Literature Search and Studies Characteristics

We initially identified 4,372 records through our search strategy and scanning the reference lists of related meta-analyses (**Figure 1**); 3,571 studies remained after excluding duplicates. Then we reviewed the titles and abstracts and obtained 678 studies for full-text scanning. We further excluded 612 studies due to studies without our concerned outcomes ($n = 371$), studies without dichotomous D-dimer ($n = 128$), review and meta-analyses ($n = 55$) and other reasons including duplicates, letters, and comments ($n = 12$). Finally, a total of 66 studies involving 40,614 COVID-19 patients were included in our meta-analysis (9, 13, 26–89).

The main characteristics of eligible studies are shown in **Table 1**. All these 66 studies were published in 2020 and from different countries including China, the United States, Italy, Turkey, Spain, Mexico, and Switzerland. In these studies, 65 studies were written in English, and one in Chinese, and 22 studies had sample size above 500 patients. What's more, 56 studies reported OR and 15 reported HR of D-dimer. Except one study, all studies of high quality had seven or more NOS scores, and details are shown in **Supplementary Table 1**.

Association of D-Dimer and COVID-19 Mortality

Fifty-six studies reported the proportion of non-survivors between high and low D-dimer groups. With heterogeneity ($I^2 = 79.6\%$, $P < 0.001$), the random-effect model was performed and suggested that patients in the high D-dimer group had higher proportion of mortality than those in the low D-dimer group [OR = 4.52, 95% CI = (3.61, 5.67), $P < 0.001$]. The conclusion did not change when using the fixed-effect model for meta-analysis [OR = 3.28, 95% CI = (3.00, 3.58), $P < 0.001$] (**Figure 2**). Sensitivity analysis did not change the conclusion (**Supplementary Figure 1**). The funnel plot was not in a form of symmetry, indicating the existence of potential publication bias (**Supplementary Figure 2A**). Then we used Egger test to detect the presence of publication bias ($P < 0.001$) (**Supplementary Figure 2B**). However, the conclusion did not change in fixed-effect model [OR = 2.92, 95% CI = (2.68, 3.18), $P < 0.001$] or random-effect model [OR = 3.33, 95% CI = (2.66, 4.16), $P < 0.001$] after filling 15 studies in the comparison. To examine whether the observed heterogeneity could be contributed by possible moderators, univariate meta-regression was performed and suggested that country and analysis of OR were possible significant moderators (**Table 2**). To further assess the stability of the conclusion, we conducted the subgroup analysis stratified by different countries, cutoffs, sample size, study design, and analysis of OR. The conclusion did not change, highlighting the independent prognostic value of D-dimer and that the cutoff of D-dimer could be determined as a series of values including 0.5 μg/ml, 1 μg/ ml, and 2 μg/ ml (**Figure 3**).

Fifteen studies reported HRs of high D-dimer vs. low D-dimer. Due to the heterogeneity among studies ($I^2 = 83.7\%$, $P < 0.001$), the random-effect model was used, and pooled data showed that patients in the high D-dimer group were significantly associated with poor overall survival [HR = 2.81, 95% CI = (1.85, 4.27), $P < 0.001$]. This result was consistent when using the fixed-effect model to analyze the pooled data [HR = 1.63, 95% CI = (1.45, 1.84), $P < 0.001$] (**Figure 4**). We further performed a sensitivity analysis through excluding any one specific study each time. We did not observe obvious decline of heterogeneity, and the conclusion was consistent (**Supplementary Figure 3A**). The funnel plot identified four studies over the pseudo 95% CI (**Supplementary Figure 3B**), and the Egger test detected the presence of publication bias ($P = 0.013$) (**Supplementary Figure 3C**). Then the Duval and Tweedie trim-and-fill method was adopted, but no studies were trimmed and filled. To explore the origin of heterogeneity, we performed the univariate meta-regression and found that analysis of HR was possible significant moderator (**Table 3**). Subgroup analysis based on different countries, cutoffs, sample size, study design, and analysis of HR did not change the conclusion, which means D-dimer is an independent indicator for COVID-19 mortality, and the cutoff of D-dimer (0.5μg/ ml or 1μg/ ml) could be used clinically (**Figure 5**).

TABLE 2 | Univariate meta regression of odds ratio (OR) of COVID-19 mortality for D-dimer.

Variables	β	95% LCI	95% UCI	*P*
Country				
China	0.561747	0.34901	0.904156	**0.018**
USA	1.572451	0.874163	2.828536	0.128
Italy	0.373506	0.131622	1.059905	0.064
Spain	1.743824	0.651697	4.666158	0.262
Cut-off				
0.5 μg/ml	1.228279	0.74308	2.030291	0.416
1 μg/ ml	1.112854	0.458536	2.700866	0.81
2 μg/ ml	1.049783	0.393859	2.798072	0.921
>2 μg/ ml	0.713175	0.381889	1.331852	0.283
Sample size	0.718948	0.430974	1.199346	0.202
Study type				
Single-center	0.686701	0.415319	1.135414	0.14
Multi-center	1.548838	0.937437	2.558998	0.086
Analysis of OR	0.531366	0.303191	0.931262	**0.028**

Trial Sequential Analysis

Trial sequential analysis has been widely used to improve the reliability of conclusion and eliminate early false positive findings due to imprecision and repeated significance testing in meta-analyses (90). We collected the numbers of death and total numbers of patients in the high and low D-dimer group from 37 studies (**Supplementary Table 2**). Trial sequential analysis on data for death supported a 20% risk ratio reduction in the low D-dimer group compared with high D-dimer group. The required information size of 42,893 was calculated based on a control event proportion of 11.5% (based on data in our meta-analysis), a risk of type I error of 5%, a power of 80%, and a diversity of 87.16%. Although the actual information size did not reach the required

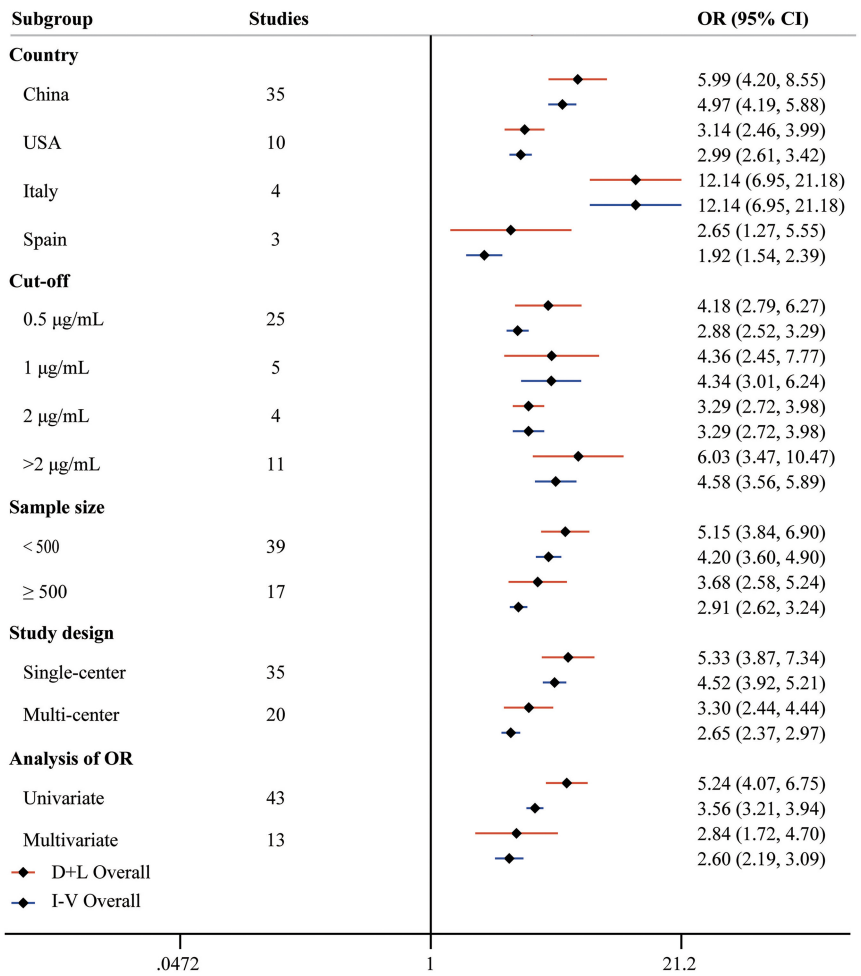

FIGURE 3 | Forest plot to assess OR of COVID-19 mortality for D-dimer stratified by different countries, cutoffs, sample size, study design, and analysis of OR.

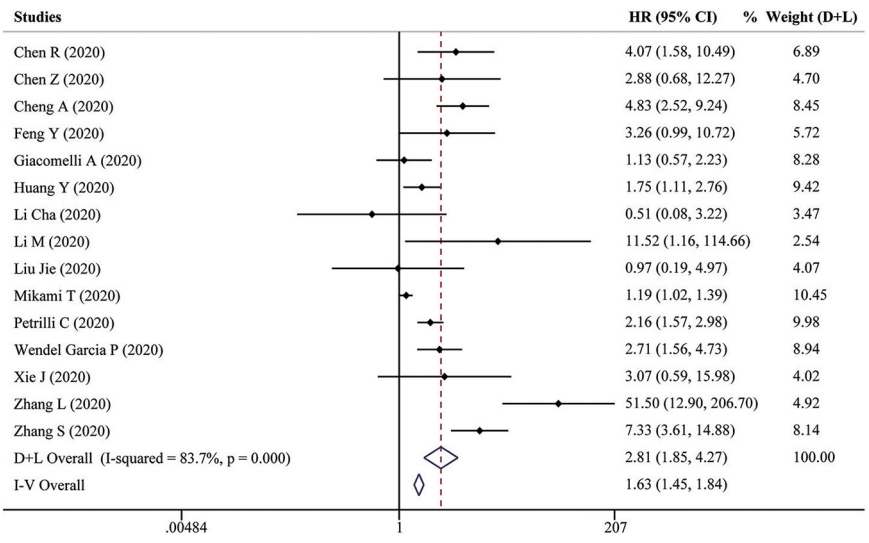

FIGURE 4 | Forest plot to assess hazard ratio (HR) of COVID-19 mortality for D-dimer.

information size, the cumulated Z-curve (blue curve) crossed the traditional boundary of 5% significance (horizontal red line) and the trial sequential monitoring boundary (red curve), implying that firm evidence was reached (**Figure 6**).

DISCUSSION

The ongoing spread of COVID-19 is posing a huge threat to global public health. Nowadays, the numbers of deaths caused by COVID-19 are still increasing while there is still no effective medication (64). Thus, it's imperative to identify the predictors for COVID-19 mortality. With regard to the role of plasma D-dimer in COVID-19 mortality, studies have reported associations that vary in strength and direction. Therefore, a comprehensive meta-analysis is necessary to illuminate the clinical significance of plasma D-dimer in COVID-19 mortality.

In this meta-analysis, a total of 66 studies involving 40,614 COVID-19 patients were enrolled. We found that patients in high D-dimer group had a poorer prognosis than those in low D-dimer group, independent of countries, cutoffs, sample size, study design, and analysis of OR/HR. Sensitivity analysis and pooled data based on different effect models were used to explore the consistency of our conclusions, and the conclusions were still consistent. Additionally, even though there exist publication bias in the combined outcomes of high D-dimer vs. low D-dimer, the conclusion still did not change after the Duval and Tweedie trim-and-fill method. Trial sequential analysis further confirmed our conclusions. Based on the above findings, we could conclude that D-dimer was an independent predictor for COVID-19 mortality. Furthermore, subgroup analyses based on cutoffs of dimer highlighted that a series of values including 0.5

TABLE 3 | Univariate meta regression of hazard ratio (HR) of COVID-19 mortality for D-dimer.

Variables	β	95% LCI	95% UCI	*P*
Country	0.436659	0.150542	1.266569	0.117
Cut-off				
0.5 μg/ml	1.119004	0.263079	4.759676	0.869
1 μg/ml	1.001067	0.275978	3.631211	0.999
Sample size	0.587985	0.18532	1.865568	0.339
Study type	0.697132	0.198794	2.444712	0.545
Analysis of HR	0.351424	0.142391	0.867322	**0.027**

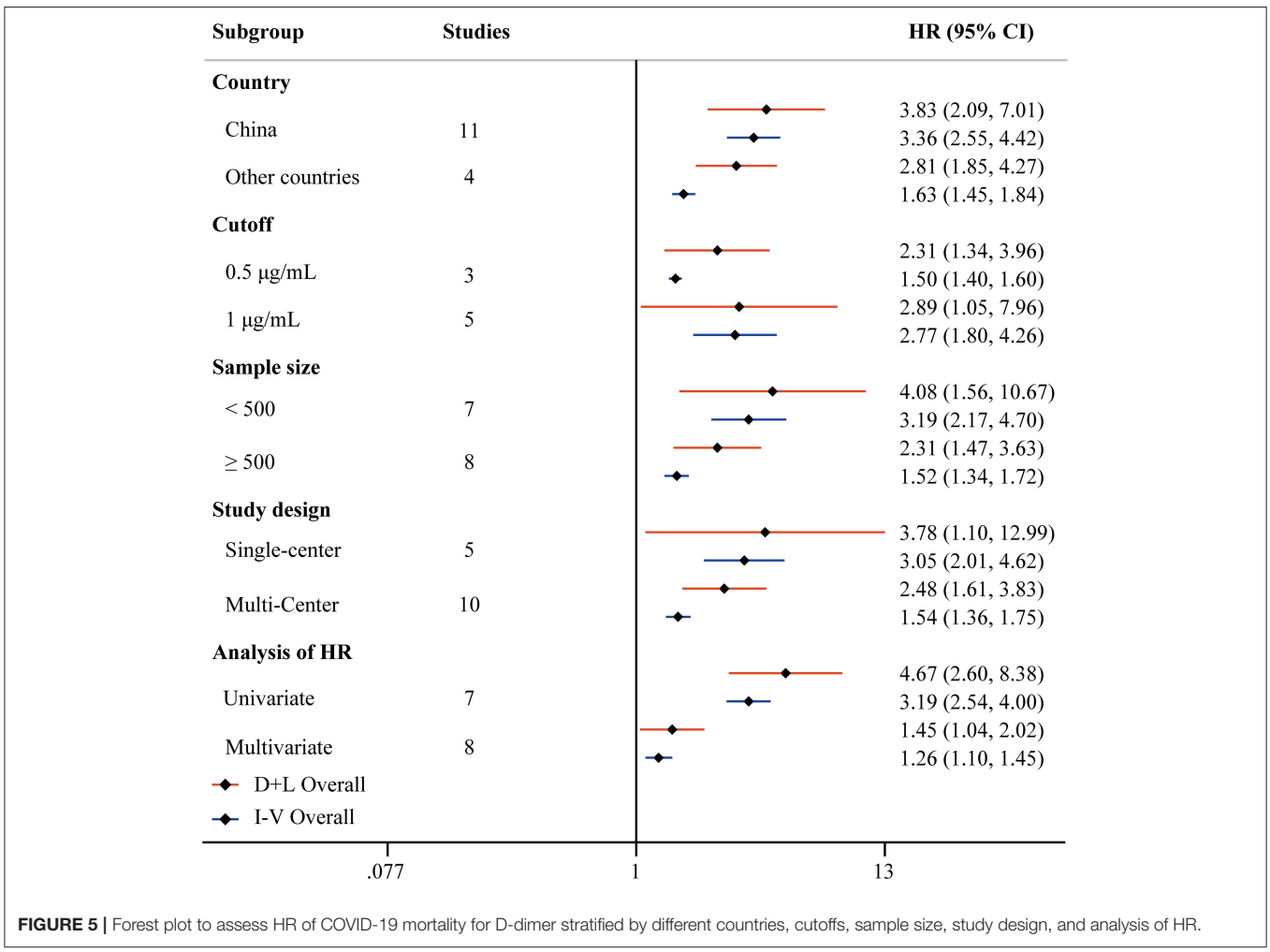

FIGURE 5 | Forest plot to assess HR of COVID-19 mortality for D-dimer stratified by different countries, cutoffs, sample size, study design, and analysis of HR.

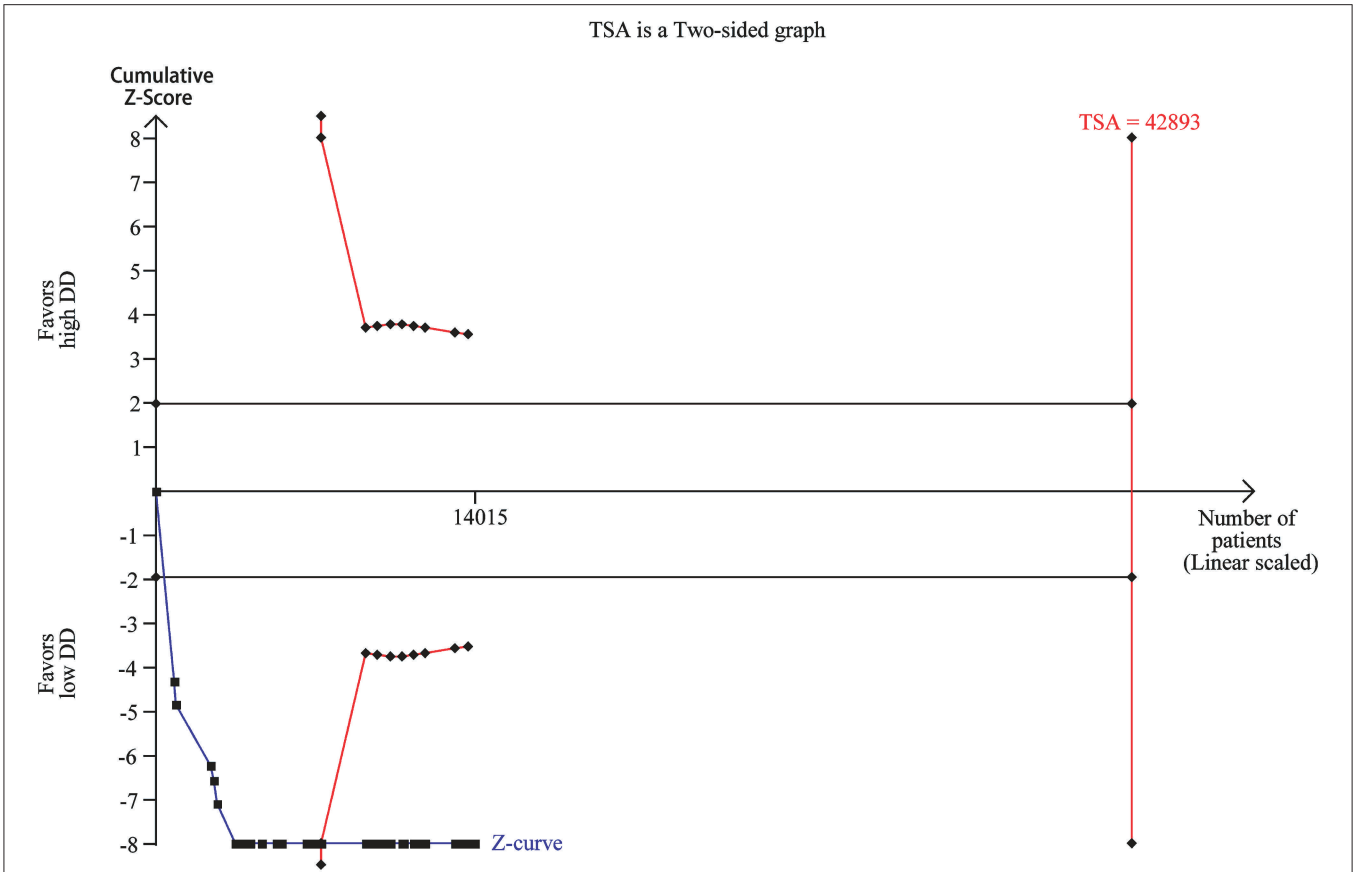

FIGURE 6 | Trail sequential analysis of COVID-19 mortality between high and low D-dimer group.

μg/ ml, 1 μg/ ml, and 2 μg/ ml could be determined as the cutoff of D-dimer for clinic use.

D-dimer is one of the commonest laboratory findings for COVID-19 patients. As early as February 2019, Guan et al. reported that severe patients had a significantly higher level of D-dimer than non-severe patients through analyzing 1,099 patients with laboratory-confirmed COVID-19 from over 550 hospitals in China (91). Moreover, Zhou and his colleague conducted a retrospective study involving 191 COVID-19 patients and found that elevated D-dimer at admission was a risk factor for death of adult patients (13). However, this conclusion was not consistent in other studies. Xie et al. found that D-dimer is not a risk factor after adjustment of age and gender through analyzing 140 COVID-19 patients (79). Besides, Liu and his team even did not find the difference in the unadjusted association between D-dimer and all-cause death in COVID-19 patients (35). Therefore, our findings are necessary to solve the problem and highlight the clinical significance of plasma D-dimer in COVID-19 mortality.

The mechanism is still unknown about the association between elevated D-dimer with COVID-19 mortality. Wang et al. previously showed that the significantly increased D-dimer and corresponding hypoxemia could induce the formation of pulmonary microthrombus in the 2009 novel influenza A(H1N1) (10). A recent study conducted by Klok and his colleague demonstrated that approximately 31% COVID-19 patients in intensive care unit had the thrombotic complications (11). Moreover, D-dimer could be used to indicate deep venous thrombosis in COVID-19 patients with cardiovascular diseases (92). That means elevated level of D-dimer, the indicator of thrombotic complications, might be the cause of COVID-19 mortality. However, other studies held different opinions that COVID-19 progress is the cause of the increase of D-dimer level. One possible mechanism is that SARS-CoV-2 infections are usually accompanied by an aggressive inflammatory response and even cytokine storm. The hyperinflammation could induce the dysfunction and damage of endothelial cells, resulting in the elevation of D-dimer and excess thrombin generation (93). Additionally, organ damage and corresponding hypoxemia caused by SARS-CoV-2 infection could stimulate thrombosis through increasing blood viscosity and activating hypoxia-inducible transcription factor-dependent signaling pathways (94, 95). Recently, Turagam et al. found that mortality is mostly associated with pulseless electrical activity. Whether D-dimer-associated thrombosis could cause pulseless electrical activity and ultimately mortality needs to be clarified (96). Overall, The underlying mechanism is unsolved about the relationship between elevated D-dimer and COVID-19 mortality. Our finding highlights the association, and more studies are needed to dig out the detailed mechanism.

To our knowledge, this is the largest meta-analysis to evaluate the clinical significance of plasma D-dimer in COVID-19 mortality. However, several limitations must be acknowledged. First, noticeable heterogeneity exists in all of the analyses. Sensitivity analysis pooled the data based on different effect models, yet the heterogeneity could not be eliminated completely. Second, publication bias exists in all the comparisons, though the conclusion did not change through the Duval and Tweedie trim-and-fill method. Finally, our study could not clarify the underlying mechanism between D-dimer with COVID-19 mortality.

In conclusion, D-dimer was identified as an independent predictor for COVID-19 mortality. A series of values including 0.5 μg/ ml, 1 μg/ ml, and 2 μg/ ml could be determined as cutoff of D-dimer for clinic use. Measurement and monitoring of D-dimer might assist clinicians to take immediate medical actions and predict the prognosis of COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

YL and YD: data curation, formal analysis, methodology, roles/writing-original draft. LY: conceptualization, data curation, formal analysis, methodology, roles/writing-original draft. HS: formal analysis, methodology, roles/writing-revised draft. SD and HH: methodology, software, validation. FZ, GD, and XC: conceptualization, data curation, formal analysis, software, supervision, validation, visualization, roles/writing—original draft, writing—review & editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.638097/full#supplementary-material

Supplementary Figure 1 | Sensitivity analyses of OR of COVID-19 mortality for D-dimer.

Supplementary Figure 2 | Funnel plot **(A)** and Egger test **(B)** of OR of COVID-19 mortality for D-dimer.

Supplementary Figure 3 | Sensitivity analyses **(A)**, funnel plot **(B)** and Egger test **(C)** of HR of COVID-19 mortality for D-dimer.

Supplementary Table 1 | Methodological quality of studies included in the meta-analysis.

Supplementary Table 2 | Raw data for all the analyses in the study.

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Epidemiology and Clinical Outcomes of COVID-19 Patients in Northwestern China Who Had a History of Exposure in Wuhan City: Departure Time-Originated Pinpoint Surveillance

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Background: Most COVID-19 patients cannot provide a clear exposure time; therefore, this study was designed to predict the progression of COVID-19 by using the definite departure time from Wuhan.

Methods: In this retrospective study, all cases were selected from Northwestern China, which has the lowest population density. As our study endpoints, the incubation period was defined as the date of departure from Wuhan City to the date of symptom onset; we defined the confirmed time as the interval from symptom onset to positive results (samples from the respiratory tract). Both of them were estimated by fitting a Weibull distribution on the departure date and symptom onset. The differences among the variables were analyzed.

Results: A total of 139 patients were ultimately enrolled, and ∼10.1% of patients (14 patients) had no symptoms during their disease course. We estimated the median incubation period to be 4.0 days (interquartile intervals, 2.0–8.0), and the 95th percentile of the distribution was 15.0 days. Moreover, ∼5.6% of patients (7 patients) experienced symptoms 2 weeks after leaving. Furthermore, the estimation median interval from symptom onset to final diagnosis was 4.0 days (interquartile intervals, 2.0–6.0), and the 95th percentile of the distribution was 12.0 days. Finally, the median hospitalization time was 16.0 days, ranging from 3.0 to 45.0 days. Univariate analysis showed that age ($P = 0.021$) and severity status ($P = 0.001$) were correlated significantly with hospitalization time.

Conclusions: We provide evidence that departure time can be used to estimate the incubation and confirmed times of patients infected with COVID-19 when they leave an epidemic area.

Keywords: outcomes, exposure history, departure time, epidemiological terms, COVID-19

INTRODUCTION

From December 2019 until March 11, 2020, 3,173 Chinese people died of COVID-19 (1). Compared with the SARS breakout in 2003, COVID-19 presented the following characteristics (2–7): (1) higher infection; (2) lower lethality; (3) infections in the incubation period; (4) asymptomatic patients are also contagious; (5) multiple organ susceptibility. Thus, it is indispensable to investigate the epidemiological characteristics of COVID-19, especially for patients with exposure histories in epidemic areas.

Definite exposure time is critical for analyzing infectious diseases, especially for respiratory tract infectious diseases that were spread through short-range droplets, such as influenza. Previous studies have indicated that the main routes of transmission of COVID-19 were droplets and aerosols (8); other researchers (9) reported that healthy carriers could also transmit the virus. Wuhan city is a megalopolis of high population density in China. Due to the aforementioned factors, we could not obtain definite exposure times, meaning that it was hard to estimate the incubation period of COVID-19. Guan et al. (10), in a retrospective study enrolling 1,099 patients, reported that only 289 patients had information on their specific date of exposure. Other scholars (11) dealt with this situation by choosing the date the first reported patient presented symptoms, which is obviously not rigorous.

Since March 11, the coronavirus has spread to more than 123 countries and regions, with ∼132,000 cases infected with COVID-19, as reported by WHO, spurring WHO to characterize the outbreak as a pandemic. With the global outbreak (12), all countries are facing hard work to prevent and control both domestic epidemics and imported cases from hardest hit areas; however, imported cases or suspicious ones cannot provide definite exposure times, making it hard to calculate the incubation period of patients and to establish the length of time for quarantine and medical observation.

For imported cases, the departure time can be accurately obtained in real-world studies. Therefore, this study was designed to predict the progression of COVID-19 by using definite departure times, which could be easily provided by patients, as the exposure time, and all patients experienced exposure history in Wuhan City. To reduce the possibility of secondary exposure caused by population density and mobility, we selected patients who received treatment at designated hospitals in Northwestern China, the area with the lowest population density.

MATERIALS AND METHODS

Study Design

This study was designed to analyze the epidemiological characteristics and clinical outcomes of patients from Northwestern China diagnosed with novel coronavirus pneumonia (NCP) who had history of exposure in Wuhan City. The degree of severity, diagnostic criteria, choice of treatment mode, and discharge standard refer to the 7th edition of the National New Coronavirus Pneumonia Diagnosis and Treatment Program. To reduce the possibility of secondary exposure caused by population density and mobility, we selected the patients who received treatment at designated hospitals distributed in Northwestern China, the area with the lowest population density. This area includes four autonomous regions and three provinces. All cases enrolled in this study fulfilled the following criteria: (1) had exposure history in Wuhan City; (2) without a definite exposure date; (3) COVID-19 virus nucleic acid results were positive; (4) no direct contact with confirmed or suspected patients after leaving Wuhan City; (5) symptom appearance after leaving Wuhan city; (6) treated at a designated hospital; (7) with definite disease outcome (death or discharge). The last follow-up time was March 11, 2020. This study was approved by the ethics committee of the First Affiliated Hospital of Xi'an Medical University (No. XYYFY2020LSK-026). Written informed consent was waived due to the nature of open-access data, and it was approved by the First Affiliated Human Research Ethics Committee of Xi'an Medical University. All procedures followed were in accordance with the Declaration of Helsinki.

Setting

These areas are located in Northwestern China, far from Wuhan City. They include four autonomous regions (Inner Mongolia Autonomous Region, Tibet Autonomous Region, Xinjiang Uygur Autonomous Region, and Ningxia Hui Autonomous Region) and three provinces (Gansu Province, Qinghai Province, and Shaanxi Province) (**Table 1**). These regions and provinces account for 57.5% of the total territory of China; however, the population density of the area is only 23.8 persons/km^2, which is lower than the national average population density (145.4 persons/km^2).

Data Collection

We obtained the data from the news and press releases reported by the provincial and local municipal Center for Disease Control and Prevention (CDC) or the Health Commission. The date of leaving Wuhan City, the date of symptom onset, the date of diagnosis, the date of discharge, age, gender, and other patient-related data were extracted from the news and press releases. Four reviewers (JZ, CJ, QZ, and SX) collected the data independently, and data were verified with the National Health Commission and Chinese CDC. Major disagreements between these four doctors were checked by all doctors together.

Definition

The residents were confirmed to have stayed in Wuhan City more than 2 weeks during the outbreak. The incubation period was defined as the date of departure from Wuhan City to the date of symptom onset or the date of final diagnostic time (asymptomatic patient); we defined the confirmed time as the interval from symptom onset to positive results (samples from the respiratory tract). Hospitalization time is recognized as from final diagnosis to date of discharge or death (**Figure 1**).

Statistical Analysis

Categorical variables are summarized as numbers and percentages. We estimated the incubation period and the confirmed time by fitting a Weibull distribution on the dates of departure and symptom onset. The relationship between

TABLE 1 | Association between clinical characteristics and severity status of patients diagnosed with COVID-19.

Characteristics		**Severe/critical cases**	***P***
Age (median: 36years, rang from 1 to 71)	***N* (139)**	***N* (%)**	
Gender			0.051
Male	92	8 (100%)	
Female	47	0 (0)	
Religion			0.645
Shaanxi province	67	5 (62.5%)	
Qinghai province	14	1 (12.5%)	
Tibet autonomous region	1	0 (0)	
Xinjiang Uygur autonomous region	1	0 (0)	
Ningxia Hui autonomous region	12	0 (0)	
Gansu province	23	0 (0)	
Inner Mongolia autonomous region	21	2 (25.0%)	
Age[a]			0.719
<36	68	3 (37.5%)	
≥36	71	5 (62.5)	
Exposure history			0.049
Resident	98	3 (37.5%)	
Traveler	41	5 (62.5%)	
Symptom			0.669
Fever	94	7 (87.5%)	
Cough	12	1 (12.5%)	
Diarrhea	3	0 (0)	
Other symptom	16	0 (0)	
Without symptom	14	0 (0)	

[a]*Age: median age.*

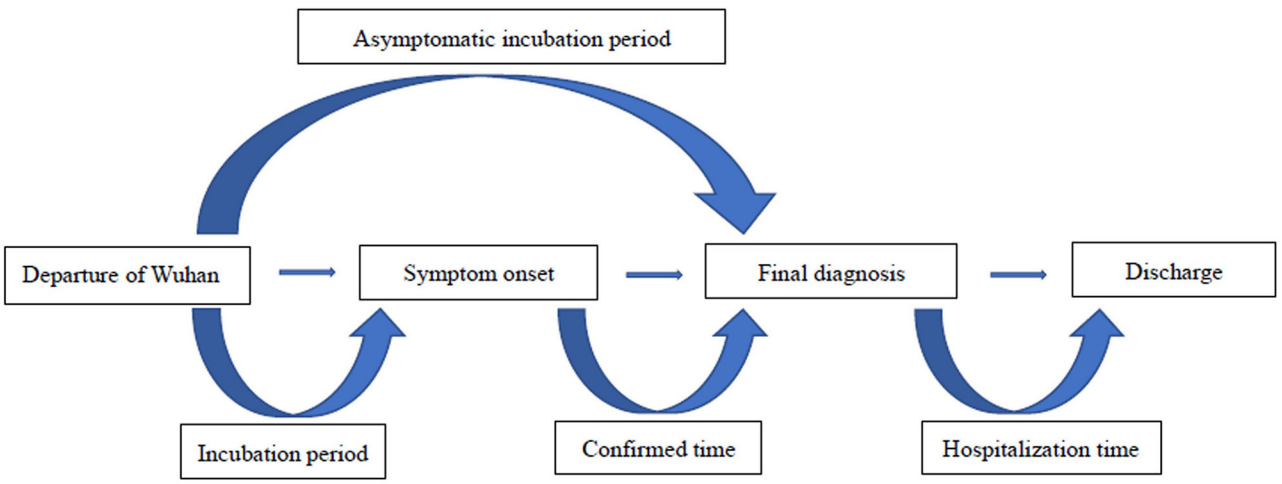


FIGURE 1 | Definitions of the main endpoints of this study.

severity status of COVID-19 and clinical characters was analyzed by using a χ^2-test. Normal distribution and homogeneity of variances were tested, *T*-test and variance analysis were performed to compare the difference among the variables, and the Mann–Whitney U-test and Kruskal–Wallis H-test were applied when the cases did not fit the normal data distribution. Bilateral $P \leq 0.05$ was considered statistically significant. All analyses were performed using SPSS software

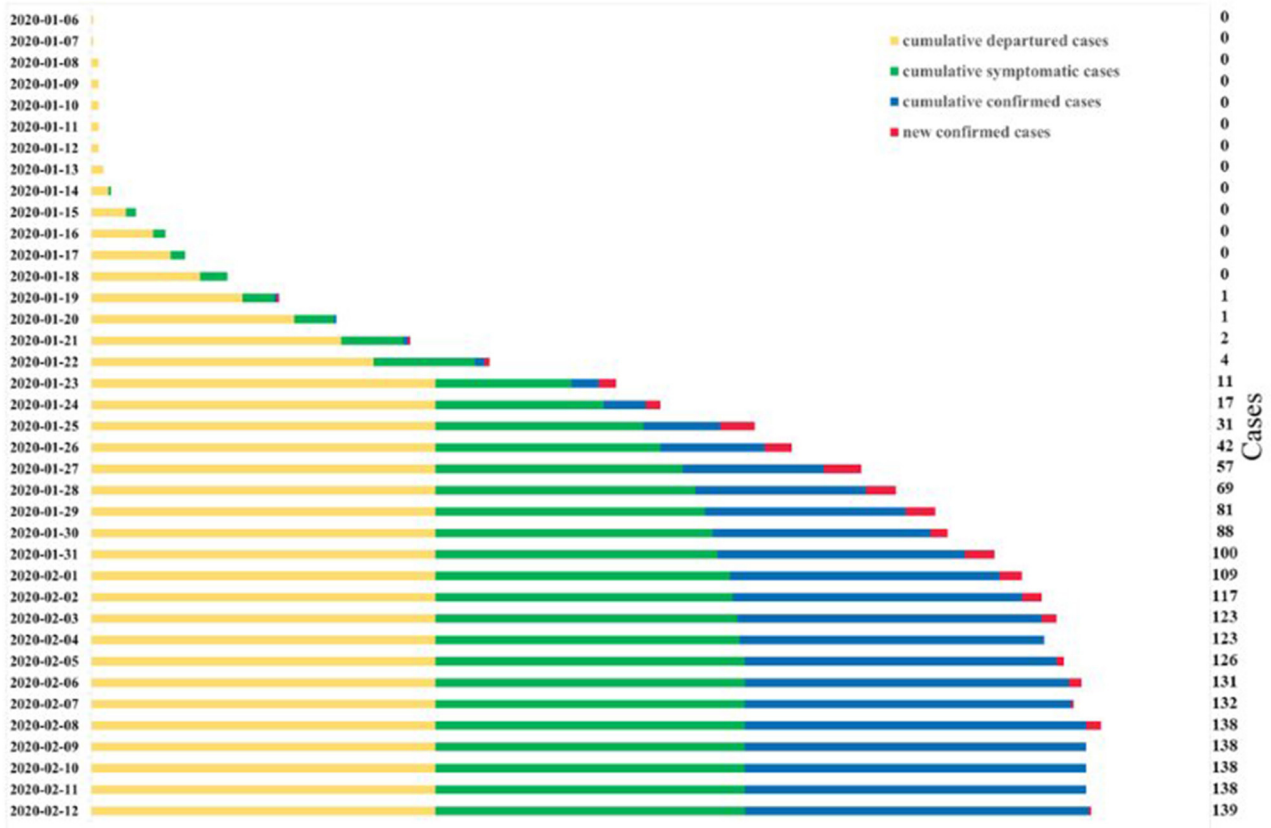
(version 22.0), and Weibull fitting distribution was estimated by MATLAB 18.0.

RESULTS

Clinical Characteristics and Severity Status

As of March 11, 2020, a total of 139 patients diagnosed with COVID-19 were enrolled in this study. All patients were from Northwestern China and were verified to have an exposure history in Wuhan City. **Figure 2** shows the time distribution of all patients; the earliest and latest times to leave Wuhan City were January 6, 2020 and January 23, 2020, respectively. Only one patient was provided by both Tibet Autonomous Region and Xinjiang Uygur Autonomous Region. The largest number of patients (67 patients) was provided by Shaanxi Province, accounting for 48.2% (**Table 1**).

Of the 139 patients, 92 patients were male and 47 patients were female; the median age of the patients was 36 years (range: 1–77 years). Approximately 70.5% of patients (98 patients) were residents, and 41 patients were travelers. A total of 94 patients had the common symptom of fever (75.2%), ∼2.4% of patients (3 patients) presented diarrhea, 12 patients presented only cough (9.6%), and 12.8% of patients presented other symptoms. According to severity status, ∼94.2% of patients were categorized as general, 4 patients (2.9%) as severe, and 4 patients (2.9%) as critical; severe and critical patients were



Date	2020-01-06	2020-01-07	2020-01-08	2020-01-09	2020-01-10	2020-01-11	2020-01-12	2020-01-13	2020-01-14	2020-01-15	2020-01-16	2020-01-17	2020-01-18	2020-01-19	2020-01-20	2020-01-21	2020-01-22	2020-01-23	2020-01-24	2020-01-25	2020-01-26	2020-01-27	2020-01-28	2020-01-29	2020-01-30	2020-01-31	2020-02-01	2020-02-02	2020-02-03	2020-02-04	2020-02-05	2020-02-06	2020-02-07	2020-02-08	2020-02-09	2020-02-10	2020-02-11	2020-02-12
cumulative departured cases	1	1	3	3	3	3	3	5	7	14	25	32	44	61	82	101	114	139	139	139	139	139	139	139	139	139	139	139	139	139	139	139	139	139	139	139	139
cumulative symptomatic cases	0	0	0	0	0	0	0	0	1	4	5	6	11	13	16	25	41	55	68	84	91	100	105	109	112	114	119	120	122	123	125	125	125	125	125	125	125	125
cumulative confirmed cases	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	4	11	17	31	42	57	69	81	88	100	109	117	123	123	126	131	132	138	138	138	138	139
new confirmed cases	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	2	7	6	14	11	15	12	12	7	12	9	8	6	0	3	5	1	6	0	0	0	1

FIGURE 2 | Time distribution of all patients after they left Wuhan City.

analyzed together. Patients with short-term exposure (travelers) were more likely to develop severe or critical status than those with long-term exposure (residents) (62.5 vs. 37.5%, $P = 0.049$). Of the eight severe or critical patients, all of them were male ($P = 0.051$) (**Table 1**).

Epidemiological Characteristics

Interestingly, ∼10.1% of patients (14 patients) were healthy carriers, without any symptoms during their disease course. For asymptomatic patients, ∼57.1% of patients (8/14) were determined to be positive for nucleic acid of COVID-19 virus within 5.0 days of leaving Wuhan City. For symptomatic patients, two patients presented onset of symptoms after they were confirmed (1.6%), and one of them presented symptoms after 9 days. Most patients (75.2%) had onset of symptoms within a week (94/125), nearly 5.6% of patients (7 patients) experienced symptoms 2 weeks after leaving, and one patient developed symptoms after 23.0 days. In addition, the peak time of symptom onset emerging after they left the epicenter was on the first day (23 patients) (**Figure 3A**). All 139 patients diagnosed with COVID-19 were estimated by fitting the Weibull distribution; the median incubation period was 4.0 days (interquartile range, 2.0–8.0), and the 95th percentile of the distribution was 15.0 days (**Figure 3B**).

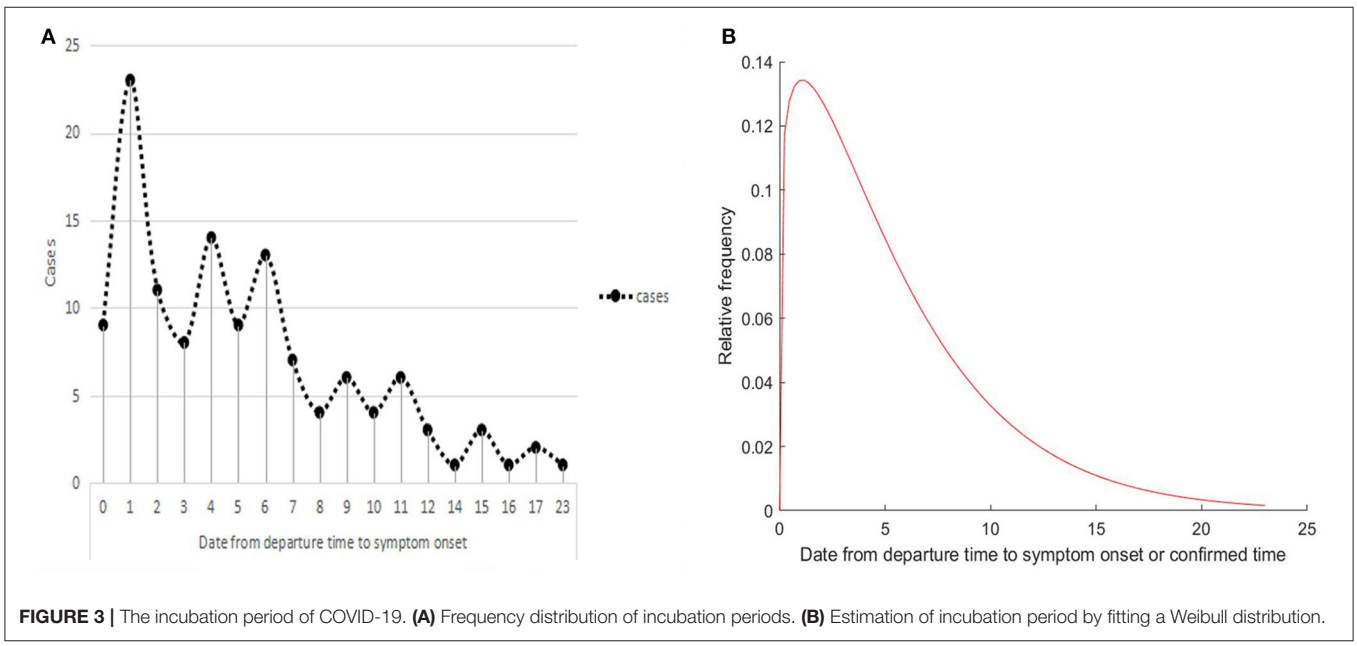


FIGURE 3 | The incubation period of COVID-19. **(A)** Frequency distribution of incubation periods. **(B)** Estimation of incubation period by fitting a Weibull distribution.

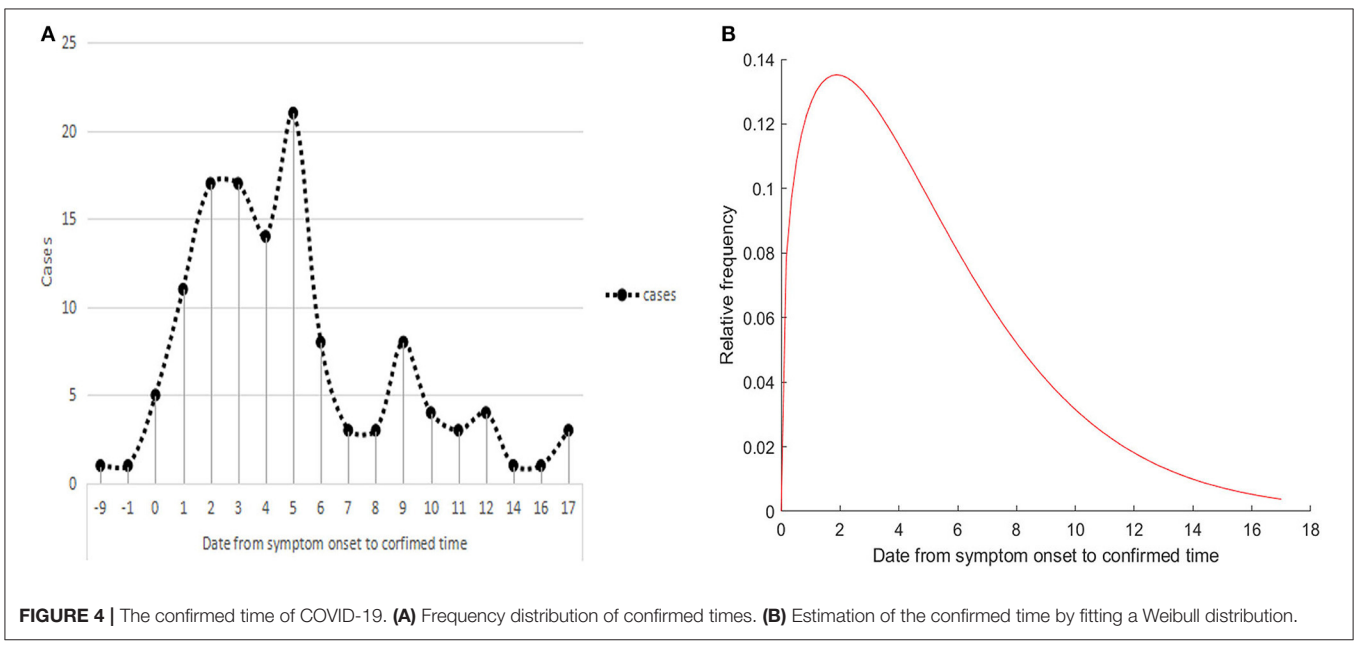


FIGURE 4 | The confirmed time of COVID-19. **(A)** Frequency distribution of confirmed times. **(B)** Estimation of the confirmed time by fitting a Weibull distribution.

Our study purpose was to address the issue of the optimal detection opportunity after symptom onset. The median confirmed time was 4.0 days (−9 to 17.0 days). **Figure 4A** shows that the peak of confirmed time was on the 5th day after symptom onset, and ∼68.0% of patients presented positive results within 5 days of symptom onset. For 125 symptomatic patients, the median interval from symptom onset to final diagnosis was 4.0 days (interquartile range, 2.0–6.0), and the 95th percentile of the distribution was 12.0 days (**Figure 4B**).

The time of positive detection of older patients (≥36 years) was later than that of younger patients (<36 years) after symptom onset (5.0 vs. 4.0 days, $P = 0.028$); in addition, male patients had later positive results detected (5.0 days, interquartile range: 3.0–7.0) compared with female patients (3.0 days, interquartile range: 2.0–5.0, $P = 0.051$).

Clinical Outcomes

Since March 11, 2020, all patients have been discharged; they all recovered. The median hospitalization time was 17.0 days, ranging from 3.0 to 45.0 days. Further analyses showed that the following variables were correlated significantly with hospitalization time: age ($P = 0.021$) and severity status ($P = 0.001$) (**Table 2**).

DISCUSSION

This retrospective study estimated the incubation period for COVID-19 by using the departure time from Wuhan City as the exposure time because in real-world studies, it is difficult to determine the definite exposure time. We collected the patients from a lower-population-density area in Northwestern China to reduce the possibility of secondary exposure; thus, our data had practical guidance value for preventing and controlling COVID-19, especially for patients or persons with suspicion of infection from the epicenter. Our data showed that the median incubation period of COVID-19 was 4.0 days after leaving the epidemic area.

Our results were basically consistent with other studies (2, 11, 13), although the definition of exposure time was different. A retrospective study from China showed that the mean incubation period of COVID-19 was 4.0 days (95% CI, 2.0–7.0); however, while a total of 1,099 patients were enrolled in this study, only 291 had a clear exposure time. All patients were Chinese. Lauer et al. (11) estimated the incubation period of COVID-19 in his study: 181 patients from 24 countries or regions were analyzed, and the median incubation period was estimated to be 5.1 days (95% CI, 4.5–5.8 days). Other related research reported longer incubation periods than ours. An epidemiological surveillance of early confirmed COVID-19 patients in Shanghai (14) showed that the mean incubation period was 6.4 days (95% CI 5.3–7.6), and the 95th percentile was 13.1 days. Data from Henan Province (15) of China estimated that the average latency of 483 patients was 7.4 days, and over 92.0% of patients had an incubation period of <2 weeks. We also found that ∼75.2% patients developed symptoms within 1 week after leaving the epidemic area (Wuhan City). It was noteworthy that 5.6% of patients presented symptoms 2 weeks later, while one patient's symptoms appeared 23.0 days later.

Interestingly, ∼10.1% of (14 patients) did not present any clinical symptoms in their disease course. It is controversial whether asymptomatic patients are contagious. A study from

TABLE 2 | The epidemiological characteristics and clinical outcomes of patients diagnosed with COVID-19.

Variable	Symptom onset to confirmed time	IQI[b]	*P*	Hospitalization time	IQI[b]	*P*
In total						
Gender			0.051			0.085
Male	5	(3, 7)		16	(11, 20)	
Female	3	(2, 5)		18	(13, 21)	
Age[a]			0.028			0.021
<36	4	(2, 5)		15.5	(12, 19)	
≥36	5	(2.5, 9)		18	(12, 22)	
Exposure history			0.145			0.881
Resident	4	(2, 6)		16	(12, 21)	
Traveler	5	(3, 9)		18	(11.5, 20)	
Symptom			0.801			0.127
Fever	4	(2, 6)		17	(13, 21)	
Cough	4	(2, 5)		20	(10.25, 27.75)	
Diarrhea	4	(2.5, 6.5)		12	(9.5, 14.75)	
Other symptom	4	(3, 9)		16	(11.25, 19.75)	
Without symptom				13.5	(9, 17.75)	
Severity status			0.414			0.001
General	4	(2, 6)		16	(12, 20)	
Severe	8	(1.5, 10.75)		23.5	(19.75, 31)	
Critical	2.5	(−0.25, 9)		34	(22.25, 44.25)	

[a] *Age: median age.*
[b] *IQI: interquartile intervals.*

Chinese researchers showed (16) that at least 59.0% of infection cases in Wuhan City might had not been identified, which may include those who are asymptomatic or who have mild symptoms. The MedRxiv platform published research from American scholars (17) that suggested that there may be a small percentage of infected individuals who are asymptomatic and can transmit the COVID-19 virus. An asymptomatic carrier from Henan Province of China transmitted COVID-19 to her five family members; her incubation period was as high as 19.0 days (9). Moreover, some research has indicated that there was no difference in the virus load between asymptomatic patients and symptomatic patients (18, 19). However, other studies showed the opposite result (20). In our study, the results of COVID-19 nucleic acid testing were positive, which indirectly proved that asymptomatic patients are contagious compared with symptomatic patients; thus, we should pay more attention to asymptomatic patients.

Further studies have shown that most asymptomatic patients are categorized as general (5, 21), meaning that these patients have better outcomes. However, the next generation of patients who become infected by these asymptomatic individuals might have worse outcomes (22); the specific mechanism of viral pathogenesis is unknown. In our study, for asymptomatic patients, ∼68.0% patients were detected as positive for COVID-19 viral nucleic acid within 5.0 days of leaving Wuhan City due to the early detection of suspicious populations by the government. Shao and Shan (23) constructed a SEIR model and suggested that medical examinations should be performed on exposed or potentially exposed individuals.

In concert with recent studies, fever was the most common symptom (75.2%) diagnosed in this study, which was consistent with the results of the meta-analysis by Sun et al. (24). For patients diagnosed with COVID-19, laboratory test results that did not match clinical symptoms were found in these studies (25–27). Two patients experienced symptoms after they were diagnosed (1.6%), and one presented symptoms after 9 days; thus, all suspicious populations should be observed dynamically.

This study also focused on the optimal time to detect COVID-19 nucleic acids. We found that the median confirmation time was 4.0 days. A recent retrospective study from Tongji Hospital (28) proved that the median time from symptom onset to confirmation was 16.0 days, longer than our data. They also found that ∼30.0% of these patients had positive results for the third time; meanwhile, they found that positive results were detected later in older patients (≥65 years) (18.0 vs. 14.0 days, $P < 0.001$), consistent with our data. Our results also indicated that most positive results appeared on the fifth day after symptom onset. Similar to our results, the clinical sensitivity of RT-PCR on swabs taken on the first day to the fifth day after symptom onset was 100% (29).

We also analyzed the factors that affect the patient's hospitalization time. In our study, the median hospitalization time was 17.0 days, ranging from 3.0 to 45.0 days. Further analyses showed that the following variables were correlated significantly with hospitalization time: age ($P = 0.021$) and severity status ($P = 0.001$), suggesting that age and severity status might been the prognostic factors for patients with COVID-19. A study from China had confirmed that older age associated with patient's in-hospital death (OR: 1.10, 95% CI: 1.03–1.17, $P = 0.0043$) (30). The possible explanation was that age and the severity of pneumonia will increase the occurrence of cardiac events after pneumonia, leading to a poor prognosis for patients (31). Therefore, we should pay more attention to these patients.

The limitations of this study should be noted. First, the laboratory results had not been analyzed because data sources from news and press releases were reported by Provincial and Autonomous CDCs. Second, because this was a retrospective study, further analysis was limited due to the small sample size of this study. Finally, although our results were broadly consistent with the related research, patients with exact exposure times should be included in future analyses for comparison.

In conclusion, we provide evidence that departure time can be used to estimate the incubation and confirmed times of patients infected with COVID-19 when they leave an epidemic area. The median of the incubation period was 4 days, and 5.6% of patients experienced symptoms 2 weeks after leaving. The longest time was 23.0 days from the date of departure, suggesting that the length of time for quarantine and medical observation, now recognized as 2 weeks, might not be sufficient. Moreover, most patients were detected to be positive for viral nucleic acid within 5.0 days of when symptoms appeared. Finally, healthy carriers should be given more attention.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the First Affiliated Hospital of Xi'an Medical University (No. XYYFY2020LSK-026). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TJ and SW participated in study design and study conception. QZ, JZ, and CJ performed data analysis and drafted the manuscript. QZ, JZ, CJ, and SX recruited patients. All authors provided critical review of the manuscript and approved the final draft for publication.

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Investigation of Coagulation Biomarkers to Assess Clinical Deterioration in SARS-CoV-2 Infection

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Since December 2019, a pandemic caused by a new coronavirus has spread to more than 170 countries around the world. Worsening infected patients requiring intensive care unit (ICU) admission associated with 30% of mortality. A part of worsening is induced by hemostasis deregulation. The aim of this study was to investigate the association of coagulation activation in COVID-19 progression. Thirty-five of the 99 patients got clinically worse. The final model of the logistic regression analysis revealed that O_2 requirement (RR = 7.27 [1.50–19.31]), monocytes below 0.2G/L (RR = 2.88 [1.67–3.19]), fibrinogen levels (RR = 1.45 [1.17–1.82] per g/L increase), prothrombin fragments 1+2 higher than 290 pM (RR = 2.39 [1.20–3.30]), and thrombin peak (RR = 1.28 [1.03–1.59] per 50 nM increase) were associated with an increased risk of clinical worsening. A fibrinogen level threshold of 5.5 g/L, a thrombin peak measurement threshold of 99 pM, and O_2 requirement associated with clinical outcome in more than 80% of our cohort. In conclusion, we identified fibrinogen and thrombin peak at admission as coagulation biomarkers associated with an increased risk of ICU admission or death. This finding allows initiating steroids and triage for worsening patients. Our results should therefore be considered as exploratory and deserve confirmation.

Keywords: COVID-19, intensive care, hypercoagulability, fibrinogen, thrombin generation

INTRODUCTION

Since December 2019, a pandemic caused by a new coronavirus has spread to more than 170 countries around the world. It started in China (1) and then spread to Europe and the United States of America. This virus called SARS-CoV-2 (2) is responsible for an infectious disease itself called COVID-19. Most patients are asymptomatic or mildly symptomatic. In symptomatic patients, the clinical manifestations are dominated by respiratory symptoms (2, 3) characterized by serious lung complications that can lead to intensive care unit admission for acute respiratory distress syndrome (4, 5) and to a less extent to cardiovascular injuries (6).

The alteration of the endothelium could originate the deregulation of hemostasis (7). In addition, sepsis promotes platelet overactivation, leading to acute respiratory distress syndrome and acute renal failure (8, 9). Recommendations from the International Society of Thrombosis and Haemostasis (ISTH) and a retrospective study suggest that preventive anticoagulation in patients would be associated with a better prognosis (10, 11).

Prediction models that combine several variables to estimate the risk of people experiencing a poor outcome from the infection could assist medical staff in triaging patients when allocating limited healthcare resources (12). Several scores exist for prediction of mortality in pneumonia such as CURB-65 and A-DROP score (13, 14). Among them, the 4C (Coronavirus Clinical Characterization Consortium) Mortality Score is an easy-to-use and validated prediction tool for in-hospital mortality, accurately categorizing patients as being at low, intermediate, high, or very high risk of death in COVID-19 (AUC = 0.79) (15). However, fewer studies focused on coagulation biomarkers to assess the risk of COVID-19 complications and intensive care unit transfer. Among them, an increase in D-dimer levels has been associated with severe forms of the pathology (16) with other markers of disseminated intravascular coagulation (DIC). Clinical manifestations of these DIC were predominantly thrombotic with high venous thromboembolism rates (5).

Since the beginning of the pandemic, many studies confirmed an increase in D-dimer level (17, 18) and a cutoff value of 2,000 μg/L in patients who were clinically worsening was determined (19). However, D-dimer level is a very sensitive but not a very specific marker of hypercoagulable state. Then, it is possible to assess coagulation globally, by measuring thrombin generation (20). This technique studies the initiation, propagation, and inhibition of coagulation allowing the observation of hypo- or hyper-coagulable risk profiles.

Thus, the aim of this study was to investigate the association of coagulation activation in COVID-19 progression and investigate how coagulation markers could be used to risk stratify patients.

METHODS

Patients

Between March 16 and May 1, 2020, 100 COVID-19 patients hospitalized in COVID-19 dedicated medical units were prospectively recruited (clinical trials registration number: NCT04367662). An informed consent was obtained from all subjects and citrated plasma from the initial blood test <24 h after the admission was collected, double centrifuged according to French Group of Hemostasis and Thrombosis (GFHT) guidelines, and frozen at −80°C within 4 h after collection. Clinical, radiological, and biological relevant data were also collected. The follow-up of patients were 15 days, with a phone call when a hospital discharged before 15 days.

The study was performed in accordance with the Declaration of Helsinki. The institutional review board (person committee protection of Rouen University Hospital) and a national ethical committee (person committee protection South Mediterranean 1) approved the study, and a national anonymous data collection was declared (Authorization protocol number: 2020-A00914-35).

Computed Tomography Imaging

As defined by the European Society of Radiology (21), finding COVID-19 pneumonia in computed tomography scan were:

- A scale of disease extension (<10, 10–25%, 25–50%, 50–75%, >75%)
- Condensation type (nodular, linear, or both)
- Radiological abnormalities localization (unilateral, bilateral).

Assays

During initial blood test, prothrombin time (PT), activated partial thrombin time (aPTT) (DIAGNOSTICA STAGO–Asnières sur Seine, France), and D-dimer (VIDAS DEX2–Biomérieux–Marcy l'étoile, France) assays were performed.

After defrost, several coagulation tests were assayed:

- Fibrinogen (STA-Liquid Fib–DIAGNOSTICA STAGO–Asnières sur Seine, France), Fibrin monomers (STA-Liatest FM–DIAGNOSTICA STAGO–Asnières sur Seine, France), and chromogenic antithrombin assays (StachromATIII–DIAGNOSTICA STAGO–Asnières sur Seine, France) were realized on STA'RMax (DIAGNOSTICA STAGO–Asnières sur Seine, France).
- VWF:GPIb-binding activity (InnovanceVWAc–Siemens Healthcare, Marburg, Germany) was assayed on BCS XP (Siemens Healthcare, Marburg, Germany).
- Prothrombin fragments 1+2 were assayed with Enzygnost F1+2 (Siemens Healthcare, Marburg, Germany) on Diasonrin Etimax.
- Complete blood count was performed on EDTA samples on XN-1000 (Sysmex, Villepinte, France).
- Thrombin generation assay (TGA) was triggered by a low concentration of tissue factor (TF) (1 pM) and a normal concentration of phospholipids (PPP low reagent, Diagnostica Stago, Asnières sur Seine, France). TGA was measured by Calibrated Automated Thrombography and Fluorocan Ascent Fluorometer (Thermoscientific Labsystems, Helsinki, Finland).

ISTH Disseminated Intravascular Coagulation Score

Disseminated intravascular coagulation score (DIC) was calculated with ISTH criteria recommendation (22). Briefly, the scoring system included platelet count, prothrombin time, fibrinogen, and D-dimer or fibrin monomer.

Data and Statistical Analysis

The primary objective of the study was to evaluate the association of baseline hemostasis and clinical worsening on admission. Patients were considered to be clinically "worsening" if they were transferred to the intensive care unit or died and clinically "improving" if not. For patient characteristics, data were expressed as median [interquartile range or IQR], n (%), or n/N (%), where N is the total number of patients with available data. P-values comparing clinical improving to clinical worsening are from χ^2 test, Fisher's exact test, χ^2 with *Yates' correction* for continuity, Spearman correlation, or Mann–Whitney U-test when appropriate. Univariate logistic

regression analysis of clinical outcome (improving or worsening) was performed using the following variables as predictors: age, sex, O_2 requirement, tobacco consumption, radiological scale of disease extension (dichotomized to lower or higher than 25%), body mass index (BMI), hypertension, diabetes, respiratory disease (including COPD and/or asthma and/or other causes of respiratory disease), aPTT ratio (higher than 1.15), blood lymphocyte count (lower than 1 G/L), blood monocyte count (lower than 0.2 G/L), neutrophil-to-monocyte ratio, neutrophil-to-leucocyte ratio, D-dimer (higher than 1,000 μg/L), fibrinogen, TGA parameters (ETP, peak, and velocity), fibrin monomers (higher than 6 μg/ml), VWF: GPIb-binding activity (higher than 250%), and F1+2 (higher than 290 pM). Significant predictors under unadjusted analysis were further analyzed by multiple logistic regression analysis (full model). Then, based on the Akaike Information Criterion (AIC), irrelevant variables were eliminated from the full model by backward variable selection to obtain the final model. Results from the logistic regressions were expressed as relative risk (RR) [95% confidence interval]. Finally, a decision tree based on the predictors retained in the logistic regression final model was built using recursive partitioning method with the following parameters: minimum number of observations that must exist in a node in order for a split to be attempted = 15, minimum number of observations in any terminal node = 5, leave-one-out cross-validation strategy, and complexity parameter that minimizes the cross-validation relative error.

Data and statistical analysis and captions were performed using *R v4.0.0* software (20) and the following software packages: *pROC* (21), *MASS* (22), *caret* (23), *sjstats* (24), *rpart* (25), and *rpart.plot* (26).

RESULTS

One hundred patients were recruited and followed up to hospital discharge or death. One patient opposed participation after analysis. With World Health Organization classification of COVID-19 severity in admission, 23 patients had pneumonia, 51 patients had severe pneumonia, and 26 patients had acute respiratory distress syndrome. During hospitalization, patients were considered to be clinically worsening ($n = 35$) if they were transferred to the intensive care unit ($n = 28$) or died ($n = 12$) and clinically improving if not. Five patients had anticoagulant treatment before admission for atrial fibrillation. During hospitalization, 46 patients had prophylactic anticoagulation. A second computed tomography was performed in case of respiratory worsening to diagnosis pulmonary embolism. Among them, nine patients developed venous thrombosis: five and three pulmonary embolisms in clinical worsening and improving group, respectively, and one superficial venous thrombosis in clinical improving group. Only one patient who had developed thrombosis did not have thromboprophylaxis. Median follow up was 20.5 days [13–27]. Each patient completed the follow up. No patient developed arterial thrombosis. Demographic and clinical data were reported in **Table 1**. Age and O_2 requirement at the time of admission were significantly different between groups. As expected, anticoagulation instauration and hospitalization duration were reported to be significantly different between groups as well as the radiological scale of disease extension.

In biological markers, we observed a non-significant difference in lymphocyte blood count <1 G/L (50 vs. 67.6%) and a significant difference between clinical worsening and improving for monocyte blood count <0.2 G/L (1.6 vs. 17.6%). Biological characteristics were resumed in **Supplementary Table 1**. Significant differences are shown in **Figure 1**. Moreover, neutrophil/lymphocyte ratio was not significantly different (4.4 [2.5–7.5] vs. 5.7 [3.4–11.4]), and we demonstrated that neutrophil/monocyte ratio was increased in worsening group (8.8 [6.7–12.1] vs. 16.7 [9.0–19.8]). Fibrinogen levels and D-dimer were also increased in worsening group. Fibrin monomers and antithrombin levels were not significantly different. ISTH DIC score was calculated at the time of admission either with D-dimer or with Fibrin monomer. We observed a significant difference between worsening and improving patients with ISTH DIC score with D-dimer, and no difference with fibrin monomer scores was significant (2 [2–3] vs. 2 [2–2], and 0 [0–0.25] vs. 0 [0–1], respectively, with D-dimer and Fibrin monomer).

VWF:GPIb-binding activity was also different ($P < 0.01$). Coagulation activation was studied thanks to thrombin generation assay and Prothrombin fragments 1+2 measurement with a significant difference among the two groups ($P < 0.05$).

Association Between Clinical–Biological Parameters and Clinical Outcome

As described in section 2.5, clinical, radiological, and biological parameters were used as predictors for logistic regression analysis in order to determine predictors of clinical worsening outcome (**Table 2**). Final model of the logistic regression analysis revealed that O_2 requirement (RR = 7.27 [1.50–19.31]; $P = 0.045$), monocytes below 0.2 G/L (RR = 2.88 [1.67–3.19]; $P = 0.015$), fibrinogen levels (RR = 1.45 [1.17–1.82] per g/L increase; $P = 0.005$), prothrombin fragments 1+2 higher than 290 pM (RR = 2.39 [1.20–3.30]; $P = 0.023$), and peak of the TGA assay (RR = 1.28 [1.03–1.59] per 50 nM increase; $P = 0.043$) were associated with an increased risk of clinical worsening (**Table 2**).

Based on the predictors of the final model of the logistic regression, a classification tree was built in order to establish a hierarchical ranking of predictors to classify patients between clinical worsening and improving. Fibrinogen levels below 5.5 g/L was associated with clinical improving ($N = 35/40$, 87.5%). For patients with higher value than 5.5 g/L, a TGA peak below 99 nM is also predictive of favorable outcome ($N = 11/12$, 90.9%). Then, for patients with fibrinogen higher than 5.5 g/L and TGA peak higher than 99 nM, patients had a better clinical outcome prognosis if they did not depend on O_2 requirement when compared with patients who need it ($N = 5/6$, 83.3% and $N = 28/42$, 66.7%, respectively). This classification tree provided an accuracy of 79%, a sensitivity of 88%, a specificity of 67%, a positive predicted value of 78%, and a negative predicted value of 80% (**Figure 2**).

TABLE 1 | Epidemiological, demographic, and clinical characteristics of the 99 hospitalized patients with COVID-19 infection.

Parameters	All ($n = 99$)	Improving ($n = 64$)	Worsening ($n = 35$)	*P*-value
Age, years	65 [51.5–75.0]	63 [48.8–74.3]	71 [59.0–76.0]	**0.044**
Male	53 (54%)	30	23	0.113
Body mass index, kg/m^2	27.8 [24.0–32.0]	26.9 [23.8–31.0]	28.4 [24.5–32.8]	0.255
Underlying comorbidity				
Chronic obstructive pulmonary disease	7 (7.1%)	4 (6.3%)	3 (8.6%)	0.695
Asthma	10 (10.1%)	6 (9.4%)	4 (11.4%)	0.739
Other respiratory disease	9 (9.1%)	7 (10.9%)	2 (5.7%)	0.486
Diabetes	30 (30.3%)	16 (25.0%)	14 (40.0%)	0.186
Hypertension	52 (52.5%)	30 (46.9%)	22 (62.9%)	0.190
Chronic kidney disease (eGFR < 30 ml/min)	9 (9.1%)	5 (7.8%)	4 (11.4%)	0.717
Chronic heart failure	4 (4.1%)	2 (3.1%)	2 (5.7%)	0.613
Previous drug use				
Immunosuppressant drugs	7 (7.1%)	3 (4.7%)	4 (11.4%)	0.240
Anticoagulation				
None	53 (53.5%)	44 (68.8%)	9 (25.7%)	**<0.001**
Standard	25 (25.3%)	13 (20.3%)	12 (34.3%)	
Enhanced	13 (13.1%)	5 (7.8%)	8 (22.9%)	
Curative	8 (8.1%)	2 (3.1%)	6 (17.1%)	
O_2 requirement on admission	73 (73.7%)	40 (62.5%)	33 (94.3%)	**<0.001**
Smoking history				
Never/Former smokers	84 (84.8%)	54 (84.4%)	30 (85.7%)	1.000
Current smokers	15 (15.2%)	10 (15.6%)	5 (14.3%)	
Hospitalization duration	12.0 [6.0–19.0]	9.0 [5.5–14.0]	18.0 [8.5–25.5]	**0.002**
Hospitalization delay since the onset of symptoms	7.0 [4.0–10.0]	7.0 [4.0–10.0]	6.0 [4.0–7.0]	0.195
Radiological injuries				
None	3/86 (3.5%)	2/54 (3.7%)	1/32 (3.1%)	**0.009**
Not suggestive	3/86 (3.5%)	3/54 (5.6%)	0/32 (0.0%)	
<10%	11/86 (12.8%)	9/54 (16.7%)	2/32 (6.3%)	
10–25%	39/86 (45.3%)	29/54 (53.7%)	10/32 (31.3%)	
25–50%	15/86 (17.4%)	5/54 (9.3%)	10/32 (31.3%)	
50–75%	13/86 (15.1%)	6/54 (11.1%)	7/32 (21.9%)	
>75%	2/86 (2.3%)	0/54 (0.0%)	2/32 (6.3%)	
Radiological condensation				
None	22/82 (26.8%)	17/52 (32.7%)	5/30 (16.7%)	0.427
Nodular	17/82 (20.7%)	10/52 (19.2%)	7/30 (23.3%)	
Linear	37/82 (45.1%)	22/52 (42.3%)	15/30 (50.0%)	
Linear and nodular	6/82 (7.3%)	3/52 (5.8%)	3/30 (10%)	
Radiological abnormalities localization				
None	10/81 (12.3%)	8/51 (15.7%)	2/30 (6.6%)	0.554
Unilateral	3/81 (3.7%)	2/51 (3.9%)	1/30 (3.3%)	
Bilateral	68/81 (84%)	41/51 (80.4%)	27/30 (90.0%)	
Thrombosis	8 (8.1%)	3 (4.7%)	5 (14.3%)	0.127
SARS-CoV-2 nucleic acid test pre-admission	81 (81.8%)	50 (78.1%)	31 (88.6%)	0.278

Data are expressed as median [IQR], n (%), or n/N (%), where N is the total number of patients with available data. Escalated anticoagulation corresponding to 4,000 UI twice a day of enoxaparin, 6,000 UI twice a day of enoxaparin if body weight > 120 kg, or 200 UI/kg of unfractionated heparin. Lymphocytes <1 G/L, monocytes <0.2 G/L, VWF, GPIb-binding activity >250%, Prothrombin fragment 1+2 >290 pM are outside values range. P-values comparing clinical improvement to clinical worsening are from χ^2 test, Fisher's exact test, or Mann–Whitney U-test. DIC, disseminated intravascular coagulation; ISTH, international society of thrombosis and haemostasis; TGA, thrombin generation assay.
Bold values are significant values.

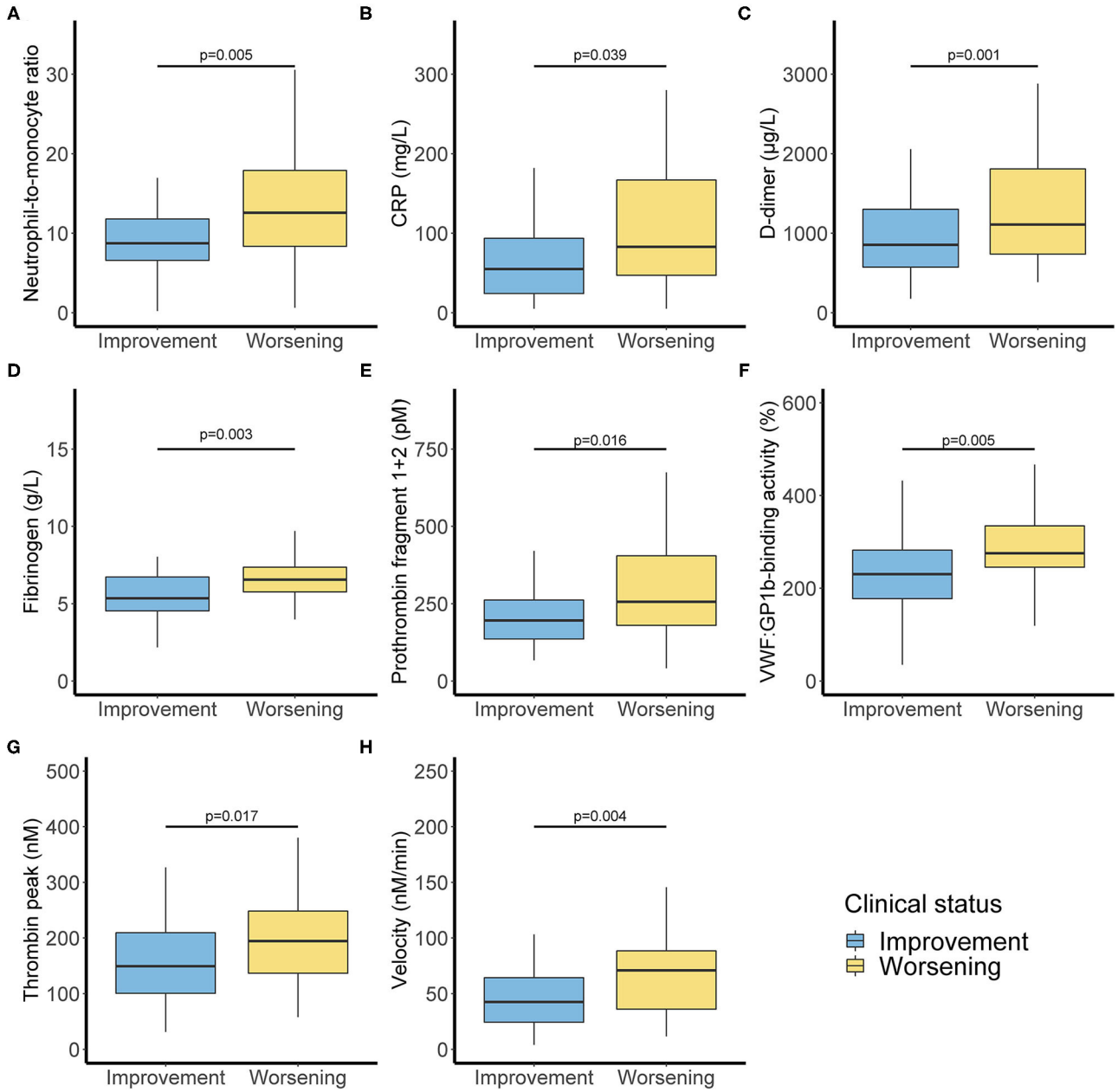

FIGURE 1 | Inflammatory and coagulation biomarkers associated with clinical worsening. Inflammation markers with neutrophil monocyte ratio **(A)** and C-reactive protein **(B)**. Activated coagulation with D-dimer **(C)**, fibrinogen **(D)**, and Prothrombin fragment 1+2 **(E)**. Increased Von Willebrand factor activity **(F)** and thrombin generation with thrombin peak **(G)** and velocity **(H)**. *P*-values comparing clinical improvement to clinical worsening are from Mann–Whitney *U*-test.

DISCUSSION

Our study aimed to demonstrate with clinical, radiological, and hemostasis markers the association of clinical worsening in COVID-19 patients. The association between fibrinogen, thrombin peak, and O_2 requirement had a good correlation with clinical outcome of patients.

Since December 2019, several clinical and biological markers were associated with poor prognosis in COVID-19 patients. Several studies predict the occurrence of critical illness (23–25) and mortality in COVID-19 infection (15, 26–28). Elderly, increased body mass index, hypertension (29), diabetes, and male gender (30) were demographics and associated comorbidity regularly included in the predictive score. As expected, O_2 requirement in preadmission was a predictive factor to develop worsening SARS-CoV-19. Among biological markers, increases in C-reactive protein and urea are regularly in the prognostic score. C-reactive protein can increase rapidly after

TABLE 2 | Association factors with clinical worsening (death or intensive care unit admission).

Patient characteristics	Unadjusted[a]		Logistic regression full model[b] (*N* = 84)		Logistic regression final model[c] (*N* = 84)	
	RR (95% CI)	*P*-value[d]	RR (95% CI)	*P*-value[d]	RR (95% CI)	*P*-value[d]
Age (per 5-year increase)	**1.10 (1.01–1.19)**	**0.035**	1.00 (0.85–1.17)	0.984		
Sex (Female)	1.67 (0.95–2.48)	0.075				
BMI (per unit increase)	1.03 (0.98–1.08)	0.261				
Chronic respiratory disease	0.82 (0.33–1.48)	0.576				
Diabetes	1.53 (0.87–2.24)	0.123				
HTA	1.53 (0.87–2.29)	0.130				
Oxygenotherapy	**5.88 (2.37–11.0)**	**0.003**	**10.6 (1.87–20.39)**	**0.033**	**7.27 (1.50–19.3)**	**0.045**
Tobacco consumption	0.93 (0.35–1.70)	0.859				
Severe radiological abnormality	**2.73 (1.73–3.55)**	**<0.001**	1.91 (0.64–3.49)	0.210		
Lymphocytes (<1 G/L)	1.64 (0.92–2.49)	0.097				
Monocytes (<0.2 G/L)	**2.79 (1.60–3.22)**	**0.018**	**2.89 (1.46–3.20)**	**0.026**	**2.88 (1.67–3.19)**	**0.015**
NL Ratio (per unit increase)	1.02 (0.98–1.06)	0.300				
NM Ratio (per unit increase)	**1.03 (1.01–1.07)**	**0.023**	0.97 (0.92–1.02)	0.219		
TCA (>1.15)	1.14 (0.61–1.77)	0.652				
D-Dimer (>1,000 μg/L)	**2.12 (1.26–3.03)**	**0.008**	1.58 (0.45–3.15)	0.419		
Fibrin monomers (>6)	1.67 (0.88–2.42)	0.102				
Fibrinogen (per unit increase)	**1.32 (1.13–1.54)**	**0.002**	**1.45 (1.10–1.88)**	**0.020**	**1.45 (1.17–1.81)**	**0.005**
VWF:GPIb-binding activity (>250%)	**2.00 (1.15–2.97)**	**0.021**	0.64 (0.14–1.89)	0.487		
Prothrombin fragment 1+2 (>290 pM)	**2.17 (1.37–2.94)**	**0.004**	**2.58 (1.23–3.46)**	**0.025**	**2.39 (1.20–3.30)**	**0.023**
ETP (per 200 unit increase)	1.12 (0.97–1.27)	0.122				
Peak (per 50 unit increase)	**1.26 (1.07–1.48)**	**0.008**	1.58 (0.59–2.47)	0.308	**1.28 (1.03–1.59)**	**0.043**
Velocity (per 50 unit increase)	**1.75 (1.28–2.20)**	**0.002**	0.64 (0.15–2.63)	0.754		

Results are expressed as relative risk (RR) (95% confidence interval). N = 84 due to missing data for retained predictors.
[a] Univariate logistic regression analysis.
[b] Multiple logistic regression analysis for variables with P-value below 0.05.
[c] Backward variable selection from the full model.
[d] Wald test.
Bold values are significant values.

the onset of inflammation, cell damage, or tissue injury. The endothelium supports an extensive repertoire of natural anticoagulant. However, during sepsis, activated endothelium increase in TF expression within the vasculature is considered a pivotal step in initiating and sustaining coagulation. The concept of sepsis induced endothelial dysfunction is known as thromboinflammation (31). Few studies evaluated coagulation biomarkers to predict intensive care unit transfer and death in COVID-19. Zhang et al. (19) described an increase of D-dimer associated with poor prognosis. However, the rise of D-dimer during hospitalization is associated with a limited performance to predict death (26). In the study of He et al. (32), the D-dimer cutoff at hospital discharge or death is 2,025 μg/L (AUC: 0.909) and associated with a poor prognosis. In our study, D-dimer >2,000 was not associated with clinical worsening. However, the aim of the study was not the same, with death for He et al. Moreover, the C-reactive protein was more correlated with disease severity compared to D-dimer (33). Our results suggest that fibrinogen had a high discriminate power and a more specific manner than D-dimer does.

Prothrombin fragments 1+2 are less impacted by inflammation than D-dimer (34). We demonstrated increase of prothrombin fragments 1+2. In a recent study evaluating prothrombin fragment 1+2 in COVID-19-associated thrombosis (35), a prothrombin fragment 1+2 >500 pmol/L was associated with venous thromboembolism (odds ratio: 4.26). Conversely, a D-dimer >2,500 ng/mL was not significantly associated with VTE (odds ratio: 5.91).

The interest of global coagulation assay has been previously demonstrated in COVID-19 (36). TGA has already been used to evaluate hypercoagulability (37–40) and acute ischemic stroke development (41). The fact that SARS-CoV-2 virus induces severe endothelial injury associated with intracellular virus and disrupted endothelial cell membranes (42) make TGA an interesting tool to predict clinical outcome of SARS-CoV-2-infected patients. Indeed, microangiopathy and occlusion of alveolar capillaries from lung patients with COVID-19 were found to be secondary to widespread vascular thrombosis (42). The monocytopenia count below 0.2 G/L could be related to COVID-19 severity. This is in accordance with the fact that a decreased monocyte count is associated with poor prognosis in sepsis (43). Recruitment of monocytes is essential for effective control and clearance of viral, bacterial, fungal, and protozoal infections (44). The inflammatory recruitment failure is also a possible explanation to aggravation.

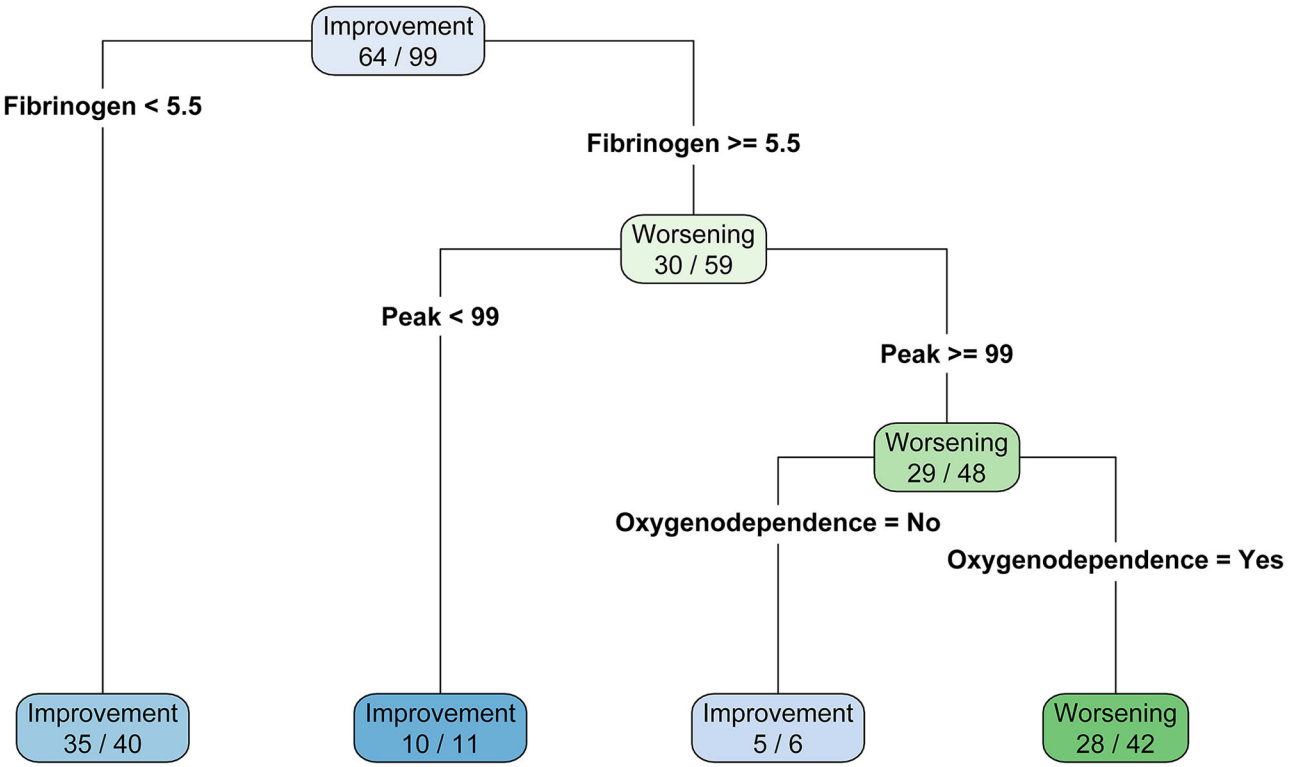

FIGURE 2 | Classification three of clinical status according to final logistic model predictors.

Several studies have described DIC in some COVID-19 patients. In the study of Fogarty et al. (45), DIC was rare and appeared in the late stage disease. In two others studies (16, 46), DIC was significantly more frequent in non-survivors than in survivors. In contrast, in the 24 patients from Panigada's report (47), DIC was not evidenced. With ISTH score, we demonstrated DIC score increase with D-dimer, in worsening patients with more than 75% with a DIC score below 3. With fibrin monomer, more than 75% worsening patients had a DIC score below of 1. Furthermore, the increase of platelet and fibrinogen, associated with normal prothrombin time in our patients, explains the normal DIC score results.

The interest to predict clinical outcome in COVID-19 leads to important increases in the demand for hospital beds and shortage of medical equipment. The urgency of diagnostic and prognostic models can assist quickly the efficient triage of patients in the COVID-19 pandemic (12). Several scores exist for the prediction of mortality in pneumonia, such as CURB-65, A-DROP score, and 4C mortality score (13–15). However, these scores are not suitable to determine intensive care unit transfer. Interestingly, our results demonstrated that the association of fibrinogen level, thrombin peak measurement, and O_2 requirement was an easy-to-apply model that could predict near than 80% of clinical outcome. Of note, we observed 33/35 patients with O_2 requirement in the clinical worsening group, among which 26 had fibrinogen level higher than 5.5 g/L and TGA peak higher than 99 nM, suggesting the ability of these last two parameters to predict clinical outcome.

The interest of predictive score to worsening, including intensive care unit transfer during hospitalization, is prompt aggressive treatment, including the initiation of steroids and early escalation to critical care if appropriate (48). A recent study demonstrate that coagulation biomarkers are independent predictors of increased oxygen requirement in COVID-19 patients (49), among them increased fibrinogen and decreased FVIII/VWF:Ag ratio. A study confirmed that D-dimer increase is not associated with intensive care unit transfer (23).

In the study of Panigada et al. (47), von Willebrand factor antigen and ristocetin cofactor activities greatly increased. In the Poissy et al. study (50), factor Willebrand antigen levels seem to be associated with a greater PE risk.

Nevertheless, our study presents several limitations. We have a limited sample size, but the aim of the study was to develop an easy-to-use score to help clinicians. Moreover, our study was prospective and each patient has completed the follow up. Furthermore, we used a robust standardized coagulation test. Finally, our predictive score was computed on our total cohort since it did not appear reasonable to split the data into a training and a test dataset. The validation of our predictive score is required to ensure the reproducibility of the developed mode.

CONCLUSION

In conclusion, we identified that high fibrinogen, O_2 requirement, and thrombin peak at admission were associated with a secondary admission in intensive care unit or death.

The score allows the initiation of steroids and triage for worsening patients. Our results should therefore be considered as exploratory and deserve confirmation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional review board (person committee protection of Rouen University Hospital) and a national ethical committee (person committee protection South Mediterranean 1; Authorization protocol number: 2020-A00914-35). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PB designed the research, performed analysis, analyzed and interpreted the data, and wrote the manuscript. VL analyzed and interpreted the data and wrote the manuscript. TD critically revised the manuscript and checked the statistical methods and results. KA, MR, SM, OG, LJ, MD, GF, VB, and ME included patients and discussed the obtained results and critically revised the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.670694/full#supplementary-material

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REVIEW
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Nanomedicine: A Diagnostic and Therapeutic Approach to COVID-19

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The SARS-CoV-2 virus is causing devastating morbidity and mortality worldwide. Nanomedicine approaches have a high potential to enhance conventional diagnostics, drugs and vaccines. In fact, lipid nanoparticle/mRNA vaccines are already widely used to protect from COVID-19. In this review, we present an overview of the taxonomy, structure, variants of concern, epidemiology, pathophysiology and detection methods of SARS-CoV-2. The efforts of repurposing, tailoring, and adapting pre-existing medications to battle COVID-19 and the state of vaccine developments are presented. Next, we discuss the broad concepts and limitations of how nanomedicine could address the COVID-19 threat. Nanomaterials are particles in the nanometer scale (10–100 nm) which possess unique properties related to their size, polarity, structural and chemical composition. Nanoparticles can be composed of precious metals (copper, silver, gold), inorganic materials (graphene, silicon), proteins, carbohydrates, lipids, RNA/DNA, or conjugates, combinations and polymers of all of the aforementioned. The advanced biochemical features of these nanoscale particles allow them to directly interact with virions and irreversibly disrupt their structure, which can render a virus incapable of replicating within the host. Virus-neutralizing coats and surfaces impregnated with nanomaterials can enhance personal protective equipment, hand sanitizers and air filter systems. Nanoparticles can enhance drug-based therapies by optimizing uptake, stability, target cell-specific delivery, and magnetic properties. In fact, recent studies have highlighted the potential of nanoparticles in different aspects of the fight against SARS-CoV-2, such as enhancing biosensors and diagnostic tests, drug therapies, designing new delivery mechanisms, and optimizing vaccines. This article summarizes the ongoing research on diagnostic strategies, treatments, and vaccines for COVID-19, while emphasizing the potential of nanoparticle-based pharmaceuticals and vaccines.

Keywords: SARS-CoV-2 virus, vaccine, nanotechnology, drug delivery systems, sepsis, acute respiratory distress syndrome

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INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection causes the ongoing pandemic of Coronavirus Disease 2019 (COVID-19). In 2019, the first confirmed and documented cases of COVID-19 in China rapidly progressed to a worldwide state of emergency unparalleled since the outbreak of the Spanish Flu in 1918. The failure to control the spread of COVID-19

has highlighted the urgency of developing diagnostic and therapeutic approaches against highly contagious pathogens. A plethora of innovative treatments is being proposed which incorporate the use of traditional and futuristic methods to minimize the pathogenicity, morbidity and mortality of SARS-CoV-2. Nanotechnology is an emerging field that has branched into the world of medicine. Due to its progressive nature, nanomedicine can overcome difficulties facing conventional medicine. Most importantly, it will hopefully contribute to revolutionizing drug-based medicine in the twenty first century.

Nanomaterials have properties that, if exploited correctly, may improve treatments and vaccines, and provide alternative and safer ways to battle diseases (1). However, emergence of side effects of these nanoparticles, such as unwanted interactions with tissues or increased inflammation, could put a temporary hold on the utilization of nanotechnology (2). The COVID-19 crisis sets the stage to evolve the concepts of nanotechnology into reality. As its potential is revealed, it can offer innovative ways of protecting healthy and infected individuals, detecting SARS-CoV-2, and helping to end the pandemic.

In this review, we present an overview of SARS-CoV-2 pathophysiology, diagnostics, treatment and vaccines followed by discussing the current and future applications of nanomedicine aiming to mitigate the COVID-19 pandemic. The nanoparticle approaches presented here will help to win the fight against SARS-CoV-2 and other pathogens.

SARS-COV-2

Origin and Transmission

In the first week of January 2020, the Chinese Center for Disease Control and Prevention (CCDC) disclosed that 27 cases of pneumonia admitted during late December of 2019, were attributed to Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), later named COVID-19 by the World Health Organization (WHO) (3). The patients had visited one of the "wet markets" in Wuhan city, located in China's Hubei province, which are known for their considerable variety of wild animals for sale (4). Recent genomic analysis has revealed that the SARS-CoV-2 genome is 96% identical to a known bat coronavirus (BatCoV RaTG13) from *Rhinolophus affinis*, a species found in Yunnan province (5, 6). The WHO declared the viral outbreak a public health emergency of global proportions at the end of January, when there were approximately 10,000 diagnosed cases around the globe (7). It was estimated that SARS-CoV-2 has a Case Fatality Rate (CFR) of 2–4% (8, 9) with substantial variation between countries, as well as a higher basic reproduction number (median R_0 range: 3.5–4.7) compared to other coronaviruses or influenza (**Figure 1**) (10–12). As of April 2021, more than 140 million people across the globe have

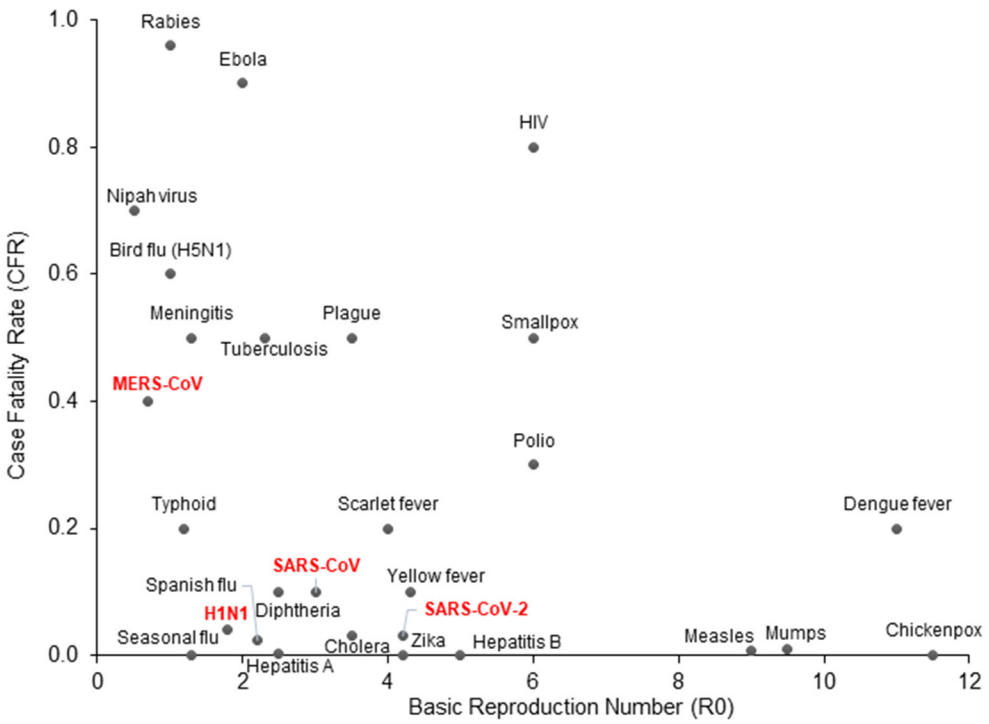

FIGURE 1 | COVID-19 epidemiologic characteristics compared to other prevalent infections. The Case Fatality Rate (CFR) for COVID-19 is estimated around 2–4% with some variation and a recent decline due to optimized supportive care. The Basic Reproduction Number (R0) shown on the x-axis is also an estimate from epidemiological data. SARS-CoV-2 is more contagious than SARS-CoV and MERS-CoV, which may be attributed to longer incubation periods and asymptomatic carriers.

TABLE 1 | Human pathogenic coronaviruses.

Name	R0	CFR	Pathophysiology	Natural host–intermediate host	Epidemiology	References
HCoV-229E	NA	NA	Sore throat, Fever, Cough, Headache, Nasal discharge	Bats–Camelids?	Global–Fall	(13)
HCoV-NL63	NA	NA	Cough, Fever, Hypoxia, Tachypnea	Bats–NA	Global–Fall	(14)
HCoV-OC43	NA	NA	Sore throat, Fever, Cough, Headache, Nasal discharge	Rodents–Bovines	Global–Fall	(15)
HCoV-HKU1	NA	NA	Fever, Cough	Rodents–NA	Global–Fall	(16)
MERS-CoV	0.7	0.4	Pneumonia, Sore throat, Fever, Cough, Chills, Dyspnea	Bats–Camels	Middle East−2011	(12, 17)
SARS-CoV	3	0.1	Respiratory distress, Fever, Dry cough, Headache, Myalgia	Bats–Palm Civets	China then Global−2003	(12, 18)
SARS-CoV-2	3.5–4.7	0.03	Pneumonia, ARDS, Fibrosis, Fever, Dry cough, Coagulopathy	Bats–Pangolins?	China then Global−2019	(7, 10, 19)
H1N1	1.7	0.04	Cough, Sore throat, Chills, Fever, Headache	Pigs–Pigs	Global–Fall (Outbreak 2011)	(12, 20, 21)

SARS-CoV, MERS-CoV, and SARS-CoV-2 can cause severe morbidity and mortality in vulnerable individuals. Infections with other coronaviruses usually only result in mild symptoms. For comparison, the influenza A subtype, H1N1, of the orthomyxovirus family is shown. R0, Basic reproduction number; CFR, Case fatality rate; NA, Not available.

contracted COVID-19, and more than 3 million of those cases resulted in fatalities.

Taxonomy and Structure

Coronaviruses (CoVs) belong to the *Cornidovirineae* suborder under the *Coronaviridae* family. CoVs are a predominant group of viruses, but of the 46 known CoVs only 7 have been confirmed to infect humans (13). Human coronavirus 229E (HCoV-229E) and human coronavirus NL63 (HCoV-NL63) are members of the genus *Alphacoronavirus* while human coronavirus OC43 (HCoV-OC43) and human coronavirus HKU1 (HCoV-HKU1) belong to the genus *Betacoronavirus*. These viruses are linked to mild upper respiratory tract diseases and can be attributed to 15–30% of common cold cases with regional/global and seasonal patterns (**Table 1**) (14, 22, 23). In contrast, SARS-CoV (sometimes referred to as SARS-CoV-1), MERS-CoV, and SARS-CoV-2 of the *Betacoronavirus* genus, are associated with severe disease pathophysiology, including respiratory disease, multi-organ failure, sepsis and death (14–16, 20, 21, 24–26).

SARS-CoV-2 is encoded by positive single-stranded RNA (ssRNA) bound to the nucleocapsid phosphoprotein (N). It is enclosed in a bilipid envelope surrounded by transmembrane proteins, such as the small envelope glycoprotein (E), membrane glycoprotein (M), and type-I trimeric spike protein (S) (**Figure 2**) (27–33). SARS-CoV-2 spike protein binds the Angiotensin Converting Enzyme 2 (ACE2) receptor located on type I and II pneumocytes and other epithelial and non-epithelial tissues, to enter host cells (34). More specifically, the spike protein monomers depend on host proteases for entry, such as the transmembrane serine protease 2 (TMPRSS2). TMPRSS2 can hydrolyze peptide bonds between the S1 and S2 subunits (35, 36). This process primes the spike protein and allows the S1 subunit, which contains the receptor binding domain (RBD) held together by several disulfide bonds, to bind with the N-terminal helix of ACE2 (17, 18, 37, 38). After internalization into the host cell, SARS-CoV-2 undergoes an uncoating process and initial viral transcription which requires supportive proteins and enzymes, including some rarely found in other RNA viruses such as (3'-to-5' exoribonuclease, 2'-O-ribose methyltransferase, ADP ribose 1'-phosphatase) (27). The viral transcripts can amass to 15–30% of the transcriptome in infected host cells (39). The translation of viral proteins occurs in the cytoplasm and viral proteins control the replication process. Viral proteins are inserted into the Golgi apparatus and are transported to the plasma membrane, where virions are released and begin infecting neighboring cells (19).

The genome sequences by next-generation sequencing (NGS) indicated that SARS-CoV-2 is more closely related to bat coronaviruses (BatCoV RaTG13 [96%], SL-CoVZXC21 [88%], SL-CoVZC45 [88%] (5), than to SARS-CoV (79% similarity) and MERS-CoV (50% similarity) (27, 40). SARS-CoV-2 spike protein has ∼75% sequence similarity to the amino acid sequence of SARS-CoV spike protein (41), including a mutation in the C-terminal RBD for enhanced binding to ACE2 (5, 42, 43). SARS-CoV-2 quasispecies have been described, although the mutation rate is slower than for influenza virus (44). Virus variants of concern with higher infectivity and pathogenicity and a risk for resistance against the first generation of vaccines have emerged (45). A variant encoding a D614G mutation (conversion of aspartic acid to glycine at position 614) in the spike protein, located in the S1 domain has become most prevalent (46). This new D614G variant is associated with increased replication and transmission when compared to other less common isolates, such as the USA-WA1/2020 variant, which contains an aspartic acid residue at this position (47, 48). There are several sub-variants, such as the D16 INMI1 isolated in Italy, the G614 PV08449/2020 isolated in New York and the G614 BavPat1/2020 isolated in Germany (49, 50). Three variants of concern each with 17 amino acid changes and all featuring a N501Y spike protein mutation have emerged in the end of 2020 (51): A VUI-202012/01 (B.1.1.7) variant was first detected in the United Kingdom (52). The 501Y.V2 (B.1.351) variant was first discovered in South Africa and the P.1 variant was initially reported in Brazil and

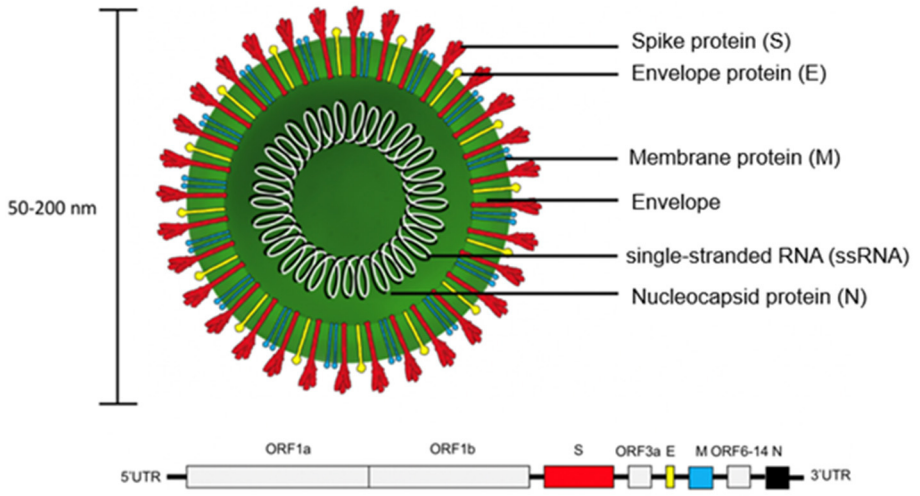

FIGURE 2 | Structure of SARS-CoV-2 virion and genome. The trimeric spike protein (S) is required for docking to the hACE2 receptor. S protein is targeted by antibody-based therapies and is used as the immunogen for vaccine candidates. The single-stranded RNA (ssRNA) genome is bound to the Nucleocapsid phosphoprotein (N) which facilitates transcription after virus entry into host cells. The large viral genome (29.9 kb) is arranged as open reading frames (ORF) encoding for about 27 non-structural proteins (e.g., replicase, protease) and the four structural proteins (S, E, M, N).

Japan (53). The P.1 and B.1.351 variants contain an E484K spike mutation.

Pathophysiology

The clinical presentation of COVID-19 can be grouped in three categories based on disease severity and progression: the asymptomatic phase/stage, the mild symptomatic stage, and the severe respiratory infection stage (**Table 2**). Most individuals do not pass through all stages and asymptomatic or mild symptoms are most common (61). It is estimated that 15–30% of cases are asymptomatic, which may contribute to herd immunity (62). Individuals in the first category, also known as "stealth carriers," do not present any symptoms and molecular testing can even be negative. If COVID-19 progresses to stage 2, mild infection symptoms are observed such as fever and coughing, and the patient typically tests positive in RT-PCR assays (59). It can take 1–3 weeks after the first symptoms for the production of antibodies against SARS-CoV-2. The third and most severe phase may present as a flu-like stage, a respiratory inflammation stage including pneumonia, acute respiratory distress syndrome (ARDS), pulmonary edema, and sometimes the complications of coagulopathy and fibrotic changes due to lung remodeling (54). This sequence of events can result in dramatically compromised gas exchange and respiratory failure (**Table 2**) (55, 63). Severe COVID-19 (stage 3) appears to be associated with a higher production of neutralizing antibodies. Additional symptoms include gastrointestinal dysfunction and secondary infections, as well as harmful tissue destruction due to pro-inflammatory leukocytes such as macrophages and granulocytes (56, 58–60).

Emerging evidence suggests that a previous infection with one of the four endemic coronaviruses that cause "common cold" (HCoV-OC43, -HKU1, -NL63, and−229E) is associated with mitigated SARS-CoV-2 illness, which may be explained by a better pre-existing immune response and heterotypic immunity to homologous viruses (64). In addition to neutralizing, cross-reactive antibodies, memory $CD4^+$ T cells have been hypothesized to reduce lung viral burden, accelerate antibody production and to enable heterotypic immunity (65–67). On the other hand, anti-SARS-CoV-2 antibodies with cross-reactivity for host proteins may contribute to pathologies such as Kawasaki-like disease and Guillain-Barré syndrome (68–70).

Many factors can influence the severity and outcome of COVID-19 infection such as age, gender, pre-existing health conditions and comorbidities (71). In general, the rates for apparent infection, hospitalization and death are higher for individuals aged 65 and above. Men have a higher risk for severe disease, an observation that has not been fully explained (72, 73). One hypothesis is centered around the higher tobacco use in men (4:1) and that long-term smokers develop cardiovascular and respiratory conditions which correlate with rapid and severe progression of COVID-19 (57). Increased vulnerability to SARS-CoV-2 is also correlated with a variety of health factors such as severe obesity, type II diabetes mellitus, serious cardiovascular conditions and immunocompromised states such as autoimmune disease or recent chemotherapy (74, 75). Last but not least, susceptibility to COVID-19 has been linked to certain genetic traits including polymorphisms for *IFNAR2*, *TYK2, TLR7, OAS1, DPP9,* and *CCR2*, and the major histocompatibility complex loci (*HLA*) (76) which also provide susceptibility to other infections such as Influenza, Hepatitis B, and leprosy (77).

Research and Detection

The WHO and the Center for Disease Control and Prevention (CDC) have established detailed protocols regarding the use,

TABLE 2 | COVID-19 pathophysiology.

	Observed symptoms	Clinical markers	Viral burden	Immune response	Therapeutic strategy
Stage 1 Early infection phase	None or mild symptoms	Lymphopenia, ↑CRP and ↑IL-6	Low (Incubation period)		No therapy needed
Stage 2 Pulmonary phase	Dry coughing, Fever, Shortness of breath, Headache	Glass opacities (CT scans), Mild hypoxia	Intermediate (Spread from lower respiratory tract)		Treat symptoms
Stage 3 Hyperinflammation phase	Pneumonia, Chest pain, Productive coughing, Multiple organ failure	Cytokine storm, ARDS, Severe hypoxemia, Acute kidney injury	Highest (Expansion throughout the respiratory tract)		Reduce inflammation, Mechanical ventilation, Hemodialysis
References	(54–57)	(54, 55, 58)	(59)	(54, 60)	(55, 60)

The time course and severity of illness can be classified into three stages (1–3). The clinical presentations are variable and most patients do not experience all stages.

TABLE 3 | Detection methods for SARS-CoV-2.

Type	RT-PCR	RT-LAMP	CRISPR-Cas12	Enzyme linked immuno- assay	Rapid diagnostic test
Detection	N gene, E gene, RdRp	N gene, S gene, ORF1ab,	N gene, E gene	IgM/IgG antibodies	IgM/IgG antibodies
Sample type	Nasopharyngeal swab, Oropharyngeal swab	Nasopharyngeal swab, Oropharyngeal swab	Nasopharyngeal swab, Oropharyngeal swab	Plasma or Serum	Plasma or Serum
Time point	Symptom onset	Symptom onset	Symptom onset	Days/weeks after symptom onset	Days/weeks after symptom onset
Advantages	High accuracy, High reliability, Direct detection	High accuracy, High reliability, Rapid detection, Color visualized by the naked eye	High accuracy High reliability	High specificity	Low cost, Ease of use, High specificity
Disadvantages	Labor intensive, Errors with sample collection	Carry-over contamination	High limit of detection	Lower sensitivity	Lower sensitivity
References	(74, 78)	(87, 88)	(89)	(78–80)	(78, 80, 90)

RT-PCR testing is highly sensitive and widely applied. Limitations are false positive results and prolonged test positivity after recovery from active COVID-19. The characteristics of serology tests for antibodies and innovative CRISPR-Cas methods are shown.
RdRp: RNA dependent RNA polymerase.

containment, culturing, and testing for SARS-CoV-2. The CDC has classified any research work with infective SARS-CoV-2 as Biosafety Level 3 (BSL-3) category, while protocols with inactivated forms of SARS-CoV-2 or pseudotyped viruses can be performed in a BSL-2 laboratory (78).

It is widely accepted that suitable cell lines to propagate SARS-CoV-2 must express sufficient numbers of ACE2 and TMPRSS2 on their surface. The Vero cell line is derived from kidney cells of the African green monkey and sublineages such as the Vero E6 and Vero CCL81 cell lines produce even higher SARS-CoV-2 titers. Other cell types such Calu-3 (a human lung cancer line), Caco-2 (a human colorectal adenocarcinoma line), HEK 293T (derived human embryonic kidney line) and Huh7 (a human hepatocellular carcinoma line) can also be used for infection studies, but are not suitable for generating high titer virus stocks (79). Genetically modified cell lines, such as an ACE2 overexpressing HEK 293T line and Air-Liquid Interface (ALI) epithelial cell models exist (80). Human induced Pluripotent Stem Cell (iPSC)-derived alveolar type 2 cells (iAT2) are susceptible to SARS-CoV-2 infection in ALI culture. SARS-CoV-2 infection of the iAT2 cells hijacks the transcriptomic machinery, deprograms host cell differentiation, while inducing the NFkB pathway and interferon-dependent host defense programs (39, 81).

SARS-CoV-2 infection can be investigated in animal models. Mice *(Mus musculus)* were genetically engineered to over-express human ACE2 because SARS-CoV-2 spike protein does not bind well to murine ACE2. In addition, non-modified Syrian hamsters *(Mesocricetus auratus)*, ferrets *(Mustela putorius furo)*, non-human primates (Cynomolgus macaques and Rhesus Macaques) and other mammalian species can be infected to study the pathobiology of COVID-19 (79, 82–86).

Molecular diagnostic test for SARS-CoV-2 were rapidly developed (**Table 3**). The first Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assay was released by the WHO and targeted three regions of the SARS-CoV-2 genome: the N gene, the E gene, and a highly conserved gene for RNA-dependent RNA polymerase (RdRp) (91, 92). Meanwhile, multiple alternative RT-PCR primer sets are available, while additional methods such as SARS-CoV-2 nucleoprotein antigen tests and antibody

detection kits have also been developed (90, 93–95). The SARS-CoV-2 specific antibodies can be detected by rapid diagnostic tests (90). CRISPR-Cas12 based assays, such as DETECTR, identify the presence of SARS-CoV-2 RNA (89, 95). Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) is a faster (30–40 min) and cheaper alternative for RT-PCR with the advantage of point-of-risk testing (87, 88). Another method is termed Specific High-sensitivity Enzymatic Reporter Unlocking (SHERLOCK) and utilizes Cas13a for the accurate and highly sensitive detection of viral RNA copies (96).

DRUGS AND VACCINES

Drugs

Chloroquine

Chloroquine is a malaria drug, which passively diffuses into acidic lysosomes, endosomes, and Golgi vesicles. While initial reports and studies were promising for chloroquine/hydroxychloroquine in COVID-19 patients, these findings were not confirmed and the NIH discontinued clinical trials investigating the efficiency of chloroquine (3, 97). In 2020, the FDA had authorized the administration of chloroquine to certain COVID-19 patients, but shortly thereafter, the agency terminated its use due to the high ambiguity regarding its efficiency and side effects (**Table 4**). In fact, hydroxychloroquine did not improve 28 day mortality in hospitalized COVID-19 patients (1,561 patients, 27% non-survivors) as compared to standard care (3,155 patients, 25% non-survivors) (98). A meta-analysis of studies on the efficiency of chloroquine for treating COVID-19 has shown that there was no significant difference in patient outcome and that the side effects posed a larger threat (99, 107).

Azithromycin

Azithromycin is classified as a broad-spectrum macrolide antibiotic. Azithromycin also amplifies antiviral immune recognition and interferon pathways in airway epithelial cells (108). A single-center study had suggested that a combination of hydroxychloroquine and azithromycin significantly reduced viral loads and time to a negative PCR test after SARS-CoV-2 infection (100). However, subsequent trials failed to reproduce these results, with no significant difference in viral burden as well as continuing positive PCR results (109). The latter study suggests that the antiviral properties of both medications have been overestimated and their side effect profiles might adversly manifest in COVID-19.

Remdesivir

Remdesivir was originally designed to target hepatitis C virus, and later studied for effectiveness against Ebola virus. It is classified as an anti-viral adenosine-tri-phosphate analog, which is incorporated into the forming viral RNA chain by the RNA-dependent polymerase and disrupts viral replication (98). Remdesivir showed some efficacy in inhibiting infection of mammalian cells by human coronaviruses (110). In clinical trials, remdesivir tended to shorten the recovery time for adult patients and reduced symptoms of upper respiratory infection (101).

Remdesivir was one of the first drugs granted emergency use authorization, and it is now approved by the FDA for use in adults children (>12 years) for the treatment of COVID-19 requiring hospitalization (102). However, remdesivir only achieves modest benefits for subgroups of hospitalized COVID-19 patients.

Dexamethasone

Dexamethasone is a potent anti-inflammatory corticosteroid that binds to the glucocorticoid receptor and depending on the dosage either reduces the expression of certain pro-inflammatory genes or boosts the transcription of a subset of anti-inflammatory regulators (103). A meta-analysis of $n = 1{,}703$ severely ill COVID-19 patients found glucocorticoids to reduce 28 day mortality (32% vs. 40%) without an increased risk for severe adverse events (111). The RECOVERY trial ($n = 2{,}104$) showed that dexamethasone decreased COVID-19 mortality (29% vs. 41%) in patients on mechanical ventilation or receiving oxygen without mechanical ventilation (23 vs. 26%) (104). No difference in survival was found in patients who did not require respiratory support. Hence, dexamethasone is recommended for severe cases of SARS-CoV-2 infection and its best role could be as part of a combination therapy (22).

Tocilizumab

Tocilizumab is a humanized monoclonal antibody against the Interleukin-6 (IL-6) receptor used in autoimmune diseases and inflammatory disorders (112). Clinical trials suggest that Tocilizumab can reduce hyperinflammation during severe COVID-19. More specifically, one trial showed that Tocilizumab reduced mortality of COVID-19 when compared to standard care while increasing the risk of secondary infections (105). Tocilizumab relieves clinical symptoms, reduces the requirement for supplementary oxygen, reverses lymphopenia and decreases C-Reactive Protein (CRP) levels (106). A direct positive correlation was found between CRP levels, lung lesions and higher severity of COVID-19 (113). While not all studies have shown significant differences in disease severity or survival of infected patients treated with Tocilizumab compared to a placebo (114), a meta-analysis of $n = 2{,}120$ patients supported a reduction of mortality in severe cases of COVID-19 (115). In a more recent analysis of $n = 4{,}116$ adults, Tocilizumab reduced COVID-19-associated mortality (29% vs. 33%) and was more effective in combination with glucocorticoids (54% vs. 47%) (116). Patients receiving Tocilizumab were less likely to require mechanical ventilation and showed improved clinical outcomes (116). Sarilumab is another blocking anti-IL-6R antibody which is studied for COVID-19.

Immunoglobulin

Neutralizing antibodies and passive immunization are a feasible approach to mitigate SARS-CoV-2 infection (117, 118). Passive immunization could be especially helpful for immunocompromised individuals at risk for severe clinical manifestations such as respiratory failure (119). Prophylaxis against infectious agents using purified polyclonal immunoglobulin (Ig), also known as polyvalent immunoglobulin, is not a new idea (120). Ranging from highly

TABLE 4 | Efforts for drug repurposing.

Name	Chloroquine	Azithromycin	Remdesivir	Dexamethasone	Tocilizumab
Target	Heme polymerase	Ribosomes	RNA-dependent polymerase	Glucocorticoid receptor	Interleukin-6 receptor
Manufacturers	10	18	6	15	2
Efficacy for COVID-19	No	No	Modest or None	Yes	Yes
Side effects	Nausea, Retinopathy, Cardiotoxicity, QT prolongation	Diarrhea, Allergies, Headaches, Liver toxicity	Nausea, Liver toxicity, Anaphylaxis	Gastrointestinal ulcers, Hyperglycemia, Osteoporosis	Headaches, ↑Lipids, Upper Respiratory Infections
References	(82, 98, 99)	(99, 100)	(98, 101, 102)	(103, 104)	(105, 106)

The medications listed are all FDA approved for other indications and were evaluated in clinical trials for efficacy in severe COVID-19.

specific to very broad, neutralizing monoclonal antibodies have been designed against a variety of viral agents such as MERS-CoV (121, 122).

Recent work on the antibody repertoire produced by infected humanized mice and recovered patients has generated a large bank of antibodies that can be used against COVID-19. Anti-SARS-CoV-2 spike antibodies were generated by immunizing mice with a DNA plasmid encoding the RBD protein. In addition, B-cells were isolated from the peripheral blood of recovered patients (117).

The antibodies generated from both studies were reported to be highly similar in function and efficacy against many spike variants. However, four of them, utilized individually or in cocktails, showed promising results against newer strains that had originated from human populations (117). A cocktail therapy was proposed to limit viral resistance to therapy by using antibodies that target two distinct, non-overlapping regions of the RBD (123). Nevertheless, the antibodies were not effective in neutralizing SARS-CoV-2 when new spike mutations arose from *in vitro* passaging or when combinations of antibodies that target overlapping regions were administered (123). Other neutralizing antibodies (LY-CoV555 and LY-CoV016) have shown promising results in the BLAZE-2 clinical trial. Bamlanivimab (LY-CoV555) alone reduced the risk of symptomatic COVID-19 by 80% (124), while in a separate study the combination of Bamlanivimab (LY-CoV555) with Etesevimab (LY-CoV016) was found to decrease hospitalization and death from COVID-19 by 70% (124–127). Furthermore, Regeneron's REGN-COV2 neutralizing antibody cocktail (Casirivimab and Imdevimab) was effective in reducing the viral load in patients with delayed immune responses or with high initial virus titers (128).

Vaccines

Vaccines are the best approach for prevention of infection. There are five types of vaccines under development (**Figure 3**) (129):

(i) Genetic Vaccines use SARS-CoV-2 specific DNA/RNA sequences to stimulate an immune response. (ii) Viral Vector Vaccines employ alternative viruses as "carriers" for SARS-CoV-2 genes. (iii) Whole Virus Vaccines present an inactivated form of the virus to the immune system. (iv) Protein-based Vaccines incorporate selected virus proteins such as the spike protein. (v) Repurposing the Bacillus Calmette-Guérin vaccine to stimulate the immune system. Recent discussions raised questions regarding the safety, long-term side effects, and social implications of a vaccine developed in a short period time without sufficient pre-clinical testing and adequate clinical trials. A major concern for a vaccine is the syndrome of acquired cellular immunopathology, a condition observed when the delivery platform for the viral proteins or genes leads to a violent pro-inflammatory response from T-cells. This results in the migration of white blood cells into target tissues, further deteriorating the health of a patient. Another concern is Antibody-Dependent Enhancement (ADE), when non-specific antibodies generated by the vaccine allow for enhanced viral internalization, thus potentially worsening the infection and pathophysiology of COVID-19 (130). A vaccine could also have low efficacy in terms of long-lasting protection from infection because of insufficient neutralizing antibodies, weak memory T cell responses or new SARS-CoV-2 variants.

In the pre-COVID-19 era, vaccine development lasted on average about a decade and required extensive funding, scientific diversity, and countless volunteers. The federal Center for Biologics Evaluation and Research is a branch of the FDA responsible for evaluating the safety and efficacy of novel medication and vaccines. The FDA and CDC have established a strict set of clinical trials (phase 1–3), through which the safety and efficacy are investigated. In the last year, there has been a race to develop the first vaccine to prevent COVID-19.

There are more than 110 potential vaccine candidates, with over 80 in human trials and almost another 80 vaccine candidates in preclinical testing. There are currently seven approved vaccines, developed mainly in the US, Russia, China, India, UK, Germany and Belgium. In some cases, the development of vaccine candidates came to a halt, such as for Merck, Imperial College London, Themis, Institut Pasteur and IAVI (131–136) (**Figure 3**).

The clinical trial of the mRNA-based vaccine (BNT162b2) from Pfizer/BioNTech enrolling 43,000 participants showed a reduced risk for SARS-CoV-2 infection by over 90%. This vaccine received FDA emergency approval in the US, while it has been fully approved in other countries (137). Further studies have shown that the BNT162b2 vaccine has 95% efficacy in preventing a COVID-19 infection 7 days after the second dose (137, 138). Of note, the nanoparticles that deliver the mRNA contain polyethylene glycol (PEG), a compound that has been linked to unwanted severe allergy-like symptoms.

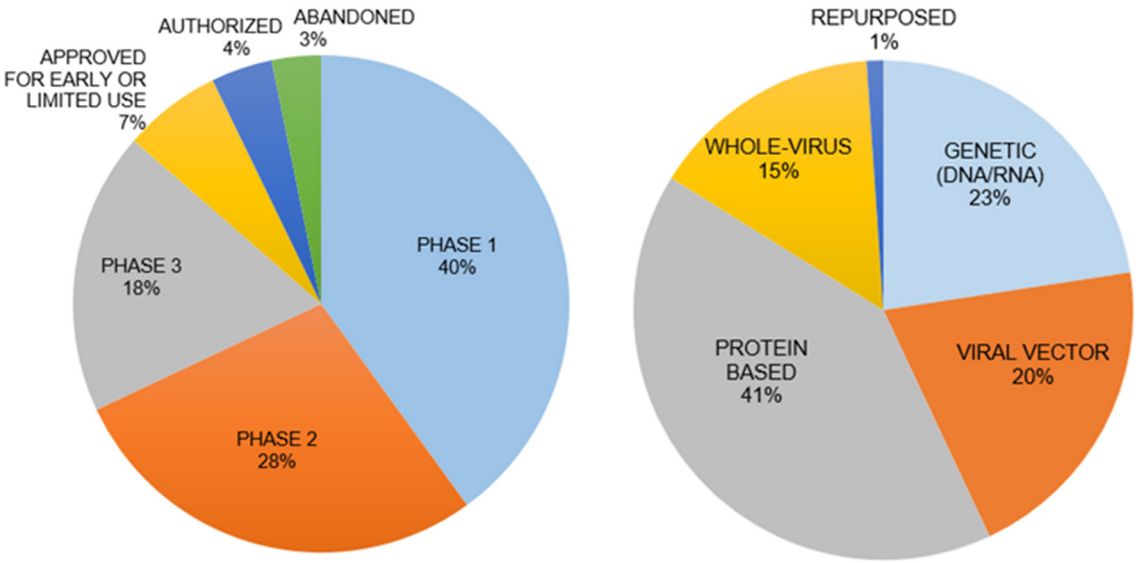

FIGURE 3 | Vaccine types and clinical phases of their development. The genetic vaccines immunize with mRNA for SARS-CoV-2 and protein-based vaccines immunize with spike protein to induce immunity. The interim analysis of phase 3 clinical trials for both types of vaccines have been disclosed with promising results in November 2020. The mRNA vaccines are a new principle with little information on how long the induced immunity will last.

Similar concerns have been raised with the nanoparticles used in the mRNA vaccine from Moderna. It consists of mRNA-1273 encapsulated in lipid nanoparticles (139). The mRNA-1273 showed similar efficiency (94%) to the Pfizer vaccine and was granted FDA emergency use authorization. The protection by a protein-based adenovirus vector vaccine from AstraZeneca was around 70% with some uncertainties about optimal dosing and recent concerns about a risk for thrombotic complications. Regardless of these promising results, the duration of long-lasting immunity induced by these vaccines has yet to be determined. The Johnson & Johnson vaccine also uses an adenovirus vector to express SARS-CoV-2 spike protein in the host cells to induce immunity. This process yields SARS-CoV-2-specific antibodies in ∼90% of individuals after the first dose (140). The Johnson & Johnson vaccine has been associate with a very rare risk for cerebral venous sinus thrombosis. Mild to moderate local (e.g., pain and swelling at injection site) and systemic (e.g., fever, chills) side effects are very common for the current COVID-19 vaccines. Seropositive participants develop higher antibody titers and experience higher rates of systemic side effects (141). It is expected that SARS-CoV-2 will eventually transition from a pandemic to an endemic disease, a change that is associated with the distribution of infected individuals. Endemic dynamics are characterized by a shift of primary infections to younger ages in the population, which for COVID-19 usually causes only mild disease or asymptomatic infection. The shift to mild endemic disease depends on the rate of virus transmission and may be accelerated by vaccination (142).

NANOMEDICINE APPROACHES

Nanomedicine approaches may provide new solutions in the fight against COVID-19. The hope is that nanotechnology can improve the effectiveness and specificity of drugs and vaccines. The nanomedical field utilizes nanomaterials: particles in the nanometer scale that possess unique chemo-physiological properties. Two key characteristics of nanoparticles are their size and polarity. Their size, ranging from 10 to 100 nm, allows them to easily interact with a biological target of similar size and pass through several types of membranes, such as the lung-blood vessel junction and the blood-brain barrier (143). In addition, the polarity of nanoparticles can be modified to facilitate a specific purpose such as binding other drugs, increasing the surface stability, or reducing aggregation and precipitation (144, 145). Specialized nanoparticles with a magnetic nature can be guided through the body via a system of external magnets and forced to increase their temperature by exposing them to an oscillating magnetic field, a technique currently used in oncology for tumor suppression (146–148). Moreover, these particles can be both organic and inorganic, used individually or aggregated, and combined with other medication or other nanoparticles. Due to the unique features of nanomaterials, widespread applications in both the prevention and treatment of SARS-CoV-2 are feasible. Nanotechnology could be applied for personal protective equipment, gene silencing, creating biosensors, developing pharmacologically active compounds and nano-vaccines, and for directly destroying SARS-CoV-2 particles (149–151).

Biosynthesis of nanoparticles by microorganisms has recently emerged as an alternative to conventional chemical and physical synthesis. Biosynthetic nanoparticles can have similar morphology and properties to their conventional counterparts (152, 153). There are several benefits of large-scale synthesis of microbe-derived nanoparticles such as avoiding hazardous chemicals, expensive reagents or toxic materials for stabilization and synthesis. Nanoparticles can bioconjugate, genetically

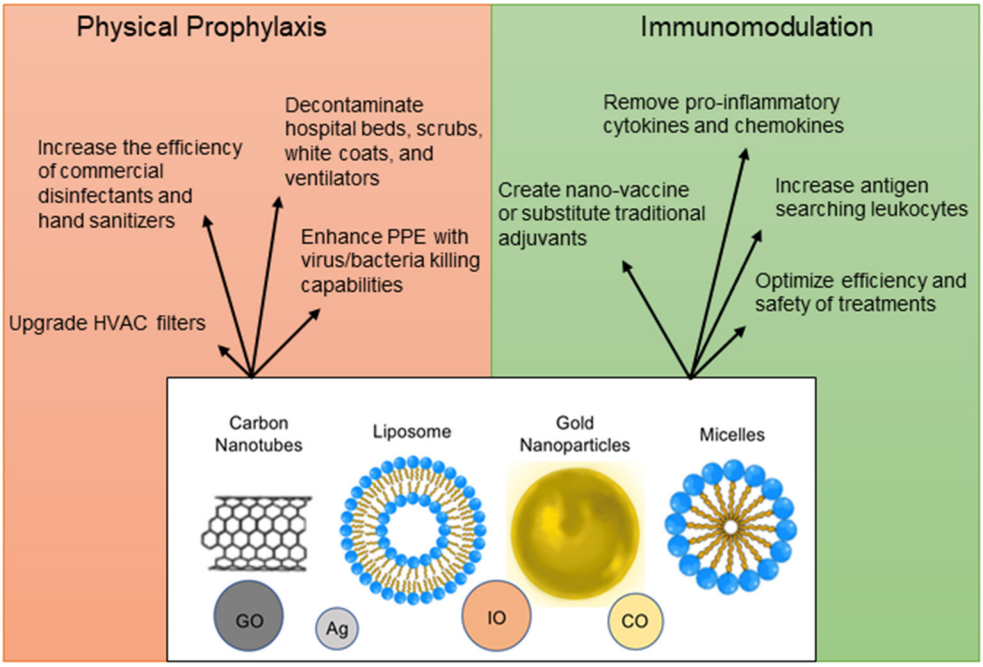

FIGURE 4 | Nanoparticle applications as prophylactic and therapeutic measures. Nanoparticles can be used in a plethora of ways for protection against infection, for immunomodulation, vaccine design, and optimization of detection methods. The structural design of nanomaterials is diverse and includes engineering of carbon nanotubes [Graphene Oxide (GO)], liposomes, micelles, precious metals [Silver (Ag), Copper Oxide (CO), and Iron Oxide (IO)].

engineer, infuse, mineralize or even assist in self-assembly of viral and bacterial particles. These techniques could be used as tools for vaccine design and production (152, 153).

There are several designs for nanoparticle-based peptide vaccines. Nanoparticles can be used to construct a multiple antigen-presenting platform. Self-assembling lipo-peptides, consisting of a lipid chain bound to an antigen, can form micelles with enhanced epitope presentation ability (154). Another safe and effective method of antigen delivery to antigen-presenting cells is encapsulation or conjugation of antigens with nanoparticles in order to preserve their structure and protect them from degradation (155). Nanoparticles designed to either deliver antigens or act as adjuvants can be administered intranasally to induce immunity against lower respiratory tract virus infections, such as influenza, RSV and adenovirus (155). Bacteriophage-derived nanoparticles from Escherichia virus Q-beta were incorporated into a H1N1 vaccine of high immunogenicity and low safety concerns in a phase 1 clinical trial (156).

Adenovirus (class I-dsDNA virus), adeno-associated virus (AAV, class II-ssDNA virus), human papilloma virus (HPV) or even human immunodeficiency virus (HIV) can be modified into carriers for targeted gene/protein delivery (153, 157–161). Bacteria can be engineered for nanoparticle biosynthesis such as *Bacillus cereus* and *Bacillus subtilis* for silver nanoparticles (162), *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* for gold nanoparticles (163), *Shewanella algae* for platinum nanoparticles (164), and *Pseudomonas aeruginosa* for Lanthanum nanoparticles (165).

Nanoparticle Applications

Nanotechnology is a fast growing industry. The current $60 billion market is expected to double to $120 billion in 5 years. The main market prospects involve the utilization of nanoparticles for medicine, food, agriculture, conductors and computers. In medicine, nanoparticles are used and developed for applications inside of the body (e.g., drug delivery, repair of tissues) and for external purposes.

To decrease the spread of SARS-CoV-2, nanoparticles with a potential to inactivate the virus can enhance physical barriers, sterilize commonly contacted surfaces or air filters, and be incorporated into hand sanitizers and disinfectants (166–169) (**Figure 4**). Personal Protective Equipment (PPE) such as masks and gloves could be upgraded with nanoparticles that have antimicrobial or antiviral capabilities. Iron-oxide nanoparticles (IO-NPs) and Silver nanoparticles (Ag-NPs) have been shown to neutralize various strains of Influenza and Coronaviruses by physically binding to the SARS-CoV-2 virion and preventing internalization into host cells (166, 167, 170, 171). Moreover, Copper Oxide nanoparticles (CO-NPs) possess antimicrobial capabilities against a plethora of respiratory tract pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (172). Antimicrobial nanoparticles use chemical and biological mechanisms to eliminate microbes such as cell membrane disruption, DNA and protein damage, gene silencing, heavy metal ion toxicity, Reactive Oxygen Species (ROS) formation, and prevention of biofilm formation (173).

One of the strongest arguments for the use of nanoparticles in drug enhancement is that modern drug delivery can lack

target specificity due to a poor cellular uptake, insufficient stability under physiological conditions, non-target effects, and excessive immunogenicity (174). A novel approach to avoid such problems uses short interactive RNA molecules bound to nanoparticles, which can interact with a biomarker on the desired cell population, thus localizing the drug's effects to avoid unnecessary contact with other cells and reduce overall toxicity (175, 176). Nanoparticles coated with specific antibodies against a cellular receptor such as human ACE2 or against SARS-CoV-2 spike protein comprise an elegant delivery system for any drug that requires cell specificity and may help reduce the dose of medication and off-target side effects (174). Many nanoparticle types can be used, such as polymers, dendrimers and quantum dots. Nanobots, microscopic robots that can carry out localized drug delivery, could be controlled by a user and might advance drug delivery even further in future (176).

The properties of the molecules mentioned above could also be engineered to reduce the chances of secondary infections that are associated with COVID-19 pathophysiology. PPE, patient gowns, scrubs, white coats and commonly contacted surfaces could be coated with a mixture of nanoparticles to protect healthy and infected individuals. Cotton fabrics can be enriched with zinc oxide nanoflowers to trap and denature SARS-CoV-2 spike protein (177). Additionally, enhancing conventional hand sanitizers and upgrading air filter systems with antimicrobial nanoparticles could be useful for disinfection and containment of SARS-CoV-2 spread. FDA-approved iron-oxide nanoparticles (IO-NPs) were recently found to bind to the envelope and spike protein subunits of SARS-CoV-2 and alter their conformation, thus inactivating the virus (178). Nanoformulations can help to reduce the needed quantities of precious elements such as gold, silver and copper.

The SpyCatcher/SpyTag technology allows irreversible conjugation of a recombinant protein by adding a sequence of the SpyTag peptide (13 amino acids) to its DNA sequence. The SpyTag spontaneously reacts with the SpyCatcher protein and allows for oligomerization (179). This system was employed to generate mosaic nanoparticles that display multivalent antigens of SARS-CoV-2 spike RBD along with RBDs from different animal betacoronaviruses to enhance B cell responses and elicit high-titers of cross-reactive, neutralizing antibodies (180).

Nanoparticle Applications for Detection, Immune Prophylaxis, and Vaccines

Nanoparticles in Diagnostics

Early and rapid detection is key for lowering the basic reproduction number of infected individuals. Nanoparticles can be engineered as biosensors for the detection of biomarkers, including nucleic acids (DNA, RNA), specific antigens (proteins, enzymes), or antibodies in order to rapidly and accurately detect SARS-CoV-2 (143, 149, 181, 182). Recent advancements in nanotechnology have allowed for the release of a SARS-CoV-2 detection platform that uses graphene conjugated to an anti-spike antibody. This novel kit requires no sample pretreatment or labeling and is impressively effective in detecting SARS-CoV-2 at very low concentrations (183). Alternative detection methods have been designed such as dual-functioning plasmonic biosensors, which tap into the energetics of DNA-RNA hybridization, as well as Graphene Oxide particles coated with fluorophore-bound DNA target strands that can detect viral helicase (184, 185).

Nanoparticles for Drug Delivery and Vaccines

Nanoparticles can be engineered to directly target SARS-CoV-2 or as immunomodulatory factors to prime and alarm the immune system and reduce the inflammatory response during COVID-19.

Small-interfering RNAs against conserved regions of SARS-CoV-2 were incorporated into lipid nanoparticle formulations and upon delivery into lungs suppressed viral replication and improved survival of infected mice (186).

Graphene Oxide Nanoparticles (GO-NPs) have been shown to increase leukocyte numbers such as macrophages and T cells. This effect boosts adaptive immunity, thus allowing for a better immune response and viral clearance, or a possible use as vaccine adjuvants. In the scenario of uncontrolled hyperinflammation, nanodiamonds elicit an anti-inflammatory state in macrophages, while carbon and graphene sheets can be repurposed to remove pro-inflammatory cytokines and interleukins from the blood of patients (149).

Most importantly, nanotechnology may offer solutions to some of the major problems of traditional vaccines and medications such as sensitivity to acidity, water insolubility, or absorption. Nanoparticles can increase drug delivery efficiency by binding or encapsulating hydrophobic or pH-sensitive drugs and creating a targeted release. For example, certain nanoparticles bound to drugs can be modified using organic molecules that provide better release characteristics, such as Cholesterol-modified-Hydroxychloroquine. Other nanoparticles can facilitate the transport of two or more drugs, thus decreasing each dose as well as the side effects, while augmenting the combined outcome (187).

Another proposal claims that a simple and unconventional vaccine design could combine layered double hydroxide (LDH-NPs) nanoparticles and a plasmid encoding short hairpin RNA to silence the expression of targeted genes, such as essential SARS-CoV-2 proteins to stop infection early. The LDH-nanoparticles are compatible with mammalian cell lines and can insulate the shRNA against degradation, thus providing a promising delivery mechanism (188).

The current COVID-19 mRNA vaccines (Moderna, Pfizer/BioNtech) contain mRNA wrapped in lipid nanoparticles. More nanoparticle vaccines are under development. For example, NVX-CoV2373 (Novavax) is a recombinant nanoparticle-based vaccine, which incorporates the full trimeric spike glycoprotein with a saponin-based adjuvant (Matrix-M1) (189). Testing on macaques and later in phase 1–2 human clinical trials revealed that this vaccine could elicit neutralizing antibodies such as anti-spike IgG antibodies, as well as a specific T-cell response (189).

Sinovac Biotec Company also designed a nanovaccine against SARS-CoV-2 and successfully tested it in mice. This NP based vaccine incorporates the RBD subunit of the spike protein

combined with two adjuvants: Monophosphoryl Lipid A (MPLA) and CpG-ODN, which stimulate TLR4 and TLR9, respectively. The vaccination of mice was achieved in three stages (original shot and two boosters) and resulted in a potent and protective T cell response accompanied by neutralizing IgA antibodies (190). This nanovaccine is currently in clinical phase 3 testing and in light of the promising results, a large-scale manufacturing plant is under construction (191).

Of note, another nanotechnology vaccine was recently found to induce a persistent antibody production and long-lasting memory response for at least 7 months in mice (192). In this vaccine design, the RBD of spike protein was conjugated via the SpyTag/SpyCatcher technology to ferritin nanoparticles. Hence, the unique capabilities of nanoparticles could revolutionize the processes of vaccine design, manufacture, and delivery.

Challenges and Limitations

While the widespread use of nanoparticles in medicine is an exciting idea, a few drawbacks may delay the realization of these endeavors. An overall examination of literature surrounding the design and application of nanoparticles in pharmacology has shown that there is a lot of variability between the results of independent research studies, and translating the efficacy of these particles from an *in vitro* to an *in vivo* situation is difficult (2). Additionally, critics have emphasized that the large-scale production of nanoparticles will be a high hurdle to overcome, especially when trying to keep these treatments affordable (193). The required sophistication of the manufacturing processes of nanoparticles and intellectual property rights can drive up their prices, although overall health care expenditures could be saved if nanomaterials and nano-vaccines accomplish to prevent COVID-19. Another limitation of nanoparticles are risks of unwanted tissue interactions and toxicity, unwanted spread and deposition in the body including unwanted crossing of the blood-brain barrier (194, 195). Accidental inhalation into the lungs is feared to cause epithelial injury, pulmonary inflammation and contribute to fibrosis depending on the size and chemical composition of the nanoparticles (196). Moreover, nanoparticles have been shown to interfere with biological processes like inflammation, oxidative stress, mitochondrial function, macrophage phagocytosis and platelet function (2). Acute or chronic toxicity of nanoparticles may be caused via ROS generation, cell membrane binding, DNA damage, altered cell cycle regulation and protein denaturation (197). Another important issue is the incomplete understanding of long-term effects of nanoparticles in humans and the environment. For example, a study on the effect of chronic administration of nanoparticles to rats resulted in structural damage in their testis, including disorganization of spermatogenic cells, misoriented testis and reduction of germ cells (198, 199). Allergic reactions and anaphylaxis to the mRNA lipid nanoparticle vaccines (Moderna, Pfizer/BioNtech) for COVID-19 have been blamed on the nanoparticle design and composition (200).

These limitations, and other unknown risks, should be taken into consideration when evaluating the actual potential of nanoparticles to form a reasonable approach toward nanomedicine.

CONCLUSIONS

Nanotechnology is an emerging field that can alter the way we approach the diagnosis, treatment, and prevention of human diseases. Nanomedicine offers unique potentials to address future epidemiological challenges with other emerging viruses. The ongoing COVID-19 pandemic has shown that health care systems were underprepared for such a large-scale event. Nanotechnology seems very promising, but one must not forget that it is a young and unexplored field. The current state of the field leans in favor of nanoparticles supporting modern medicine, but risks and long-term side effects remain hard to assess.

Effective therapies of COVID-19 remain elusive, but fortunately, the widespread public distribution of vaccines has begun. The promising potential of nanoparticles is not limited to diagnostic and therapeutic approaches but can also be applied to global prophylactic measures that aim toward limiting the spread and symptoms of SARS-CoV-2 infection. Theranostics, a new discipline of medical science, focuses on detecting and eliminating new viral or bacterial threats using nanomedicine and nanodrugs for diagnostics and therapy. This field has demonstrated futuristic applications of nanotechnology, such as spike protein-specific nanoparticles and neutralizing nanomaterials. It may even become a pharmacological standard of care once the side effects are well-understood and mitigated. While true benefit of nanomedicine in the fight against COVID-19 remains to be seen, it is worthy of in-depth considerations and efforts.

AUTHOR CONTRIBUTIONS

AS and KK wrote the manuscript and prepared the figures. MB wrote and edited the manuscript and supervised and funded the work. All the authors are responsible for the contents of this publication.

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Health Protection of CT Radiographers During the Outbreak of COVID-19: Application of Automatic Positioning Technology for Relocatable CT in the Fang Cang Hospital

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Background: To investigate the value of automatic positioning technology in improving the protection of radiographers in the relocatable CT room of a Fang Cang hospital during the outbreak of coronavirus disease 2019 (COVID-19).

Methods: The National Emergency Medical Team of our hospital assumed command of Wuchang Fang Cang Hospital and treated confirmed COVID-19 patients with mild symptoms. Relocatable CT was used to examine patients in this hospital. Automatic positioning technology was applied to avoid close contact between medical staff and patients and to protect medical staff more effectively.

Results: Seven hundred lung CT scans acquired from 269 patients were completed from February 17 to 26, 2020 with automatic positioning technology for relocatable CT in a Fang Cang hospital. All scans were conducted successfully using automatic positioning technology. All patients entered the scanning room from a separate door. All the position lines were accurate, and all images met the requirement for diagnosis of COVID-19, with satisfied quality. None of our medical staff had any close contact with patients.

Conclusion: Automatic positioning technology applied to relocatable CT can minimize the close contact between technologists and patients and effectively improve the protection of medical staff without sacrificing image quality.

Keywords: automatic positioning technology, COVID-19, Fang Cang hospital, health protection, relocatable CT

INTRODUCTION

In December 2019, the outbreak of coronavirus disease 2019 (COVID-19) was reported in Wuhan City, Hubei Province, China (1). Due to its high infectivity and lethal potential, COVID-19 quickly spread worldwide (2, 3). The number of confirmed patients rapidly increased, and not all patients could be treated in the hospital in a timely manner, which could lead to cross-infection

or secondary infection. In this context, the outbreak of COVID-19 may be exacerbated in a short time. Meanwhile, medical workers treating a substantial number of patients every day are facing extreme danger of infection. As of February 15, 2020, healthcare workers account for 1,716 confirmed cases of COVID-19, including 6 deaths in China (4). To treat confirmed patients in a timely manner and better control the outbreak, the Chinese government decided to build mobile cabin hospitals as temporary treatment centers in Wuhan, named Fang Cang hospitals. These mobile cabin hospitals were transformed from large indoor venues, such as stadiums or convention centers, into temporary hospitals that met the requirements of health protection for infectious diseases. Fang Cang hospitals were used to treat confirmed mild or common types of patients with COVID-19 (5).

Although the real-time reverse-transcriptase polymerase chain reaction (RT-PCR) assay remains the gold standard for the diagnosis of COVID-19, false-negative issues, low stability, and long test times relatively limit its application in clinical practice. CT, the most frequently used modality in clinical practice, could provide important imaging information for the detection, follow-up, and prognosis of COVID-19 (6). Relocatable CT is the best substitute for routine CT to be installed in mobile cabin hospitals to date (7). The protection of medical staff in the mobile cabin hospital during relocatable CT scanning matters, which requires high standard protective measures. Automatic positioning technology used in relocatable CT plays a vital role in the protection of medical staff and substantially avoids cross-infection.

Thus, the aim of the study was to introduce our experience in the protection of medical staff operating relocatable CT in a Fang Cang hospital during the outbreak of COVID-19 and provide a reference for peers who may use this equipment in the future.

MATERIALS AND METHODS

This study was approved by Medical Ethical Committee (Approval No. 2020002), which waived the requirement for patients' informed consent referring to the CIOMS guideline.

Study Population

The National Emergency Medical Team of our hospital assumed command of Wuchang Fang Cang Hospital and treated confirmed COVID-19 patients with mild symptoms. From February 17 to 26, 2020, 700 lung CT scans of 269 patients completed using automatic positioning technology were included in our study.

Relocatable CT

A relocatable CT system is a kind of temporary large medical equipment with the same internal structure as a routine CT system (**Figure 1**). It contains a series of necessary facilities, including an independent scanning room, a controlling room, and an ultraviolet disinfection device. The relocatable CT can be not only installed and dismantled quickly but also transferred easily, with the advantage of an independent box-like design. The scanning room covers an area of only $\sim$20 m^2 and can be conveniently used with electricity. In addition, the waterproofness, thermal insulation, and constant temperature make it suitable for different extreme environments to fight the outbreak of major infectious diseases or execute rescue work.

Health Protection

Although a relocatable CT system is not installed and used in conventional medical institutions, protective measures require the same standard during the outbreak of COVID-19 (8–10).

Health Protection of Medical Staff

CT radiographers should apply necessary protection including disposable work caps, protective glasses or masks (anti-fog type), medical protective masks (N95), protective clothing, isolation clothing, disposable latex gloves, and disposable shoe covers (11). They must apply hand hygiene strictly according to the national hygienic standard. All protective supplies must be changed if radiographers change shifts. The health protection of medical staff must be managed strictly in accordance with the Medical Waste Management Regulations and Medical Waste Management Measures of Medical Institutions.

Radiologists review the CT images far away from the relocatable CT or review CT images online to minimize the number of medical staff entering the relocatable CT. Traditional CT requires a radiographer to position the machine in the scanning room, which inevitably leads to close contact with patients (12, 13). The mobile cabin hospital implements paperless communication to reduce unnecessary contact between medical staff and patients. An electronic communication system was applied instead.

Prevention Measures for The Equipment and Environment

The equipment in the relocatable CT system should be wiped and disinfected with 75% ethanol at least two to four times per day. Disposable materials should be used to remove pollutants when visible pollutants are present, and then routine disinfection should be performed.

The floor of the scanning room and controlling room shall be disinfected with 2,000 mg/L chlorine disinfectant (14). When there are visible pollutants, disposable absorbent material shall be used to remove the pollutants completely, and then disinfection shall be performed at least twice per day.

A circulating air disinfector is used for continuous disinfection during operating hours, and a hydrogen peroxide air disinfector is used for spray disinfection after work is completed. An ultraviolet radiation system is used continuously for disinfection when no one is in the room four times per day (lasting for 60 min each time).

Management of Patients

All patients should enter and exit the imaging examination area through a special channel wearing masks and try to avoid coughing during the whole process of examination.

According to the guideline from the China National Health Commission, patients with negative nucleic acid test obtained for two consecutive respiratory pathogens (sampling interval $\geq$1

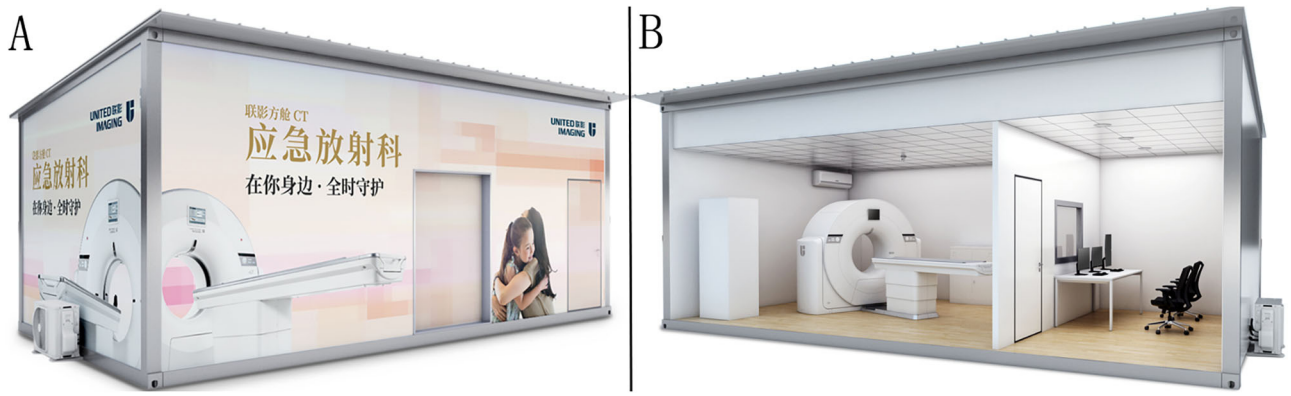

FIGURE 1 | The structure of the relocatable CT system. **(A)** The outward appearance of the relocatable CT system. **(B)** The interior structure of the relocatable CT system. The figures were provided through the courtesy of Shanghai United Imaging Healthcare Co., Ltd.

day) need to undergo a follow-up CT to determine whether they are eligible for discharge (15). Because these patients had PCR results turned negative, they were considered possible recovered cases. Therefore, they have priority for examination and should undergo CT scans separately from those with positive RT-PCR results to avoid possible cross-infection. Also, the scanning room should be sterilized before and after each examination for every individual.

Application of Automatic Technology

To avoid close contact between radiographers and patients as much as possible, automatic positioning technology was applied to the relocatable CT system in the Fang Cang hospital.

Introduction of Automatic Positioning Technology

The automatic positioning technology system includes camera-based intelligent auxiliary positioning (**Figure 2**) and positioning box self-adaption.

Camera-Based Intelligent Auxiliary Positioning

After patients remained in the examination bed with head-first supine position, the camera can automatically detect the natural image of patients intelligently and then calculate the scanning range according to the examination site and the body type of different patients. The positioning information of the patient will be presented to the radiographer on a synchronized screen. Radiographers can adjust the position parameters or confirm them directly and then move the examination bed using a controlling button to finalize the patient's positioning.

Positioning Box Self-Adaption

Automatic positioning system can identify and segment the structure in the positioning image, adjust the range of the positioning box according to the scanning area, and further display the shape of the positioning box to the radiographer. Radiographers can adjust the position if needed or confirm it directly and then start the scanning.

Operation Process

The patients enter the scanning room from a separate door by themselves or with the help of medical workers (**Figures 3A,B**). Radiographers confirmed patient's information and instructed them to lie on the examination bed through a microphone in the controlling room.

With the help of the camera-based intelligent auxiliary positioning system, the examination bed moved to the ideal position automatically according to the patient's size and the area to be examined. Then, the patient's real-time image was transmitted to a computer in the controlling room, and the technologist determined whether fine-tuning was required (**Figures 3C,D**).

After the radiographer confirmed the patient's position and finished the scanning of the positioning image, the automatic positioning system started the positioning box self-adaptive function and automatically set up the scan range. Radiographers could fine-tune the range or confirm it directly and then start scanning (**Figures 3E,F**).

After finishing the scanning, the radiographer moved the examination bed using a controlling button. The patient left the scanning room according to the radiographer's instructions.

Scanning Parameters

All included patients were examined with the following scanner: uCT-550 (Shanghai United Imaging Healthcare Co., Ltd.). The parameters were as follows: spiral scanning, 100–120 kV or automatic tube voltage, intelligent milliampere seconds (50–350 mAs), 0.5–1.5 mm collimator width, 1–5 mm layer thickness, and layer spacing.

Evaluation of Automatic Positioning Technology

We designated the inner positioning line aligned with the lower edge of the mandible and the horizontal positioning line parallel with the mid-axillary line as accurate position.

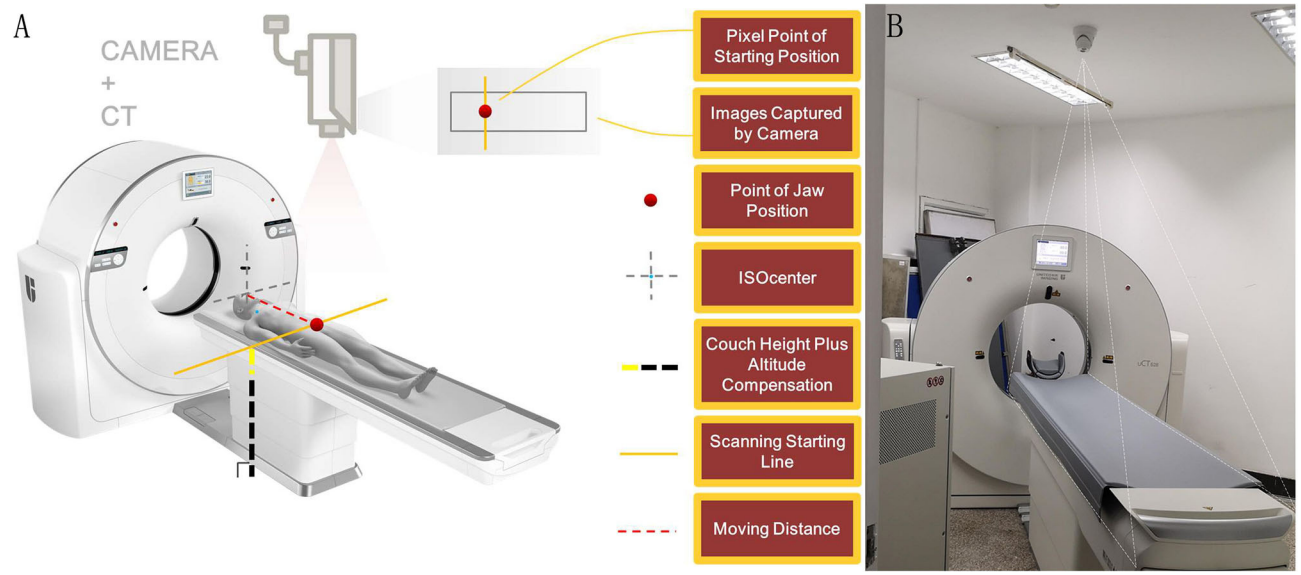

FIGURE 2 | Camera-based intelligent auxiliary positioning system. **(A)** The working principle of the camera-based intelligent auxiliary positioning system. **(B)** The relocatable CT system with AI positioning technology in Wuchang Fang Cang Temporary Hospital at Hongshan Stadium in Wuchang district, Wuhan. The figures were provided through the courtesy of Shanghai United Imaging Healthcare Co., Ltd.

According to the expert consensus, we defined the accurate scanning range as from the apex pulmonis to the right costophrenic angle.

Imaging quality were evaluated by a senior radiologist. We designated images be of high quality with following points: (1) images were clear without artifacts; (2) scanning range was accurate; (3) scanning parameters were consistent with conventional pulmonary CT.

RESULTS

From February 17 to 26, 2020, 269 patients (166 women, 103 men) diagnosed with mild or common types of COVID-19 admitted to Wuchang Fang Cang Hospital were included in this study. The demographic characteristics are shown in **Table 1**. Seven hundred lung CT scans of the 269 patients were completed with automatic positioning technology and a relocatable CT system in the Fang Cang Hospital. All scans were completed successfully with the assistance of the automatic positioning technology. Among 700 times camera-based intelligent auxiliary positioning, 642 (91.7%) times got the accurate position lines automatically; only 58 (8.3%) times needed artificial adjustments. Among 700 times positioning box self-adaption, 660 (94.3%) times got the accurate scanning range; 40 (5.7%) times fine adjustments were made. All images (100%) had high quality for reviewing (**Table 2**). Two CT radiographers and one radiologist worked in this mobile cabin hospital. None of them had any close contact with patients and none of them confirmed COVID-19 during work.

DISCUSSION

All 269 patients seen at the mobile cabin hospital were all confirmed cases of COVID-19 with mild symptoms. They could move freely and were able to complete the CT examination according to the radiographer's requirements alone. During the entire examination process, the radiographers could monitor the patients through the intelligent camera and communicate with the patient by the voice system. Therefore, it is completely feasible to perform CT examination without contact with patients closely aided by automatic positioning technology.

Complete CT examination usually requires two radiographers. One operates the machine in the controlling room and another positions the patient in the scanning room. By using automatic positioning technology, only one radiographer is needed to perform the CT scan. On the other hand, patients and medical staff go to different areas of the CT room through different channels. Therefore, the design of the relocatable CT and automatic positioning technology system can minimize the close contact between technologists and patients as much as possible, which may reduce or even avoid the occupational exposure of medical staff.

Previous studies demonstrated that CT played a vital role in the screening, diagnosis, and evaluation of treatment response of COVID-19 (16–18). Furthermore, the reported sensitivity of CT is higher than that of RT-PCR in detecting COVID-19 cases (19, 20). Therefore, CT was considered a standard clinical diagnostic tool in China and helped us to screen out suspected cases and evaluate the treatment response of patients. During the outbreak of COVID-2019 in China, the mobile cabin hospital, also called Fang Cang hospital, efficiently helped in controlling the spread of the epidemic by treating enormous numbers of patients with mild

FIGURE 3 | Operation process of relocatable CT aided by automatic positioning technology. **(A)** The inner structure of the relocatable CT system. **(B)** The patients enter the scanning room from a separate door by themselves. **(C)** The examination bed moves to the ideal position automatically with the help of the camera-based intelligent auxiliary positioning system. **(D)** The patient's real-time image is transmitted to a computer screen in the controlling room. **(E)** The automatic positioning system determines the scan range automatically. **(F)** The technologist starts scanning.

TABLE 1 | Demographic characteristics of 269 patients treated in a Fang Cang hospital.

Basic characteristics	All patients ($n = 269$)
Sex (%)	
Female	166 (61.7%)
Male	103 (38.3%)
Age	
All population	48.35 ± 12.38
Female group	48.15 ± 12.84
Male group	48.66 ± 11.65
Age range (%)	
≤20	5 (2%)
21–30	14 (5.2%)
31–40	56 (20.8%)
41–50	71 (26.4%)
51–60	77 (29%)
61–70	44 (16.5%)
>70	2 (0.1%)

TABLE 2 | Imaging analysis of 700 scans conducted by re-locatable CT.

	All images ($n = 700$)
Position accuracy	
Accurate position group	642 (91.7%)
Artificial adjustment group	58 (8.3%)
Scanning range accuracy	
Accurate range group	660 (94.3%)
Adjustment group	40 (5.7%)
Imaging quality (%)	
High quality	700 (100%)
Poor quality	0

symptoms in a short time. In this new treatment mode, the relocatable CT system is a useful equipment for diagnosis due to its convenience of installment. Moreover, since we have reached the post-pandemic era, relocatable CT using automatic positioning technology can be applied for community screening or medical supporting program for remote areas, assisting disease control.

In the routine process of CT examination, medical staff, such as radiographers, need close and frequent contact with different patients. Approximately 3.5% of the confirmed patients with COVID-19 are medical staff (21). Therefore, it is very meaningful to apply automatic positioning technology to relocatable CT in Fang Cang hospitals to minimize the contact between radiographers and confirmed patients. Our study indicated that relocatable CT can effectively protect medical workers from direct exposure of confirmed cases without sacrificing image quality, consisting with other studies (22).

The relocatable CT still exhibited limitation. All patients with COVID-19 in Wuhan Fang Cang Hospital underwent non-contrast pulmonary CT examination. Considering the need for needle docking when injecting contrast agents which requires human participants, the system has not been used in contrast-enhanced examination. For further application in conventional hospitals, the automatic positioning technology may still need an operator to go inside the controlling room for contrast agents. Despite this, the technology can still benefit radiographers from reducing contact with patients, completing accurate examination automatically.

Our study presents some limitations. First, this study applied one radiologist to evaluate the quality of CT images because of the shortage of medical workers in Fang Cang hospitals. Further studies on application of relocatable CT in community screening or medical supporting program for remote areas in the post-pandemic era will be conducted by using several assessors as well as quantitative methods. Also, it is a single-center study and the results of our study shall be improved by cooperating with other centers.

In summary, automatic positioning technology applied to relocatable CT can minimize the close contact between radiographers and patients and effectively improves the protection of medical staff without sacrificing image quality.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethical Committee of Second Xiangya Hospital (Approved No. 2020002). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

ZC, SJ, ZJ, and L-HL designed the research. SJ, ZJ, and KY performed the research and acquired the data. ZC, SJ, and ZJ drafted and wrote the paper. YC, XX, WZ, WS, and JL revised the paper. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Major Characteristics of Severity and Mortality in Diabetic Patients With COVID-19 and Establishment of Severity Risk Score

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Objectives: Diabetes is a risk factor for poor COVID-19 prognosis. The analysis of related prognostic factors in diabetic patients with COVID-19 would be helpful for further treatment of such patients.

Methods: This retrospective study involved 3623 patients with COVID-19 (325 with diabetes). Clinical characteristics and laboratory tests were collected and compared between the diabetic group and the non-diabetic group. Binary logistic regression analysis was applied to explore risk factors associated in diabetic patients with COVID-19. A prediction model was built based on these risk factors.

Results: The risk factors for higher mortality in diabetic patients with COVID-19 were dyspnea, lung disease, cardiovascular diseases, neutrophil, PLT count, and CKMB. Similarly, dyspnea, cardiovascular diseases, neutrophil, PLT count, and CKMB were risk factors related to the severity of diabetes with COVID-19. Based on these factors, a risk score was built to predict the severity of disease in diabetic patients with COVID-19. Patients with a score of 7 or higher had an odds ratio of 7.616.

Conclusions: Dyspnea is a critical clinical manifestation that is closely related to the severity of disease in diabetic patients with COVID-19. Attention should also be paid to the neutrophil, PLT count and CKMB levels after admission.

Keywords: diabetes, COVID-19, severity, mortality, risk score

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HIGHLIGHTS

- Dyspnea is a critical clinical manifestation that is closely related to the severity of disease in diabetic patients with COVID-19. Attention should also be paid to the neutrophil, PLT count and CKMB levels after admission.
- Different from previous study, our study found that CRP did not predict the severity and death of diabetic patients after infected with COVID-19.
- Based on these factors, a risk score was built to predict the severity of disease in diabetic patients with COVID-19. Patients with a score of 7 or higher had an odds ratio of 7.616.

INTRODUCTION

At the end of 2019, a newly identified virus, termed COVID-19, began spreading rapidly through China and the rest of the world, which has become a global catastrophe. As of August 1, 2020, at least 17 million patients worldwide have been diagnosed with COVID-19, with more than 670,000 deaths, and the global epidemic has not stopped yet. COVID-19 is highly infectious, and many patients worsen very quickly after infection. Acute respiratory distress syndrome (ARDS), multiple organ dysfunction syndrome (MODS) and septic shock are commonly found in severe cases (1).

Diabetes has been reported as a frequent comorbidity in COVID-19 patients (2). Recent studies have shown that diabetic patients with COVID-19 may have a more than 50% higher rate of experiencing a fatal outcome than those who do not have diabetes (3). Guo et al. (4) reported that diabetes should be considered a risk factor for a rapid progression and poor prognosis of COVID-19. Yan et al. (5) found that of 193 patients with severe COVID-19, 48 (24.9%) had diabetes, and diabetics had a higher risk of death compared with those who did not have diabetes. Chen and colleagues demonstrated that older diabetic patients with COVID-19 were at increased risk of death (6). Interestingly, a recent study showed that diabetic patients with well-controlled blood glucose had markedly lower mortality compared to those with poorly controlled blood glucose in diabetics with COVID-19(4). However, these recent studies did not clarify why diabetic patients with COVID-19 had different outcomes or what factors contribute to the increased severity and risk of death in diabetic patients with COVID-19. Few studies integrated multiple risk factors into the risk prediction score which was capable of accurately stratifying diabetic patients with COVID-19 into different risk groups on the basis of clinical data. It would be undoubtedly of great significance for clinical work if a clinician could prejudge which patients have a higher risk of severe disease and death at the time at which the patient begins clinical treatment.

Based on the above expectations, a retrospective multicenter study of a cohort of 3623 patients diagnosed with COVID-19 from three different hospitals in Hubei, China, was performed. The basic information, clinical manifestations and laboratory tests at admission were collected into the database of study indicators, and the factors leading to different outcomes in diabetic patients after infection with COVID-19 were evaluated. Based on these clinical data, we sought to develop a risk stratification score capable of identifying severity of diabetic patients with COVID-19 using clinical data to facilitate the target of rapid evaluation of patients' risk of critical and death, providing guidance for subsequent treatment.

Abbreviations: COVID-19, coronavirus disease 19; PLT, platelet; CKMB, creatine kinase-MB; IQR, interquartile range; WBC, white blood cell; CRP, C-reactive protein; MG, mild group; SG, severe group; SurG, survival group; DG, deceased group; ACE2, angiotensin-converting enzyme 2; TMPRSS2, transmembrane serine protease 2.

METHODS

Study Design and Participants

This retrospective study included 3,623 patients who were admitted to three hospitals (HuoShenShan Hospital, Jinyintan Hospital and Taikang Tongji Hospital) in Wuhan, Hubei Province, China. There were 2271 COVID-19 patients admitted to HSS Hospital from Febryary 4, 2020 to March 31, 2020; they were retrospectively screened and followed until April 15, 2020 or until HSS Hospital closed. A total of 152 patients were excluded due to duplicate data, and 2119 patients were ultimately included. Ninety-five COVID-19 patients were admitted to Jinyintan Hospital from January 26, 2020 to February 1, 2020; two patients were excluded due to missing data, and one patient was excluded due to death upon arrival. A total of 1412 COVID-19 patients from Taikang Tongji Hospital, admitted from February 19, 2020 to April 2, 2020, were also included in this study. No patients were excluded from this cohort. These three hospitals were class A tertiary comprehensive hospitals designated to treat patients with COVID-19. Most patients included in this study were local residents. Patients diagnosed according to the World Health Organization (WHO) interim guidance for COVID-19 were included in the study. Diabetes was ascertained through previous medical records or self-reported diagnosis confirmed by clinicians. Diabetes was diagnosed according to WHO diagnostic criteria: blood glucose (>11.1 mmol/L) (200 mg/dl) at any time of the day, fasting blood glucose (>7.0 mmol/L) (126 mg/dl), or oral glucose tolerance test (>11.1 mmol/L) (200 mg/dl) at 2 h. We did not further classify diabetes in the 325 diabetic patients.

Ethics Statement

The study was approved by the ethics committee of Xinqiao Hospital (2020-yd073-01) with written informed consent waived due to the retrospective nature of the study. This study was carried out according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines.

Inclusion and Exclusion Criteria

Inclusion criteria: Patients were diagnosed with COVID-19, according to the standard WHO (World Health Organization) diagnostic criteria.

Exclusion criteria: (1) Suspected patients were diagnosed and excluded from COVID-19 infection; (2) patients had died upon arrival without treatment; (3) missing data.

Data Collection

Patients enrolled in this study were divided into two groups according to the diagnosis of diabetes. The demographic data (such as sex and age), clinical symptoms (such as fever, cough, sputum, dyspnea(According to the "New Coronavirus Infection Pneumonia Diagnosis and Treatment Plan," released by the National Health and Health Council of China, dyspnea is defined as respiratory distress (frequency >30 times/min), resting state oxygen saturation is <93%), chest tightness, hemoptysis, fatigue, nausea, abdominalgia, diarrhea, anorexia), vital signs (body temperature, breathing rate, heart rate, blood pressure) and basic laboratory test (which were carried out in approved labs with internal quality controls) results at admission were reviewed and extracted by experienced clinicians using a standardized data collection form. The COVID-19 severity grading (mild, moderate, severe, or critical) was defined according to the Diagnosis and Treatment Plan for COVID-19 issued by the NHC of China.

Sample Size Evaluation

In this study, 325 cases of diabetic patients infected with COVID-19, who met the inclusion and exclusion criteria, were included for analysis. Among these patients, severe group and the mild group contained 115 and 210 cases, respectively. According to the p value, the final 10 factors were selected for logistic regression analysis. Finally, based on the statistical results, five factors were used as the scoring indicators, which was in conformity with the principle of beyond the 10 events per variable (EPV) rule of thumb (Richard, DR, et al. BMJ. 2020). The minimum sample size of each group is $5^{*}10 = 50$.

Statistical Analysis

Continuous variables were expressed as the median (interquartile range [IQR]) or mean ± SD, and categorical variables were presented as n (%). The differences between groups were compared by using the Mann-Whitney U test, t test, $\chi 2$ test, or Fisher's exact test, as appropriate. The determination of odds ratios (ORs) and 95% CIs for factors associated with clinical outcomes were analyzed by binary logistic regression analysis. A prediction model was built based on the results of binary logistic regression analysis. Each predictor in the final model was weighted based on the estimated coefficient. An ROC curve was created by using the risk scores to evaluate the sensitivity and specificity. The Hosmer–Lemeshow goodness-of-fit test was applied to assess the risk score model calibration. Statistical analyses were performed using IBM SPSS 23 statistics software (SPSS Inc., Chicago, IL, United States) and R software (version 3.4, R Foundation, Vienna, Austria. www. R-project. org). All p-values were two-sided, and $P < 0.05$ was considered statistically significant.

RESULTS

Clinical Characteristics of Patients With COVID-19 Upon Admission

Clinical characteristics of the 3,623 patients with confirmed COVID-19 from three hospitals in Wuhan, Hubei, China were collected (**Figure 1**); this cohort included 325 patients with pre-existing diabetes and 3,298 non-diabetic patients (**Table 1**). The median ages were 66 (58–72) and 61 (49–69) in the diabetic and non-diabetic groups, respectively. There were no significant differences between the diabetic and non-diabetic groups in terms of clinical basic vital signs, such as body temperature (37.69 vs. 37.61°C), respiratory rate (20/min vs. 20/min), blood pressure (133/80 vs. 130/80 mmHg) or pulse (86/min vs. 84/min). The diabetic group exhibited significantly higher incidence rates of dyspnea (29.85 vs. 23.65%, $p = 0.013$) than the non-diabetic groups, while no significant difference was found in the prevalence of cough (69.54 vs. 64.43%), expectoration (13.54 vs. 12.25%), chest tightness (20.92 vs. 20.16%), or hemoptysis (0 vs. 0.24%) between the two groups. It seems that diabetic patients exhibited a higher incidence of loss of appetite (31.38 vs. 25.86%, $p = 0.031$) than non-diabetic patients, and no significant differences were observed in other clinical manifestations of the digestive tract, such as vomiting (2.15 vs. 2.33%), abdominal pain (0.31 vs. 1.33%), and diarrhea (4 vs. 5.25%), between these two groups. Interestingly, pre-existing cardiovascular diseases (including hypertension and coronary heart disease) had a higher frequency in the diabetic group than in the non-diabetic group (55.38 vs. 23.53%, $p < 0.001$), similar to a previous report.

The two groups showed greater differences in laboratory test results. There was no significant difference in WBC (white blood cell) count between the diabetic and non-diabetic groups ($5.8 \times 10\text{^}9$/L vs. $5.8 \times 10\text{^}9$/L); however, according to our findings, patients in the diabetic group had higher neutrophil ($3.68 \times 10\text{^}9$/L vs. $3.45 \times 10\text{^}9$/L, $p = 0.007$) and lower lymphocyte ($1.38 \times 10\text{^}9$/L vs. $1.53 \times 10\text{^}9$/L, $p < 0.001$). In addition, CRP (C-reactive protein) also showed higher levels in the diabetic group than in the non-diabetic group (4.64 vs. 1.81 mg/L, $p < 0.001$). These results may indicate that diabetic patients have a higher inflammatory response base, and their autoimmune function is different from that of non-diabetic patients. Surprisingly, the liver function indexes [ALT (20.1 vs. 24.7 IU/L, $p < 0.001$), AST (19.1 vs. 23.1 IU/L, $p < 0.001$) and total bilirubin (9.5 vs. 10.28 umol/L, $p = 0.001$)] levels of diabetic patients were better than those of non-diabetic patients. At the same time, creatinine (64.9 vs. 62.1 umol/L, $p = 0.007$) and creatine kinase-MB (CKMB) (9.6 vs. 8.89 ng/mL, $p < 0.001$) were higher in the diabetic group than in the non-diabetic group. Furthermore, diabetic patients gained a higher MuLBSTA score than non-diabetic patients (9 vs. 5, $p < 0.001$). Importantly, we also found that the diabetic group also showed a higher rate of severe cases (35.38 vs. 23.74%, $p < 0.001$) and deaths (5.23 vs. 2.40%, $p = 0.002$) compared with the non-diabetic group, suggesting that diabetic patients may need more intensive care during their in-hospital treatment.

Different Clinical Characteristics Risk Factors for COVID-19 Severity Between Diabetic and Non-diabetic Patients

Based on previous studies and our results, diabetic patients have a significantly higher probability of severe disease and death after infection with COVID-19 than non-diabetic patients. However, the factors that could lead to this worse result in the diabetic

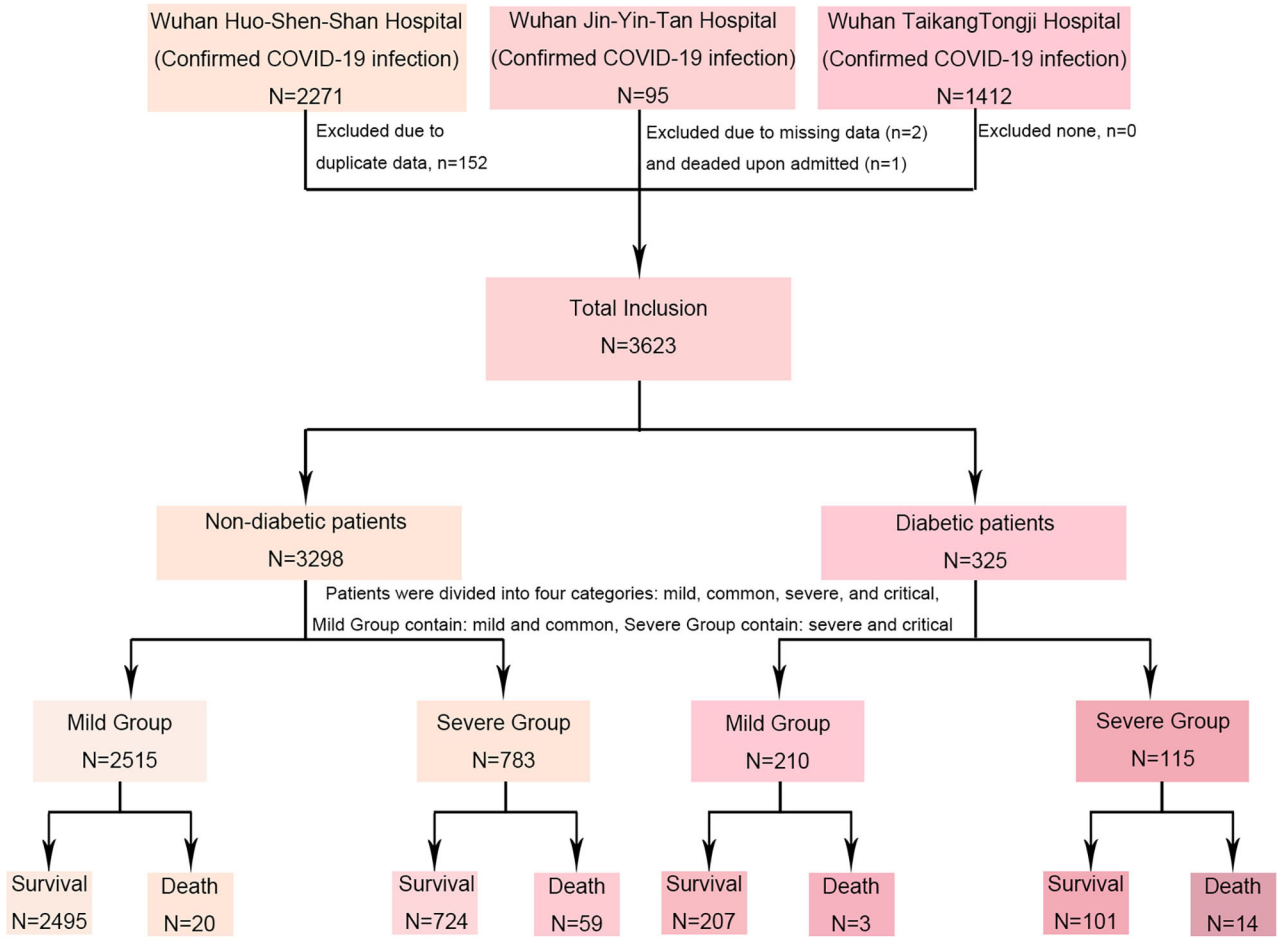

FIGURE 1 | Enrollment flow chart of study population.

group have not been confirmed by relevant studies, so we further analyzed the diabetic patients infected with COVID-19. Diabetic patients were divided into the mild group (MG, contain mild and common type) and severe group (SG, contain severe and critical type) according to the Clinical Characteristics of Coronavirus Disease 2019 in China (1), and differences between the two groups were further discussed (**Table 2**).

The median ages were 68 (60–74) and 65 (57–72) in the SG and MG, respectively. The MG exhibited a quicker pulse than the SG [89/min (80–100) vs. 85/min (78–94), $p = 0.012$]. There was no significant difference between the two groups in terms of body temperature, fever, respiratory rate, blood pressure, fatigue or duration of first symptoms. In addition, we also found that the SG showed a higher rate of dyspnea than the MG (42.61 vs. 22.86%, $p < 0.001$), while no significant difference was found in terms of cough (72.17 vs. 68.1%), expectoration (15.65 vs. 12.38%), chest tightness (25.22 vs. 18.57%), or hemoptysis (0 vs. 0%) between the two groups. There was no difference in the incidence of gastrointestinal symptoms between the two groups. Pre-existing cardiovascular diseases were more frequent in the SG than in the MG (66.09 vs. 49.52%, $p = 0.004$). Moreover, the SG showed more severe inflammatory markers of infection than the MG, such as WBC count (6.40 × 10^9/L vs. 5.51 × 10^9/L, $p = 0.001$), neutrophil (4.15 × 10^9/L vs. 3.50 × 10^9/L, $p < 0.001$), and CRP (7.76 vs. 3.18 mg/L, $p < 0.001$). The SG also showed a higher level of CKMB than the MG (11.31 vs. 9.2 ng/mL, $p = 0.003$).

To further explore the risk factors associated with the severe progression of diabetic patients infected with COVID-19, binary logistic regression analysis was applied (**Figure 2**). We found that dyspnea ($p = 0.002$, OR = 2.309), cardiovascular diseases ($p = 0.019$, OR = 1.850), neutrophil ($p < 0.001$, OR = 1.288), PLT count ($p = 0.002$, OR = 0.995), and CKMB ($p = 0.010$, OR = 1.015) were more common in the SG, suggesting that these five factors may strongly related with the severity of diabetic patients when infected with COVID-19. In addition, we also analyzed the risk factors in the non-diabetic group (**Supplementary Tables 1, 2**). Interestingly, the risk factors leading to COVID-19 severity of the non-diabetic groups were different from those of the diabetic group. Sex ($p = 0.019$, OR = 1.244), age ($p < 0.001$, OR = 1.040), dyspnea ($p < 0.001$, OR = 1.752), cardiovascular diseases ($p = 0.009$, OR = 1.298),

TABLE 1 | Basic characteristics of diabetic and non-diabetic patients with COVID-19 ($N = 3,623$).

	Total ($N = 3,623$)	Non-diabetic ($n = 3,298$)	Diabetic ($n = 325$)	*P*-value
Male, *n* (%)	1,766(48.74)	1,589(48.18)	177(54.46)	0.031
Female, *n* (%)	1,857(51.26)	1,709(51.82)	148(45.54)	0.031
Age, median (IQR)	61(50–69)	61(49∼69)	66(58–72)	0.000
Body temperature, Mean ± SD, °C	37.62 ± 1.04	37.61 ± 1.04	37.69 ± 1.04	0.203
Fever, *n* (%)	2,319(64.01)	2,099(63.64)	220(67.69)	0.147
Respiratory rate, *n*/min	20(18–21)	20(18∼21)	20(19∼22)	0.007
Pulse, *n*/min	84(78–96)	84(78∼95)	86(78∼96)	0.066
SBP, median (IQR), mmHg	130(120–140)	130(120∼140)	133(124∼143)	0.000
DBP, median (IQR), mmHg	80(73–88)	80(73∼88)	80(74.5∼89)	0.279
Fatigue, *n* (%)	1,799(49.65)	1,620(49.12)	179(55.08)	0.040
Duration of first symptom, day (IQR)	20.5(14–30)	21.0(14–30)	20.0(13–30)	0.793
Respiratory symptoms				
Cough, *n* (%)	2,351(64.89)	2,125(64.43)	226(69.54)	0.066
Expectoration, *n* (%)	448(12.37)	404(12.25)	44(13.54)	0.501
Dyspnea, *n* (%)	877(24.21)	780(23.65)	97(29.85)	0.013
Chest tightness, *n* (%)	733(20.23)	665(20.16)	68(20.92)	0.745
Hemoptysis, *n* (%)	8(0.22)	8(0.24)	0(0)	1.000
Digestive tract symptoms				
Vomiting, *n* (%)	84(2.32)	77(2.33)	7(2.15)	0.836
Abdominal pain, *n* (%)	45(1.24)	44(1.33)	1(0.31)	0.175
Diarrhea, *n* (%)	186(5.13)	173(5.25)	13(4.00)	0.332
Anorexia, *n* (%)	955(26.36)	853(25.86)	102(31.38)	0.031
Past medical history				
Cardiovascular disease[a], *n* (%)	956(26.39)	776(23.53)	180(55.38)	0.000
Lung diseases[b], *n* (%)	159(4.39)	147(4.46)	12(3.69)	0.521
Liver disease[c], *n* (%)	107(2.95)	99(3.00)	8(2.46)	0.583
WBC, median (IQR), 10^9/L	5.80(4.80∼7.00)	5.80(4.8∼6.98)	5.80(4.8∼7.2)	0.525
Neutrophil, median (IQR),10^9/L	3.48(2.68∼4.54)	3.45(2.66∼4.52)	3.68(2.86∼5.08)	0.007
Lymphocyte, median (IQR), 10^9/L	1.52(1.12∼1.88)	1.53(1.14∼1.89)	1.38(0.99∼1.74)	0.000
Proportion of neutral lymph, median (IQR)	2.31(1.65∼3.33)	2.26(1.63∼3.26)	2.67(1.84∼4.3)	0.000
HGB, median (IQR), g/L	122(111∼133)	122(111∼133)	120(108.5∼131)	0.044
PLT, median (IQR), 10^9/L	225(183∼272)	226(184∼272)	215(176∼269)	0.058
Bilirubin, median (IQR), umol/L	10.28(7.80∼13.25)	10.30(7.90∼13.25)	9.50(6.88∼12.98)	0.001
ALT, median (IQR), IU/L	24.40(15.10∼37.00)	24.70(15.20∼37.83)	20.10(13.95∼33.15)	0.000
AST, median (IQR), IU/L	22.80(17.1∼37.35)	23.10(17.30∼37.35)	19.10(14.50∼27.98)	0.000
ALB, median (IQR), g/L	37.85(34.72∼40.21)	37.85(34.84∼40.30)	36.96(33.50∼39.55)	0.000
CRP, median (IQR), mg/L	1.99(0.50–7.00)	1.81(0.50∼6.42)	4.64(1.21∼17.05)	0.000
CREA, median (IQR), umol/L	62.40(51.94–75.70)	62.10(51.58∼75.23)	64.90(54.40∼79.60)	0.007
CKMB, median (IQR), ng/mL	8.90(6.70–11.63)	8.89(6.63∼11.42)	9.60(7.60∼13.95)	0.000
MuLBSTA score, median (IQR)	5(7–9)	5(7–9)	9(7–11)	0.000
Diagnosis type				
Mild and Common, *n* (%)	2,725(75.21)	2,515(76.26)	210(64.62)	0.000
Severe and Critical, *n* (%)	898(24.79)	783(23.74)	115(35.38)	
Death, *n* (%)	96(2.65)	79(2.40)	17(5.23)	0.002

SBP, systolic blood pressure; DBP, diastolic blood pressure; WBC, white blood cell; HGB, hemoglobin; PLT, platelet; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; CREA, creatinine; CKMB, creatine phosphokinase isoenzyme.

[a]*Cardiovascular disease includes coronary heart disease and hypertension and etc.*

[b]*Lung disease includes chronic bronchitis, COPD, tuberculosis and lung cancer and etc.*

[c]*Liver disease includes hepatitis B, hepatitis C, fatty liver, cirrhosis, liver cancer, hepatitis A, hepatic hemangioma, schistosomiasis liver disease and etc.*

respiratory rate ($p < 0.001$, OR = 1.028), WBC ($p = 0.022$, OR = 1.037), HGB ($p < 0.001$, OR = 0.987), ALB ($p = 0.001$, OR = 0.966) and CRP ($p < 0.001$, OR = 1.007) were related to COVID-19 severity in the non-diabetic group. Although dyspnea and cardiovascular diseases were related to disease severity in both the diabetic group and non-diabetic group, dyspnea and

TABLE 2 | Univariate analysis of severity-related factors in diabetic patients with COVID-19, (*N* = 325).

	Total (N = 325)	MG (*N* = 210)	SG (*N* = 115)	*P*-value
Male, *n* (%)	177(54.46)	107(50.95)	70(60.87)	0.086
Female, *n* (%)	148(45.54)	103(49.05)	45(39.13)	0.086
Age, median (IQR)	66(58∼72)	65(57∼72)	68(60∼74)	0.039
Body temperature, mean ± SD, ◦C	37.69 ± 1.04	37.73 ± 1.02	37.61 ± 1.08	0.345
Fever, *n* (%)	220(67.69)	147(70.00)	73(63.48)	0.229
Respiratory rate, *n*/min	20(19∼22)	20(19∼21)	20(19∼22)	0.004
Pulse, *n*/min	86(78∼96)	85(78∼94)	89(80∼100)	0.012
SBP, median (IQR), mmHg	133(124∼143)	132(124∼143)	133(125∼144)	0.547
DBP, median (IQR), mmHg	80(74.5∼89)	80(75∼89)	80(73∼88)	0.577
Fatigue, *n* (%)	179(55.08)	121(57.62)	58(50.43)	0.213
Duration of first symptom, day (IQR)	20(13∼30)	20(12.75∼30)	23(14∼30)	0.313
Respiratory symptoms				
Cough, *n* (%)	226(69.54)	143(68.10)	83(72.17)	0.445
Expectoration, *n* (%)	44(13.54)	26(12.38)	18(15.65)	0.410
Dyspnea, *n* (%)	97(29.85)	48(22.86)	49(42.61)	0.000
Chest tightness, *n* (%)	68(20.92)	39(18.57)	29(25.22)	0.159
Hemoptysis, *n* (%)	0(0)	0(0)	0(0)	
Digestive tract symptoms				
Vomiting, *n* (%)	7(2.15)	5(2.38)	2(1.74)	1.000
Abdominal pain, *n* (%)	1(0.31)	1(0.48)	0(0.00)	1.000
Diarrhea, *n* (%)	13(4.00)	8(3.81)	5(4.35)	0.776
Anorexia, *n* (%)	102(31.38)	62(29.52)	40(34.78)	0.329
Past medical history				
Cardiovascular disease[a], *n* (%)	180(55.38)	104(49.52)	76(66.09)	0.004
Lung diseases[b], *n* (%)	12(3.69)	7(3.33)	5(4.35)	0.643
Liver disease[c], *n* (%)	8(2.46)	6(2.86)	2(1.74)	0.717
WBC, 10^9/L	5.80(4.80∼7.20)	5.51(4.70∼6.80)	6.40(5.00∼8.18)	0.001
Neutrophil, 10^9/L	3.68(2.86∼5.08)	3.50(2.79∼4.37)	4.15(3.20∼6.30)	0.000
Lymphocyte, 10^9/L	1.38(0.99∼1.74)	1.41(1.05∼1.75)	1.33(0.77∼1.72)	0.058
Proportion of neutral lymph	2.67(1.84∼4.30)	2.54(1.72∼3.32)	3.03(1.98∼6.21)	0.000
HGB, g/L	120(108.50∼131.00)	120(111.75∼131)	120(103.5∼132)	0.159
PLT, 10^9/L	215(176∼269)	221.5(184∼274.5)	205(157∼255)	0.014
Bilirubin, umol/L	9.50(6.88∼12.98)	9.35(6.90∼12.76)	10.30(6.40∼13.50)	0.352
ALT, IU/L	20.10(13.95∼33.15)	21.70(14.5∼32.88)	18.00(12.30∼34.40)	0.047
AST, IU/L	19.10(14.50∼27.98)	19.60(15.38∼27.96)	18.50(13.70∼28.90)	0.214
ALB, g/L	36.96(33.50∼39.55)	37.40(34.39∼39.83)	35.90(31.80∼38.90)	0.002
CRP, mg/L	4.64(1.21∼17.05)	3.18(0.88∼12.30)	7.76(1.86∼34.09)	0.000
CREA, umol/L	64.90(54.40∼79.60)	63.49(54.55∼77.63)	66.80(53.90∼81.60)	0.315
CKMB, ng/mL	9.60(7.60∼13.95)	9.20(7.38∼12.23)	11.31(7.80∼21.80)	0.003
MuLBSTA score	9(7∼11)	7(5∼9)	9(8∼13)	0.000

[a] *Cardiovascular disease includes coronary heart disease and hypertension and etc.*
[b] *Lung disease includes chronic bronchitis, COPD, tuberculosis and lung cancer and etc.*
[c] *Liver disease includes hepatitis B, hepatitis C, fatty liver, cirrhosis, liver cancer, hepatitis A, hepatic hemangioma, schistosomiasis liver disease and etc.*

cardiovascular diseases might bring a greater risk of exacerbation in diabetic patients than in non-diabetic patients.

Risk Factors for Diabetic and Non-diabetic in-hospital Mortality

We have discussed the risk factors that contributed to the severity of COVID-19 in diabetic patients. Previously, we also found that diabetic patients had a higher risk of death after infection with COVID-19 than non-diabetic patients. What were the risk factors that contributed to the high in-hospital mortality of diabetic patients?

The data from the 325 hospitalized diabetic patients with COVID-19 were collected and analyzed (**Table 3**). Patients with diabetes were divided into a survival group (SurG, n = 308)

Intercept and Variable	β Confficient(SE)	P value	Odds ratio(95% CI)
Intercept	-1.472(0.473)	0.002	
Dyspnea	0.837(0.269)	0.002	2.309(1.362-3.915)
Cardiovascular disease	0.615(0.262)	0.019	1.850(1.107-3.091)
Neutrophil	0.253(0.061)	0.000	1.288(1.142-1.452)
PLT	-0.005(0.002)	0.002	0.995(0.992-0.998)
CKMB	0.015(0.006)	0.010	1.015(1.004-1.027)

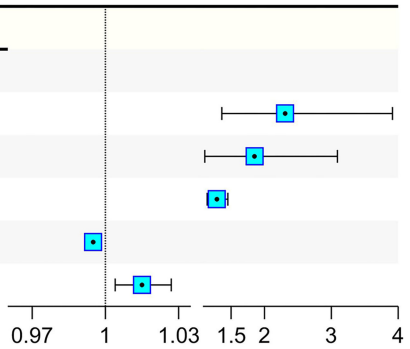

FIGURE 2 | Binary logistic regression analysis of severity-related factors in diabetic patients with COVID-19.

and a deceased group (DG, $n = 17$) according to patient outcome. The median age was 77 (66.5–82) and 65 (58–72) years in the DG and SurG, respectively, and the age of the DG was older than that of SurG ($p = 0.001$). No significant differences were found in body temperature, fever, respiratory rate, pulse, blood pressure, fatigue symptoms, or duration of first symptom between the two groups. Similar to **Table 2**, we also found that the frequency of dyspnea in the DG ($n = 13$, 76.47%) was much higher than that in SurG ($n = 84$, 27.27%) ($p < 0.001$); however, there was no significant difference in the incidence of cough, expectoration, chest tightness, hemoptysis and gastrointestinal symptoms (including vomiting, abdominal pain, diarrhea, anorexia) between the two groups. Interestingly, although no significant difference was found in the prevalence of pre-existing cardiovascular disease between the SurG and DG, the DG had a higher rate of coronary heart disease ($n = 5$, 29.41%) compared with the SG ($n = 29$, 8.92%). Our data also showed that the DG had a higher frequency of pre-existing lung disease ($n = 3$, 17.65%) than the SG ($n = 9$, 2.92) ($p = 0.002$), while no significant difference was found in liver disease. Similar to previous findings, the DG showed a higher WBC count [8.5 $\times$ 10^9/L (6.65–12) vs. 5.7 $\times$ 10^9/L (4.78–7), $p < 0.001$] and neutrophil [7.53 $\times$ 10^9/L (5.16–10.88) vs. 3.64$\times$10^9/L (2.81–4.69), $p < 0.001$] but a lower lymphocyte (0.69 $\times$ 10^9/L vs. 1.41 $\times$ 10^9/L, $p = 0.001$), HGB level (103.50 vs. 120.50 g/L, $p = 0.021$), and PLT count (168.00 $\times$ 10^9/L vs. 216.50 $\times$ 10^9/L, $p = 0.027$). Higher levels of CRP (57.79 vs. 4.30 mg/ml, $p < 0.001$), CKMB (22.60 vs. 9.40 ng/ml, $p < 0.001$) and creatinine (82.60 vs. 64.43 umol/L, $p = 0.031$) were also observed in the DG than in the SurG.

Binary logistic regression analysis was also applied to explore the risk factors associated with death in diabetic patients infected with COVID-19 (**Figure 3**). Dyspnea ($p = 0.003$, OR = 17.492), coronary heart disease ($p = 0.019$, OR = 8.343), neutrophil ($p < 0.001$, OR = 1.775), PLT count ($p = 0.022$, OR = 0.991), and CKMB ($p = 0.004$, OR = 1.014) were related to the risk of death among diabetic patients who were infected with COVID-19. Compared with the risk factors for non-diabetic patient death (**Supplementary Tables 3, 4**), we found that age ($p = 0.002$, OR = 1.037), dyspnea ($p = 0.002$, OR = 2.347), WBC count ($p < 0.001$, OR = 1.037), albumin ($p < 0.001$, OR = 0.987), PLT count ($p = 0.001$, OR = 0.966) and CRP ($p < 0.001$, OR = 1.007) were related to the risk of death in non-diabetic patients infected with COVID-19. Although no significant difference was found in CRP and cough in diabetic patients, these two indicators are extremely important for mortality risk prediction in non-diabetic patients. Regardless of diabetes mellitus, the independent risk factors for death included dyspnea, neutrophil, PLT count, and CRP. Previous studies suggested that CRP could be an independent risk factor for death in diabetic patients with COVID-19; however, according to our data, we believe that CRP may be an important risk factor for mortality risk in all patients with COVID-19, independent of diabetes mellitus status.

Establishment of Risk Score of Diabetic Patients With COVID-19 (DPCR Score)

Although many studies have reported that different risk factors, both clinical and laboratory, were correlated with the progression of diabetic patients with COVID-19, few studies have put these risk factors into a risk score to predict the severity of diabetic patients with COVID-19. Based on our previous data, we took dyspnea, cardiovascular diseases, neutrophil, PLT count, and CKMB as the scoring indicators, and conducted two classification calculation to obtain the weight coefficient (**Figure 4**). For the convenience of calculation, we expand the weight coefficient of each index by 10 times, an integer value for each risk factor was used to calculate a total score capable of quantifying the risk of severity progression in diabetic patients, that is, DPCR score (**Table 4**). Dyspnea, cardiovascular disease, PLT count and CKMB each earned a patient 2 points, whereas the neutrophil was worth 3 points in diabetic patients. The maximum score was 11.

Then, the model's ability to accurately differentiate the risk of progression in diabetic patients with COVID-19 was tested. The area under the receiver operator curve (ROC) was 0.724 (**Figure 5A**). Predicted and observed rates of progression for

TABLE 3 | Univariate analysis of death-related factors in diabetic patients with COVID-19 ($N = 325$).

	Total ($N = 325$)	**Survival ($n = 308$)**	**Death ($n = 17$)**	***P*-value**
Male, *n* (%)	177(54.46)	165(53.57)	12(70.59)	0.710
Female, *n* (%)	148(45.54)	143(46.43)	5(29.41)	0.710
Age, median (IQR)	66(58∼72)	65(58∼72)	77(66∼82)	0.001
Body temperature, Mean ± SD, ◦C	37.8 ± 1.04	37.8 ± 1.03	38.03 ± 1.17	0.170
Fever, *n* (%)	220(67.69)	208(67.53)	12(70.59)	0.793
Respiratory rate, *n*/min	20(19∼22)	20(19∼22)	20(19∼22)	0.009
Pulse, *n*/min	86(78∼96)	86(78∼96)	86(78∼96)	0.030
SBP, median (IQR), mmHg	133(124∼143)	133(124∼143)	133(124∼143)	0.855
DBP, median (IQR), mmHg	80(74.5∼89)	80(74.5∼89)	80(74.5∼89)	0.763
Fatigue, *n* (%)	179(55.08)	168(54.55)	11(64.71)	0.412
Duration of first symptom, day (IQR)	20(13∼30)	20(13∼30)	20(13∼30)	0.165
Respiratory symptoms				
Cough, *n* (%)	226(69.54)	211(68.51)	15(88.24)	0.085
Expectoration, *n* (%)	43(13.23)	41(13.31)	3(17.65)	0.611
Dyspnea, *n* (%)	97(29.85)	84(27.27)	13(76.47)	0.000
Chest tightness, *n* (%)	68(20.92)	64(20.78)	4(23.53)	0.786
Hemoptysis, *n* (%)	0(0)	0(0)	0(0)	
Digestive tract symptoms				
Vomiting, *n* (%)	7(2.15)	7(2.27)	0(0)	1.000
Abdominal pain, *n* (%)	1(0.31)	1(0.32)	0(0)	1.000
Diarrhea, *n* (%)	13(4.00)	13(4.22)	0(0)	1.000
Anorexia, *n* (%)	102(31.38)	95(30.84)	7(41.18)	0.371
Past medical history				
Cardiovascular disease[a], *n* (%)	180(55.38)	170(55.19)	10(58.82)	0.770
Lung diseases[b], *n* (%)	12(3.69)	9(2.92)	3(17.65)	0.002
Liver disease[c], *n* (%)	8(2.46)	7(2.27)	1(5.88)	0.352
WBC, 10^9/L	5.80(4.80∼7.20)	5.70(4.78∼7.00)	8.50(6.65∼12.00)	0.000
Neutrophil, 10^9/L	3.68(2.86∼5.08)	3.64(2.81∼4.69)	7.53(5.16∼10.88)	0.000
Lymphocyte, 10^9/L	1.38(0.99∼1.74)	1.41(1.00∼1.75)	0.69(0.36∼1.28)	0.001
Proportion of neutral lymph	2.67 (1.84∼4.30)	2.65 (1.80∼3.80)	16.09(3.84∼17.93)	0.000
HGB, g/L	120.00(108.50∼131.00)	120.50(110.25∼131.00)	103.50(87.00∼128.00)	0.021
PLT, 10^9/L	215.00(176.00∼269.00)	216.50(178.13∼269.50)	168.00(72.00∼263.50)	0.027
Bilirubin, umol/L	9.50(6.88∼12.98)	9.40(6.81∼12.78)	12.60(7.70∼22.40)	0.050
ALT, IU/L	20.10(13.95∼33.15)	19.75(13.93∼33.03)	24.90(15.55∼41.40)	0.323
AST, IU/L	19.10(14.50∼27.98)	18.83(14.43∼26.85)	31.80(19.90∼59.05)	0.001
ALB, g/L	36.96(33.50∼39.55)	37.15(33.83∼39.60)	32.00(27.10∼37.25)	0.002
CRP, mg/L	4.64(1.21∼17.05)	4.30(1.17∼15.80)	57.79(7.14∼169.28)	0.000
CREA, umol/L	64.90(54.40∼79.60)	64.43(54.00∼77.45)	82.60(56.40∼157.30)	0.031
CKMB, ng/ml	9.60(7.60∼13.95)	9.40(7.43∼13.48)	22.60(10.40∼44.23)	0.000
MuLBSTA Score	9(7∼11)	9(7∼10)	13(10∼15)	0.000
Diagnosis type				
Mild and Common	210(64.62)	207(67.21)	3(17.65)	0.000
Severe and Critical	115(35.38)	101(32.79)	14(82.35)	

[a]*Cardiovascular disease includes coronary heart disease and hypertension and etc.*
[b]*Lung disease includes chronic bronchitis, COPD, tuberculosis and lung cancer and etc.*
[c]*Liver disease includes hepatitis B, hepatitis C, fatty liver, cirrhosis, liver cancer, hepatitis A, hepatic hemangioma, schistosomiasis liver disease and etc.*

each risk score were also compared (**Supplementary Table 5**). In addition, the Hosmer-Lemeshow test resulted in a *p*-value of 0.194, suggesting that there was no statistical difference between the predicted and observed rates of progression. In addition, a calibration plot was also applied in **Figure 5B**. Based on the ROC curve, an optimal risk score cutoff of 7 or higher was

Intercept and Variable	β Confficient(SE)	P value	Odds ratio(95% CI)
Intercept	-10.262(2.464)	0.000	
Dyspnea	2.862(0.962)	0.003	17.492(2.652-115.359)
Cough	2.547(1.309)	0.052	12.774(0.982-166.119)
Lung disease	2.888(1.106)	0.009	17.957(2.053-157.058)
Cardiovascular disease	2.121(0.902)	0.019	8.343(1.425-48.829)
Neutrophil	0.574(0.146)	0.000	1.775(1.335-2.361)
PLT	-0.010(0.004)	0.022	0.991(0.983-0.999)
CRP	0.009(0.005)	0.063	1.009(1.000-1.018)
CKMB	0.042(0.015)	0.004	1.043(1.014-1.074)

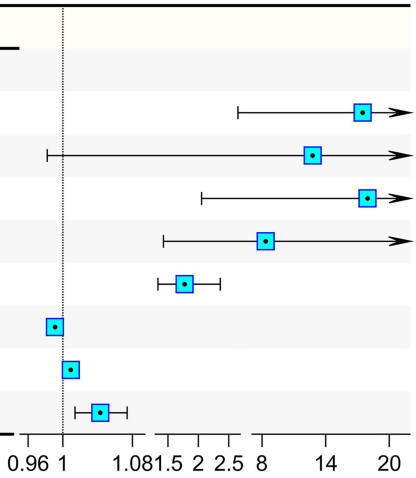

FIGURE 3 | Binary logistic regression analysis of death-related factors in diabetic patients with COVID-19.

Intercept and Variable	β Confficient(SE)	P value	Odds ratio(95% CI)
Intercept	-2.335(0.314)	0.000	
Dyspnea(Yes)	0.923(0.268)	0.001	2.516(1.487-4.258)
Cardiovascular disease(Yes)	0.613(0.259)	0.018	1.847(1.113-3.066)
Neutrophil(>3.85)	0.953(0.258)	0.000	2.592(1.562-4.302)
PLT(⩽200)	0.744(0.264)	0.005	2.104(1.254-3.531)
CKMB(>11.2)	0.836(0.257)	0.001	2.306(1.394-3.815)

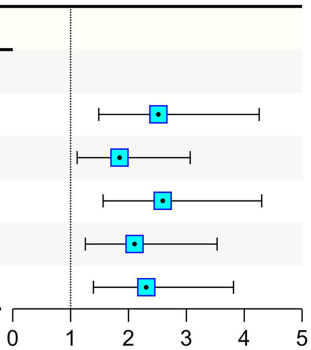

FIGURE 4 | The weight coefficient was calculated by two classification.

TABLE 4 | Establishment of risk score of diabetic patients with COVID-19 (DPCR score).

	Weighted beta coefficient	Score
Dyspnea (yes)	0.233	2
Cardiovascular disease (yes)	0.168	2
Neutrophil (>3.85 10^9/L)	0.261	3
PLT (≤200 10^9/L)	0.199	2
CKMB (>11.2 ng/ml)	0.225	2

associated with a high risk of severity progression in diabetic patients with COVID-19. If a diabetic patient had a score of 4 or lower, they were considered to have a low risk of severity progression. The sensitivity and specificity of the model were 0.496 and 0.886, respectively.

DISCUSSION

Recent studies have shown that patients infected with COVID-19 who also have underlying chronic diseases (7, 8), such as hypertension, cancer, cardiovascular diseases and diabetes, have a higher risk of severe disease and death. Among these chronic diseases, diabetes stood out, not only because it was reported to be the second most common comorbidity of COVID-19, but also because it had been proven by many other studies that diabetes contributes to the risk of mortality following COVID-19 infection.

The potential mechanism by which COVID-19 infection increases the susceptibility and mortality of diabetic patients has also been discussed by many researchers. Muniyappa et al. (9) suspected that higher affinity cellular binding and efficient virus entry, decreased viral clearance, diminished T cell function, increased susceptibility to hyperinflammation and cytokine storm syndrome, and the presence of cardiovascular disease were possible mechanisms leading to the increased severity

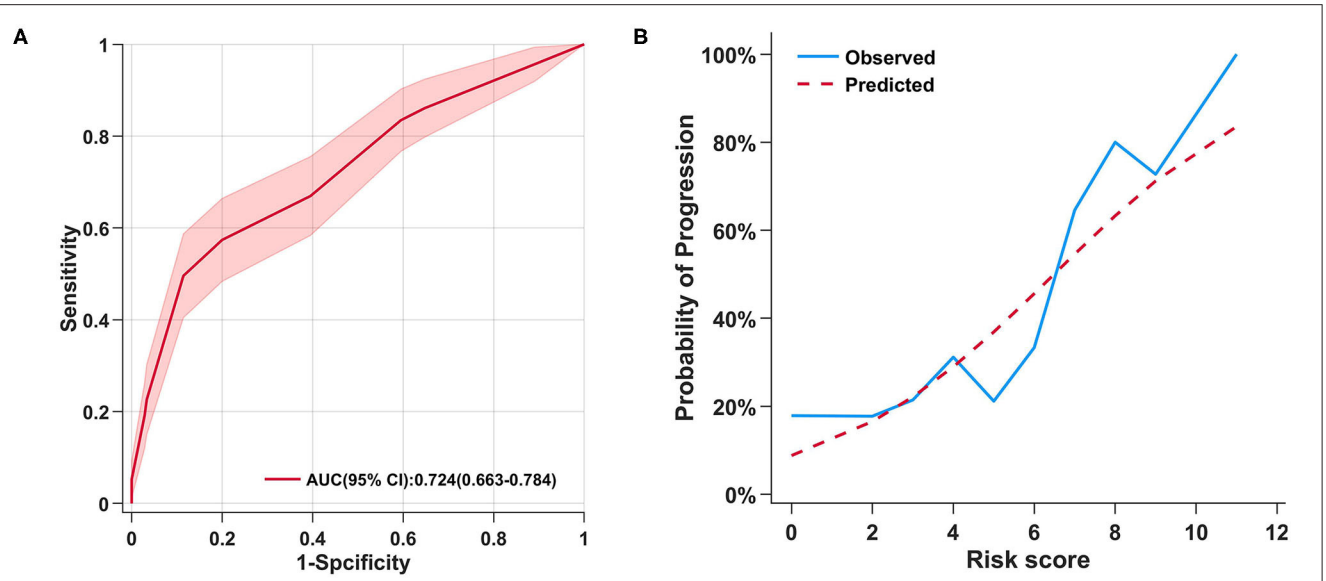

FIGURE 5 | (A) ROC curve for risk score as a predictor of severity of diabetic patients with COVID-19; **(B)** Observed vs. predicted probabilities of progression by risk score.

observed among diabetic patients with COVID-19 (9). Similarly, recent studies showed that angiotensin-converting enzyme 2 (ACE2), transmembrane serine protease 2 (TMPRSS2), sialic acid receptors, matrix metalloproteinase inducer (CD147), catepsin B and L were reported as potential key entry factors in the pathogenesis of COVID-19 (10). ACE2 is also highly expressed in pancreatic beta cells. In addition, previous studies also showed that the state and function of immune cells in diabetic patients were different from those in healthy persons (11, 12). Moreover, senescent T cells showed strong similarities to those in patients with hyperglycemia, and the accumulation of highly differentiated end-stage memory T cells was also found in these patients, which has a detrimental impact on immune function in diabetes (13). T cell senescence in turn contributes to abnormal glucose homeostasis (14), causing a vicious cycle. The above studies demonstrated that the decrease in T cell function laid the foundation for the difference in the immune environment in diabetic patients infected with COVID-19. In our study, we found that the absolute lymphocyte count in diabetic patients was significantly lower than that of normal patients ($p<0.001$, OR=4.264), suggesting that the decrease in lymphocytes in diabetic patients is related to poor prognosis.

We also found that the absolute value of neutrophils was significantly higher in diabetic patients, which also suggested that the inflammatory response in diabetic patients was significantly higher than that in non-diabetic patients. Recently, Chen et al. found that CRP may help to identify patients with diabetes who were at greater risk of dying during hospitalization (6). They found that high CRP (OR = 1.16, $p = 0.033$) and low albumin (OR = 0.91, $p = 0.030$) were risk factors for poor prognosis in patients with diabetes and COVID-19. Similarly, in our study, we found that CRP was highly expressed in diabetic patients. In addition, the level of CRP was much higher in the DG of diabetic patients than in the SurG of diabetic patients [57.79 mg/L (7.14–169.28) vs. 4.295 mg/L (1.1675–15.7975)]. The high level may be related to the immune function and response of diabetic patients. However, further binary logistic regression analysis revealed that CRP could not be used as a risk factor related to the severity and risk of death of diabetic patients with COVID-19, although the OR value was relatively high. Therefore, we believe that CRP has a certain suggestive role, but it is not significantly related to the severity and death of diabetic patients infected with COVID-19. Conversely, through binary regression analysis, we found that CRP could be a relevant factor for severity and mortality risk in non-diabetic patients.

The level of CKMB increases after myocardial necrosis, which has made it the gold standard for the diagnosis of acute myocardial infarction for many years (15, 16). However, elevations of CKMB were never intended to be synonymous with myocardial infarction, only indicative of cardiac injury, because the expression of CKMB in other tissues impairs specificity (15). A recent study showed that an increase in CKMB, along with other cardiac-specific biomarkers (such as CK, myoglobin, troponin, and NT-proBNP), can play a crucial role in identifying patients vulnerable to developing cardiovascular manifestations of COVID-19(17). Interestingly, we found that the level of CKMB was much higher in diabetic patients than in non-diabetic patients [8.89 ng/ml (6.63–11.42) vs. 9.6 ng/ml (7.6–13.95), $p < 0.0001$, OR = 4.808]. In addition, binary logistic regression analysis also showed that a high level of CKMB was related to the risk of death and disease severity in diabetic patients. The increase in CKMB may be related to hyperglycemia in diabetic patients (18). Qiu et al. Demonstrated, in a diabetic model, that hyperglycemia could induce NLRP3

inflammasome activation, which may lead to pyroptosis and aggravated myocardial ischemia/reperfusion injury. This basic study demonstrated the importance of glycemic control in diabetic patients with COVID-19.

Recently, studies have shown that diabetes is a risk factor for the progression and prognosis of COVID-19, and many laboratory and clinical data have been analyzed. Some indicators have been considered potential targets related to the prognosis of diabetic patients with COVID-19. However, no risk score has been applied to predict the severity of diabetic patients with COVID-19. Here, based on our data, independent risk factors associated with the severity of diabetes mellitus were determined by binary logistic regression analysis: dyspnea, cardiovascular disease, neutrophil, PLT count and CKMB. The risk score was built according to these five factors, which may strongly relate to the severity of disease in diabetic patients with COVID-19. In our risk score, all the data were available to the clinician immediately upon admission to the hospital. In addition, predicted and observed rates of progression for each risk score were also compared.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Xinqiao Hospital (2020-yd073-01) with written informed consent waived due to the retrospective nature of the study. This study was carried out according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Y-FX, J-LH, and YX interpreted results and drafted the manuscript. XL, QL, ZX, M-DH, X-BR, CZ, W-JZ, WD, Y-FT, PL, and HW collected the data. HL analyzed the data. EL built and evaluated the risk score. C-PS and S-MY conceived the study, interpreted results and supervised research. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.655604/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Lopinavir/Ritonavir and Darunavir/Cobicistat in Hospitalized COVID-19 Patients: Findings From the Multicenter Italian CORIST Study

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Background: Protease inhibitors have been considered as possible therapeutic agents for COVID-19 patients.

Objectives: To describe the association between lopinavir/ritonavir (LPV/r) or darunavir/cobicistat (DRV/c) use and in-hospital mortality in COVID-19 patients.

Study Design: Multicenter observational study of COVID-19 patients admitted in 33 Italian hospitals. Medications, preexisting conditions, clinical measures, and outcomes were extracted from medical records. Patients were retrospectively divided in three groups, according to use of LPV/r, DRV/c or none of them. Primary outcome in a time-to event analysis was death. We used Cox proportional-hazards models with inverse probability of treatment weighting by multinomial propensity scores.

Results: Out of 3,451 patients, 33.3% LPV/r and 13.9% received DRV/c. Patients receiving LPV/r or DRV/c were more likely younger, men, had higher C-reactive protein levels while less likely had hypertension, cardiovascular, pulmonary or kidney disease. After adjustment for propensity scores, LPV/r use was not associated with mortality (HR = 0.94, 95% CI 0.78 to 1.13), whereas treatment with DRV/c was associated with a higher death risk (HR = 1.89, 1.53 to 2.34, E-value = 2.43). This increased risk was more marked in women, in elderly, in patients with higher severity of COVID-19 and in patients receiving other COVID-19 drugs.

Conclusions: In a large cohort of Italian patients hospitalized for COVID-19 in a real-life setting, the use of LPV/r treatment did not change death rate, while DRV/c was associated with increased mortality. Within the limits of an observational study, these data do not support the use of LPV/r or DRV/c in COVID-19 patients.

Keywords: COVID-19, SARS-CoV-2, darunavir, lopinavir, in-hospital mortality

INTRODUCTION

After more than 1 year of COVID-19 pandemic there are still no solid certainties on the efficacy of the therapies variously proposed. The urgency to intervene has induced drug agencies to allow the use of off-label drugs, although only few clinical trials have already been published.

Protease inhibitors have been considered as a candidate therapy because they inhibit enzymes that activate envelope glycoproteins as part of the process of viral entry into cells (1).

Lopinavir is a human immunodeficiency virus (HIV) type-1 aspartate protease inhibitor, with an *in vitro* inhibitory activity against the coronaviruses causing severe acute respiratory syndrome (SARS) (2) and Middle-East respiratory syndrome (MERS) (3). It is administered in combination with ritonavir to increase its plasma half-life. Both drugs have been shown to be able to bind well to the SARS-CoV 3C-like protease (3CLpro) (4), which is involved in the proteolytic processing of the replicase polyprotein and is crucial for viral replication (5). However, the efficacy of this combination in patients with SARS or MERS was based on scarce data (6).

Both the Recovery (7) and the Solidarity trial (8) failed to observe any clinical benefit of lopinavir/ritonavir (LPV/r) treatment beyond standard care in hospitalized patients with severe COVID-19. Null efficacy of LPV/r was also observed in other clinical trials (9) or retrospective studies, as systematically reviewed (10).

Given the structural similarity with lopinavir, darunavir, another protease inhibitor used in HIV therapy (11, 12), with cobicistat as a pharmaco-enhancer, has also been proposed as a COVID-19 treatment (13). In the emergency phase of COVID-19 pandemic the Italian Drug Agency (AIFA) (14) allowed the therapeutic use of both LPV/r and darunavir/cobicistat (DRV/c). However, evidence for the efficacy of DRV/c in COVID-19 patients is scarce, and findings from randomized clinical trials are lacking. In this context of uncertainty, sufficiently powered retrospective observational studies may be useful to shed light on the efficacy of these drugs in the SARS-CoV-2 pandemic.

We analyzed the association between DRV/c or LPV/r use and mortality in 3,451 COVID-19 patients from 33 clinical centers all over Italy.

MATERIALS AND METHODS

Setting

This national retrospective observational study was conceived within the CORIST Project (ClinicalTrials.gov ID: NCT04318418), which is a multicenter study launched in March 2020 (15) and aimed at testing the association of risk factors (16) and therapies with in-hospital COVID-19 mortality (17, 18). The study was approved by the institutional ethics board of all recruiting centers. Data for the present analyses were provided by 33 hospitals distributed throughout Italy (**Appendix**). Each hospital provided data from hospitalized patients (≥18 years of age) who had a positive test result for the SARS-CoV-2 virus at any time during their hospitalization from February 19 to May 23, 2020. The follow-up continued through May 29, 2020.

Data Sources

We obtained data from a cohort comprising 3,971 COVID-19 patients. The SARS-CoV-2 status was based on polymerase chain reaction on nasopharyngeal swab. Data were extracted at one-time point from electronic medical records or charts. Data included patients' demographics, laboratory tests, historical and current medication lists and diagnoses. Information on the most severe manifestation of COVID-19 occurred during hospitalization was retrospectively captured (16). We obtained the following information for each patient: date of admission and date of discharge or death; age; sex; the first recorded laboratory tests at entry; past comorbidities (coronary disease, diabetes, hypertension, respiratory disease and cancer) and current drug therapies for COVID-19—DRV/c, LPV/r, hydroxychloroquine (HCQ), remdesivir, tocilizumab, sarilumab, corticosteroids. Chronic kidney disease was classified by using of glomerular filtration rate (GFR) as reported in footnote of **Table 1**. Patients were defined as receiving LPV/r or DRV/c if they were receiving it at admission to hospital or received it during the follow-up period. Every physician in each hospital decided for him or herself if and how to treat their patient. According to the AIFA guidance (13, 14), LPV/r was administered at the dose of 400/100 mg × 2/day and DRV/c at the dose of 800/150 mg/day, both for at least 5–7 days, according to the clinical evolution of disease.

Statistical Analyses

The study index date was defined as the date of hospital admission. Index dates ranged from February 19, 2020 to May 23, 2020. The study end point was the time from study index to death. The number of patients who either died, or had been discharged alive, or were still admitted to hospital as of May 29, 2020, were recorded, and hospital length of stay was determined. Patients alive had their data censored on the date of discharge. Data were censored at 35 days in $N = 330$ (8.3%) patients with a follow up >35 days.

Of the initial cohort of 3,971 patients, 350 patients were excluded from the analysis because of missing data on LPV/r or DRV/c use ($N = 112$), other drug COVID-19 therapies (hydroxychloroquine, tocilizumab or sarilumab, remdesivir or corticosteroids, $N = 247$), time to event ($N = 59$), outcome ($N = 8$), COVID-19 severity ($N = 4$), age ($N = 4$), or sex ($N = 2$). Of the remaining 3,621 patients, 170 patients died or were discharged within 24 h after presentation, and were also excluded from the analysis.

At the end, the analyzed cohort consisted of $N = 3,451$ patients. Among them, 8.5% had at least a missing value for covariates. Distribution of missing values was as follows: C-reactive protein ($N = 178$); GFR ($N = 69$); ischemic disease ($N = 74$); chronic pulmonary disease ($N = 64$); diabetes ($N = 51$); hypertension ($N = 51$); and cancer ($N = 56$). We used multiple imputation techniques ($N = 10$ imputed datasets) to maximize data availability. We also conducted a case-complete analysis on 3,156 patients.

TABLE 1 | General characteristics of COVID-19 patients at baseline, according to lopinavir/ritonavir (LPV/r) or darunavir/cobicistat (DRV/c) use.

Characteristic	Controls*(*N* = 1,824)	LPV/r (*N* = 1,148)	DRV/c (*N* = 479)	*P*-value unadjusted^	*P*-value adjusted#
Age-median (IQR-yr.)	69 (56–80)	65 (55–76)	65 (58–77)	<0.0001	0.65
Gender-no (%)				<0.0001	0.65
Women	774 (42.4%)	386 (33.6%)	141 (29.4%)		
Men	1,050 (57.6%)	762 (66.4%)	338 (70.6%)		
Diabetes-no (%)‡				0.12	0.91
No	1,422 (79.0%)	937 (82.0%)	364 (79.3%)		
Yes	377 (21.0%)	205 (18.0%)	95 (20.7%)		
Hypertension-no (%)‡				0.0068	0.63
No	853 (47.4%)	564 (49.3%)	255 (55.7%)		
Yes	946 (52.6%)	579 (50.7%)	203 (44.3%)		
Ischemic heart disease-no (%)‡				0.0046	0.76
No	1,449 (80.9%)	962 (84.8%)	389 (86.1%)		
Yes	341 (19.1%)	173 (15.2%)	63 (13.9%)		
Chronic pulmonary disease-no (%)‡				0.0003	0.64
No	1,489 (83.1%)	1,000 (87.7%)	402 (88.6%)		
Yes	304 (16.9%)	140 (12.3%)	52 (11.4%)		
Cancer-no (%)‡				0.17	0.75
No	1,590 (88.4%)	1,034 (90.6%)	408 (89.5%)		
Yes	208 (11.6%)	107 (9.4%)	48 (10.5%)		
CKD stage¶-no (%)‡				<0.0001	0.57
Stage 1	629 (35.4%)	399 (35.2%)	183 (39.0%)		
Stage 2	615 (34.6%)	490 (43.2%)	167 (35.6%)		
Stage 3a or stage 3b	377 (21.2%)	202 (17.8%)	88 (18.8%)		
Stage 4 or stage 5	158 (8.9%)	43 (3.4%)	31 (6.6%)		
C Reactive Protein-no (%)‡				<0.0001	0.10
<1 mg/L	235 (13.7%)	95 (8.7%)	30 (6.4%)		
1-3 mg/L	215 (12.6%)	153 (14.0%)	53 (11.4%)		
>3 mg/L	1,263 (73.7%)	845 (77.3%)	384 (82.2%)		
Hydroxychloroquine use				<0.0001	0.18
No	621 (34.0%)	170 (14.8%)	26 (5.4%)		
Yes	1,203 (66.0%)	978 (85.2%)	453 (94.6%)		
Tocilizumab or Sarilumab use				0.38	0.93
No	1,526 (83.7%)	981 (85.4%)	408 (85.2%)		
Yes	298 (16.3%)	167 (14.6%)	71 (14.8%)		
Remdesivir use				0.35	0.19
No	1,781 (97.6%)	1,111 (96.8%)	467 (97.5%)		
Yes	43 (2.4%)	37 (3.2%)	12 (2.5%)		
Corticosteroids use				0.11	0.25
No	1,163 (63.8%)	775 (67.5%)	313 (65.3%)		
Yes	661 (36.2%)	373 (32.5%)	166 (34.7%)		
Clusters of hospitals				<0.0001	0.19
Northern regions (except Milan) (n)	414 (22.7%)	268 (23.3%)	103 (21.5%)		
Milan (m)	340 (18.6%)	224 (19.5%)	122 (25.5%)		
Center regions (except Rome) (c)	674 (36.9%)	186 (16.2%)	190 (39.7%)		
Rome (r)	109 (6.0%)	321 (28.0%)	54 (11.3%)		
Southern regions (s)	287 (15.7%)	149 (13.0%)	10 (2.1%)		

Control group was formed by patients with neither LPV/r nor DRV/c. ^Chi-square test. #Adjusted by inverse probability by treatment weighting as obtained by multinomial propensity score. ‡Missing values were N = 51 for diabetes, N = 51 for hypertension, N = 74 for ischemic heart disease, N = 64 for chronic pulmonary disease, N = 56 for cancer, N = 69 for CKD stage and N = 178 for C reactive protein. ¶Stage 1: Kidney damage with normal or increased glomerular filtration rate (GFR) (> 90mL/min/1.73m^2); Stage 2: Mild reduction in GFR (60–89 mL/min/1.73 m^2); Stage 3a: Moderate reduction in GFR (45–59 mL/min/1.73 m^2); Stage 3b: Moderate reduction in GFR (30–44 mL/min/1.73 m^2); Stage 4: Severe reduction in GFR (15–29 mL/min/1.73 m^2); Stage 5: Kidney failure (GFR < 15mL/min/1.73m^2 ordialysis). GFR was calculated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-Epi) equation. (n) includes hospitals of 5–10; (m) includes hospitals 1–4; (c) includes hospitals 11–17; (r) includes hospitals 18–20; (s) includes hospitals 21–33 (see **Appendix).*

Cox proportional-hazards regression models were used to estimate the association between drugs use and death. Since multiple imputation was applied, the final standard error was obtained using the Rubin's rule (19). The proportional hazards assumption was assessed using weighed Schoenfeld residuals, and no violation was identified. To account for the non-randomized drugs administration, we used the multinomial propensity-score method (20). Individual propensities for receiving LPV/r or DRV/c treatment were assessed with the use of a multivariable logistic-regression model based on the generalized logit and including age, sex, diabetes, hypertension, history of ischemic heart disease, chronic pulmonary disease, GFR, C-reactive protein, use of hydroxychloroquine, tocilizumab or sarilumab, remdesivir or corticosteroids and hospitals clustering. Associations between drug treatments and death were then appraised by multivariable Cox regression models with the use of propensity-score and further controlling for hospitals clustering as random effect [frailty model (21)]. The primary analysis used inverse probability by treatment weighting (22). Secondary analyses used multivariable Cox regression analysis or multivariable logistic regression analyses, or accounted for hospitals clustering via stratification or by robust sandwich estimator. Hospitals were clustered according to their geographical distribution, as illustrated in **Table 1**. To quantify the potential for an unmeasured confounder to render apparent statistically significant hazard ratio non-significant, the E-value was calculated (23). Analyses were performed with the aid of the SAS version 9.4 statistical software for Windows.

RESULTS

We included in the final analyses 3,451 COVID-19 patients; of these, 1,824 (52.9%, range among hospitals 22.5–64.3%) received neither LPV/r nor DRV/c, 1,148 (33.3%, range 17.7–66.3%) received LPV/r and 479 (13.9%, range 2.2–18.1%) received DRV/c. For both drugs, treatment started as soon as possible after diagnosis confirmation and was 7–15 days long. Half of patients were hospitalized before 22 March 2020. In this first period, the prevalence of patients who received or not LPV/r or DRV/c was 38.5% (neither LPV/r nor DRV/c), 42.7% (LPV/r) and 18.8% (DRV/c). In the second period, the use of protease inhibitors clearly decreased (prevalence became 67.3, 23.8, 8.9%, respectively). However, among patients who received protease inhibitors, the percentage of individuals who were allocated to DRV/c unchanged in the two periods (30.6 and 27.4%, in the first and in the second period, respectively).

Baseline characteristics of the 3 groups are shown in **Table 1**. Patients receiving LPV/r or DRV/c were more likely younger, men, had higher C-reactive protein but less likely had hypertension, ischemic heart or chronic pulmonary or severe kidney disease. Patients in the LPV/r or DRV/c group more likely received hydroxychloroquine. As expected, all the pre-treatment differences disappeared after adjustment by propensity score weighting (**Table 1,** c-statistic = 0.72). Percentage of patients who needed of intensive care was 9.5% (in the group with neither LPV/r nor DRV/c), 13.9% (LPV/r) and 10.5% (DRV/c), $P = 0.0010$ for difference.

Primary Outcome

Out of 3,222 patients, 486 died (15.1%), 2,269 were discharged alive (70.4%) and 467 (14.5%) were still at the hospital. The median follow-up was 14 days (interquartile range 8–23). Death rate (per 1,000 person-days) was 8.2, 15.1, and 10.8 in LPV/r, DRV/c and control group, respectively (**Table 2**).

As compared to control group, univariable hazard ratios for death were 0.76 (95% CI: 0.62–0.91) and 1.35 (95% CI: 1.07–1.69) for LPV/r and DRV/c, respectively (**Table 2**). The association with mortality for the LPV/r group disappeared in multivariable analysis (HR = 0.94, 95% CI 0.78–1.13). (**Figure 1**, **Table 2**). On the contrary, the increased risk of death associated with DRV/c was reinforced in primary analysis (HR = 1.89, 95% CI 1.53–2.34, E-value for confidence interval = 2.43) (**Figure 1**, **Table 2**).

Secondary multivariable analyses yielded very similar results (**Table 2**), as also happened for the case-complete analyses restricted to the 3,156 patients without missing data or when the association with death was quantified by logistic regression analysis (**Table 2**).

Control of hospitals clustering with different approaches also yielded similar results (LPV/r group HR = 0.94, 95% CI: 0.78–1.14 and DRV/c group HR = 1.93, 95% CI: 1.55–2.38 when hospitals clustering was stratified for and LPV/r group HR = 0.95, 95% CI: 0.71–1.29 and DRV/c group HR = 1.84, 95% CI: 1.28–2.65 with the robust sandwich estimator). Considering secondary multivariable analyses overall, HR for mortality associated with LPV/r ranged between 0.94 and 1.12, and that associated with DRV/c ranged between 1.44 and 1.93.

Sensitivity analyses are presented in **Table 3**. LPV/r treatment was not associated with mortality in any subgroup, with the exception of patients with less severe COVID-19 (this finding is plagued by very large uncertainty due to small sample size) and in patients not treated with other anti COVID-19 drugs. The increased mortality risk associated with use of DRV/c was more marked in women, in elderly, in patients with higher severity of COVID-19 and in patients treated for other COVID-19 drugs.

DISCUSSION

In a large cohort of 3,451 patients hospitalized for COVID-19 in 33 clinical centers all over Italy, treatment with LPV/r did not modify the risk of death, while administration of DRV/c was associated with an increased risk.

Though taking into consideration the limitations of the observational design of our study, our results do not support the use of LPV/r or DRV/c in patients with COVID-19.

Concerning LPV/r use, our findings are in agreement with findings from clinical trials (7–9, 24) and with results of a systematic review pooling data on 6 clinical trials and 10 observational studies (10). In our study, LPV/r was given according to Italian official guidelines, mostly to less severe patients and as early as possible after hospital admission. We performed a series of sensitivity analyses, all confirming the absence of association between LPV/r and risk of death.

TABLE 2 | Incidence rates and hazard ratios for death in COVID-19 patients, according to lopinavir/ritonavir (LPV/r) or darunavir/cobicistat (DRV/c) use.

Multiple Imputation analysis (*N* = 3,451)	**Death (*N* = 576)**	**Patient at risk (*N* = 3,451)**	**Person-days**	**Death Rate (x1,000 person-days)**
Controls (neither LPV/r nor DRV/c)- no. (%)	319 (17.5%)	1,824 (100%)	29,665	10.8
LPV/r- no. (%)	158 (13.8%)	1,148 (100%)	19,172	8.2
DRV/c- no. (%)	99 (20.7%)	479 (100%)	6,551	15.1
Hazard ratio for death (Cox regression analysis)			**LPV/r vs. controls HR (95% CI)**	**DRV/c vs. controls HR (95% CI)**
Crude analysis			0.76 (0.62 to 0.91)	1.35 (1.07 to 1.69)
Multivariable analysis*			1.12 (0.91 to 1.37)	1.67 (1.31 to 2.14)
Propensity score analysis, inverse probability weighting (primary analysis)**			**0.94 (0.78 to 1.13)**	**1.89 (1.53 to 2.34)**
Odds ratio for death (logistic regression analysis)			**OR (95% CI)**	**OR (95% CI)**
Propensity score analysis, inverse probability weighting**			1.04 (0.85 to 1.27)	1.87 (1.47 to 2.38)
Case Complete analysis (*N* = 3,156)	**Death (*N* = 510)**	**Patient at risk (*N* = 3,156)**	**Person-days**	**Death Rate (x1,000 person-days)**
Controls (neither LPV/r nor DRV/c)-no. (%)	286 (17.3%)	1,657 (100%)	28,380	10.1
LPV/r-no. (%)	146 (13.7%)	1,063 (100%)	18,776	7.8
DRV/c- no. (%)	78 (17.9%)	436 (100%)	6,275	12.4
Hazard ratio for death (Cox regression analysis)			**LPV/r vs. controls HR (95% CI)**	**DRV/c vs. controls HR (95% CI)**
Crude analysis			0.75 (0.61 to 0.92)	1.14 (0.89 to 1.47)
Multivariable analysis*			1.11 (0.89 to 1.37)	1.44 (1.10 to 1.89)
Propensity score analysis, inverse probability weighting**			0.94 (0.77 to 1.13)	1.86 (1.49 to 2.32)
Odds ratio for death (logistic regression analysis)			**OR (95% CI)**	**OR (95% CI)**
Propensity score analysis, inverse probability weighting**			1.05 (0.85 to 1.29)	1.82 (1.41 to 2.34)

*HR, hazard ratios; CI, confidence intervals. *Controlling for age, sex, diabetes, hypertension, history of ischemic heart disease, chronic pulmonary disease, chronic kidney disease, C-reactive protein and use of hydroxychloroquine, tocilizumab or sarilumab, Remdesivir or corticosteroids as fixed effects and hospitals clustering as random effect. **Including hospitals clustering as random effect covariate.*

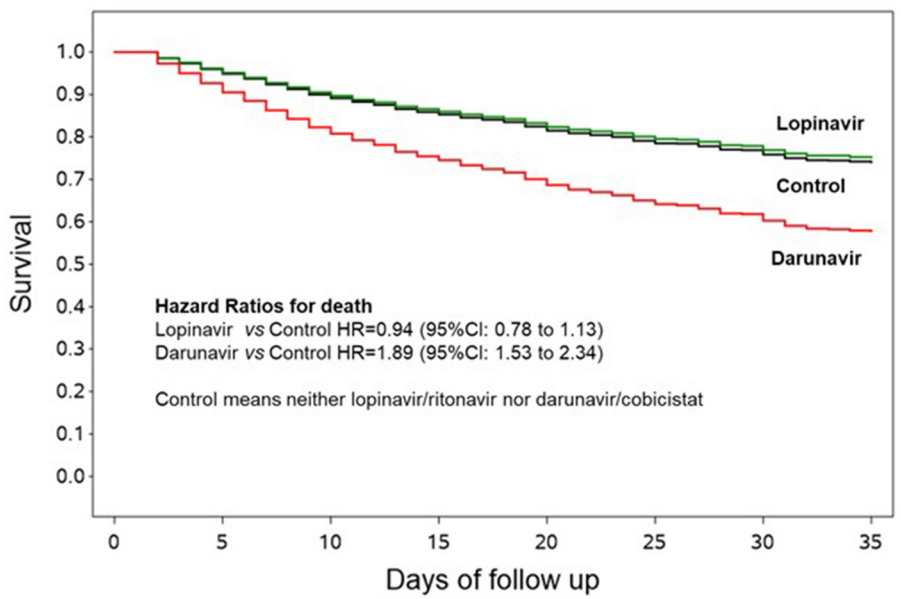

FIGURE 1 | Survival curves according to lopinavir/ritonavir or darunavir/cobicistat use. The curves are adjusted by propensity score analysis (inverse probability by treatment weighting) and hospitals clustering as random effect, and are generated using the first imputed dataset. The other imputed datasets are similar and thus omitted.

TABLE 3 | Hazard ratios for mortality according to lopinavir/ritonavir (LPV/r) or darunavir/cobicistat (DRV/c) use, in different subgroups.

	Controls* (*N* = 817)	LPV/r (*N* = 2,634)	DRV/c (*N* = 450)	HR (95% CI)¶	
Subgroups	**No. death /patient at risk**	**No. death /patient at risk**	**No. death /patient at risk**	**Lopinavir vs. controls**	**Darunavir vs. controls**
Women	124/774	47/386	25/141	0.88 (0.63 to 1.21)	2.41 (1.69 to 3.42)
Men	195/1,050	111/762	74/338	0.96 (0.77 to 1.21)	1.55 (1.18 to 2.03)
Age <70 years	57/922	38/693	20/284	0.86 (0.57 to 1.29)	1.39 (0.84 to 2.33)
Age ≥70 years	262/902	120/455	79/195	1.04 (0.84 to 1.28)	2.20 (1.73 to 2.79)
Highest degree of COVID-19 severity experienced at hospital					
Mild pneumonia or less	53/962	13/616	2/204	0.52 (0.29 to 0.92)	0.30 (0.08 to 1.16)
Severe pneumonia	133/514	72/327	47/176	0.94 (0.72 to 1.24)	1.26 (0.89 to 1.77)
Acute respiratory distress syndrome	133/348	73/205	50/99	1.07 (0.80 to 1.42)	2.09 (1.50 to 2.89)
Use of hydroxychloroquine					
No	149/621	35/170	6/26	0.58 (0.39 to 0.86)	1.50 (0.99 to 2.29)
Yes	170/1,203	123/978	93/453	0.97 (0.77 to 1.22)	2.02 (1.57 to 2.61)
Use of other COVID-19 treatments^					
No	101/439	20/104	3/17	0.50 (0.31 to 0.82)	1.25 (0.74 to 2.12)
Yes	218/1,385	138/1,044	96/462	0.99 (0.80 to 1.22)	1.99 (1.57 to 2.53)

**Control group was formed by patients with neither LPV/r nor DRV/c. HR, hazard ratios; CI, confidence intervals; ¶Propensity score analysis, inverse probability weighting, including hospital clustering as random effect covariate; multiple imputed analysis. ^tocilizumab or sarilumab or remdesivir or corticosteroids.*

In our study DRV/c was associated with a mean 89% increased risk of death, particularly in women, older people, more severely affected or HCQ treated patients, probably due to an increased cardiotoxicity of the drug in these conditions (25).

Although Lopinavir and Darunavir are no longer the gold standard of HIV therapy, their efficacy and safety profile has been well-established in HIV infected patients (11, 26, 27), while there are no clear evidence supporting their use in other viral diseases (28, 29). In fact, the target enzymes involved by HIV and SARS-CoV-2 are quite different: HIV protease is an aspartic protease, whereas SARS-CoV-2 3C-like proteinase is a cysteine protease. Unfortunately, no X-ray crystal structures of 3CLpro complexes including either lopinavir or darunavir are available. Nevertheless, a limited series of computational studies have so far produced contrasting results. In some articles lopinavir was found to have a higher theoretical affinity for SARS-CoV-2 3CLpro than that of darunavir (30, 31). Other articles, instead, describe darunavir as showing large binding free energies to SARS-CoV-2 3CLpro (32–34). These contrasting computational results do not definitely establish whether lopinavir or darunavir is more or less active on the specific SARS-CoV-2 main protease. Nevertheless, when compared to their original indication, both compounds are likely to behave quite differently in the treatment of COVID-19 patients and also to display dissimilar side effects.

Use of DRV/c in COVID-19 patients has been associated with severe drug-drug interactions with concomitant medications that may contribute to death (35). Interestingly, we found an increased relative risk for death associated with DRV/c in older patients (more likely taking other drugs), in patients who experienced at hospital highest degree of COVID-19 severity (more likely taking other drugs) and in patients taking hydroxychloroquine, tocilizumab, sarilumab, remdesivir, or corticosteroids.

Moreover, in spite of the fact that both LPV/r and DRV/c include CYP3A4 inhibitors (ritonavir and cobicistat, respectively) with similar *in vitro* inhibition potencies and subtype selectivities (36), they present remarkable differences in their overall DDI profiles (37); these differences are quite difficult to be placed in a rational correlation with the final clinical outcome, but they should be acknowledged as a possible factor explaining different results in total mortality when comparing LPV/r and DRV/c, as obtained in our study. Furthermore, serious concerns about the possibility that cobicistat, in particular, could produce relevant undesired DDIs were recently raised in analyzing drug combinations for the treatment of HIV infection (38). As already mentioned, lopinavir was found to have a higher theoretical affinity for SARS-CoV-2 3CLpro than darunavir. Therefore, theoretically, lopinavir efficacy might be greater. On the other hand, both drugs have side effects. It is possible that efficacy and side effects balanced for lopinavir (giving a null net effect on mortality) but not for darunavir (giving a net negative effect). Of interest, in an Italian cohort of 689 COVID-19 hospitalized patients followed for negative outcomes (39), it was found that the incidence of in-hospital pulmonary embolism was higher in patients using DRV/c but not LPV/r. On the contrary, other studies did not find an increased rate of severe adverse effects associated with DRV/c (28, 40).

We cannot exclude that patients on DRV/c had a more advanced disease (and then a higher risk of mortality) because DRV/c was used after the run out/shortage of LPV/r. However, we found that among patients who received protease inhibitors (LPV/r or DRV/c), the proportion of individuals who were allocated to DRV/c unchanged during recruitment (from February 2020 to May 2020). This finding suggest that, at least in the CORIST Collaboration, is unlikely that allocation of patients to DRV/c was temporarily biased.

STRENGTHS AND LIMITATIONS

A major strength of this study is the large, unselected, real-life patient sample from 33 hospitals, covering the entire Italian territory. This study has, however, several recognized limitations. First of all, we are well aware of the limits of an observational study. However, the CORIST Collaboration was launched at the very beginning of the pandemic, when the general situation in Italy was dramatic and the organization of a controlled clinical trial was considered to be quite difficult. In the absence of any solid data, a prompt, real-life observational study appeared to be the best option at that moment. We took a number of precautions to account for the non-randomized drugs administration procedure and to reduce the effects of confounders by using a propensity-score method. Due to the critical conditions in which the project was launched and the retrospective nature of the study, some parameters were not available in all patients, and not all in-hospital medications and clinical conditions have been recorded. As a consequence, a fully evaluation of disease severity at entry in hospital has not been possible. This is mainly due to our decision to interfere in a quite soft way with the dramatic clinical situation present in the majority of participating hospitals by proposing a relatively simple protocol, asking to report an essential data set information. Use of LPV/r or DRV/c was a missing data for only 2.8% of the whole cohort. For differing reasons, timing of the first dose of LPV/r or DRV/c after presentation to the hospital and duration of treatment could not be provided at individual level by some clinical centers. Although guidelines on the use of LPV/r and DRV/c in COVID-19 patients had been published in Italy since the first phase of the pandemic, individual centers could have deviated from recommendations and used different doses or treatment schemes. Reason for stopping drug therapies and adverse events possibly related to drug therapy were not collected, thus we cannot exclude bias due to therapy interruption because of side effects.

Finally, the possibility of unmeasured residual confounding cannot be completely ruled-out. However, the E-value for the lower boundary of the confidence interval for the detrimental association of DRV/c with death has the large value equal to 2.43, indicating that the confidence interval could be moved to include the null by a strong unmeasured confounder associated with both DRV/c treatment and death with a risk ratio of 2.43-fold for each, above and beyond all the measured confounders. Weaker confounders, however, could not do so.

CONCLUSION

In conclusion, in a large cohort of patients with COVID-19 we found no association between LPV/r treatment and risk of death but an increased risk of death related to treatment with DRV/c. Although these data are not conclusive, the inappropriate use of this drug combination in the present pandemic entails the risk of shortage of a drug that is currently used as a second-line treatment for people with HIV.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because a consensus by the authors is needed. Requests to access the datasets should be directed to corresponding author.

ETHICS STATEMENT

The study was approved by the institutional ethics board of all recruiting centers ($N = 33$). The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

LI and AD: had full access to all data in the study, took responsibility for the integrity of the data and the accuracy of the data analysis, drafting of the manuscript, and supervision. AD, LI, and RD: concept and design. AD, SC, AG, RA, GV, and GS: statistical analysis. All authors: acquisition, analysis, interpretation of data, critical revision of the manuscript for important intellectual content, administrative, technical, or material support.

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APPENDIX

Clinical Centers

1. Centro Cardiologico Monzino IRCCS, Milano.
2. Humanitas Clinical and Research Hospital IRCCS, Rozzano-Milano.
3. IRCCS Policlinico San Donato, San Donato Milanese (MI).
4. ASST Milano Nord - Ospedale Edoardo Bassini. Cinisello Balsamo (MI).
5. Fondazione IRCCS Policlinico San Matteo, Pavia.
6. Ospedale di Circolo e Fondazione Macchi di Varese. Varese.
7. Ospedale San Gerardo, ASST Monza. Monza.
8. Ospedale di Cremona, Cremona.
9. Ospedale Maggiore della Carità. Novara.
10. Azienda Ospedaliera Universitaria di Padova. Padova.
11. Azienda Ospedaliero - Universitaria di Modena. Modena.
12. Ospedale Morgagni-Pierantoni Forlì.
13. Ospedale di Ravenna. AUSL della Romagna. Ravenna.
14. Azienda ospedaliero-universitaria Careggi. Firenze.
15. Azienda Ospedaliero-Universitaria Pisana. Pisa.
16. Azienda Sanitaria Locale (AUSL) di Pescara, Pescara.
17. Ospedale Clinicizzato SS. Annunziata. Chieti.
18. Istituto nazionale per le malattie infettive Lazzaro Spallanzani, IRCCS. Roma.
19. Fondazione Policlinico Universitario A. Gemelli IRCCS, Roma.
20. Columbus Clinic Center. Roma.
21. Fondazione I.R.C.C.S "Casa Sollievo della Sofferenza", San Giovanni Rotondo, Foggia.
22. IRCCS Neuromed, Pozzilli (IS).
23. Azienda Ospedaliera Universitaria "Federico II". Napoli.
24. Ospedale del Mare, ASL NA1. Napoli.
25. PO S. Maria di Loreto Nuovo -ASL Napoli 1 Centro. Napoli.
26. Azienda Ospedaliera dei Colli, Ospedale Cotugno, Napoli.
27. Ospedale di Boscotrecase - ASL Napoli 3. Napoli.
28. EE Ospedale Regionale F. Miulli, Acquaviva delle Fonti (BA).
29. P.O. San Giuseppe Moscati, Taranto.
30. Azienda Ospedaliero Universitaria Mater Domini. Catanzaro.
31. P.O. "San Marco", AOU Policlinico-Vittorio Emanuele. Catania.
32. Azienda Ospedaliera Universitaria. Policlinico-Vittorio Emanuele. Catania.
33. Azienda Universitaria Policlinico Paolo Giaccone. Palermo

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A Quantitative Estimate of the Expected Shortening of the Median Isolation Period of Patients With COVID-19 After the Adoption of a Symptom-Based Strategy

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A long period of isolation was observed in patients hospitalized for COVID-19 in Milan over March-September 2020 (45; IQR: 37–54 days). A significantly shorter period would have been observed by the application of May-WHO (22, IQR: 17–30 days, $P < 0.001$) and October-Italian (26, IQR: 21–34 days, $P < 0.001$) Guidelines. The adoption of the new symptom-based criteria is likely to lead to a significant reduction in the length of the isolation period with potential social, economic and psychological benefits, particularly in the younger population with mild/moderate disease and no comorbidities. In our opinion, the release from isolation after 21 days from symptoms onset, even without a PCR diagnostic test, in most cases seems the most adequate strategy that could balance precautions to prevent SARS CoV-2 transmission and unnecessary prolonged isolation or overuse of diagnostic testing.

Keywords: COVID-19, SARS CoV-2, molecular diagnosis, isolation and quarantine, criteria for releasing COVID-19 patients from isolation

INTRODUCTION

Accumulating data show that a replication competent SARS CoV-2 virus is rarely found in respiratory samples after 9–10 days from symptoms onset, while rRT-PCR on oro- or naso-pharyngeal swabs may remain persistently positive up to 3 months from the onset of SARS CoV-2 infection (1, 2).

Furthermore, worldwide cases of SARS CoV-2 RNA turning to positive, with or without recurrent symptoms after clinical recovery, are not associated with the isolation of competent virus in culture in most cases (3–5). As a consequence, WHO recommendations to release COVID-19 patients from isolation changed overtime, according to new evidence, as well as other International and Italian Guidelines (6–8) (**Table 1**).

In this analysis, we calculated the median isolation period using real data of COVID-19 patients admitted to two tertiary hospitals in Milan over the period March-September 2020 and we provided an estimate of the shortening of this time under the hypothetical scenario of an isolation period as recommended by the current WHO and October-Italian guidelines (6, 7). We aimed to predict the median isolation period for people with similar characteristics during the second wave of epidemic in Milan and to identify the patients who are likely to most benefit from the reduction of their isolation period.

METHODS

Study Population

In this prospective observational study we included patients fulfilling the following criteria:

- hospitalized for COVID-19 symptomatic infection (from March 1st to September 30th, 2020) at San Paolo and San Carlo hospital, Milan, Italy;m
- discharged from hospital with clinical recovery (apyrexia from ≥72 h and normal respiratory rate in room air);
- two negative rT-PCR for SARS CoV-2 (ELITeInGenius® system and the GeneFinder COVID-19 Plus RealAmp Kitassay; ELITechGroup, France) on naso-pharingeal swabs, according to the February-Italian Guidelines (9).

After hospital discharge, patients were followed-up in an outpatient service to monitor the virological clearance. Naso-pharingeal swabs were repeated every 7 days till two consecutive negative tests. Patients who obtained two RNA negative swabs were given a certificate of virological recovery, attesting end of isolation.

Patients who performed nasopharyngeal swabs to document virological clearance outside our outpatient services and for whom data of end of isolation was unknown were excluded from the analyses.

TABLE 1 | Criteria for releasing COVID-19 patients from isolation.

Old criteria	Updated criteria
WHO, 12 January 2020	**WHO, 27 May 2020**
Clinical recovery and two negative RT-PCR results on sequential samples taken at least 24 hours apart.	10 days after symptom onset and at least 3 additional days without symptoms (including without fever and respiratory symptoms)
Italian Ministry of Health, 28 February 2020	**Italian Ministry of Health, 12 October 2020**
After clinical recovery: Two consecutive negative SARS-CoV-2 RT-PCR tests in a 24-h interval from respiratory specimens.	One negative SARS-CoV-2 RT-PCR test from respiratory specimens after 10 days from symptom onset including at least 3 days without symptoms Persistent RNA positive patients: 21 days from symptom onset (without repeating SARS-CoV-2 RT-PCR test) and at least 7 days without symptoms

We considered the following patients' characteristics: age (<50, 50–69, and ≥70 years), Charlson comorbidity index (CCI) (score 0, 1, 2, and ≥3) (10) and the maximum grade of respiratory support, as proxy of disease severity: no O_2 therapy (mild disease); low/high O_2 flows (moderate disease); Continuous Positive Airway Pressure (cPAP) (severe disease); Non Invasive Ventilation (NIV); and Invasive Mechanical Ventilation (IMV) (critical disease).

Estimation of the Median Time From Symptoms Onset to Release From Isolation Under Three Possible Scenarios

We calculated the median time from symptoms onset to release from isolation under three scenarios: (i) the factual scenario (what has actually happened in March–September 2020); (ii) counterfactual scenario A: if the May-WHO criteria were adopted in March (7); (iii) counterfactual scenario B: if the October-Italian criteria (6) were adopted in March.

Median time to end of isolation under the three scenarios was determined for the whole cohort and compared using non parametric Wilcoxon test for paired data. Mean (±standard deviation, SD) of isolation time was also calculated for specific subgroups (according to age, CCI, and disease severity). We calculated marginal means of estimated time spent in isolation under the two counterfactual scenarios and the average treatment effect with bootsrap 95% Confidence Intervals (CI) using the factual scenario as the comparator.

We classified participants according to whether the isolation time under the WHO scenario was >20 days shorter than the actual time. This threshold was chosen under the assumption that 20 days of shorter isolation was enough to have a significant impact on quality of life and utilization of health resources.

We then calculated marginal probabilities by fitting a logistic regression using age strata (<50, 50–69, and ≥70 years), CCI (score 0, 1, 2, and ≥3) and disease severity (no O_2 therapy; low/high O_2 flows; cPAP; NIV; and IMV) as covariates without interactions and estimated the probability of a shortening of time spent in isolation by more than 20 days according to participants profiles; marginal plots by subgroups were shown.

The same logistic regression model has been used to calculate crude and adjusted odds ratios (OR/AdjOR) of a shortening of more than 20 days with 95% CI for the three variables (age, CCI, and disease severity); AdjOR were corrected for all the three variables included in the model. All analyses were performed using Stata (version 14, StataCorp, USA).

Informed consent from study participants was obtained; the study was approved by Ethic Committee-Area 1, Milan (2020/ST/049-2020/ST/049_BIS, 11/03/2020).

RESULTS

Four hundred and thirty patients were discharged from March 1st to September 30th, 2020 and kept in isolation until virological clearance.

Table 2 shows demographic and clinical characteristics of the study population. Median age was 59 years (IQR: 50–71) and

268 (62.3%) were males. Fifty-four (12.6%) and 109 (25.3%) received IMV/NIV or CPAP as highest grade of respiratory support during hospitalization, respectively. Median days from symptoms onset to clinical recovery were 19 (IQR: 14–27) and median length of hospitalization was 12 (IQR: 7–21) days (**Table 2**).

Median time from symptoms onset to isolation release was 45 (IQR: 37–54) days; median time from clinical recovery to isolation release was 23 (IQR: 19–31) days.

A shorter time would have been observed by the application of the May-WHO (22, IQR: 17–30 days, $P < 0.001$) and the 12 October-Italian criteria (26, IQR: 21–34, $P < 0.001$; **Figure 1A**). The estimate using WHO counterfactual scenario A was significantly shorter also compared to scenario B ($P < 0.001$; **Figure 1A**).

The estimated mean days of isolation in the three scenarios according to age, CCI, and severity of the diseases are shown in **Figure 1B**. A significant reduction of isolation could have been occurred, regardless of patient's characteristics, under both counterfactual scenarios.

Nevertheless, some small differences have been detected; the estimated probability of observing a reduction of time spent in isolation by more than 20 days under the WHO scenario, compared to the actual scenario, was the highest in patients <50 years, without significant comorbidities (CCI = 0) and mild disease severity (low/high O_2 flow; **Figure 2**). Patients aged >70 years old, with CCI ≥ 3 and severe disease were the group with the least estimated benefit (**Figure 2**).

By fitting a univariable logistic regression analysis, a higher probability of shortening the time spent in isolation by more than 20 days under the adoption of May-WHO criteria (counterfactual scenario A) compared to actual scenario were younger age (<50 vs. ≥70 years, OR = 2.04, 95%CI: 1.2–3.47, and $P = 0.009$), no comorbidities (age-unadjusted CCI = 0 vs. ≥3, OR = 2.84, 95%CI: 1.28–6.29, and $P = 0.01$) and mild severity (no O_2 therapy vs. NIV/IMV, OR = 2.73, 95%CI: 1.31–5.7, and $P = 0.008$; low/high flows of O_2 therapy vs. NIV/IMV, OR = 2.80, 95%CI: 1.45–5.44, and $P = 0.002$; **Table 3**).

A medical history without significant comorbidities (age-unadjusted CCI = 0 vs. ≥3, AOR = 2.53, 95%CI: 1.06–6.04, and $P = 0.036$) and lower grades of respiratory support during hospitalization (low/high O_2 flows vs. NIV/IMV, AOR = 3.05, 95%CI: 1.55–6.03, and $P = 0.001$ and no O_2 therapy vs. NIV/IMV, AOR = 2.67, 95%CI: 1.25–5.72, and $P = 0.012$) were confirmed independently associated with a higher probability of reducing time in isolation by at least 20 days in the multivariable analysis (mutually adjusting for age, CCI, and severity of disease; **Table 3**).

TABLE 2 | Characteristics of study population.

Characteristics	Study population (*N* = 430)
Age, years, median (IQR)	59 (50–71)
Age, *n* (%)	
<50 years	104 (24.1%)
50–69 years	203 (47.1%)
≥70 years	123 (28.6%)
Gender, males, *n* (%)	268 (62.3%)
BMI >30, *n* (%)	64 (14.9%)
Italian, *n* (%)	324 (75.3%)
Age-unadjusted Charlson score, median (IQR)	0 (0–1)
Age-unadjusted Charlson score, *n* (%)	
0	283 (65.8%)
1	80 (18.6%)
2	33 (7.68%)
≥3	34 (7.92%)
Symptoms at hospital admission, *n* (%)	
Anosmia/dysgeusia	27 (6.3%)
Arthromyalgia	26 (6.1%)
Chest pain	18 (4.2%)
Cough	265 (61.6%)
Dyspnea	226 (52.6%)
Fatigue	79 (18.4%)
Fever	372 (86.5%)
Gastro-intestinal symptoms	82 (19.1%)
Highest grade of respiratory support during hospitalization, *n* (%)	
IMV or NIV	54 (12.6%)
CPAP	109 (25.3%)
O_2 low/high flows	185 (43.0%)
No O_2 therapy	82 (19.1%)
Blood exams at hospital admission, median (IQR)	
C Reactive Protein, mg/L	45.6 (18.7–88.3)
Lactate dehydrogenase (LDH), U/L	286 (222–364)
Lymphocytes, cells/mmc	1.070 (760–1460)
Hemoglobin, g/dL	13.7 (12.5–14.8)
Creatinine, mg/dL	0.9 (0.7–1.1)
Days from symptoms onset to clinical recovery, median (IQR)	19 (14–27)
Days of hospitalization, median (IQR)	12 (7–21)

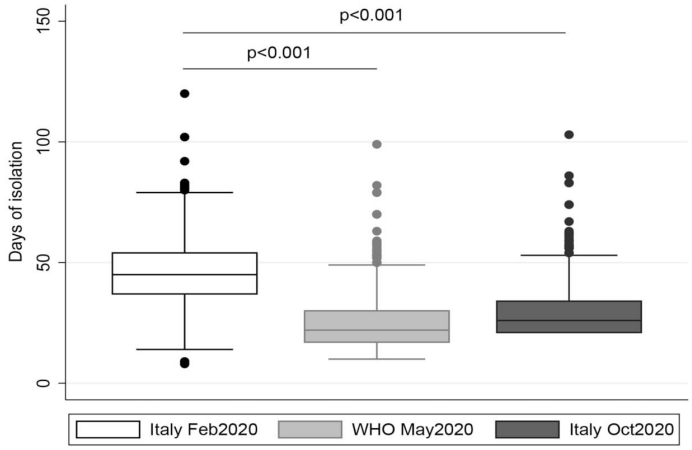
Quantitative data are presented as median (Interquartile Range), categorical data as absolute numbers (percentages). IMV, Invasive mechanical ventilation; NIV, Non Invasive Ventilation; CPAP, Continuous Positive Airway Pressure; Immunomodulating drugs, IL-6 receptors antagonists and JAK inhibitors.

DISCUSSION

With a view to the second wave of COVID-19 epidemic in Milan, our study suggests that the application of the recent and less restrictive Guidelines for releasing COVID-19 patients from isolation will result in a significant reduction of time spent in isolation in our setting.

Under the two counterfactuals scenarios (6, 7), a median of 15–20 days of isolation would be saved compared to the Italian criteria of February 2020. In fact, before 12 October 2020, the virological recovery, corresponding to the end of isolation, was defined only in case of two consecutive SARS-CoV-2 RNA negative swabs, taken 24–48 h apart, after clinical recovery (9); adopting this strategy, isolation period in March-September 2020 resulted extremely long as a substantial proportion of

A



B

	Factual scenario (Italy Feb2020)	Counterfactual scenario A (WHO May2020)			Counterfactual scenario B (Italy Oct2020)		
	median (IQR)	median (IQR)	Δdays, median (IQR)	p-value	median (IQR)	Δdays, median (IQR)	p-value
Overall	45 (37, 54)	22 (17, 30)	20 (16, 18)	<0.001	26 (21, 34)	15 (11, 22)	<0.001
Age strata, years							
<50	43 (34, 51)	18 (13, 23)	22 (17, 30)	<0.001	22 (21, 27)	16 (10, 25)	<0.001
50-69	46 (39, 56)	23 (18, 31)	19 (17, 27)	<0.001	27 (22, 35)	15 (12, 21)	<0.001
≥70	45 (36, 54)	23 (18, 34)	19 (13, 27)	<0.001	27 (22, 38)	14 (8, 20)	<0.001
Age unadjusted CCI							
0	45 (38, 54)	21 (16, 29)	21 (17, 29)	<0.001	25 (21, 33)	16 (12, 24)	<0.001
1	45 (39, 55)	22 (18, 34)	19 (16, 28)	<0.001	26 (22, 38)	15 (12, 21)	<0.001
2	44 (36, 51)	22 (17, 28)	18 (13, 23)	<0.001	26 (21, 32)	14 (7, 19)	<0.001
≥3	36 (28, 57)	24 (15, 36)	13 (-1, 21)	<0.001	28 (21, 40)	7 (-6, 15)	<0.001
Max. grade ventilatory support							
No O2 therapy	41 (30, 52)	14 (10, 19)	22 (16, 31)	<0.001	32 (26, 39)	15 (8, 24)	<0.001
low/high flows of O2	43 (36, 51)	20 (16, 24)	21 (18, 29)	<0.001	39 (32, 52)	16 (13, 24)	<0.001
cPAP	49 (42, 58)	28 (22, 35)	19 (16, 25)	<0.001	21 (21, 23)	15 (12, 20)	<0.001
NIV or IMV	52 (44, 62)	35 (28, 48)	17 (10, 21)	<0.001	24 (21, 28)	13 (6, 17)	0.003

FIGURE 1 | Comparison of time from symptoms onset to release from isolation by application of February-Italian, May-WHO, and October-Italian criteria. **(A)** Box plot representing median days spent in isolation by application of Italian and WHO criteria for releasing COVID-19 patients from isolation. *P*-values by Wilcoxon signed rank test for paired data. **(B)** Mean (±standard deviation) of the isolation time under the three scenarios (February-Italian, May-WHO, and October-Italian guidelines) according to age, CCI, and maximum grade of respiratory support. Average treatment effects (95%CI) for WHO May 2020 and Italian October 2020 criteria, using the factual scenario (Italian February 2020) as the comparator, were shown.

patients was persistently positive and went on repeating the nasopharyngeal swabs each week until reaching the virological clearance, in some cases months later.

Fear of transmission and unknown contagiousness period were the main determinants of these early recommendations resulting in prolonged isolation.

The consequences of retained isolation are both social and psychological (11). Considering that 52% of our patients is aged 60 or younger, the impact of prolonged isolation on their ability to keep their job can be dramatic.

Further, consistently with other studies (12, 13) we previously demonstrated that 30% of patients recovered from COVID-19 showed anxiety symptoms and had abnormal scores in the Hospital Anxiety and Depression Scale (HADS) 1–3 months after recovery (14).

Assuming that clinical characteristics of hospitalized COVID-19 patients in the first and second wave of the epidemic were comparable, the median isolation period for those patients can be estimated between 22 (17–30) and 26 (11–22) days. The lower estimate is calculated using the WHO counterfactual scenario A, which corresponds exactly to the best-case scenario of the new Italian guideline of October 2020 (negative SARS CoV-2 swab 10 days after symptoms onsets, 3 of which without symptoms). The highest estimate corresponds to the counterfactual scenario B, the worst-case scenario according to new Italian guidelines, for patients with persistent long-term SARS CoV-2 RNA positivity. This would amount to a shortening of the isolation period by a significant 15–20 days.

By adopting the new recommendations for releasing patients from isolation, the isolation period would shorten especially for patients without comorbidities and diagnosed with a not severe disease; in the first wave of epidemic in Milan also young subjects with mild disease who obtained early clinical recovery remained in home isolation for a long period pending virological clearance.

Other advantages of the shortening of the isolation period in hospital is the reduced burden on national health resources and the greater availability of extra space for people with acute disease who need urgent care.

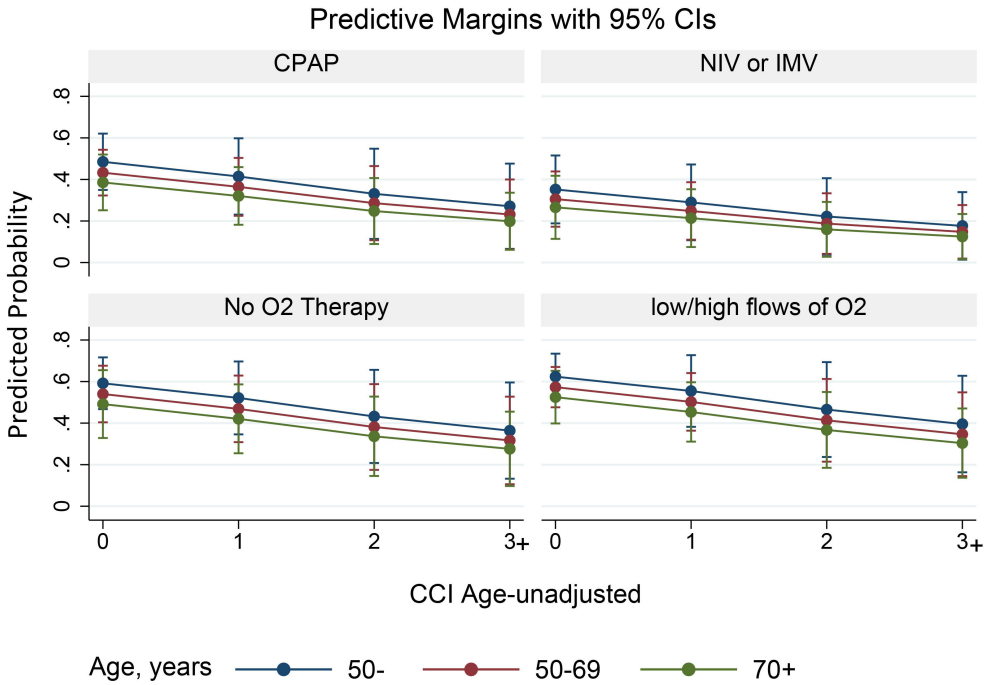


FIGURE 2 | Plots of the marginal predictions of shortening the time spent in isolation by more than 20 days. Predictive margins with 95% CI by subgroups (age classes, CCI, and O_2 therapy) are represented.

TABLE 3 | Factors associated with the reduction of time spent in isolation by more than 20 days under 27-may WHO criteria.

Parameters	*N* (%)	OR	95%CI	*p*-value	AOR*	95%CI	*p*-value
Age strata, years							
≥70	(*N* = 123, 28.6%)	1			1		
50–69	(*N* = 203, 47.1%)	1.31	0.83–2.07	0.242	1.21	0.73–2.01	0.452
<50	(*N* = 104, 24.1%)	2.04	1.20–3.47	0.009	1.50	0.81–2.77	0.196
Age unadjusted Charlson comorbidity index							
≥3	(*N* = 34, 7.92%)	1			1		
2	(*N* = 33, 7.68%)	1.39	0.49–3.97	0.54	1.33	0.46–3.88	0.601
1	(*N* = 80, 18.6%)	1.95	0.81–4.71	0.138	1.90	0.76–4.76	0.169
0	(*N* = 283, 65.8%)	2.84	1.28–6.29	0.01	2.53	1.06–6.04	0.036
Max grade of respiratory support							
NIV or IMV	(*N* = 54, 12.6%)	1			1		
cPAP	(*N* = 109, 25.3%)	1.69	0.83–3.44	0.145	1.74	0.84–3.57	0.134
Low/high flows of O_2 therapy	(*N* = 185, 43.0%)	2.80	1.45–5.44	0.002	3.05	1.55–6.03	0.001
No O_2 therapy	(*N* = 82, 19.1%)	2.73	1.31–5.70	0.008	2.67	1.25–5.72	0.012

**Adjusted for all the factors shown in the table.*

Univariable and multivariable logistic regression analysis. OR, odds ratio; AOR, adjusted odds ratio; 95%CI, 95% confidence interval; IMV, Invasive mechanical ventilation; NIV, Non Invasive Ventilation; cPAP, Continuous Positive Airway Pressure.

Considering the growing evidence that, after 10 days following symptoms onset, rRT-PCR on upper respiratory samples could remain positive, but no replication-competent virus is recovered in viral cultures (1, 2, 15, 16), a test-based strategy appears to be inadequate at the current time.

However, a minimal residual risk of transmission exists when adopting the new criteria, as in few cases of severe COVID-19 disease and immunosuppression, competent virus, and thus contagiousness, has been reported till 20 days from symptoms onset (17). Furthermore, there might be situations in which

this minimal risk is not acceptable (e.g., if a PCR-positive patient needs to be transferred into COVID-negative department together with immunocompromised/vulnerable patients). In these situations a laboratory-based approach can still be useful.

Finally, worldwide cases of recurrent symptoms after clinical recovery are scarce and in most cases are not associated with the isolation of competent virus in culture, but with a persistent positive RNA (3–5).

Possible limitations of our study are: (i) the lack of actual data on other cohorts that adopted WHO criteria for releasing patients from isolation for comparisons with our study population; in fact, we simulated the isolation time we would have had on our cohort of patients by adopting WHO and October 2020 Italian criteria. However, this approach has the advantage of better control for confounding in the logistic regression analysis as characteristics of patients in different pandemic waves can be dramatically different and this could bias the comparison; (ii) limited generalizability of the results to patients actually enrolled in subsequent waves as these might differ for key effect measure modifiers; (iii) missing data about health and financial outcomes associated with the reduction of time spent in home isolation; (iv) our data are related to a particular time-period of the COVID-19 pandemic and it might need to be adjusted as new variants of concern might arise in the future.

In conclusion, the use of a test-based strategy during the first wave of the pandemic in all COVID-19 patients, including young and mildly ill patients, led to long periods of hospital and home isolation with consequent economic and psychological damage. More and more data report the absence of contagiousness after 10 days following onset of symptoms, making symptoms-based criteria the most appropriate strategy currently. In our opinion a symptom-based strategy will lead to significant benefits in terms of quality of life and optimization of resources with little consequences in terms of risk of transmission, which should be however monitored.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethic Committee-Area 1, Milan (2020/ST/049-2020/ST/049_BIS, 11/03/2020). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Ad'A, AC-L, FB, and AT developed the concept of this study. FB, AT, GM, CF, MAu, DB, DM, DTe, MAl, GT, and DTo collected data on Case Report Form and performed data entry. AT did the statistical analyses. FB wrote the manuscript. FB, AT, AC-L, GMa, and Ad'A contributed to the final text. All the authors revised the text critically and have read and approved the final text.

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frontiers in Medicine

SYSTEMATIC REVIEW
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Quality of and Recommendations for Relevant Clinical Practice Guidelines for COVID-19 Management: A Systematic Review and Critical Appraisal

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Background: The morbidity and mortality of coronavirus disease 2019 (COVID-19) are still increasing. This study aimed to assess the quality of relevant COVID-19 clinical practice guidelines (CPGs) and to compare the similarities and differences between recommendations.

Methods: A comprehensive search was conducted using electronic databases (PubMed, Embase, and Web of Science) and representative guidelines repositories from December 1, 2019, to August 11, 2020 (updated to April 5, 2021), to obtain eligible CPGs. The Appraisal of Guidelines for Research and Evaluation (AGREE II) tool was used to evaluate the quality of CPGs. Four authors extracted relevant information and completed data extraction forms. All data were analyzed using R version 3.6.0 software.

Results: In total, 39 CPGs were identified and the quality was not encouragingly high. The median score (interquartile range, IQR) of every domain from AGREE II for evidence-based CPGs (EB-CPGs) versus (vs.) consensus-based CPG (CB-CPGs) was 81.94% (75.00–84.72) vs. 58.33% (52.78–68.06) in scope and purpose, 59.72% (38.89–75.00) vs. 36.11% (33.33–36.11) in stakeholder involvement, 64.58% (32.29–71.88) vs. 22.92% (16.67–26.56) in rigor of development, 75.00% (52.78–86.81) vs. 52.78% (50.00–63.89) in clarity of presentation, 40.63% (22.40–62.50) vs. 20.83% (13.54–25.00) in applicability, and 58.33% (50.00–100.00) vs. 50.00% (50.00–77.08) in editorial independence, respectively. The methodological quality of EB-CPGs were significantly superior to the CB-CPGs in the majority of domains ($P < 0.05$). There was no agreement on diagnosis criteria of COVID-19. But a few guidelines show Remdesivir may be beneficial for the patients, hydroxychloroquine +/– azithromycin may not, and there were more consistent suggestions regarding discharge management. For instance, after discharge, isolation management and health status monitoring may be continued.

Conclusions: In general, the methodological quality of EB-CPGs is greater than CB-CPGs. However, it is still required to be further improved. Besides, the consistency of COVID-19 recommendations on topics such as diagnosis criteria is different. Of them, hydroxychloroquine +/– azithromycin may be not beneficial to treat patients with COVID-19, but remdesivir may be a favorable risk-benefit in severe COVID-19 infection; isolation management and health status monitoring after discharge may be still necessary. Chemoprophylaxis, including SARS-CoV 2 vaccines and antiviral drugs of COVID-19, still require more trials to confirm this.

Keywords: COVID-19, SARS-CoV-2, guideline, AGREE II, prophylaxis, diagnosis, treatments, discharge management

INTRODUCTION

The morbidity and mortality associated with coronavirus disease 2019 (COVID-19) are still increasing at present. According to the official website of World Health Organization (WHO), by 10 April 2021, there have been 134,308,070 confirmed cases of COVID-19, including 2,907,944 deaths worldwide (1). Containing the spread poses a challenge because of the rising number of infected people with high mortality and the highly contagious nature of COVID-19. Clinical practice guidelines (CPGs) have been defined as "statements that include recommendations intended to optimize patient care that are informed by a systematic review of evidence and a risk-benefit assessment of alternative care options" (2), and they play an important role in guiding clinical decisions about prevention, diagnosis, treatment and care. Some professional association, guideline development groups have issued successively COVID-19 management guidelines.

Previous reviews have also concentrated on methodological quality and recommendations for COVID-19 guidelines, but these have covered a narrow range of topics (3–6). The methods and reporting quality of practice guidelines for five different viruses causing public health emergencies of international concern, including the severe acute respiratory syndrome coronavirus 2, tended to be low, particularly in stakeholder involvement and applicability. There was also poor quality of recommendations for the use of antiviral drugs such as lopinavir-ritonavir, convalescent plasma, and intravenous immunoglobulins. Reverse transcription-polymerase chain reaction (RT-PCR) and Computed tomography (CT) were the most common diagnostic methods for COVID-19. Besides, there was no effective treatment against COVID-19; supportive therapy (mainly rest in bed, ensuring adequate calories, maintaining water-electrolyte balance, oxygen therapy, etc.) is the most significant treatment plan. Live evidence related to COVID-19 is still appearing on a daily basis, and live recommendations on chemoprophylaxis, diagnosis, and antiviral therapy are also being continuously updated. As for discharged patients, a small proportion of patients experienced reappearance of a positive test for SARS-CoV-2 during convalescence (7–9). As the number of cured patients increases, criteria for discharge management is also an important issue.

Thus, this review, based on a comprehensive literature search, has been conducted to compare the variations in recommendations within prophylaxis, diagnosis, antiviral treatment, and discharge management of COVID-19 and to assess their methodological quality. We aim to provide relatively more reliable suggestions for decision-making bodies regarding possible health problems to satisfy the needs of the public, providing guidance for government departments and COVID-19 prevention and control institutions.

METHODS

The review was performed according to preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines (10).

Search Strategy

We searched PubMed, Embase, and Web of Science. Additionally, eight representative guideline repositories were searched: World Health Organization (WHO), National Institute for Health and Care Excellence (NICE), Guidelines International Network (GIN), National Institutes of Health (NIH), Scottish Intercollegiate Guidelines Network (SIGN), Association of American Medical Colleges (AAMC), ECRI Guideline Trust, and Biochemical Genetic and Genomic (BIGG). A list of the websites with COVID-19 guidelines is presented in **Supplementary Table 1**. The search dates were from December 1, 2019, to August 11, 2020 (updated to April 5, 2021). The key words mainly included "severe acute respiratory syndrome coronavirus 2 or SARS-COV-2 or COVID-19 or COVID19 or 2019 coronavirus or 2019 novel coronavirus or 2019-nCoV or Novel coronavirus pneumonia or NCP or coronavirus disease-19 or coronavirus disease 2019" AND "guideline or guidance or recommendation or clinical practice guideline or consensus." MeSH terms were used to search Title/Abstract. Furthermore, taking PubMed as an example, the retrieval strategy is shown in **Supplementary Figure 1**.

Guidelines Identification

All guidelines related to COVID-19 published in English were included if they met the following criteria: (1) explicit recommendations on COVID-19 management (Which kind of

agent can prevent COVID-19? Which strategy can be used to diagnose COVID-19 and identify and risk stratify patients with suspected or confirmed COVID-19? Which drugs can be used to treat patients with COVID-19? What are the discharge criteria for COVID-19, and what indicators are there for follow-up attention after discharge?); (2) evidence-based clinical practice guidelines (EB-CPGs) or consensus-based guidelines (CB-CPGs); and (3) updated versions of CPGs if multiple versions of the guidelines exists. To determine the eligible guidelines, EB-CPGs were defined as having recommendations based on a systematic literature search and literature quality assessment or grade for evidence and recommendation; CB-CPGs were defined as having recommendations developed by multidisciplinary experts (such as frontline clinicians) based on their experience or the existing literature using a consensus method rather than a systematic review.

We excluded (1) translated versions, interpretations, and summaries of existing CPGs; (2) regional or hospital protocols for COVID-19; and (3) CPGs without full text access.

Data Extraction

Four reviewers independently extracted the details of the guidelines relevant to their characteristics using a standardized data collection form. Extracted data included guideline title, date of publication, publication country/region, guideline developers, target population, development method (evidence-based or consensus-based), topic, funding, and the related recommendations. Another reviewer examined the data extraction forms to make sure no errors had occurred. Disagreements were resolved by consensus.

Methodological Quality Appraisal

Two reviewers independently evaluated the quality of each included guideline using the widely accepted CPG assessment tool—AGREE II, which is composed of 23 items within 6 domains including "scope and purpose," "stakeholder involvement," "rigor of development," "clarity of presentation," "applicability," and "editorial independence" (11, 12). Details of each domain are shown in **Supplementary Table 2**. Each item was scored from 1 (strongly disagree) to 7 (strongly agree). We calculated each domain score for every eligible CPG individually using the following formula provided by the AGREE II tool: (obtained score–minimal possible score)/(maximal possible score–minimal possible score) × 100% (11).

Guideline Recommendations Synthesis

We performed a textual descriptive synthesis to analyze eligible CPGs from four aspects: chemoprophylaxis; diagnosis; antiviral therapy; and discharge management.

Statistical Analysis

Descriptive statistical analyses were performed. Data for each AGREE II domain of every included CPG were presented as medians and interquartile ranges (IQRs). The scores of EB-CPGs and CB-CPGs in each domain were compared using Wilcoxon Rank-Sum Test. A $P < 0.05$ was regarded as significance. Intraclass correlation coefficients (ICCs) with a 95% confidence interval (CI) were calculated to evaluate the agreement among two assessors for each domain. The degree of agreement between 0.00 and 0.40 was considered poor, 0.41 to 0.75 was good, and 0.75 to 1.00 was excellent (13). All the data were analyzed using R version 3.6.0 software (The R Foundation for Statistical Computing, Vienna, Austria) for Windows.

RESULTS

Guidelines Identification and Selection

Figure 1 presents the flow chart of guidelines identification, and 39 CPGs were eventually included (14–52).

Characteristics of Included Guidelines

As **Supplementary Table 3** shows, the guidelines were published from February 6, 2020, to April 5, 2021. Of them, 15 guidelines were CB-CPGs and 24 were EB-CPGs, and 15 received funding support. Among the recommendations in these 39 CPGs, 8 were on chemoprophylaxis, 18 on diagnosis, 1 on identification and triage of patients with COVID-19, 25 on antiviral drugs, and 6 on discharge. The guidelines were mainly developed by the United States, China, or other international organization or cooperation (See **Figure 2**).

Guidelines' Quality

The ICC values for all six domains of AGREE II were over 0.75, indicating a high consistency on the scores between the two assessors. As shown in **Supplementary Table 4**, **Table 1**, **Figure 3**, the final domain score of every guideline across all domains ranged from 0% (Domain 6 of editorial independence in 1 guidelines) (51) to 100% (Domain 6 in 11 guidelines) (16, 19, 25, 29, 31, 32, 34, 44, 46–48). Regarding the score of each domain across all guidelines, for EB-CPGs, the score of Domain 5 (applicability) was the lowest with a median score of 40.63% (IQR 22.40–62.50), the median scores of Domains 1, 2, 3, 4, 6 (scope and purpose, stakeholder involvement, rigor of development, clarity of presentation, editorial independence) were 81.94% (IQR 75.00–84.72), 59.72% (IQR 38.89–75.00), 64.58% (IQR 32.29–71.88), 75.00% (IQR 52.78–86.81), and 58.33% (IQR 50.00–100.00), respectively. For CB-CPGs, Domain 1 scored highest with a median score of 58.33% (IQR 52.78–68.06), Domain 5 scored lowest with median scores of 20.83% (13.54–25.00), and the median scores of Domains 2, 3, 4, 6 were 36.11% (33.33–36.11), 22.92% (16.67–26.56), 52.78% (50.00–63.89), and 50.00% (50.00–77.08), respectively. In addition, EB-CPGs were significantly superior to the CB-CPGs in the domain 1, 2, 3, 4, 5($P < 0.05$).

Synthesis of Recommendations

Five EB-CPGs (15, 19, 20, 24, 34) and three CB-CPGs (17, 27, 41) focused on the chemoprophylaxis of COVID-19. Two EB-CPGs of them recommended not to use hydroxychloroquine for COVID-19 pre-exposure prophylaxis or post-exposure prophylaxis outside the setting of a clinical trial (15, 19); two EB-CPG (20, 24) and two CB-CPGs (17, 27) recommended SARS-CoV vaccine for COVID-19 prevention; one CB-CPG (41) suggested that a few traditional Chinese medicine

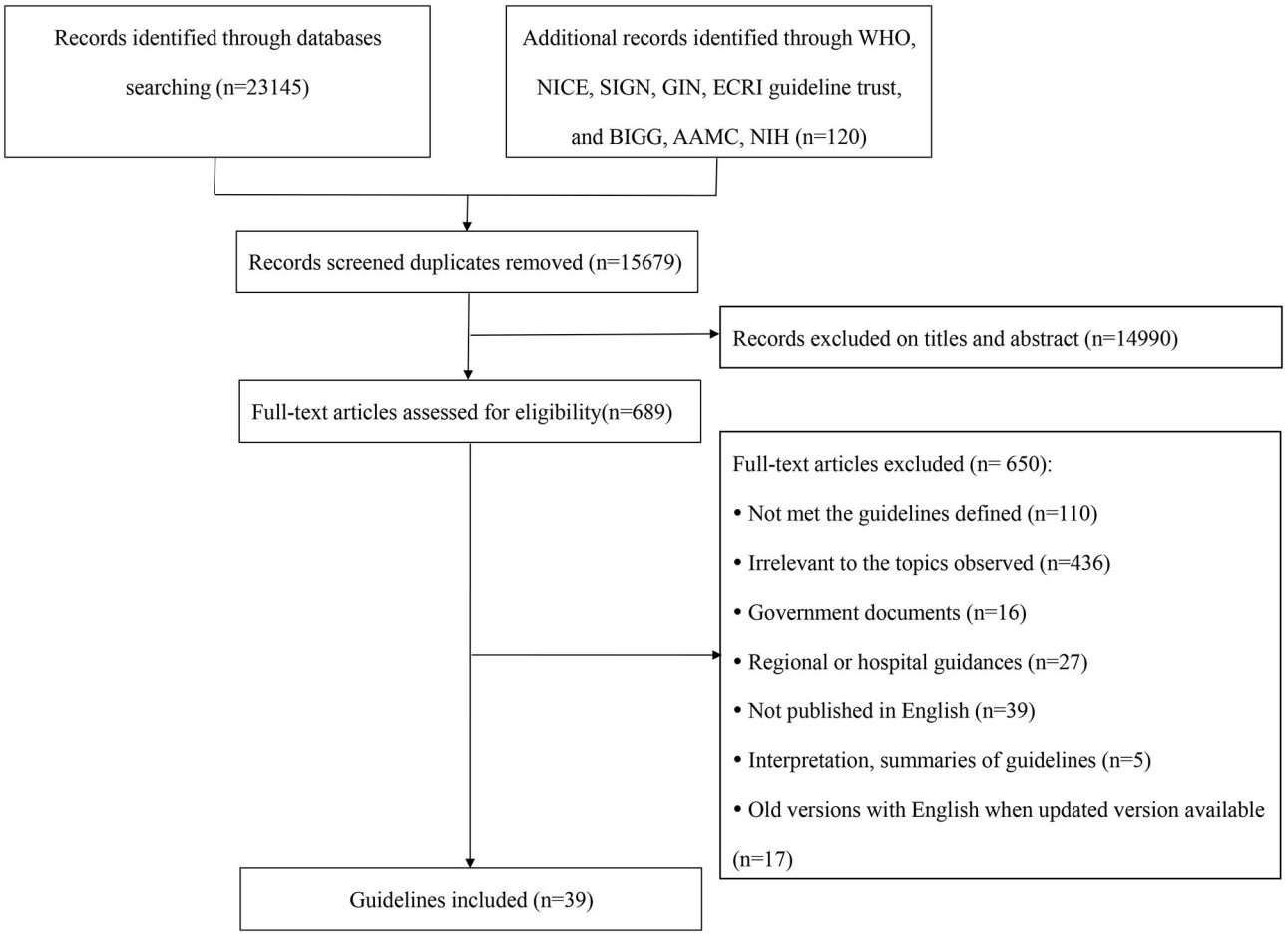


FIGURE 1 | Flow chart of guidelines identification and selection.

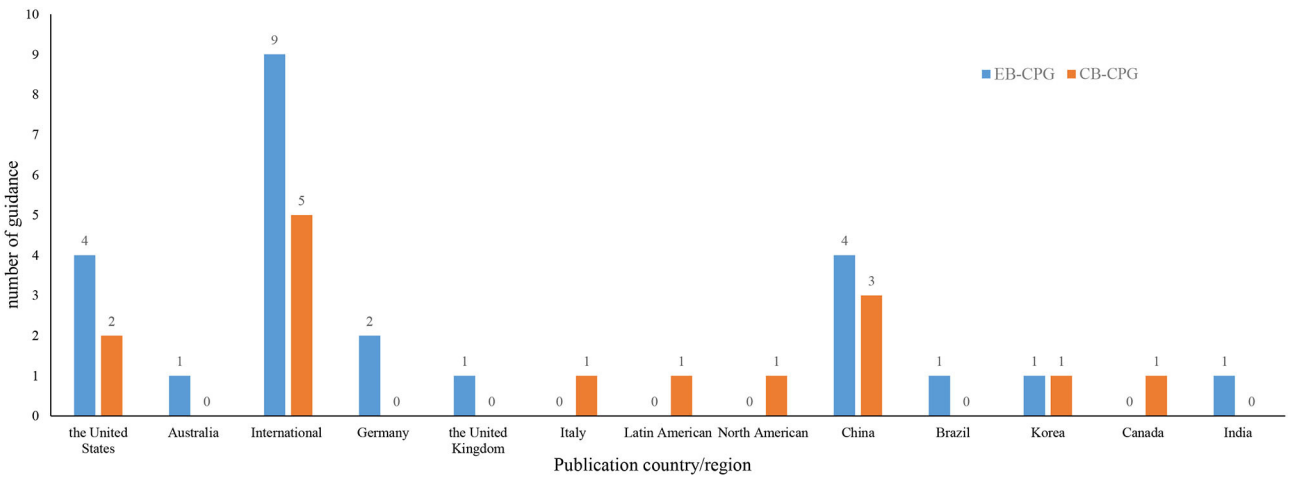


FIGURE 2 | Distribution of publication country/region in the guidelines included.

TABLE 1 | The difference of quality between EB-CPGs and CB-CPGs.

Domains	Scope and purpose	Stakeholder involvement	Rigor of development	Clarity of presentation	Applicability	Editorial independence
Z	−3.493	−3.744	−4.102	−2.828	−2.905	−0.714
P	0.000	0.000	0.000	0.005	0.004	0.475

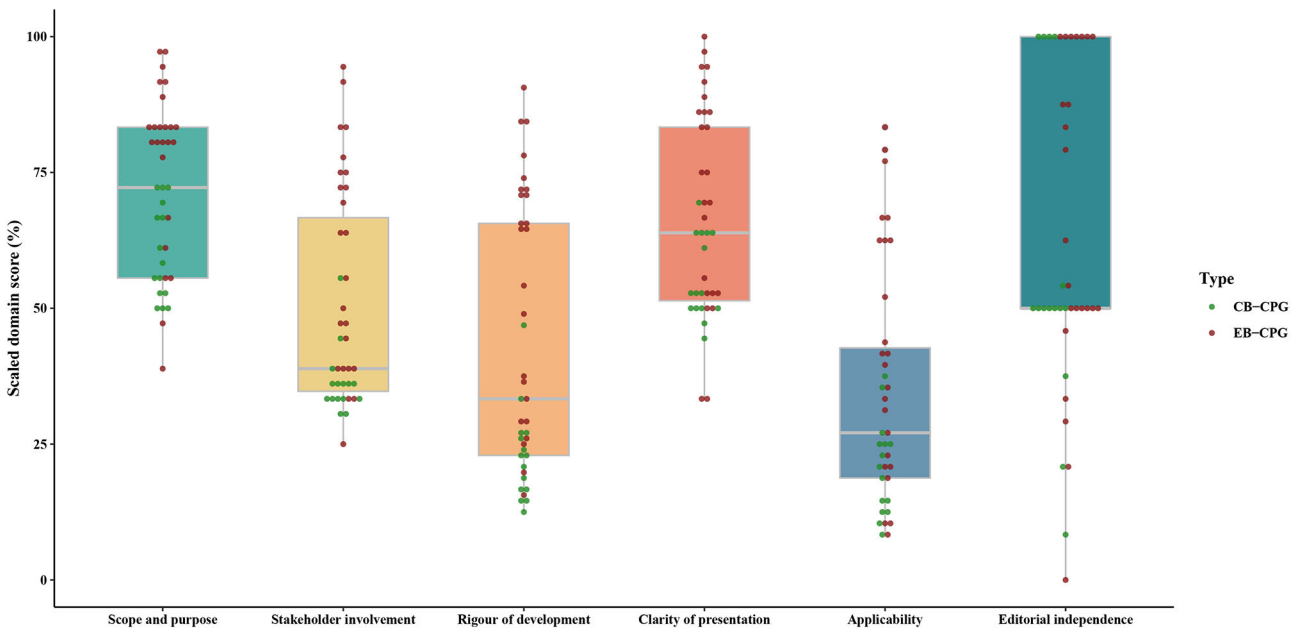


FIGURE 3 | The summary of scores for each domain over all included guidelines.

may be beneficial for COVID-19 prevention, for example, Youngyopaedoc-san plus Bojungikgitang, Youngyopaedoc-san plus Saengmaek-san (See **Table 2**).

In total, 11 EB-CPGs (14, 20, 21, 24, 26, 30, 34, 36, 42, 43, 51) and 7 CB-CPGs (32, 33, 44, 46, 48, 49, 52) reported the diagnostic criteria for COVID-19 (See **Table 3**). The diagnosis of SARS-CoV-2 infection was mainly based on RT-PCR test, serum-specific antibodies IgM and IgG test, epidemiological history, and clinical manifestations in one EB-CPG (34) and one CB-CPG (46). However, nine EB-CPGs (14, 20, 21, 24, 26, 30, 36, 43, 51) and six CB-CPGs (32, 33, 44, 48, 49, 52) only focus on one or two of the above criteria. Three CPGs (two EB-CPGs, one CB-CPG) (20, 26, 32) did not suggest SARS-CoV-2 antibody tests for diagnosis of current infection with COVID-19 or as the sole basis or to routinely to diagnose active COVID-19 in symptomatic pregnant women with negative RT-PCR. Two CPGs (one EB-CPG, one CB-CPG) (32, 42) did not recommend that CT scan were used routinely in the diagnosis of COVID-19 in children or symptomatic pregnant women. In addition, one EB-CPG (31) provided some suggestions on how to predict whether a patient is COVID-19 positive, validated triage and severity of illness, risk stratify patients with suspected or confirmed COVID-19 in low- and middle-income countries.

In total, 18 EB-CPGs (14–16, 18, 20, 21, 24–26, 34, 37, 39, 40, 42, 45, 47, 50, 51) and 7 CB-CPGs (22, 23, 28, 32, 38, 41, 46) provided suggestions on antivirals treatment for COVID-19. As shown in **Table 4**, there were no consistent views on effective and validated antiviral drugs such as hydroxychloroquine/chloroquine plus azithromycin, lopinavir/ritonavir, convalescent plasma for the treatment in clinical scenarios. The majority of guidelines agreed that some antiviral drugs such as Remdesivir can be used in the context of clinical trials or under special conditions such as severe and critical patients. Two EB-CPGs (34, 39) and one CB-CPG (41) provided a traditional Chinese medicine treatment plan for COVID-19.

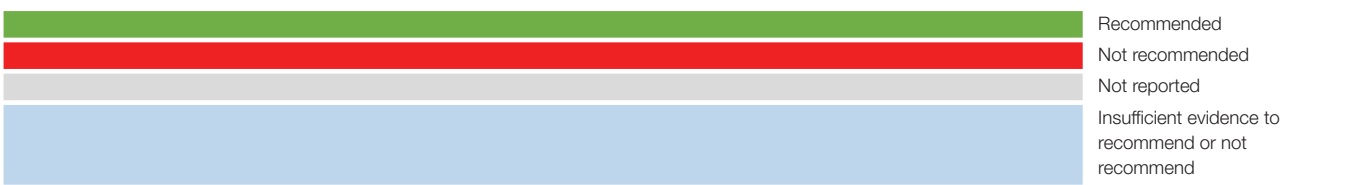
As presented in **Tables 5A,B**, four EB-CPGs (29, 34, 36, 51) and two CB-CPGs (35, 52) concentrated on the discharge management of COVID-19. The criteria were mainly based on temperature returning to normal more than 3 days, improvement in respiratory symptoms and negative results from two successive nucleic acid test of respiratory samples (with a sampling interval of at least 1 day). Besides, three EB-CPGs (29, 34, 51) and one CB-CPG (35) described the relevant precautions after discharge. For example, isolation management should be continued, and the patients should wear a mask if necessary.

DISCUSSION

EB-CPGs and CB-CPGs play an important role in this pandemic, which is constantly being updated. The first EB-CPGs were published on Feb 6, 2020 (53); the first protocol of the updated

TABLE 2 | Recommendations on chemoprophylaxis of COVID-19.

Guidelines title	Drugs	Pre-exposure prophylaxis	Post-exposure prophylaxis
EB-CPG			
Australian guidelines for the clinical care of people with COVID-19 (15)	Hydroxychloroquine	*	**
WHO living guideline: drugs to prevent COVID-19 (19)	Hydroxychloroquine	***	***
Coronavirus disease 2019 (COVID-19) treatment guidelines (20)	Any drugs	****	*****
	Vaccine	***	***
2021 update of the AGIHO guideline on evidence-based management of COVID-19 in patients with cancer regarding diagnostics, viral shedding, vaccination and therapy (24)	Vaccine		
Chemoprophylaxis, diagnosis, treatments, and discharge management of COVID-19: an evidence-based clinical practice guideline (updated version) (34)	Any drugs		
CB-CPG			
American College of Rheumatology guidance for COVID-19 vaccination in patients with rheumatic and musculoskeletal diseases–Version 1 (17)	Vaccine	***	***
SARS-CoV-2 vaccination for patients with inflammatory bowel diseases: recommendations from an international consensus meeting (27)	Vaccine	******	
A consensus guideline of herbal medicine for coronavirus disease 2019 (41)	Youngyopaedoc-san + Bojungikgitang (Lianqiao baidu san + Buzhong Yiqi Tang)		
	Youngyopaedoc-san + Saengmaek-san (Lianqiao baidu san + Shengmai Yin)		
	Youngyopaedoc-san + Bulhwangeumjeonggi-san (Lianqiao baidu san + Buhuanjin Zhengqi San)		
	Youngyopaedoc-san + Bojungikgi-tang (Lianqiao baidu san + Buzhong Yiqi Tang)		



**For healthcare workers with no active COVID-19, do not use hydroxychloroquine for pre-exposure prophylaxis outside of randomized trials with appropriate ethical approval; **For people exposed to individuals with severe acute respiratory syndrome coronavirus 2 infection, do not use hydroxychloroquine for post-exposure prophylaxis outside of randomized trials with appropriate ethical approval; ***No specific indication of pre-exposure or post-exposure prophylaxis; ****Recommending against the use of any drugs for severe acute respiratory syndrome coronavirus 2 pre-exposure prophylaxis, except in a clinical trial; *****Recommending against the use of hydroxychloroquine for SARS-CoV-2 post-exposure prophylaxis,against the use of other drugs for SARS-CoV-2 post-exposure prophylaxis, except in a clinical trial; ******Patients with inflammatory bowel diseases who are receiving immune-modifying therapies should not receive live virus vaccines.*

EB-CPGs, Evidence-based clinical practice guidelines; CB-CPGs, Consensus-based guidelines.

EB-CPG was released on March 7, 2020 (54). Finally, 39 CPGs were included in this review. The methodological quality of EB-CPGs is better than CB-CPGs because the median score with IQR is statistically significantly higher in EB-CPGs for domains of the AGREE II assessment tool in general. However, they all still need to be further improved, especially in the areas of gathering and synthesizing reliable the latest up-to-date information, involving the target population in guideline development and improving the implementability of the recommendations. Recommendations relevant to chemoprophylaxis, diagnosis, antiviral drugs, and discharge management of COVID-19 showed small differences.

COVID-19 is a newly identified infectious disease, which poses a significant threat to both the general population and

TABLE 3 | Recommendations on diagnosis criteria of COVID-19.

Guidelines title	Etiological criteria	Serological criteria	Epidemiological history and clinical manifestations	CXR or chest CT
EB-CPG				
IDSA guidelines on the treatment and management of patients with COVID-19 (14)		*		
Coronavirus disease 2019 (COVID-19) treatment guidelines (20)		**		
Surviving Sepsis Campaign guidelines on the management of adults with coronavirus disease 2019 (COVID-19) in the ICU: first update (21)				
2021 update of the AGIHO guideline on evidence-based management of COVID-19 in patients with cancer regarding diagnostics, viral shedding, vaccination, and therapy (24)				
Clinical management of COVID-19 patients: living guidance (26)		***		
Clinical practice guideline: recommendations on inpatient treatment of patients with COVID-19 (30)				
Chemoprophylaxis, diagnosis, treatments, and discharge management of COVID-19: an evidence-based clinical practice guideline (updated version) (34)				
Use of chest imaging in the diagnosis and management of COVID-19: a WHO rapid advice guide (36)				****
Rapid advice guidelines for management of children with COVID-19 (42)				*****
Expert consensus for managing pregnant women and neonates born to mothers with suspected or confirmed novel coronavirus (COVID-19) infection (43)				******
Perinatal-neonatal management of COVID-19 infection (51)				
CB-CPG				
Clinical management of coronavirus disease 2019 (COVID-19) in pregnancy: recommendations of WAPM-World association of perinatal medicine (32)		*******		********
Algorithms for testing COVID-19 focused on use of RT-PCR and high-affinity serological testing: a consensus statement from apanel of Latin American experts (33)				
Canadian society of thoracic radiology/Canadian association of radiologists consensus statement regarding chest imaging in suspected and confirmed COVID-19 (44)				
Updated diagnosis, treatment and prevention of COVID-19 in children: experts' consensus statement (condensed version of the second edition) (46)				
Imaging of coronavirus disease 2019: a Chinese expert consensus statement (48)				
The role of chest imaging in patient management during the COVID-19 pandemic (49)				
Chinese expert consensus on the perinatal and neonatal management for the prevention and control of the 2019 novel coronavirus infection (first edition) (52)				

**When SARS-CoV-2 infection requires laboratory confirmation for clinical or epidemiological purposes, testing for SARS-CoV-2 IgG or total antibody 3 to 4 weeks after symptom onset to detect evidence of past SARS-CoV-2 infection;using IgG antibody to provide evidence of COVID-19 infection in symptomatic patients with a high clinical suspicion and repeatedly negative NAAT testing; In pediatric patients with multisystem inflammatory syndrome, using both IgG antibody and NAAT to provide evidence of current or past COVID-19 infection; **Not recommended as the sole basis; ***SARS-CoV-2 antibody tests are not recommended for diagnosis of current infection with COVID-19; ****For symptomatic patients with suspected COVID-19, using chest imaging for the diagnostic workup of COVID-19 when RT-PCR testing is not available; RT-PCR testing is available but results are delayed; and initial RT-PCR testing is negative but with high clinical of suspicion of COVID-19; *****CT scan should not be used routinely in the diagnosis of COVID-19 in children; ******Pregnant women with suspected COVID-19 infection; *******Not recommend routine serological testing to diagnose active COVID-19 in symptomatic pregnant women with negative RT-PCR; ********Not currently recommend using chest CT scans or X-rays as a first-line test for diagnosing COVID-19 in symptomatic pregnant women.*

EB-CPG, Evidence-based guideline; CB-CPG, Consensus-based guideline; CXR, chest radiography; chest CT, chest computed tomography. Etiological criteria: testing positive for SARS-CoV-2 by real-time polymerase chain reaction (PCR) and highly homologous genetic sequencing of respiratory tract or blood samples with the known SARS-CoV-2; Serological criteria: positive results of serum-specific antibodies IgM and IgG test, specifying serum-specific antibody IgG changed from negative to positive or increased four-fold or higher from that in the acute phase during the recovery period; Epidemiological history: involved noting whether the patients had a travel or residence history in a community with infected cases reported in China or a country or region with a serious epidemic, a history of contacting patients infected with SARS-Cov-2, a history of contacting patients with fever or respiratory symptoms from communities with reported cases in China or countries or regions with serious epidemics, clustered cases within 14 days prior to disease onset. Clinical manifestations: mainly consisted of fever, fatigue, dry cough, and/or other respiratory symptoms; COVID 19 imaging features and, in the early stage of the disease, the total number of leukocytes was normal or decreased, and the lymphocyte count was decreased.

TABLE 4 | Recommendations on antivirals drugs for COVID-19.

Guidelines title	Hydroxychloroquine +/– azithromycin	Lopinavir/ritonavir	Corticosteroids	Tocilizumab	Convalescent plasma	Remdesivir	Antibiotics	Famotidine	Bamlanivimab+/– etesevimab	Azithromycin	Baloxavir marboxil	Chloroquine	Favipiravir	Recombinant human granulocyte colony-stimulating factor	Sarilumab	Umifenovir	Interferon alfa	Interferon beta	Immunoglobulins	Traditional Chinese Medicine
EB-CPG																				
IDSA guidelines on the treatment and management of patients with COVID-19 (14)			*	**	***	****		*****	******											
Australian guidelines for the clinical care of people with COVID-19 (15)			*** ****	**** ****		**** *** **			**** *** ***		**** **** **	*** *** ****	*** **** ***	**** **** **	*** **** ***	**** *** ***				
COVID-19 rapid guideline: managing COVID-19 (16)			**** **** ***	**** **** ****		**** **** *****									**** ****** ****						
Management of hospitalized adults with coronavirus disease-19 (COVID-19): a European Respiratory Society living guideline (18)			**** ****** *****																	
Coronavirus disease 2019 (COVID-19) treatment guidelines (20)			***** **** **** ***			***** **** **** ***														
Surviving Sepsis Campaign guidelines on the management of adults with coronavirus disease 2019 (COVID-19) in the ICU: first update (21)			**** ******* ******		****** ***** *******	***** **** **** ******													***** ***** ***** *****	

(Continued)

TABLE 4 | Continued

Guidelines title	Hydroxychloroquine +/− azithromycin	Lopinavir/ritonavir	Corticosteroids	Tocilizumab	Convalescent plasma	Remdesivir	Antibiotics	Famotidine	Bamlanivimab+/− etesevimab	Azithromycin	Baloxavir marboxil	Chloroquine	Favipiravir	Recombinant human granulocyte colony-stimulating factor	Sarilumab	Umifenovir	Interferon alfa	Interferon beta	Immunoglobulins	Traditional Chinese Medicine
2021 update of the AGIHO guideline on evidence-based management of COVID-19 in patients with cancer regarding diagnostics, viral shedding, vaccination and therapy (24)																				
Should remdesivir be used for the treatment of patients with COVID-19? rapid, living practice points from the American College of Physicians (version 2) (25)																				
Clinical management of COVID-19 patients: living guidance (26)																				
Chemoprophylaxis, diagnosis, treatments, and discharge management of COVID-19: an evidence-based clinical practice guideline (updated version) (34)																				
Remdesivir for severe covid-19: a clinical practice guideline (37)																				

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TABLE 4 | Continued

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TABLE 4 | Continued

Guidelines title	Hydroxychloroquine +/– azithromycin	Lopinavir/ritonavir	Corticosteroids	Tocilizumab	Convalescent plasma	Remdesivir	Antibiotics	Famotidine	Bamlanivimab+/– etesevimab	Azithromycin	Baloxavir marboxil	Chloroquine	Favipiravir	Recombinant human granulocyte colony-stimulating factor	Sarilumab	Umifenovir	Interferon alfa	Interferon beta	Immunoglobulins	Traditional Chinese Medicine
Perinatal-neonatal management of COVID-19 infection (51)		****** ***** ****** ***** ***** ***																		
CB-CPG																				
COVID-19 convalescent plasma: interim recommendations from the AABB (22)																				
Multicenter interim guidance on use of antivirals for children with coronavirus disease 2019/severe acute respiratory syndrome coronavirus 2 (23)						***** ***** ****** ***** ***** *****														
Therapeutic strategies for severe COVID-19: a position paper from the Italian Society of Infectious and Tropical Diseases (SIMIT) (28)						***** ***** ****** ****** **********	***** ****** ****** ***** ***													

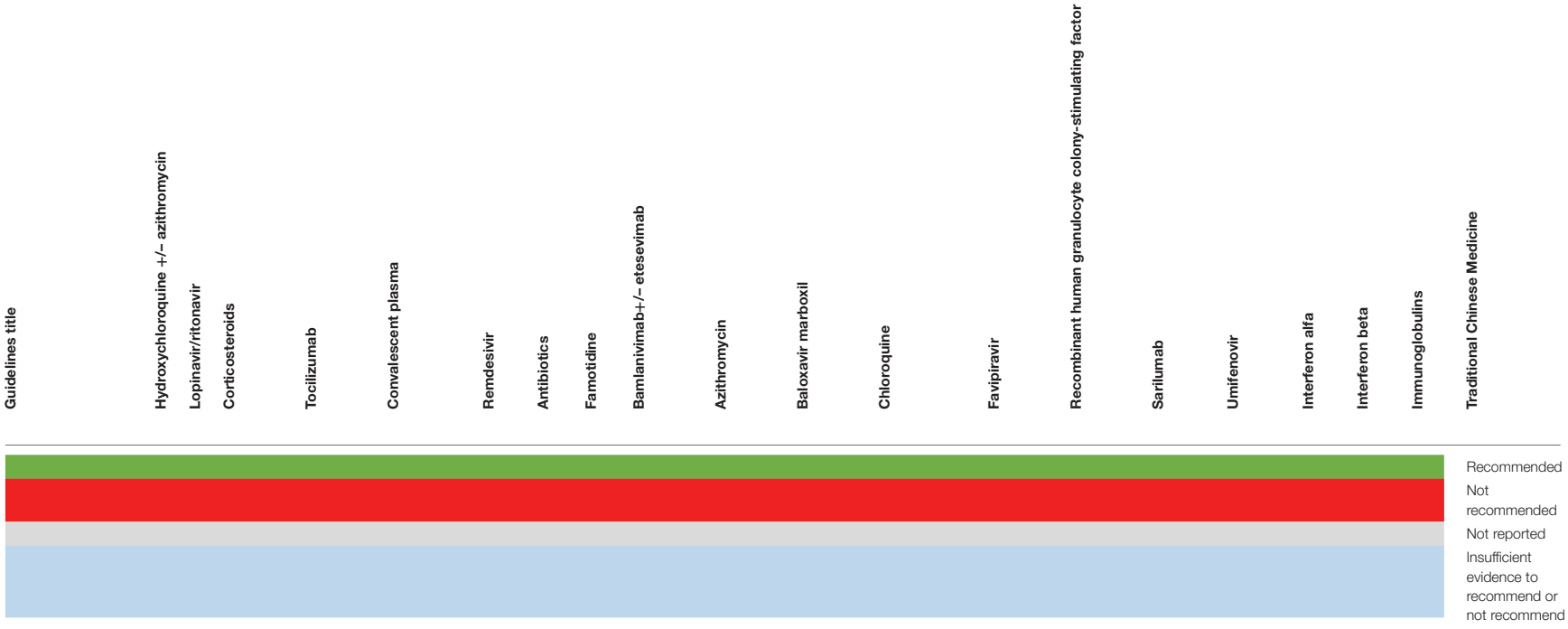
(Continued)

TABLE 4 | Continued

Guidelines title	Hydroxychloroquine +/– azithromycin	Lopinavir/ritonavir	Corticosteroids	Tocilizumab	Convalescent plasma	Remdesivir	Antibiotics	Famotidine	Bamlanivimab+/– etesevimab	Azithromycin	Baloxavir marboxil	Chloroquine	Favipiravir	Recombinant human granulocyte colony-stimulating factor	Sarilumab	Umifenovir	Interferon alfa	Interferon beta	Immunoglobulins	Traditional Chinese Medicine
Clinical management of coronavirus disease 2019 (COVID-19) in pregnancy: recommendations of WAPM-World Association of Perinatal Medicine (32)				***** ***** ***** **** *** **********		** **** **** ***** ***** ****							***** ***** **** ****** ****** ***** ***							
Updated guidance on the management of COVID-19: from an American thoracic society/European respiratory society coordinated international task force (38)																				
A consensus guideline of herbal medicine for coronavirus disease 2019 (41)																				
Updated diagnosis, treatment and prevention of COVID-19 in children: experts' consensus statement (condensed version of the second edition) (46)							***** ***** ***** ***** *****												***** ******* ***** **** ***	

(Continued)

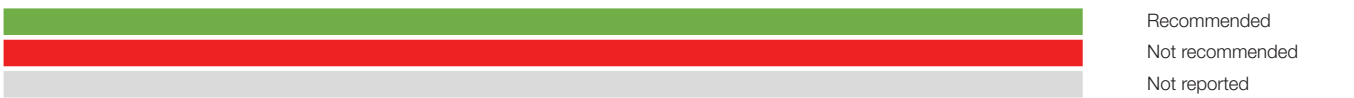
TABLE 4 | Continued



Among hospitalized severe or critically ill patients with COVID-19; **Among hospitalized adults with progressive severe or critical COVID-19 who have elevated markers of systemic inflammation; ***Only in the context of a clinical trial; ****In hospitalized patients with severe COVID-19; *****Hospitalized patients with severe COVID-19, not using famotidine use for the sole purpose of treating COVID-19 outside of the context of a clinical trial; ******Ambulatory patients with mild to moderate COVID-19 at high risk for progression to severe disease; *******Adults with COVID-19 or pregnant or breastfeeding women with COVID-19 or children and adolescents with acute COVID-19 who are receiving oxygen (including mechanically ventilated patients); ********Adults or children and adolescents who require supplemental oxygen; *********Adults or pregnant or breastfeeding women hospitalized with moderate to severe COVID-19 who do not require ventilation; **********Not use outside of the context of a clinical trial; **********People with COVID-19 who: need supplemental oxygen to meet their prescribed oxygen saturation levels or have a level of hypoxia that needs supplemental oxygen but who are unable to have or tolerate it; *********Adults in hospital with COVID-19 if all of the following apply: having or have completed a course of corticosteroids such as dexamethasone, unless they cannot have corticosteroids they have not had another interleukin-6 inhibitor during this admission there is no evidence of a bacterial or viral infection (other than SARS-CoV-2) that might be worsened by tocilizumab. And they either: need supplemental oxygen and have a C-reactive protein level of 75 mg/l or more, or are within 48 h of starting high-flow nasal oxygen, continuous positive airway pressure, non-invasive ventilation or invasive mechanical ventilation; ************COVID-19 pneumonia in adults, and young people 12 years and over weighing 40 kg or more, who are in hospital and on supplemental oxygen but not on invasive mechanical ventilation; ************Adults in hospital with COVID-19 only if tocilizumab cannot be used or is unavailable. Use the same eligibility criteria as those for tocilizumab. That is, if all of the following apply: they are having or have completed a course of corticosteroids such as dexamethasone, unless they cannot have corticosteroids they have not had another interleukin-6 inhibitor during this admission there is no evidence of a bacterial or viral infection (other than SARS-CoV-2) that might be worsened by sarilumab. And they either need supplemental oxygen and have a C-reactive protein level of 75 mg/l or more or are within 48 h of starting high-flow nasal oxygen, continuous positive airway pressure, non-invasive ventilation or invasive mechanical ventilation; **************Patients with COVID-19 requiring oxygen, non-invasive ventilation or invasive mechanical ventilation; ***************Hospitalized but requires supplemental oxygen; ***************For adults with severe or critical COVID-19; ****************For adults with severe or critical COVID-19 outside clinical trials; *****************For adults with severe COVID-19 who do not require mechanical ventilation; ******************In critically ill adults with COVID-19 or Children; *******************Hospitalized patients with COVID-19 who do not require mechanical ventilation or ECMO or hospitalized patients with COVID-19 who require mechanical ventilation or ECMO within a 5-day course; *********************Not using unproven drugs not be administered as treatment or prophylaxis for COVID-19, outside of the context of clinical trials; **********************Not using the combination of HCQ and azithromycin; **********************In severe covid-19; ***********************COVID-19 patients with suspected bacterial coinfection; ***********************Patients with severe coronavirus disease 2019 and acute respiratory distress syndrome; ************************Not using convalescent plasma in patients with severe COVID-19; ************************In mechanically ventilated adults with COVID-19 and respiratory failure (without ARDS), suggesting against the use of systemic corticosteroids; **************************The administration of antibiotics should be initiated within an hour of assessing the patient. Antibiotic therapy should be deescalated on the basis of microbiological results and clinical judgment; ***************************If any of the following criteria are met: hypoxia, hypotension, new onset organ dysfunction (one or more of Increase in creatinine by 50% from baseline, GFR reduction by >25% from baseline or urine output of <0.5 ml/kg for 6 h), Reduction of GCS by 2 or more, or Any other organ dysfunction; ****************************Only in children with positive SARS-CoV-2 viral testing; used only within the context of a clinical trial in outpatients and hospitalized patients with asymptomatic, mild, or moderate COVID; suggested for children with severe COVID-19; ****************************Oxygen support only no mechanical ventilation; ****************************Tocilizumab may be considered for off-label use in pregnant women who have severe or critical COVID-19 with the suspicion of cytokine activation syndrome with elevated IL-6 levels as a last resort or based on a clinical research protocol; ******************************In pregnancy.*
EB-CPG, Evidence-based guideline; CB-CPG, Consensus-based guideline.

TABLE 5A | Recommendations on discharge criteria of COVID-19.

Guidelines title	Body temperature	Respiratory symptoms	Pulmonary imaging	Detection of SARS-CoV-2 nucleic acid
EB-CPG				
Pragmatic recommendations for tracheostomy, discharge, and rehabilitation measures in hospitalized patients recovering from severe COVID-19 in low- and middle-income countries (29)	*	**		
Chemoprophylaxis, diagnosis, treatments, and discharge management of COVID-19: an evidence-based clinical practice guideline (updated version) (34)	***	**	****	*****
Use of chest imaging in the diagnosis and management of COVID-19: a WHO rapid advice guide (36)			******	
CB-CPG				
Chinese expert consensus on the perinatal and neonatal management for the prevention and control of the 2019 novel coronavirus infection (first edition) (52)	***	**	****	*****



**Afebrile for ≥24 h; **Substantially improved respiratory symptoms; ***Temperature returned to normal for more than 3 days; ****Significant absorption of pulmonary chest lesions; *****Two consecutive negative nucleic acid tests from sputum, nasopharyngeal swabs, or other respiratory tract samples (at least 24 h between samples); ******For hospitalized patients with COVID-19 whose symptoms are resolved, not using chest imaging in addition to clinical and/or laboratory assessment to inform the decision regarding discharge.*
EB-CPG, Evidence-based guideline; CB-CPG, Consensus-based guideline.

health care workers. In the early stage of the pandemic, the absolute lack of direct evidence is the biggest challenge for guideline development. A large number of CB-CPGs and EB-CPGs in accordance with experience of frontline health professionals, such as experts in infectious disease, medical imaging, and clinical immunology, have put forward valuable suggestions to guide clinical practice. Although the methodological quality of EB-CPGs is higher than CB-CPGs in general, they all have deficiencies in the following aspects, including obtaining the views and preferences of the target population, considering benefits and risks when formulating recommendations, introducing a detailed update plan, and providing implementation strategy for the recommendations or methods for managing potential conflicts of interest, similar to Dagens', Luo', and Zhao' studies (3, 4, 6). In view of the above topics, there are some examples of good practice, for example, conducting interviews and group surveys to collect information on treatment evidence from frontline experts fighting the disease (34); inviting patients recovering from COVID-19 to get involved in the guideline development panel (45); critically assessing new studies where these supersede previous outdated recommendations (14); providing available recommendation summaries in user-friendly and multilayered formats for clinicians and patients through the MAGIC app (55) or the provision of consultation decision aids to facilitate shared decision-making (45); and using the GRADEpro guideline development tool online software to conduct evidence-based CPGs (56). The methodology for guideline development to deal with public health emergencies is still a challenge, and methods for their development which ensure the rigor, timeliness and implementability of recommendations is a problem to be further explored by methodologists.

Recommendations relevant to chemoprophylaxis, diagnosis, antiviral treatments, and discharge management of COVID-19 varied in the guidelines. Chemoprophylaxis may be beneficial to reduce COVID-19 spread, which is important when lacking specific vaccines due to the high social and economic costs caused by social distancing of entire populations and blockade of entire cities. This method has been applied to other respiratory viruses; for example, healthcare workers who were exposed to high risk groups fought against the Middle East respiratory syndrome coronavirus using lopinavir-ritonavir plus ribavirin in South Korea (57). Unfortunately, there are still no effective and verified drugs for COVID-19 prophylaxis in the guidelines. However, a

TABLE 5B | Recommendations on precautions after discharge of COVID-19.

Guidelines title	Isolation management	Health examination	Personal prevention	Points for attention
EB-CPG				
Pragmatic recommendations for tracheostomy, discharge, and rehabilitation measures in hospitalized patients recovering from severe COVID-19 in low- and middle-income countries (29)	*		**	***
Chemoprophylaxis, diagnosis, treatments, and discharge management of COVID-19: an evidence-based clinical practice guideline (updated version) (34)	****	*****		Not reported
Perinatal-neonatal management of COVID-19 infection (51)		******	*******	
CB-CPG				
COVID-19: interim guidance on rehabilitation in the hospital and post-hospital phase from a European respiratory society- and American thoracic society-coordinated international task force (35)		********		*********

Recommended
Not reported

**Following local/regional/national deisolation, or ability to self-isolate adequately for a minimum of 10 days following the onset of symptoms, if applicable; **All patients and caregivers receive comprehensive education on adequate hygiene and the importance of mask-wearing, including for close contacts; ***Taking into consideration the capability of primary caregivers to provide the necessary care to meet the psychological, physical, and neurocognitive needs; ****Discharged patients may be quarantined for 2 weeks; *****PCR tests can be performed at 2 and 4 weeks after discharge; ******Early discharge to home may be followed by a telephonic follow-up or home visit by a designated nurse; *******Mothers should practice respiratory hygiene and wear a mask while breastfeeding and providing other care to the baby; they should routinely clean and disinfect all the surfaces; ********At 6–8 weeks following discharge, a formal assessment of physical and emotional functioning for patients with COVID-19; a formal psychological assessment for COVID-19 survivors with symptoms of psychological distress; *********At 6–8 weeks following discharge, doing regular daily activities in the first 6–8 weeks after hospital discharge; nutritional support for COVID-19 survivors with loss of lower-limb muscle mass, a musclestrengthening programme for COVID-19 survivors with loss of lower-limb muscle mass and/or function; a comprehensive pulmonary rehabilitation programme for COVID-19 survivors with pre-existing/ongoing lung function impairment; a comprehensive rehabilitation programme for COVID-19 survivors with a need for rehabilitative interventions. EB-CPG, Evidence-based guideline; CB-CPG, Consensus-based guideline.*

retrospective cohort study on family members and health care workers who were exposed to patients diagnosed with SARS-CoV-2 suggested that Arbidol could reduce risk of infection with the disease in hospital and family settings (58). SARS-CoV 2 vaccines may be beneficial for the prevention of COVID-19. The effectiveness and safety of them are still continuously ongoing trials. For instance, estimated BNT162b2 and mRNA-1273 COVID-19 vaccines effectiveness for prevention of infection was 90% for full immunization and 80% for partial immunization (59). Most commonly reported adverse effects of COVID-19 mRNA-1273 vaccine were localized pain, generalized weakness, headache, and myalgia (60). New evidence may inform decision making on chemoprophylaxis for healthcare personnel by policy makers in the future.

Diagnostic criteria for COVID-19 were not identical across the guidelines. What is more consistent is confirmation of diagnosis by testing positive for SARS-CoV-2 by real-time PCR. The main differences are the inclusion of other features, such as epidemiological history, serological tests, and clinical manifestations, as one of the bases for the diagnosis. Early studies have confirmed that 49–66% patients had contact with personnel in outbreak area (61). Up to now, asymptomatic infection of SARS-CoV-2 has become a worldwide concern. A recent study indicated that these cases may account for 60% of all infections and may trigger new outbreaks (62). Asymptomatic cases were significantly younger than those with symptomatic patients, had similar common incidence rate, and were more likely to come from high altitude and low mobility areas, with better history of epidemiology (63). Careful examination of the epidemiological history would help to identify asymptomatic patients that may have delayed symptoms after diagnosis. In addition, stability issues of RT-PCR testing of COVID-19 for hospitalized patients clinically diagnosed with SARS-CoV-2 are a problem. Li et al. reported a potentially high false negative rate of RT-PCR where results from several tests from the same patients at different points were inconsistent during the course of their diagnosis and treatment (64). Current systematic reviews have confirmed that the detection of anti-SARS-CoV-2 IgG and IgM had high diagnostic efficiency (2,282 patients with SARS-CoV-2 and 1,485 healthy persons or patients without SARS-CoV-2) (65) and a high

sensitivity of chest CT for the detection of COVID-19 in regions with severe (3,186 patients) (66). The presentation of COVID-19 symptoms (such as fever, cough, myalgia/fatigue, leukocyte, and neutrophil counts) might be regarded as a surrogate marker for the disease' presence and severity (67, 68). Therefore, serological criteria, epidemiological history, clinical manifestations, and chest x-ray/CT should also be used for to assist diagnosis for COVID-19 infection during the current epidemic, counteracting possible false negative RT-PCR results if available.

Studies published after the deadline for analysis have been included here. Although there were no consistent recommendations on the usage of antiviral drugs, it does offer a few valuable suggestions, including antiviral drugs, such as hydroxychloroquine and remdesivir, for COVID-19. The majority of EB-CPGs did not recommend hydroxychloroquine +/– azithromycin to treat patients with COVID-19 because higher certainty benefits (e.g., mortality reduction) are now highly unlikely even if additional high quality randomized controlled trials would become available (14, 15, 18, 20, 21, 40, 45). Remdesivir is an antiviral drug with potent *in vitro* activity against a range of RNA viruses including MERS-CoV, SARS-CoV, there may be a favorable risk-benefit profile for remdesivir compared with no antiviral treatment in severe COVID-19 infection with limited safety data currently available for the drug (14–16, 20, 21, 24, 25, 34, 37, 47). In addition, Traditional Chinese medicine treatment may be beneficial for the treatment of COVID-19, including Lianhua Qingwen granules/capsules and Huashi Baidu granules. More new evidence concentrating on antiviral therapy continuously emerges. For example, early application of lopinavir / ritonavir+interferon-α can reduce the shedding time of sars-cov-2 (69); Early initiation with interferon) β- 1b, lopinavir, ribavirin combination therapy were more safe and effective than lopinavir alone in relieving symptoms, shortening length of stay in patients with mild to moderate COVID-19 (70); Lianhua Qingwen combined with Western medicine may have a significant effect and fewer side effects in the treatment of common patients with new coronavirus pneumonia (71). The new evidence above will help to update the recommendations of the guidelines.

The phenomenon that some discharge patients have tested positive for COVID-19 again after recovery has attracted a lot of attention. The included guidelines provided different suggestions on discharge criteria and precautions after discharge. As previously stated, Chest CT and X ray can be beneficial for COVID-19 diagnosis. Viral RNA was detected in 48.1% of patients' feces, even in the feces who have been diagnosed with negative results in respiratory tract samples (72). Thus, a nucleic acid test of upper airway specimens (nasopharyngeal and pharyngeal swabs) and fecal stool can be considered along with other criteria. Additionally, it may be necessary to continue isolation management and health status monitoring. A follow-up study for 651 patients recovered from COVID-19 revealed that 3% of the patients were positive for SARS-CoV-2 by RTqPCR in routine physical examination and the median time from discharge to retest with postive results was 15.0 days (73). Thus, the COVID-19 pandemic is a rapidly changing situation. The recommendations in the guidelines are also continuously changing. The evidence-based living guidelines are pursed (74, 75).

A strength of this review lies in the updated study (up to April 5, 2021) concentrating on hot topics, including chemoprophylaxis, diagnosis, antiviral therapy and discharge management of COVID-19 guidelines, at the same time and summarizing the recommendations. In addition, we defined the CPGs, distinguished EB-CPGs and CB-CPGs in order to gain the valuable recommendations developed by multidisciplinary experts and based on best evidence. However, there are several inevitable limitations in this current study. First, we did not compare the evidence and recommendation levels or different grade systems used in EB-CPGs. With new evidence emerging over time, some CPGs will be updated and evidence and recommendation levels may be changed or improved later. Second, we only searched the three medical databases and eight representative guidelines repositories, and some eligible EB-CPGs and CB-CPGs will thus have been missed.

CONCLUSION

In general, the methodological quality of EB-CPGs is greater than CB-CPGs. But we still need to pay attention to gathering and synthesizing reliable the latest up-to-date information, involving the target population in guideline development and improving the implementability of the recommendations. As for the recommendations of COVID-19, SARS-CoV 2 vaccines are still going through ongoing trials; various diagnosis strategies, including serological criteria and CT for COVID-19, may be more effective if available; hydroxychloroquine +/– azithromycin may be not beneficial to treat patients with COVID-19, but remdesivir may be a favorable risk-benefit in severe COVID-19 infection; and isolation management and health status monitoring after discharge may be still necessary. Thus, chemoprophylaxis and antiviral drugs of COVID-19 still need more trials for confirmation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

Y-YW and X-TZ designed the study and formulated inclusion criteria. Y-YW, QS, HZ, QH, and B-HL searched and identified eligible guidelines. HZ, B-HL, M-ZL, and S-HH extracted important information. M-ZL and QS evaluated the quality of each included guideline using AGREE II. X-TZ and Y-HJ examined the data extraction forms. Y-YW, QH, and S-HH analyzed the data. Y-YW, X-TZ, XY, and Y-HJ contributed to discussed the findings. Y-YW, X-TZ, XY, and Y-HJ developed the final manuscript. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.630765/full#supplementary-material

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Hospitalization Costs of COVID-19 Cases and Their Associated Factors in Guangdong, China: A Cross-Sectional Study

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Background: The ongoing COVID-19 pandemic has brought significant challenges to health system and consumed a lot of health resources. However, evidence on the hospitalization costs and their associated factors in COVID-19 cases is scarce.

Objectives: To describe the total and components of hospitalization costs of COVID-19 cases, and investigate the associated factors of costs.

Methods: We included 876 confirmed COVID-19 cases admitted to 33 designated hospitals from January 15th to April 27th, 2020 in Guangdong, China, and collected their demographic and clinical information. A multiple linear regression model was performed to estimate the associations of hospitalization costs with potential associated factors.

Results: The median of total hospitalization costs of COVID-19 cases was $2,869.4 (IQR: $3,916.8). We found higher total costs in male (% difference: 29.7, 95% CI: 15.5, 45.6) than in female cases, in older cases than in younger ones, in severe cases (% difference: 344.8, 95% CI: 222.5, 513.6) than in mild ones, in cases with clinical aggravation than those without, in cases with clinical symptoms (% difference: 47.7, 95% CI: 26.2, 72.9) than those without, and in cases with comorbidities (% difference: 21.1%, 21.1, 95% CI: 4.4, 40.6) than those without. We also found lower non-pharmacologic therapy costs in cases treated with traditional Chinese medicine (TCM) therapy (% difference: −47.4, 95% CI: −64.5 to −22.0) than cases without.

Conclusion: The hospitalization costs of COVID-19 cases in Guangdong were comparable to the national level. Factors associated with higher hospitalization costs included sex, older age, clinical severity and aggravation, clinical symptoms and comorbidities at admission. TCM therapy was found to be associated with lower costs for some non-pharmacologic therapies.

Keywords: COVID-19, hospitalization costs, associated factors, traditional Chinese medicine therapy, China

INTRODUCTION

The ongoing COVID-19 pandemic, an emerging infectious disease caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has now affected more than 210 countries, areas or territories worldwide. As of October 26th, 2020, more than 42 million confirmed cases and more than 1.1 million deaths have been reported (1). In order to "flatten the epidemic curve," the global community has enforced border shutdowns, travel restrictions and quarantine, which has severely affected global socio-economic development (2). It was estimated that the current outbreak of COVID-19 leads to at least 1 trillion U.S. dollars ($1 trillion) loss to world's economy during year 2020, which is even worse than the 2008 Great Financial Crisis (3). In addition, the COVID-19 pandemic brought significant challenges to health system and consumed a lot of health resources. The large number of COVID-19 cases have occupied most heath care resources and caused many health care workers infected in some countries.

Understanding the hospitalization costs of COVID-19 cases and their associated factors has important implications for estimating the direct costs of COVID-19, and for clinical doctors to develop treatment strategies. However, few studies have reported such information in China where the COVID-19 epidemic was firstly reported (4, 5). A Chinese national report showed that the mean hospitalization costs of confirmed COVID-19 cases were $3,083.0, and the mean costs of severe cases was more than $21,500.0 (6). In Li et al. investigation conducted in 105 COVID-19 cases in Shenzhen, China, they found that the mean hospitalization costs were $1,762.0, and that the hospitalization costs were associated with age and duration of hospitalization (5). In addition, one report in the United States of America estimated that a single symptomatic COVID-19 infection would cost a median of $3,045 in direct medical costs incurred, and that patients' age, ICU admission and hospitalization would affect the costs (7). Some of these studies did not analyze the components of hospitalization costs, and did not account for the impacts of other factors such as presence of symptoms, comorbidities at admission, clinical aggravation during hospitalization, and strategies of therapy. Therefore, more investigations are needed.

As SARS-CoV-2 is a novel virus, there is no specific effective treatment particularly in the early stage of pandemic. Based on past experiences in the treatment of infectious diseases in China, Traditional Chinese Medicine (TCM), including herbal formulas, was widely used to treat COVID-19 cases (8). It was reported that early intervention with TCM in mild cases can effectively prevent them from progressing to severe conditions (9). Hence, it is plausible that the usage of TCM may reduce hospitalization costs of COVID-19 cases. However, few (if any) investigated the impacts of TCM on hospitalization costs.

METHODS

Study Setting

Guangdong is a province with a large population size located in southern China, which is a place early affected by COVID-19. The first confirmed case was reported on January 15th, 2020, and a total of 1,819 confirmed cases were reported as of September 28th, 2020. We retrospectively selected admitted confirmed COVID-19 cases from 33 designated hospitals from January 15th, 2020 to April 27th, 2020 in Guangdong, China. These hospitals were designated by governments to receive and treat confirmed COVID-19 cases. There is at least one designated hospital in each city in Guangdong Province, China. Once a COVID-19 case was confirmed in a general hospital according to the Diagnosis and Treatment scheme of COVID-19 (10, 11), he/she would be immediately admitted to a near designated hospital. There were few cases after May, 2020 (Out of the total 1,819 confirmed cases up to September, 2020, only 231 cases were confirmed after May), and most of them were imported cases. Some important information of hospitalization costs in those imported cases was not available. Therefore, we selected days from January 15th, 2020 to April 27th, 2020 as our study period. Moreover, those confirmed cases (712 cases) without information of key variables such as hospitalization costs, drug usage and non-pharmacologic therapy were excluded.

Data Collection

Information of all included cases were obtained from the Guangdong Provincial Office of COVID-19 Control and Prevention, which was set and designated by the government. Following the Law of the China on the Prevention and Treatment of Infection Diseases, and the Emergency Regulations Regarding Emergency Public Health Incidents, each confirmed COVID-19 case's information must be reported to the office, and was used for making and adjusting policies.

We collected the following information of each included case from medical record system and treatment system of all designated hospitals after they discharged from hospital: demographic characteristics (sex, age, and days of hospitalization), hospitalization costs, including total costs, drug usage costs (the cost of TCM and western medicine), examination costs (such as blood routine, urine routine, liver function, D-dimer, and B-ultrasound), non-pharmacologic therapy costs (the fee charged by the medical staff for the relevant operation, such as infusion fee, suture removal fee, atomization inhalation fee, physical therapy fee, and nursing fee) and others costs (the cost of materials, such as disposable infusion set, and disposable syringe) (**Supplementary Table 1**), symptoms at admission, comorbidities, severity, clinical aggravation during hospitalization, drug usage, and non-pharmacologic therapy information, TCM therapy, and general information of the designated hospital. All data used in this study were anonymous and without identifiable private information.

Definitions of Key Covariates

The hospital level was divided into two categories: tertiary hospital (typically larger and comprehensive), and secondary hospital (often regional, relatively smaller) (12). Clinical severity at admission was divided into three categories according to the Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia released by the National Health Commission of the People's Republic of China: mild, moderate and severe (13). A case would be defined as aggravation by clinical doctors if his/her clinical situation becomes severer during hospitalization

such as from mild to moderate. Symptoms and comorbidities at admission were also collected by clinical doctors.

Statistical Analysis

For categorical variables, we calculated the percentages (%) of cases in each category. Because the distribution of hospitalization costs was positive skewedness distribution, we used the median and interquartile range (IQR) to describe the hospitalization costs of COVID-19 cases in each category. A multiple linear regression model was performed to evaluate the associations between hospitalization costs and potential associated factors including demographic characteristics, symptoms at admission, severity of cases at admission, clinical aggravation during hospitalization, comorbidities, drug usage, non-pharmacologic therapy, TCM therapy, and other factors. In the multiple linear regression model, three variables were adjusted for as potential confounders including sex, age, and hospital level at admission. Collinearity diagnosis was performed to test the potential collinearity among the three adjusted for confounders and independent variables, and only variables with variance expansion factor (VIF) < 2 were included in multiple linear regression (14). The hospitalization costs were transformed by natural logarithm (ln), and the natural log-scaled partial coefficient of linear regression can be exponentiated to express the percentage changes [% difference and its 95% confidence interval (CI)] of the hospitalization costs (15).

In particular, we tested the differences in drug usage costs and non-pharmacological therapy costs between TCM group and non TCM group after adjustment for potential confounders (sex, age, clinical severity, and hospital level at admission) in the total cases and several subgroups, which was used to particularly examine the effects of TCM on hospitalization costs. All data analyses were conducted by R software (version 3.6.3, R Foundation for Statistical Computing).

Ethics Approval Statement

This study was approved by the Ethics Committee of Guangdong Provincial Center for Disease Control and Prevention (No. W96-027E-2020004). The data analysis was carried out at a population level after data aggregation. We didn't contact any individual subjects.

RESULTS

General Characteristics of COVID-19 Cases

A total of 876 COVID-19 cases were included for analysis in this study. **Table 1** shows the general characteristics of all participants. Out of the total participants, 442 (50.5%) were males, 621 (70.9%) were aged 20–59 years, and 802 (91.6%) were admitted to tertiary hospitals. At the time of admission, 73 (8.3%), 760 (86.8%), and 43 (4.9%) cases were categorized to mild, moderate, and severe groups, respectively, 733 (83.7%) cases had clinical symptoms, and 222 (25.3%) cases had comorbidities at admission. During the hospitalization, 150 (17.1%) patients aggravated in which four (0.5%) cases died, 647 (73.9%) were hospitalized for more than 15 days, and 45 (5.9%) cases were treated with TCM therapy (**Table 1**).

Figure 1 shows the distribution of total hospitalization costs and its components in the total cases. The median of total hospitalization costs in all cases was $2,869.4 IQR: $3,916.7 with the maximum of $0.4 million, and the minimum of $53.0. Out of the total cases, 94% had total hospitalization cost <$14,347.2. The total costs were consisted of drug usage (26.3%), examination (17.3%), non-pharmacologic therapy (10.5%), and other costs (45.9%). The median costs of drug usage, examination, non-pharmacologic therapy, and others were $631.3 (IQR: $2,539.5), $416.1 (IQR: $789.1), $258.3 (IQR: $401.7), and $1,047.3 (IQR: $1,477.8), respectively. **Figure 2** demonstrates the weekly average hospitalization cost in all cases from January 11th, 2020 to April 27th, 2020. A general decreasing trend was found during the study period. Several cases with extremely high costs were observed before February 15th, after which the costs of most cases were <$14,347.2.

Associated Factors of the Total Hospitalization Costs in COVID-19 Cases

Tables 2, **3** shows the factors that influence the hospitalization costs of COVID-19 cases. We found higher hospitalization costs in male cases (% difference: 29.7, 95% CI: 15.5, 45.6) than in female cases, in older cases than in younger cases (e.g., % difference: 323.0, 95% CI: 202.9, 490.7 for cases over 70 years compared with cases under 19 years), in cases admitted in the tertiary hospitals (% difference: 51.3, 95% CI: 22.8, 86.3) than in the secondary hospitals, and in cases with longer days of hospitalization (e.g., % difference: 273.1, 95% CI: 222.8, 331.3 for cases hospitalized over 28 days compared with cases hospitalized under 14 days). Higher costs were also found in severe cases (% difference: 344.8, 95% CI: 222.5, 513.6) than in mild cases, and in cases whose clinical conditions aggravated during hospitalization (% difference: 123.7, 95% CI: 92.6, 159.9) than cases without aggravation.

Cases with symptoms at admission had 47.7% (95% CI: 26.2%, 72.9%) higher hospitalization costs than those without symptoms. In particular, cases with fever at admission had 42.1% (95% CI: 25.4%, 61.1%) higher hospitalization costs than those without fever. Compared with cases without comorbidities, cases with comorbidities had higher hospitalization costs (% difference: 21.1, 95% CI: 4.4, 40.6), particularly in cases with diabetes (% difference: 33.4, 95% CI: 2.3, 74.0), cardiovascular diseases (% difference: 49.2, 95% CI: 7.3, 107.5), and chronic kidney diseases (% difference: 74.2, 95% CI: 14.2, 165.5).

We also observed higher costs in cases who received oxygen inhalation therapy (% difference: 41.1, 95% CI: 24.4, 60.0), oxyhydrogen atomizer therapy (% difference: 142.1, 95% CI: 72.7, 239.4), non-invasive ventilator therapy (% difference: 194.1, 95% CI: 137.8, 263.9), tracheal cannula therapy (% difference: 1142.3, 95% CI: 799.9, 1615.0), extracorporeal membrane oxygenation (ECMO) therapy (% difference: 1524.8, 95% CI: 887.3, 2574.0), intensive care unit (ICU) therapy (% difference: 353.0, 95% CI: 253.8, 479.9), anti-infective drug (% difference: 58.4, 95% CI: 40.8, 78.1), angiotensin drugs (% difference: 898.9, 95% CI:

TABLE 1 | General characteristics of COVID-19 cases in Guangdong Province, China.

Characteristics	*N* (%)	Characteristics	*N* (%)
Sex		Clinical aggravation during hospitalization	
Female	434 (49.5)	No	726 (82.9)
Male	442 (50.5)	Yes	150 (17.1)
Age (years)		Death during hospitalization	
<19	79 (9.0)	No	872 (99.5)
20–29	124 (14.2)	Yes	4 (0.5)
30–39	203 (23.2)	Comorbidity	
40–49	129 (14.7)	No	654 (74.7)
50–59	165 (18.8)	Yes	222 (25.3)
60–69	137 (15.6)	Clinical symptoms at admission	
≥70	39 (4.5)	No	143 (16.3)
Hospital level of admission		Yes	733 (83.7)
Secondary hospital	74 (8.4)	Days of hospitalization	
Tertiary hospital	802 (91.6)	0–14	227 (25.9)
Clinical severity at admission		15–21	289 (33.0)
Mild	73 (8.3)	22–28	163 (18.6)
Moderate	760 (86.8)	>28	195 (22.3)
Severe	43 (4.9)	Unknown	2 (0.2)
Antiviral drugs usage		TCM therapy[b]	
No	812 (92.7)	No	831 (94.9)
Yes	64 (7.3)	Yes	45 (5.1)
ECMO therapy[a]		ICU therapy[c]	
No	865 (98.7)	No	827 (94.4)
Yes	11 (1.3)	Yes	49 (5.6)

[a] *ECMO, Extracorporeal membrane oxygenation.*
[b] *TCM, Traditional Chinese Medicine.*
[c] *ICU, Intensive care unit.*

562.8, 1405.5), and hormone (% difference: 298.6, 95% CI: 219.5, 397.5) than cases who did not receive the corresponding therapy, respectively. Similar associated factors were found for drug usage costs, examination costs, and non-pharmacologic therapy costs (**Supplementary Tables 2–4**).

The Associations of TCM With Drug Usage and Non-pharmacologic Therapy Costs

We observed significantly negative associations of TCM with non-pharmacologic therapy costs in the total cases (% difference: −47.4, 95% CI: −64.5 to −22.0), in moderate cases (% difference: −51.0, 95% CI: −69.1 to −22.3), and in cases without comorbidities (% difference: −49.1, 95% CI: −66.5 to −22.5). However, we did not find a significant association of TCM with drug usage costs (**Table 4**).

DISCUSSION

In this study, we investigated the total and components of hospitalization costs of COVID-19 cases in Guangdong province, China, and examined the associated factors of costs. We observed that the median of total hospitalization costs was $2,869.4, in which the median costs of drug usage, examination, and non-pharmacologic therapy were $631.3, $416.1, and $258.3, respectively. Factors that increase the hospitalization cost included male, older age, higher level of hospital, longer days of hospitalization, severe clinical conditions, clinical aggravation during hospitalization, clinical symptoms at admission, drug usage such as angiotensin drugs, and non-pharmacologic therapy ICU care, and ECMO therapy. Furthermore, the TCM therapy could reduce the non-pharmacologic costs. Our findings could help clinical doctors and health care managers understand the factors which influence the hospitalization costs, better allocate limited medical resources, and improve treatment strategies in early stage. In addition, we provided information for medical insurance department to determine reimbursement standards, make relevant policies, and optimize the utilization of medical sources.

The total hospitalization cost of COVID-19 cases in this study had a large variation, ranged from $0.4 million to $53.0. The case with the maximum cost of $0.4 million was a 74-year-old male clinical severe cases with hypertension, cardiovascular disease and chronic kidney disease, who was hospitalized for 37 days, and was treated with continuous renal replacement therapy (CRRT), ECMO, and ICU cares. By contrast, the case with the minimum cost of $53.1 was a 59-year-old male mild case without any comorbidity, who was discharged without any specific treatments. Although very few studies have reported

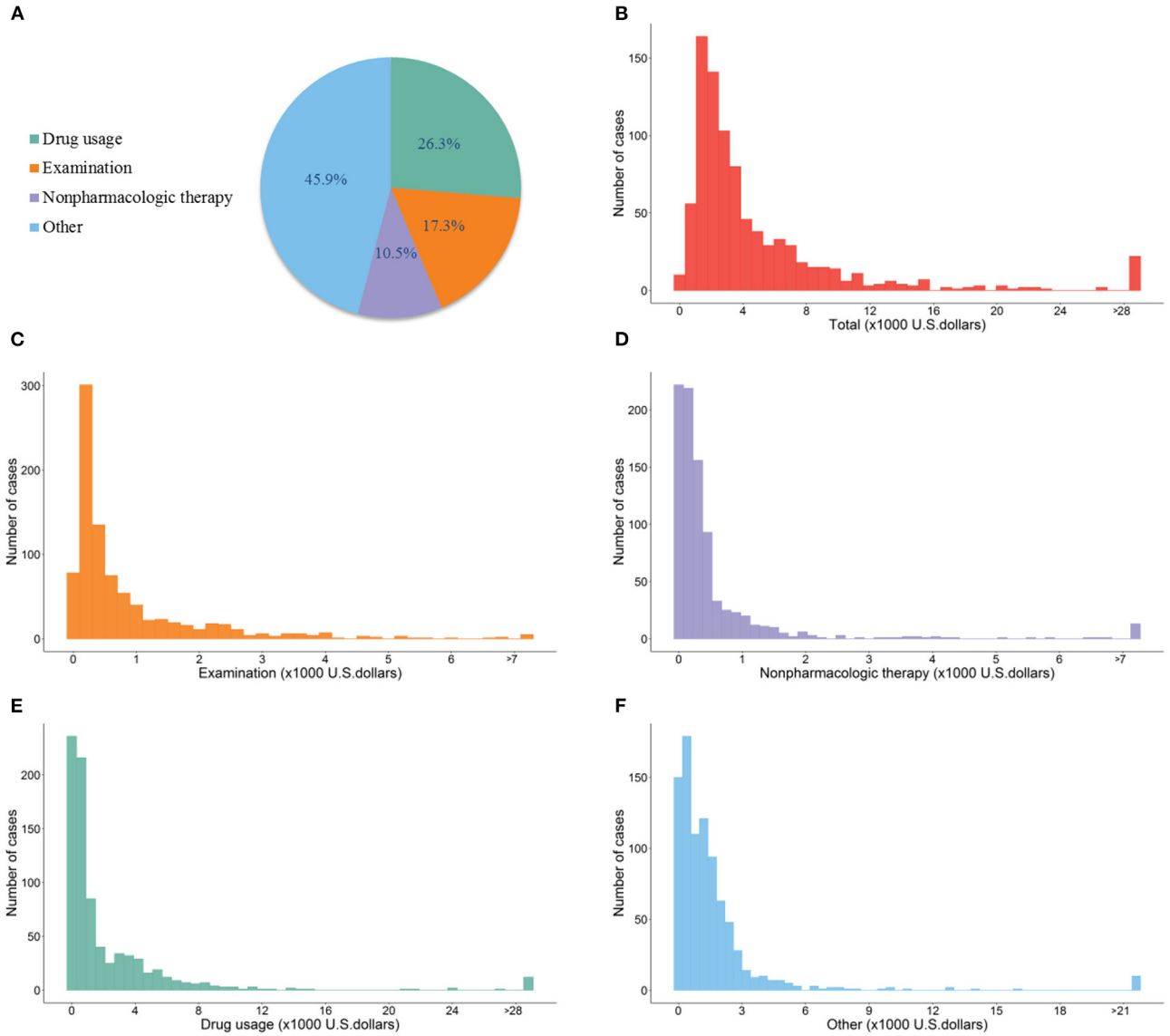

FIGURE 1 | Distribution of hospitalization costs of COVID-19 cases in Guangdong Province, China. **(A)** (Pie graph) shows the components of the total hospitalization costs, including examination costs, non-pharmacologic therapy costs, drug usage costs, and other costs. **(B–F)** Show the frequency of total hospitalization costs, examination costs, non-pharmacologic therapy costs, drug usage costs, and other costs in all included COVID-19 cases in Guangdong Province, China. The costs were presented in U.S. dollars (×1,000). The bars in these panels represent the number of cases.

that hospitalization costs of COVID-19 cases in China (4, 6), a national report showed that the mean hospitalization costs of COVID-19 cases were $3,084.6 (6), which is comparable to our findings ($2,869.4). This comparability may be partially explained by the similar distribution of clinical severity between COVID-19 cases in the present study and at nationwide. For example, the percentage of severe cases in this study was 17.0%, which was comparable to the national level (19.0% of severe rate) (16). These results further suggest the good representativeness of our study subjects.

We further found that the hospitalization costs of COVID-19 cases in this study were lower than in most countries globally (**Supplementary Table 5**). For example, the median of total cost was lower than that in the USA, India and Indonesia, and slightly higher than in Kenya (17–21). After categorizing COVID-19 cases by clinical severity, we found lower hospitalization costs in mild, moderate, and severe COVID-19 cases in this study than in South Korea and the USA, but higher than in Russia and in Kenya (21–24). The differences between China and other countries may be related to different level of development, policy, drug, and non-pharmacologic therapy costs, and therapeutic regimes. Since the very early stage of COVID-19 epidemic, inspection teams consisting of academicians and experts were organized to regularly inspect designated hospitals and discuss

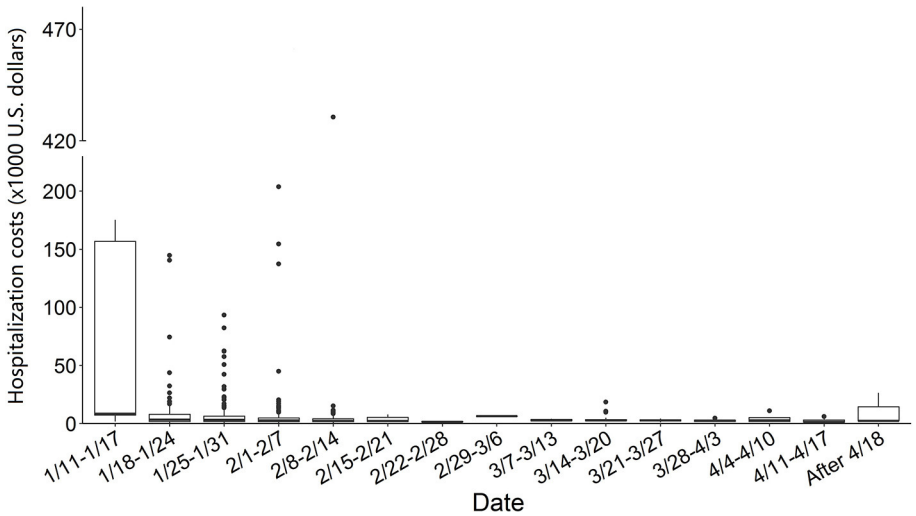

FIGURE 2 | Weekly hospitalization costs distribution of COVID-19 cases from January 11th to April 27th in Guangdong Province, China. The central line represents the median of distribution, boxes span the 25th to the 75th centiles, and the error bars represent the minimum and maximum values after excluding those outliers which were marked by dots.

and evaluate the treatment plans for COVID-19 cases. The diagnosis and treatment protocols of COVID-19 cases were constantly improved, which has substantially declined the hospitalization costs of COVID-19 cases in the late period of epidemic (**Figure 2**), and set an example in effective treatment of COVID-19 cases. In contrast, higher hospitalization costs were found before February 14, 2020 (22 out of 796 cases had more than $30,000 hospitalization costs, and the highest costs was more than $430.0 thousand during this period). The probable causes might be related to the poorer understanding on SARS-CoV-2 virus and having no specific clinical therapies in the early period of epidemic of the COVID-19. Some cases might be delayed care during this period, which would lead to the aggravation of the illness, and substantially increase the hospitalization costs because those severe cases are more likely to use expensive therapy such as ECMO.

As we expected, significantly higher hospitalization costs were observed in severe cases than in mild or moderate cases, and in cases with clinical aggregation during hospitalization. Previous studies have reported that severe and critically severe cases had higher risks of clinical aggregation, multiple organ failure, and fatality (25, 26), which was associated with higher medical costs. To maximally improve the cure rate of COVID-19 cases, all severe cases in China were treated following the principle: on the basis of symptomatic treatment, complications should be proactively prevented, comorbidities should be treated, secondary infections also be prevented, and organ function support should be provided timely (13). Based on the principle, severe cases were more frequently treated with high-flow nasal-catheter oxygenation, non-invasive mechanical ventilation, ICU care, ECMO, and CRRT, which hence increase their hospitalization costs. In this study, we also found higher costs in cases treated with ECMO, tracheal cannula, non-invasive ventilator, oxyhydrogen atomizer, and oxygen inhalation therapy.

We also found higher hospitalization costs in cases with clinical symptoms particularly for fever at admission than those without symptoms. Cases with symptoms usually need more support therapy, antiviral and antibiotic therapy during their hospitalization (**Supplementary Table 6**), which can create additional costs. Both previous studies and the present study have found that clinical symptoms such as fever were common in admitted COVID-19 cases (27–29). Thus, the symptoms at admission, especially fever, can be used as one of the important predictors of hospitalization costs because of the high proportion in clinical features.

Many studies reported that the comorbidities such as hypertension, diabetes, cardiovascular disease, and respiratory diseases could greatly affect the prognosis and mortality of the COVID-19 (25, 30–32). For example, a meta-analysis including 13 studies found that cases with hypertension had 1.72 times higher critical/mortal risk than those without hypertension (31). Therefore, it is reasonable to hypothesize that cases with comorbidities may have higher hospitalization costs than those without comorbidities. As expected, our findings found higher costs in cases with comorbidities at admission. These cases had higher proportions of severe conditions, and clinical aggravation during hospitalization, resulting in the increase of their hospitalization costs (**Supplementary Table 7**). This finding also indicates that comorbidities could also be used as predictors of hospitalization costs for COVID-19 cases.

TCM has a long history and played an indispensable role in the prevention and treatment of several epidemic diseases in China, such as severe acute respiratory syndrome (SARS) (33). TCM scheme was included in the guideline on diagnosis and treatment of COVID-19 cases as a major feature in China (13). It was widely used in patients with mild symptoms, and was also used in combination with western medicines in patients with severe symptoms. It was reported that more than 90% of COVID-19

TABLE 2 | Associated factors of the total hospitalization costs in COVID-19 cases.

Characteristics	Median (IQR) (× $1000)	% Difference[a,b] (95%CI)	Characteristics	Median (IQR) (× $1,000)	% Difference[a,b] (95%CI)
Sex			**Muscle pain**		
Female	2.7 (3.3)	Reference	No	2.8 (3.7)	Reference
Male	3.2 (4.9)	29.7 (15.5, 45.6)	Yes	3.7 (5.6)	15.0 (−8.0, 43.8)
Age (years)			**Comorbidity**		
0–19	1.7 (1.4)	Reference	No	2.6 (2.9)	Reference
20–29	2.1 (2.0)	44.0 (12.6, 84.1)	Yes	4.4 (6.3)	21.1 (4.4, 40.6)
30–39	2.4 (2.3)	50.8 (20.3, 89.1)	Diabetes		
40–49	2.6 (2.9)	82.0 (42.6, 132.4)	No	2.7 (3.7)	Reference
50–59	4.0 (4.6)	134.4 (85.6, 196.1)	Yes	5.8 (11.2)	33.4 (2.3, 74.0)
60–69	6.3 (8.9)	280.6 (199.1, 384.3)	Chronic kidney disease		
≥70	5.1 (9.7)	323.0 (202.9, 490.7)	No	2.8 (3.8)	Reference
Hospital level of admission			Yes	7.5 (10.5)	74.2 (14.2, 165.6)
Secondary hospital	1.8 (1.8)	Reference	Chronic lung disease		
Tertiary hospital	3.0 (4.1)	51.3 (22.8, 86.3)	No	2.8 (3.8)	Reference
Days of hospitalization			Yes	4.2 (7.2)	11.2 (−15.9, 47.1)
0–14	1.5 (1.0)	Reference	Hypertension		
15–21	2.6 (2.8)	62.5 (42.7, 85.1)	No	2.7 (3.4)	Reference
22–28	3.5 (4.3)	119.2 (88.6, 154.8)	Yes	5.6 (9.2)	23.2 (0.7, 50.7)
>28	5.7 (8.7)	273.1 (222.8, 331.3)	Cardiovascular disease		
Clinical severity at admission			No	2.8 (3.6)	Reference
Mild	2.2 (1.8)	Reference	Yes	7.3 (9.9)	49.2 (7.3, 107.5)
Moderate	2.8 (3.6)	8.8 (−11.0, 33.1)	Oxygen inhalation therapy		
Severe	15.1 (30.1)	344.8 (222.5, 513.6)	No	3.6 (5.1)	Reference
Clinical aggravation during hospitalization			Yes	2.2 (2.0)	41.1 (24.4, 60.0)
No	2.6 (2.7)	Reference	Oxyhydrogen atomizer therapy		
Yes	7.3 (10.0)	123.7 (92.6, 159.9)	No	2.7 (3.6)	Reference
Death during hospitalization			Yes	9.3 (6.1)	142.1 (72.7, 239.4)
No	2.8 (3.9)	Reference	Non-invasive ventilator therapy		
Yes	162.9 (37.3)	1753.2 (697.4, 4207.2)	No	2.7 (3.1)	Reference
Clinical symptoms at admission			Yes	13.1 (13.6)	194.1 (137.8, 263.9)
No	2.1 (1.5)	Reference	Tracheal cannula therapy		
Yes	3.2 (4.5)	47.7 (26.2, 72.9)	No	2.8 (3.6)	Reference
Fever			Yes	62.3 (109.9)	1142.3 (799.9, 1615.0)
No	2.3 (2.1)	Reference	Hormone usage		
Yes	3.3 (4.7)	42.1 (25.4, 61.1)	No	2.7 (3.2)	Reference
Cough			Yes	13.8 (1.5)	298.6 (219.5, 397.5)
No	2.7 (3.0)	Reference			
Yes	3.1 (4.8)	14.6 (2.1, 28.7)			

[a] *Adjusted for sex, age and hospital level of admission.*
[b] *The costs were calculated at the exchange rate between RMB and U.S dollars on August 1, 2020.*

cases both in Hubei and the rest of China were treated with TCM (34). In this study, we observed significantly negative associations of TCM usage with non-pharmacologic therapy costs in the total cases, moderate cases, and in cases without comorbidities. Previous studies showed that TCM strategies showed apparent advantages in improving symptoms, promoting virus clearance, increasing cure rate, shortening hospitalization, and reducing patient progression from mild to severe (9, 35, 36). These findings suggest the potential of TCM in saving hospitalization costs in COVID-19 cases, although dedicated and more rigorous studies are desirable before wide application of TCM for treatment of COVID-19 cases to other countries.

In addition, we found higher hospitalization costs in male cases than in female cases, which may be related to the sex differences in genes and health status. Studies have reported that male COVID-19 cases had higher prevalence of comorbidities

TABLE 3 | Associated factors of the total hospitalization costs in COVID-19 cases.

Characteristics	Median (IQR) (× $1,000)	% Difference[a,d] (95%CI)	Characteristics	Median (IQR) (× $1,000)	% Difference[a,d] (95%CI)
ICU therapy[b]			**ECMO therapy[c]**		
No	2.7 (3.3)	Reference	No	2.8 (3.7)	Reference
Yes	19.0 (53.1)	353.0 (253.8, 479.9)	Yes	154.5 (88.3)	1524.8 (887.3, 2574.0)
Anti-infective drug usage			**Angiotensin drugs usage**		
No	2.4 (2.2)	Reference	No	2.8 (3.7)	Reference
Yes	4.1 (6.4)	58.4 (40.8, 78.1)	Yes	92.5 (137.6)	898.9 (562.8, 1405.5)
Antiviral drug usage			**Chloroquine phosphate usage**		
No	2.9 (4.2)	Reference	No	2.9 (4.0)	Reference
Yes	2.6 (1.4)	16.0 (−7.9, 46.0)	Yes	2.7 (0.6)	13.4 (−36.3, 102.1)

[a] *Adjusted for sex, age, and hospital level of admission.*
[b] *ICU, Intensive care unit.*
[c] *ECMO, Extracorporeal membrane oxygenation.*
[d] *The costs were calculated at the exchange rate between RMB and U.S dollars on August 1, 2020.*

TABLE 4 | Associations (% Difference, 95% CI) of drug usage and non-pharmacologic therapy costs with TCM therapy.

Costs type	All cases[a]	Clinical severity at admission		Comorbidity[a]		Antiviral drugs usage[a]	
		Mild	Moderate	Yes	No	Yes	No
Drug usage costs							
Non TCM therapy	Reference	Reference	Reference	Reference	Reference	Reference	Reference
TCM therapy	34.1 (−17.6, 118.4)	159.7 (−13.1, 675.6)	10.2 (−37.7, 95.1)	20.3 (−63.1, 292.2)	33.4 (−22.7, 130.1)	16.0 (−48.3, 160.3)	−8.1 (−89.4, 694.9)
Non-pharmacologic therapy costs							
Non TCM therapy	Reference	Reference	Reference	Reference	Reference	Reference	Reference
TCM therapy	−47.4 (−64.5 to −22.0)	−35.8 (−73.3 to 54.3)	−51.0 (−69.1 to −22.3)	−37.8 (−79.2, 86.1)	−49.1 (−66.5 to −22.5)	−43.5 (−77.2, 39.6)	−8.7 (−83.3, 400.6)

[a] *Adjusted for sex, age, hospital level of admission and clinical severity at admission.*

(31), and hence had greater risk of severer clinical condition and mortality than female COVID-19 cases (25, 31, 32, 37, 38). Moreover, X chromosome and sex hormones in females play an important role in innate and adaptive immunity, which could protect them from clinical aggregation (39). The higher cost in older cases was related to their higher risk of COVID-19 infection, morbidity, aggregation, and death (25, 31, 40, 41). Older cases often have comorbidities like hypertension, diabetes and cardiovascular disease, and as the body's immunity declines with age, they are more likely to develop critical illness or even die, which also increase their hospitalization costs.

Strengths and Limitations

This study has several strengths. First, this study provides the total and components of hospitalization costs of COVID-19 cases in China. This result could provide clear information for systematically evaluating the direct economic burden of COVID-19 in China and worldwide. Second, we investigated associated factors of hospitalization costs including demographic characteristics, clinical severity, clinical symptoms, comorbidities, and specific clinical therapies, which could help clinical doctors to make treatment strategies, and medical insurance department to determine reimbursement standards. Third, we estimated the associations between TCM and hospitalization costs, which provides more evidence for the potential of TCM in saving costs for COVID-19 cases.

Several limitations also should be noted. First, we only obtained the information of COVID-19 cases in Guangdong Province, China, and the 876 cases' dataset was relatively small, which may limit the generalization of our findings. However, the median of total hospitalization cost in this study was comparable to the national level, which may indicate the good representativeness of our findings. Second, the costs information was from the designed hospital where the cases stayed at the time of recruitment, and costs incurred in other hospitals due to hospital transfer and other reasons were not included in this study, which may lead potential information bias. Third, hospitalization costs of COVID-19 cases in other countries were mainly obtained from news or report. The information was not peer reviewed, and may provide biased information.

CONCLUSIONS

We observed a comparable hospitalization costs of COVID-19 cases in Guangdong with the national level. Factors leading

to higher hospitalization costs included sex, older age, clinical severity and aggravation, clinical symptoms and comorbidities at admission, drug usage, and some non-pharmacologic therapies. In addition, TCM therapy may reduce non-pharmacologic therapy costs in mild and moderate cases. Our findings have both clinical and public health implications for containing the spread of COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Guangdong Provincial Center for Disease Control and Prevention (No. W96-027E-2020004). The data analysis was carried out at a population level after data aggregation. We didn't contact any individual subjects.

AUTHOR CONTRIBUTIONS

TL and MF designed the study, collected and standardized the data, and coordinated the work. MD and ZY performed the statistical analysis and drafted the manuscript and interpreted the results. TL provided substantial scientific insight into the interpretation of the results and drafting of the manuscript. YC, JS, WM, SC XS, JX, GH, JH, JW, GC, HZ, LY, JL, HX, XL, DC, and RW provided the data, and contributed to the interpretation of the results and the preparation of the submitted version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.655231/full#supplementary-material

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Short-Term Forecasting of Daily Confirmed COVID-19 Cases in Malaysia Using RF-SSA Model

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Novel coronavirus (COVID-19) was discovered in Wuhan, China in December 2019, and has affected millions of lives worldwide. On 29th April 2020, Malaysia reported more than 5,000 COVID-19 cases; the second highest in the Southeast Asian region after Singapore. Recently, a forecasting model was developed to measure and predict COVID-19 cases in Malaysia on daily basis for the next 10 days using previously-confirmed cases. A Recurrent Forecasting-Singular Spectrum Analysis (RF-SSA) is proposed by establishing L and *ET* parameters via several tests. The advantage of using this forecasting model is it would discriminate noise in a time series trend and produce significant forecasting results. The RF-SSA model assessment was based on the official COVID-19 data released by the World Health Organization (WHO) to predict daily confirmed cases between 30th April and 31st May, 2020. These results revealed that parameter $L = 5$ (T/20) for the RF-SSA model was indeed suitable for short-time series outbreak data, while the appropriate number of eigentriples was integral as it influenced the forecasting results. Evidently, the RF-SSA had over-forecasted the cases by 0.36%. This signifies the competence of RF-SSA in predicting the impending number of COVID-19 cases. Nonetheless, an enhanced RF-SSA algorithm should be developed for higher effectivity of capturing any extreme data changes.

Keywords: COVID-19, eigentriples, forecasting, recurrent forecasting, singular spectrum analysis, trend, window length

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INTRODUCTION

In 2020, Malaysia has witnessed the outbreak of a virus called Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) or COVID-19 that is highly infectious to human's respiratory system, hepatic system, gastrointestinal system, and neurological disorders. This virus can spread between humans, livestock, and wild animals, such as birds, bats, and mice (1, 2). Belonging to the coronavirus family, this novel virus type is accountable as a cause for mild to moderate colds. The SARS-CoV-2 may cause severe acute respiratory illnesses that result in fatality for various cases. The symptoms of COVID-19 are cough, fever, nose congestion, shortness of breath, and occasionally, diarrhea (3). In Malaysia, the virus started to spread swiftly by the end of January

2020. Since then, the Crisis Preparedness Response Centre (CPRC) of Malaysia's Ministry of Health (MOH) has begun recording and reporting the cases. The COVID-19 statistics is updated based on the total active cases, recoveries, and casualties attained daily from the MOH website.

The worst scenario of SARS-CoV-2 infection to individuals is fatality. Nevertheless, information on the mechanism of the spread of the virus or how it affects a patient seems to be in scarcity. The Centres for Disease Control and Prevention (CDC) has verified the COVID-19 human-to-human transmission on 30th January 2020. As noted by the CDC, COVID-19 can spread via droplet, close contact with infected patients, and contact with surfaces or objects that has the particles of the virus. It has been stipulated that 2–14 days or longer as the incubation period of COVID-19 with 5 days on average (4).

As the impact of this virus is severe, therefore it is important to be able to detect the pattern and forecast the spread of confirmed cases is very crucial. For an instance, Zhao et al. (5) had proposed a mathematical model to approximate the actual COVID-19 cases, including those unreported, for the first half of January 2020. It was deduced that the unreported cases count was 469 between 1st and 15th January 2020. Next, the estimation of cases from 17th January 2020 onwards revealed that the case numbers astonishingly encountered a 21-fold upsurge. This epidemic was predicted to reach its peak in late February and subside by late April based on the SEIR model combined with a machine-learning artificial intelligence (AI) method (6). Subsequently, Tang et al. (7) prescribed a mathematical model that could estimate the risk of COVID-19 transmission. Based on this, the potential number of the basic reproduction was 6.47. It also forecasted the total of 7 day confirmed cases with 23rd−29th January 2020 time interval. Consequently, the estimated peak was after 2 weeks from the initial date of 23rd January 2020.

In order to estimate the prolonged COVID-19 human-to-human transmission, data obtained from 47 patients were analyzed and resulted in a transmission rate of 0.4 (8). If the duration between the symptom detection and the patient hospitalization was halved from the tested study data, the transmission rate could reduce to 0.012. In another study, an estimation of SIR model was exhibited for the COVID-19 outbreak in Malaysia to predict the short-term daily COVID-19 cases (9). The study reported a transmission rate of 0.22 by considering that an infected individual can spread the virus to another individual within 4 days. This human-to-human transmission rate of 4 days should be highly considered, or even viewed as conservative.

Furthermore, various researchers have employed Box-Jenkins time series analysis model in predicting future cases of COVID-19 (10–12). For an instance, Rauf and Hannah (12) found out ARIMA (2, 2, 2) model produced the most accurate results compare to others for cases in India. Meanwhile, Jibrin et al. (11) recommended that the Autoregressive Fractional Integral Moving Average (ARFIMA) model should be used for further analysis of daily COVID-19 new cases. Rauf and Hannah (12) found an upward trend of the spread of COVID-19 in Nigeria based on ARIMA (1,1,0) model and more. According to Jianxi (13), the developed predictive model of COVID-19 cases must be considered on several factors such as intertwined human, social, and political factors. Due to that, predictive monitoring paradigm was proposed, which synthesized the prediction and monitoring of the daily COVID-19 cases in the study area. Another forecasting method to predict COVID-19 cases is based on machine learning approaches (14–17). Jianxi (13) stated that the hybridization model of machine learning approaches produces better performances in predicting cumulative COVID-19 cases with high daily incidence. In addition, the climatic variables were employed as inputs for proposed forecasting machine learning models.

Most of the previous studies focuses on the forecasting of future cases COVID-19. However, the analysis of this pandemic pattern is equally important. The proposed method suggested by Yogesh (18) considered the trend of new cases of COVID-19 in developing forecasting model. Nevertheless, this model didn't ensure that the trend and noise components in the data were clearly separated before the forecasting values were generated. The suitable analytical tools to assess the global change pattern with uncertainty metrics seem to be rather limited and seldom applied systematically, as it is often presented as an operational pattern worldwide. Systematically tracking and observing the infectious disease in a specific population and presented chronologically at high temporal resolution can lead to a modern and sophisticated methodology to perform in-depth data analysis. Hence, suitable analytical methods for time series data may be used if cases of health outcomes are assembled and aggregated with time units (e.g., weekly or daily basis).

Singular Spectrum Analysis (SSA) is a superb and effective alternative to address trend components, substantially minimize noise, and unravel the temporal structure of data minus preliminary manipulation (19). Generally, SSA represents univariate time series transformed into eigenvectors and eigenvalues of any trajectory matrix. The SSA refers to a multidimensional analog of principal component analysis (PCA), which is transformed into time series. One function of the SSA is to separate the time series data into noise, trend, and seasonal categories by decomposing the time series eigen, and later, reconstructing them into group selection (20).

The SSA, essentially, transforms a single dimension time series into trajectories with multiple dimensions via PCA [Singular Value Decomposition (SVD)], as well as reconstruction (approximation) of chosen Principal Components. However, the separation of the components in this approach depends on the parameters, which is the selection of window length, L, to form trajectory matrix and identifying the number of leading eigentriples (ET), based on eigenvector plot (21). This separation is crucial in this model to ensure that the trend, seasonal, and noise components are easily separated.

Although SSA lacks parametric description and highly relies on the length of time series, these flexible SSA models can recreate the asymmetric shapes of a trend, hence allowing better prediction of seasonal peaks than can harmonic models. This model, when compared to others, is easy to use, dismisses specification of models of time series and trend, enables extraction of trend in the presence of noise and oscillations,

and involves only two parameters to determine the accuracy and flexibility in predicting outcomes (22).

As the SSA models are seldom used to assess epidemiological data, this study is set to introduce the SSA model based on combining forecasting elements of time series analysis known as Recurrent Forecasting-Singular Spectrum Analysis (RF-SSA). To ensure that this developed model produces significant forecasting results, the selection of the parameter for this model, which are the window length, L and the amount of leading eigentriples used, ET, was identified using several tests. The SSA was used in this study as a base approach to build the forecasting model. The next sections describe the data in detail, followed by several sections that present the methodology, the results and discussion, and finally, the conclusion.

DATA

Daily COVID-19 prevalence data from 25th January to 29th April 2020 were gathered from MOH records. As this COVID-19 is a newly-founded virus; no COVID-19 data was available from the previous year. The suspected COVID-19 cases were diagnosed by using the Reverse Transcription Polymerase Chain Reaction (RT-PCR) technique and were confirmed as COVID-19 case-counts. All fully anonymized, laboratory-confirmed cases were abstracted on COVID-19, in which 5,945 cases represented COVID-19 infections in all 13 states and 3 federal territories in Malaysia, as recorded by MOH.

Figure 1 illustrates the total positive cases for COVID-19. The figure displays a significant spike in the number of positive cases that resulted in the 2nd wave of COVID-19 pandemic in Malaysia. With this substantial number, the Malaysian Government had announced a Movement Control Order (MCO) that took place from 18th to 31st March 2020. The MCO was later extended to the 4th phase.

Figure 2 portrays the observed number of cases for COVID-19 for the last 96 days in Malaysia. The MOH had categorized four zones of COVID-19 areas in Malaysia based on the areal cases number. According to the National Security Council (MKN), the four zones are: (i) green zone for areas with no positive case, (ii) yellow zone for areas with one to 20 positive cases, (iii) orange zone for areas with 21 to 40 positive cases, and (iv) red zone for areas with more than 40 positive cases (23).

The projection and estimate daily cases of COVID-19 obtained were impacted by the definition of the case reported to CPRC daily, whereby a large number of pending result test daily was definitely influential to a non-consistent increase in the number of confirmed cases. The increased prediction cases are supported by several of the biggest clusters identified by the MOH, such as Seri Petaling Tabligh Cluster, Wedding Kenduri in Bandar Baru Bangi, Seri Petaling Sub-Cluster in Rembau, Italy Cluster in Kuching, and Church Fellowship Cluster in Sarawak. The new confirmed cases were extremely spiking as the target of biology samples were taken directly from highly susceptible infected population.

MATERIALS AND METHODS

This section elaborates on the specifics of SSA model and its components.

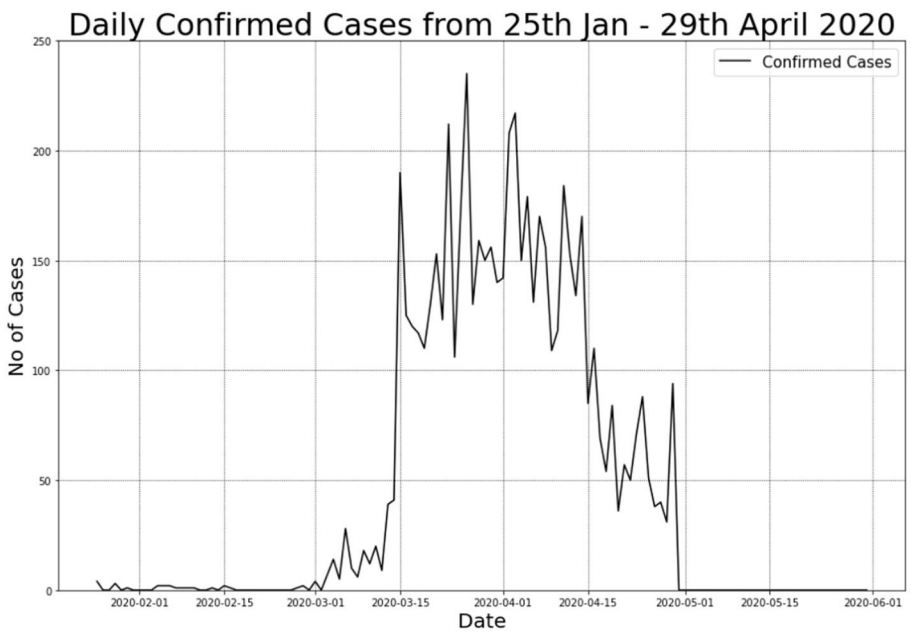


FIGURE 1 | COVID-19 daily confirmed cases in Malaysia from 25th January to 29th April 2020.

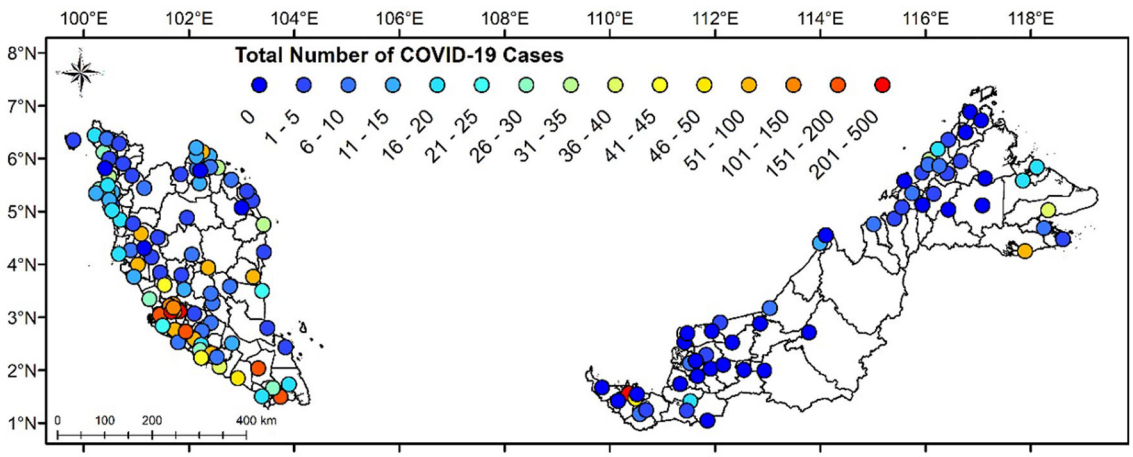


FIGURE 2 | State classification based on number of COVID-19 cases in Malaysia from 25th January to 29th April 2020.

Singular Spectrum Analysis (SSA) Model

The SSA is a model-free approach that can be applied to all types of data, regardless of Gaussian or non-Gaussian, linear or non-linear, and stationary or non-stationary (24). The daily COVID-19 data can be decomposed into several additive components via SSA, which could be defined in the forms of trend, seasonal, and noise components (25). The possible application areas of SSA are diverse (26–28). The SSA is composed of two complementary stages, known as the stages of decomposition and reconstruction (29).

Stage 1: Decomposition

The two steps in the decomposition stage are embedding and SVD. This stage decomposes the series to obtain eigen time series data.

Step I: Embedding. The first step in basic SSA algorithm is embedding, which refers to constructing the original time series into a sequence of lagged vector of size window length, L by forming lagged vectors, $K = T - L + 1$ of size L. $X_i = (x_i, \ldots, x_{i+L-1})^T \ (1 \leq i \leq K)$.

The trajectory matrix of the series $\mathbb{X}$ is

$$X = (X_1, \ldots, X_K) = \left(x_{ij}\right)_{i,j=1}^{L,K} = \begin{pmatrix} x_1 & x_2 & x_3 & \cdots & x_K \\ x_2 & x_3 & x_4 & \cdots & x_{K+1} \\ x_3 & x_4 & x_5 & \ldots & x_{K+2} \\ \vdots & \vdots & \vdots & \ddots & \vdots \\ x_L & x_{L+1} & x_{L+2} & \cdots & x_T \end{pmatrix} \quad (1)$$

The rows and columns of **X** are subseries of the original one-dimensional time series data and lagged vectors X_i are the columns of the trajectory matrix **X.**

Step II: Singular Value Decomposition (SVD). In the second step, the trajectory matrix in Step I is decomposed to obtain the eigen time series based on their singular values using SVD. The following represents the SVD of the trajectory matrix, X_i where $\lambda_1, \ldots, \lambda_L$ are denoted as the eigenvalues of XX^T where singular values are arranged in a descending order such that $(\sigma_1 \geq \sigma_2 \geq \cdots \geq \sigma_L)$ and by $U_1, \ldots, U_L$the corresponding eigenvectors. The SVD of X can be represented as $X = X_1 + \cdots + X_L$, where $X_i = \sqrt{\lambda_i} U_i V_i^T$ and $V_i = \frac{X^T U_i}{\sqrt{\lambda_i}}$ if ($\lambda_i = 0$ we set $X_i = 0$). The set of $\left(\sqrt{\lambda_i}, U_i, V_i\right)$ is called the $i - th$ eigentriple (ET) of the matrix X_i, and $\sqrt{\lambda_i}$ are the singular values of the matrix X_i.

Stage 2: Reconstruction

Grouping and diagonal averaging are the two steps in the reconstruction phase. Here, the original series are reconstructed for further analysis, including forecasting.

Step 1: Grouping. Here, the trajectory matrix is divided into dual groups—trend, seasonal and noise components. Upon setting $I = \left\{i_1, \ldots, i_p\right\}$ be a group indices, $i_1, \ldots, i_p$ where $\left(p < L\right)$. Then the matrix $\mathbf{X}_I$ corresponding to the group I is defined $\mathbf{X}_I = \mathbf{X}_{i1} + \ldots + \mathbf{X}_{ip}$. The indices set $\{1, \ldots, L\}$ is divided into m disjoint subsets; $I_1, \ldots, I_m$, based on the division of elementary matrices into groups of m. The retrieved matrices are calculated for $I = I_1, \ldots, I_m$ which called is eigentriple grouping corresponding to the representation of $\mathbf{X} = \mathbf{X}_{I1} + \ldots + \mathbf{X}_{Im}$.

Step 2: Diagonal averaging. The last step in SSA refers to the transformation of each matrix in the grouped decomposition into new series of length, T.

- Let **Z** be $L \times K$ matrix with z_{ij}, $1 \leq i \leq L$elements, $1 \leq j \leq K$. Set $L^* = \min(L, K)$, $K^* = \max(L, K)$, and $N = L + K - 1$. Let ${z_{ij}}^* = z_{ij}$ if $L < K$ and ${z_{ij}}^* = z_{ji}$ otherwise. With diagonal averaging, matrix **Z** is transferred into $z_1, \ldots, z_T$ based on the

following formula:

$$z_k \begin{cases} \frac{1}{k}\sum_{m=1}^{k} z^*_{m,k-m+1} & 1 \le k < L^* \\ \frac{1}{L^*}\sum_{m=1}^{L^*} z^*_{m,k-m+1} & L^* \le k \le K^* \\ \frac{1}{T-k+1}\sum_{m=k-k^*+1}^{T-K^*+1} z^*_{m,\ k-m+1} & K^* < k \le N \end{cases} \quad (2)$$

- Upon applying the diagonal averaging in equation above to the resultant matrix, $\mathbf{X}_{Ik}$, reconstructed series of $\tilde{\mathbb{Y}}_T^{(k)} = (\tilde{y}_1^{(k)}, \ldots, \tilde{y}_T^{(k)})$ is produced. The initial series of $\mathbb{Y}_T = \{y_1, y_2, \ldots, y_T\}$ is decomposed into the total of m reconstructed series, $y_t = \sum_{k=1}^{m} \tilde{y}_t^{(k)}$. The reconstructed series generated by elementary grouping refers to 'elementary reconstructed series.

Stage 3: Forecasting

To perform SSA forecasting, the time series should satisfy the linear recurrent formula (LRF). Time series $Y_T = (y_1, \ldots, y_T)$ satisfies LRF of order d if:

$$y_t = a_1 y_{t-1} + a_2 y_{t-2} + \ldots + a_d y_{t-d}, \qquad t = d+1, \ldots, T \quad (3)$$

In this study, Recurrent SSA (RSSA) was used for forecasting purpose because it is a popular approach to predict data (30, 31). The algorithms described below are detailed in Golyandina et al. (32).

Let us assume that U_j^{∇} is the vector of the first $L-1$ components of eigenvector U_j, while π_j is the last component of $U_j (j = 1, \ldots, r)$. Denoting $\nu^2 = \sum_{j-1}^{r} \pi_j^2$, coefficient vector $\Re$ is defined as follows:

$$\Re = \frac{1}{1-\nu^2} \sum_{j=1}^{r} \pi_j \, U_j^{\nabla} \quad (4)$$

Upon considering the prior notation, the forecast of RSSA $(\hat{y}_{T+1}, \ldots, \hat{y}_{T+M})$ can be attained by

$$\hat{y}_i = \begin{cases} \tilde{y}_i, & i = 1, \ldots, T \\ \Re^T Z_i, & i = T+1, \ldots, T+M \end{cases} \quad (5)$$

where, $Z_i = \left[\hat{y}_{i-L+1}, \ldots, \hat{y}_{i-1}\right]^T$ and $\tilde{y}_1, \ldots, \tilde{y}_T$, are the values of reconstructed time series (noise reduced series).

SSA Parameter Selection

Extraction of trend from the original time series data relies on the window length, L, to form the trajectory matrix in SSA. Improper values selection for parameter L may yield unfinished reconstruction, which may potentially mislead the forecasting results. It has been stipulated that L should be large enough, but not greater than half of the number of observations understudy at $\frac{T}{2}$ (33). The appropriate window length selection depends on the structure of time series data and the current problems (34). Generally, there is no guide to determine the proper L in a dataset. The separability conditions for shorter time series may be restrictive due to the SVD properties used in estimating the signal component in SSA. Therefore, in this study, several L namely $\frac{T}{2}, \frac{T}{5}, \frac{T}{10}, \frac{T}{20}$, were investigated on COVID-19 data based on performance error, which refers to Root Mean Square Error (RMSE).

Another parameter to be considered when using the SSA approach is the amount of leading *ET*by inspecting the eigenvector plot. This plot is the eigenvector of the SVD of trajectory matrix for time series data. The one-dimensional graphs of eigenvectors were inspected to identify the trend components. The trend has a complex form when both the trend and noise components were not properly distinguished. It is highly possible that lack of separability caused the mix-up between the components. This information may serve as a guideline to identify proper grouping for component separation of the trend and noise appropriately. This reflects a link between the stages of decomposition and reconstruction.

Evaluating Separabality in Time Series Data

A key concept when studying SSA is separability, which signifies how the varied components of time series may be differentiated from each other to enable further analysis. When working with SSA method in numerous study fields, separability becomes a vital mean (35). The separability impact can result in appropriate decomposition and component extraction. The *w-correlation* technique measures the separability between two distinct components of the reconstructed time series.

The *w-correlation* reflects the weighted correlation among components of reconstructed time series that offers highly useful information to both separate and identify groups for the reconstructed components (36). The elements of the time series terms are indicated by the weights into trajectory matrix. This ranges between 0 and 1, whereby components that are well-separated slant toward 0, whereas slant toward 1 for otherwise. The *w-correlation* matrix looks into grouped decomposition among the reconstructed components. The matrix formulation of *w-correlation* is as follows:

$$\rho_{12}^{w} = \frac{\left\langle X^{(1)}, X^{(2)} \right\rangle w}{\left\| X^{(1)} \right\| w \left\| X^{(2)} \right\| w} \quad (6)$$

where $\left\| X^{(i)} \right\| w = \sqrt{\left\langle X^{(i)}, X^{(i)} \right\rangle w}$, $i = 1, 2$, $\left\langle X^{(1)}, X^{(2)} \right\rangle w = \sum_{i=0}^{N-1} w_i x_i^{(1)} w_i^{(2)}$, and weights w_i are defined below:

Let $L^* = \min(L, K)$ and $K^* = \max(L, K)$. As a result,

$$w_i = \begin{cases} i+1 & for\ 0 \le i \le L^* - 1, \\ L^* & for\ L^* \le i \le K^*, \\ T - i & for\ K^* \le i \le T - 1. \end{cases} \quad (7)$$

The graphic illustration of *w-correlation* is composed of white-black scale, whereby white represents correlation that is small, whereas black denotes correlation between the series components near to value 1.

Evaluation Performances

In this study, four types of evaluation performances are applied to evaluate the accuracy of the predicted output for the

forecasting models. The measurements used in this study are Mean Absolute Error (MAE), Mean Forecast Error (MFE), and Root Mean Square Error (RMSE), whereby, the best model is selected based on the smallest values for that measurements. Meanwhile, the Pearson Correlation Coefficient (*r)* value is based on a range from +1 to −1. A value of *r* that close to +1 or −1 indicated that the two observed variables are related to each other. Concurrently, a value of 0 indicates that there is no association between two observed variables. The equations for each of the evaluation performances are shown

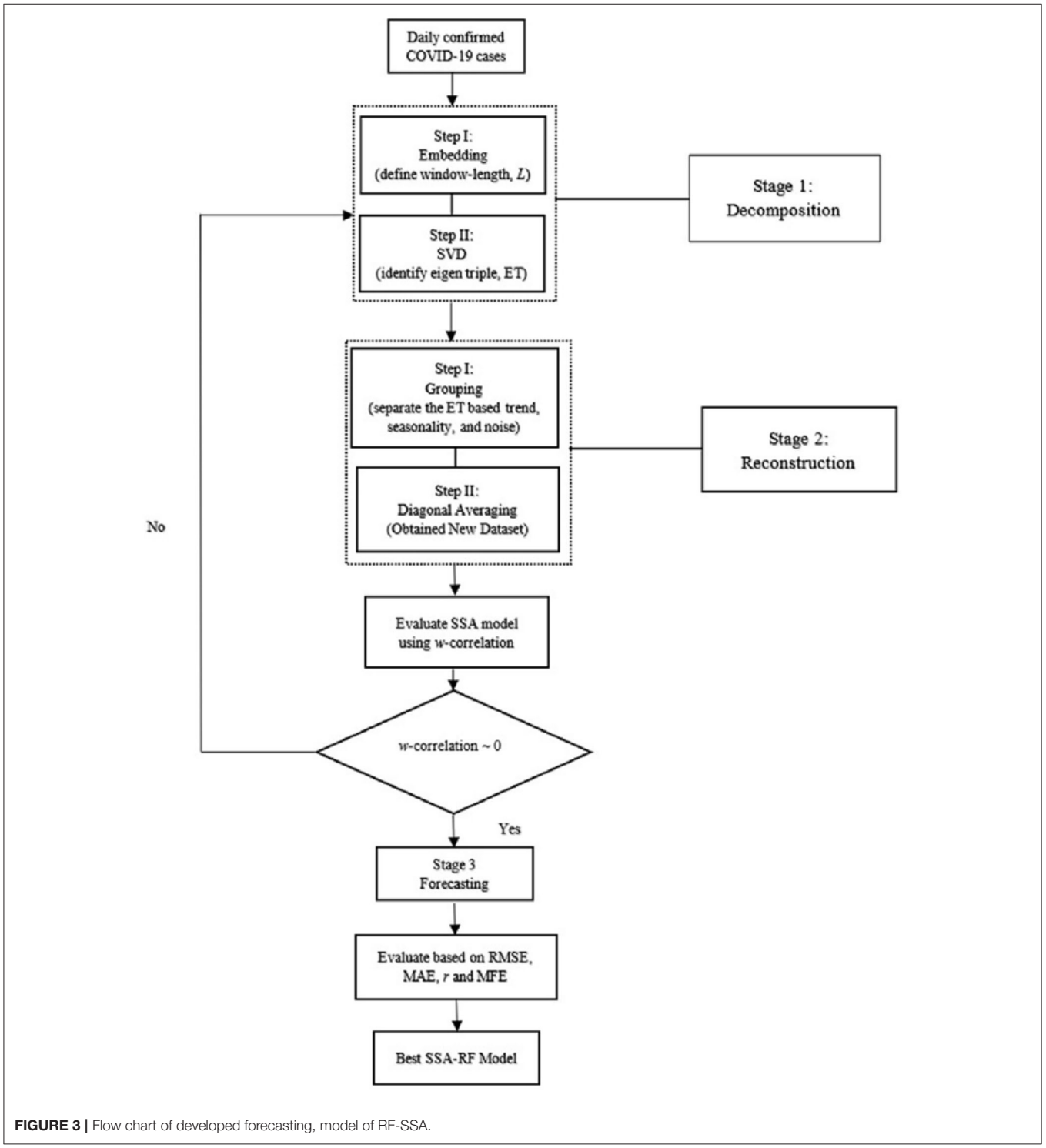


FIGURE 3 | Flow chart of developed forecasting, model of RF-SSA.

as follows:

$$MAE = n^{-1}\left[\sum_{i=1}^{n}|y_t - \hat{y}|\right] \quad (8)$$

$$MFE = n^{-1}\left[\sum_{i=1}^{n}(y_t - \hat{y})\right] \quad (9)$$

$$RMSE = n^{-2}\left[\sum_{i=1}^{n}(y_t - \hat{y})^2\right]^{-0.5} \quad (10)$$

$$r = \frac{n(\sum_{i=1}^{n} x_t y_t) - (\sum_{i=1}^{n} x_t)(\sum_{i=1}^{n} y_t)}{\sqrt{\left[n(\sum_{i=1}^{n} x_t{}^2) - (\sum_{i=1}^{n} x_t)^2\right]\left[n(\sum_{i=1}^{n} y_t{}^2) - (\sum_{i=1}^{n} y_t)^2\right]}} \quad (11)$$

where y_t is the actual values at time t; y_t is the predicted values at time t; n is the number of observations. Flow chart of developed forecasting model based on SSA as shown in **Figure 3**.

RESULTS AND DISCUSSION

Decomposition and Reconstruction

In the initial stage of this study, COVID-19 data were decomposed into components by using the SSA model, which required identification of (L, ET) parameter pair. Here, L denotes the compromise between statistical confidence and information. The suitable L value should resolve the varied oscillations embedded in the original signal.

The performance of the SSA results was determined by assessing the *w-correlation* at distinct window length, L. The *w-correlation* calculated the separability among noise, trend, and seasonal (components of reconstructed time series). Here, $L = T/2$, $T/5$, $T/10$, and $\frac{T}{20}$, which represent $L = 48$, 19, 10, and 5, respectively, for T based on 96 daily cases on COVID-19 data had been selected. The scales were selected to fit the data of the time series, apart from striking a balance to achieve a proper lag vector sequence.

In **Figure 4**, the *w-correlation* is presented based on SSA using daily cases of COVID-19 data at varying window lengths. The *w-correlation* displayed a declining trend when the total window length declined for SSA approach. The correlations among trend and other components should be close to zero for extraction of trend. This means; the distinct window lengths have an impact on the component's separability. Besides, the SSA was directed to the lowest *w-correlation* at $L = T/20$; signifying the best separability among the reconstructed components as it was the closest to zero.

The graphs in **Figures 5A–D** illustrate the heat-plot of different window lengths, L, based on *w-correlations* using the SSA approach. The heat-plot of *w-correlation* for the reconstructed components based on white-black scale ranges between 0 and 1 (37). Huge correlation values among the reconstructed components exhibited the possibility of the components to form a group while corresponding to the same component. As illustrated in **Figure 5**, the shade of each square represents the *w-correlation* strength between two components. Meanwhile, **Figures 5A–C** portrays the tendency of the components to form correlation with other components despite signifying weak correlation. Subsequently, this denotes that the components of trends are still, to some extent, mixed with the noise and seasonal components in SSA and it was rectified by the small window length, $L = 5$, which is evidently demonstrated in **Figure 5D** for better separability.

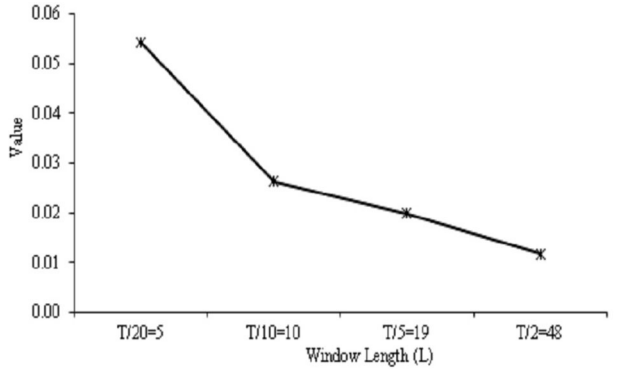


FIGURE 4 | Effect of *w*-correlation based on SSA using COVID-19 data at varied window lengths.

TABLE 1 | Comparison of Singular Spectrum Analysis Prediction Performance for Several Window Length (L).

Window Length, L	RMSE
$T/2 = 48$	29.51
$T/5 = 19$	29.67
$T/10 = 10$	23.97
$T/20 = 5$	19.12

Table 1 presents the reconstructed time series components varied window length. The lowest RMSE was observed from $L = \frac{T}{20}$, which had the smallest value amongst other L, indicating its suitability based on short-time series of the outbreak data. Meanwhile, the high RMSE values were reported in this study due to the high model variance for small sample set.

The plot of five main eigenvectors is displayed in **Figure 6**. Such plot is beneficial to choose an appropriate group for the components of time series data, especially to separate the components of noise, trend, and seasonal. The retrieved information may be further analyzed in the step of grouping in RF-SSA. The component of trend was identified from eigenvector plot, in which seasonal and trend components have sine waves indicated by the slow cycles found in the graph (high frequency). Meanwhile, the component of noise was represented by the saw-tooth found in the graph (low frequency). The leading eigenvector has nearly continual coordinates, thus corresponding to a pure smoothing by Bartlett filter (38, 39). The reconstruction result by each of the five ET is presented in **Figure 7**. The two figures verified the compatibility of the first and second ET with

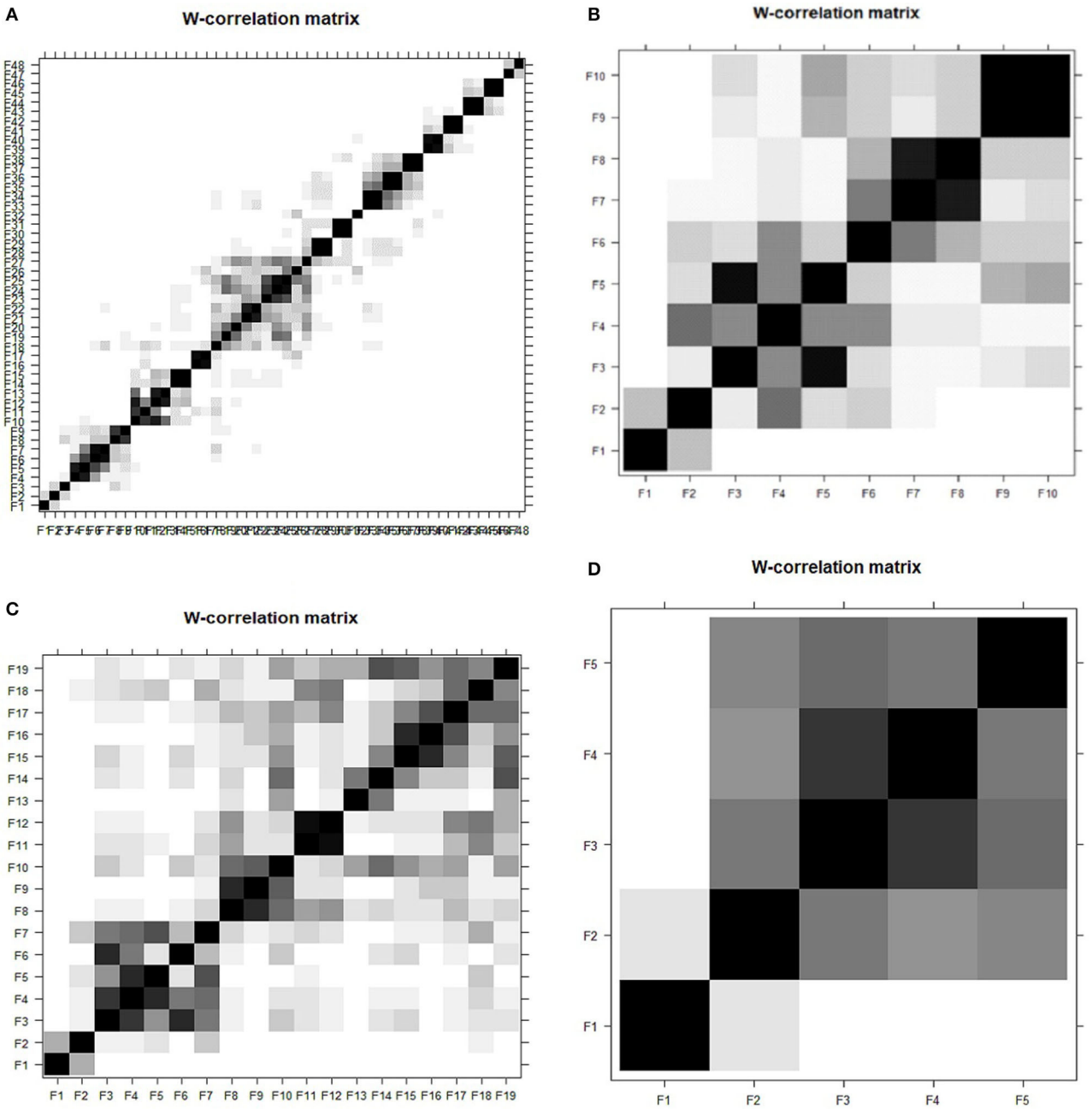


FIGURE 5 | (A–D) *w-correlation* plot using SSA with varied windows length **(A)** $L = 48$ **(B)** $L = 19$ **(C)** $L = 10$ **(D)** $L = 5$.

the trend, whereas the remaining *ET* had the noise component, thus irrelevant to trend.

Figure 8 demonstrates the components of the reconstructed time series plot from the trend extracted via RF-SSA for daily COVID-19 cases in Malaysia. The reconstructed series is the new dataset derived from the original data, which is clear from noise. It is a crucial aspect in SSA to ensure that the forecasting results are precise and accurate (40). The component of trend in the time series data was used to observe the occurrence of the cases trend and pattern, as it was randomly-tabulated as per daily cases (see **Figure 8**). In **Figures 8A**, **7**, the trend was precisely generated by a leading *ET*, which coincided with the initial reconstructed component exhibited in **Figure 8**. The trend in **Figure 8B** was precisely generated by both leading *ET*, which coincided with the first and second reconstructed components shown in **Figure 8**. The dashed and straight lines on the plot denote the reconstructed series based on the extracted trend component from SSA and the COVID-19 original time

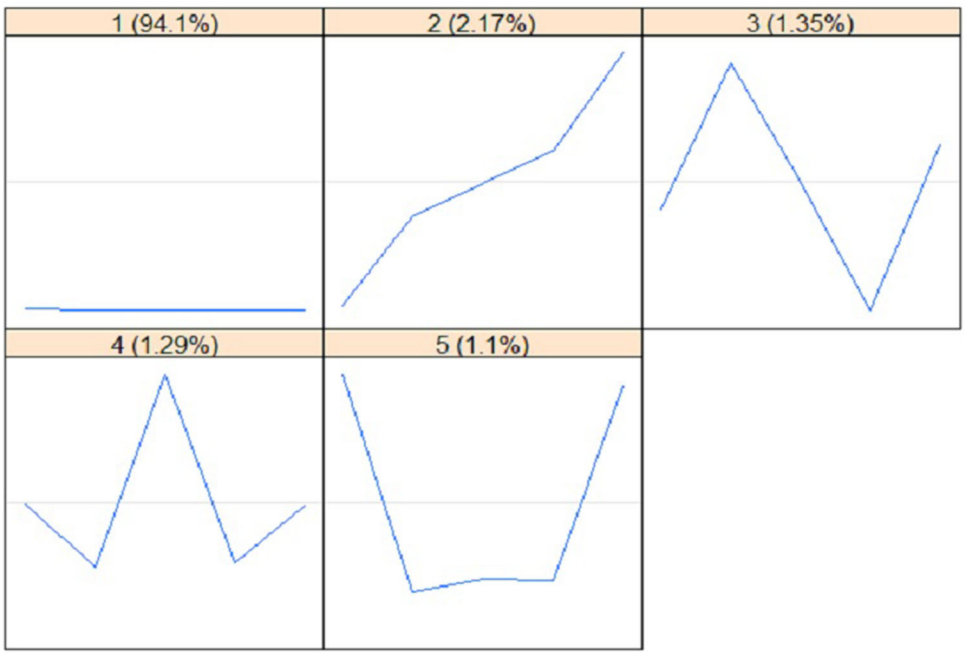


FIGURE 6 | Eigenvectors Plot using Singular Spectrum Analysis.

series data, respectively. The plot of reconstructed time series components, produced by both leading *ET*, abides by the original COVID-19 data although noise component was omitted for $L = 5$ for daily COVID-19 cases in Malaysia.

For proper identification of seasonal series components, the graph of eigenvalues and scatterplots of eigenvectors were applied. In order to determine the seasonal series components using eigenvalues plot, several steps were produced by approximately equal eigenvalues. **Figure 9** portrays the plot of the logarithms of the five singular values for the COVID-19 cases in Malaysia. It clearly showed that no step produced by approximately equal eigenvalues that corresponded to a sine wave. The scatterplot of eigenvectors displays the regular polygons yielded by a pair of eigenvectors to demonstrate that the series components have produced seasonality components. Based on **Figure 10**, no pair of eigenvectors produced regular polygons. This confirmed that the COVID-19 data in Malaysia were not influenced by the seasonality since both figures did not have sine wave.

Forecasting Daily COVID-19 Cases Using SSA-RF

As mentioned in the previous section, the daily COVID-19 cases in Malaysia were first decomposed and reconstructed using SSA model. The next step in this study is to predict the future cases of COVID-19 in Malaysia. In this stage, an SSA forecasting algorithm known as Recurrent Forecasting were used accordingly. From hereafter, the model are known as SSA-RF. **Table 2** presents the summary statistics from the experiment analysis of SSA-RF at several windows length.

Looking at **Table 2**, it is apparent that the best performances can be obtained from $L = 5$ that has the lowest MAE of 11.2549 with the highest r of 0.9619, indicating superb correlation between confirmed and predicted cases. Moreover, the MFE shows that the SSA-RF algorithm with L = 5, tends to under-forecast daily COVID-19 cases by 0.1920%. Meanwhile, the second-best model is observed from SSA-RF with L = 10 where RMSE is 23.9652, MAE of 14.8890, r of 0.9402 with MFE of 0.0067%. Meanwhile, $L = 19$ and $L = 48$ has the worst performances among all models whereby MAE and r for both models are 19.3706 and 0.9086, respectively. Furthermore, MFE statistical results showed that both models are over-forecast by 2.82%. Visual inspection on these models performances are presented in **Figures 11A–D**.

Based on **Figures 11A–D**, it is a clear indication that SSA-RF models able to capture general pattern of non-linear increasing trend of daily confirmed cases of COVID-19 in Malaysia. Detailed analysis from **Figure 11A** found out that model with $L = 5$ performed better that other models whereby the model able to follow the actual pattern of daily confirmed cases of COVID-19. Meanwhile, as can be seen from **Figures 11B–D**, other models which are $L = 10$, $L = 19$, and $L = 48$ unable to follow the actual pattern of the observed data. This is a clear indication that the models performed poorly as compared to $L = 5$ model.

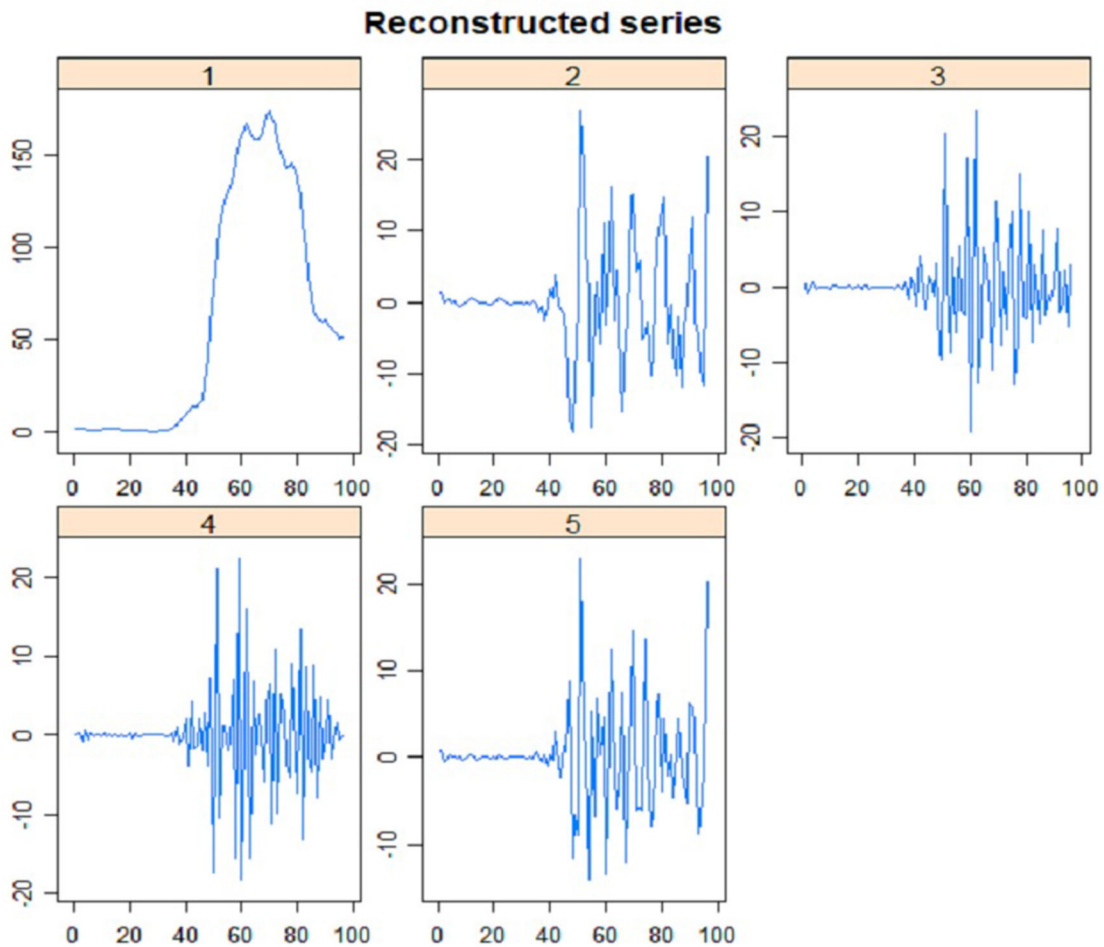
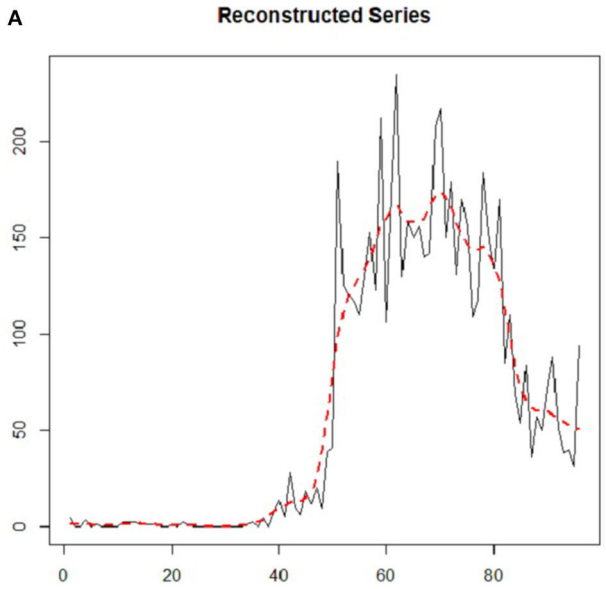


FIGURE 7 | First stage: Elementary Reconstructed Series (L = 5).

Next, the SSA-RF models were used to predict future cases starting from 30th April to 31st May 2020. At the time of this study, the historical cases from 25th January to 29th April 2020 were used and the future 32 days ahead of COVID-19 cases had been predicted accordingly. **Figures 11A–D** illustrates the confirmed cases from 25th January to 29th April 2020 and the forecasted daily cases until 31st May 2020. It is worth noting that the figures display a noticeable but faint decreasing pattern from 5th April 2020 onwards. One of the contributing factors for the decreasing trend was due to the MCO announced by the Malaysian Government which took place on 18th March 2020. The above figures also illustrate the predicted values of 32 day ahead using SSA-RF algorithm against confirmed cases of COVID-19 in Malaysia. Despite the encouraging statistical finding based from the historical data and lower under-forecast value; the SSA-RF models failed to capture the sudden drop in the COVID-19 cases, which is considered to have never happened before. This sudden drop was highly likely due to the MCO that was extended to phase-4, which ended on 12th May 2020.

During the MCO, Malaysians were advised to stay at home as much as possible to minimize the spread of further COVID-19 infections. All schools and most workplaces were closed, and they were directed to work from home except for essential services. Traveling ban, restriction movement order including interstate movement, restriction on gatherings, and public transport closure were imposed strictly by the government. Active case detection was continued, followed by isolation of the cases, and the close contacts were tested and quarantined to further curb the spread of COVID-19. All these actions successfully plateaued and reduced the number of COVID-19 cases (**Figures 11A–D**). In addition, the cases were reduced due to the incubation period of the virus between 2 to 14 days, and the recent findings from WHO has stated that after 5–10 days of the infection, the infected individual starts to gradually produce neutralizing antibodies which will decrease the risk of transmission to others (41, 42). WHO has also reported three research that found the inability of SARS-CoV-2 virus to be cultured after 7–9 days of onset of symptoms (43, 44). From all the latest findings, WHO has concluded that after 14 days, the patients are not likely to be



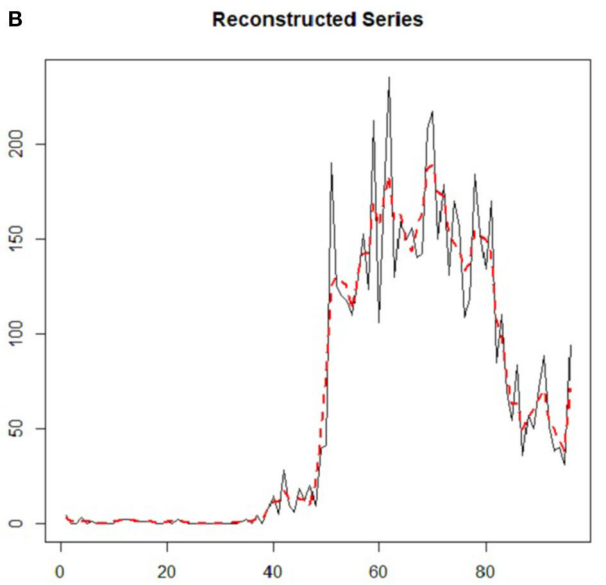


FIGURE 8 | (A,B) Daily COVID-19 cases of reconstructed components from extracted trends using SSA at **(A)** L = 5, ET 1 **(B)** *L = 5,* ET 2.

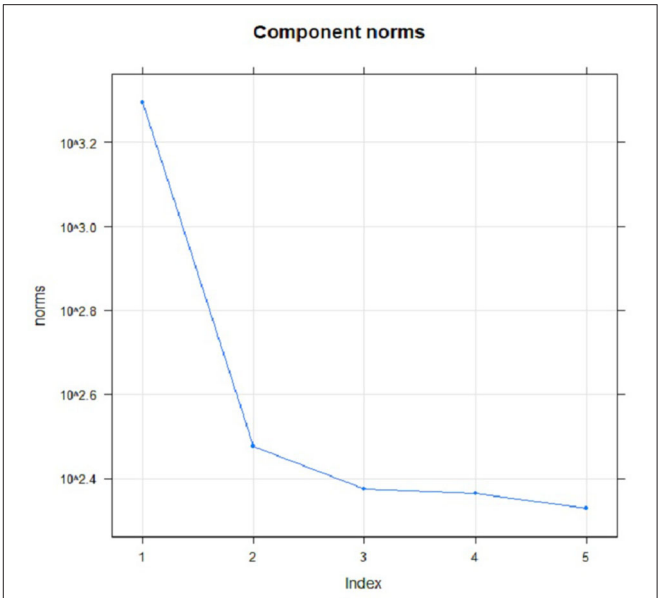


FIGURE 9 | Logarithms of five eigenvalues.

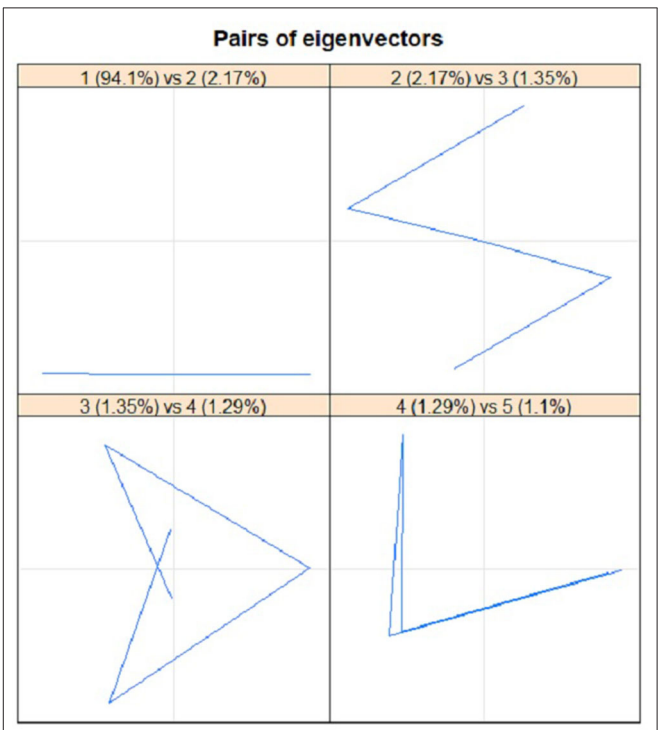


FIGURE 10 | Plots of eigenvectors (EV) pairs: 1-EV and 2-EV, 2-EV and 3-EV, 3-EV and 4-EV, as well as 4-EV and 5-EV for COVID-19 cases.

infectious (45). The government's decision to extend the MCO up to 12th May had successfully plateaued and reduced the curve as it provides sufficient time to break the virus transmission.

Furthermore, the figures showed that different window length suggested a different forecasted value of future cases. For an instance, SSA-RF with L = 48. Nineteen and 10 predicted that there will be insignificant changes in the number of future cases, while SSA-RF with L = 5 showed there will be a significant drop in the future cases. Other than that, the model also suggested that Malaysia will reach single digit in COVID-19 cases by early June 2020. However, the model unable to predict the date for total eradication of COVID-19 cases. This is consistent with WHO which indicated that this virus will not be eradicated even after the vaccine is found. It might persist to be endemic in

certain countries and will need cooperation on a global scale and leveraging tools such as contact tracing and disease surveillance to defeat COVID-19.

Limitation of SSA-RF Model

Some limitations of this study, which should be emphasized when using the SSA-RF model in assessing the pandemic data in Malaysia, are as follows:

- The SSA-RF model works best when the data exhibit a stable or consistent pattern over time with a minimum amount of outlier. This can help to obtain accurate and precise results for future predictive cases.
- The sudden spike in data leads to low performance of forecasting results using this predictive SSA-RF model.
- The SSA-RF model is mainly used to project future values using historical time series data for short-term forecast.
- Recurrent forecasting approach is a better contender than vector approach for forecasting both short and medium time series data of SSA. However, under such scenarios, it is advisable that users also evaluate the performance of forecasting SSA approach on their data to arrive at a complete picture.
- Although SSA able to capture the pattern of the Coronavirus COVID-19 cases, however, its ability in predicting the cases accurately is still need to be investigated further.
- Different observed behavior of a dataset might influence the selection of window length.
- This model did not take into account the effect of incubation period in transmission of the virus, the effect of the government measures to curb the spread of COVID-19.

TABLE 2 | SSA-RF Prediction Performance Several Window Length (*L*).

L	MAE	*r*	MSE	
$T/2 = 48$	19.3706	0.9086	−2.8249	Over-forecast
$T/5 = 19$	19.3706	0.9086	−2.8249	Over-forecast
$T/10 = 10$	14.8890	0.9402	0.0067	Under-forecast
$T/20 = 5$	11.2549	0.9619	0.1920	Under-forecast

CONCLUSION

This study assessed the applicability of SSA-RF model in predicting the COVID-19 cases in Malaysia. The application of this model is specifically advantageous for the health authorities in terms of flattening the curve by devising prompt and effective strategies. This model allows the health authorities to comprehend the outbreak pattern better. The pattern retrieved from the SSA-RF model can be applied to forecast the outbreak cases growth pattern in Malaysia. The parameters used in this model were window length, L, and the total of *ET* employed for reconstruction, r. The results revealed that parameter $L = 5$ ($T/20$) was suitable for short time series outbreak data and the appropriate number of leading *ET* s to obtain was crucial as it affected the forecasting outcomes. Overall, the results showed that the SSA-RF model could forecast this pandemic

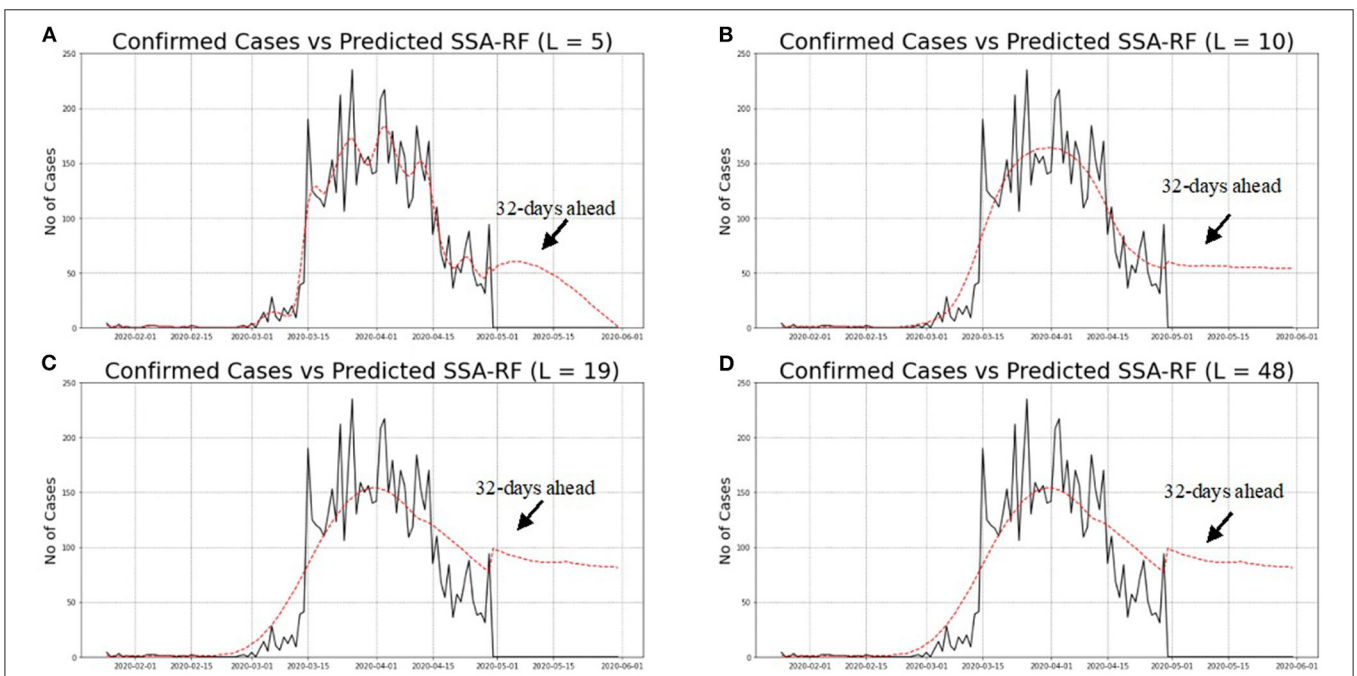


FIGURE 11 | (A–D) Predicted SSA-RF and confirmed cases of COVID-19 in Malaysia for Various Windows Length *(L)*.

with reasonable accuracy as the model had under-forecasted by 0.1920% with high correlation values between confirmed and predicted cases. Nevertheless, the SSA-RF model failed to capture the sudden drop in COVID-19 cases, likely due to the MCO that was extended to 12th May 2020. In order to improve the accuracy of the model, more information is required to better predict the COVID-19 cases for a long period. In the meantime, case definition and data collection must be maintained in real-time to enhance the RF-SSA for further evaluation. It is suggested that the SSA-RF model is enhanced to enable the model to capture sudden and rapid changes in the dataset.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SS and SI conceived the presented idea, developed the theory, and performed the computations. NH, MT, and NS verified the analytical methods and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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The Epidemiological and Spatiotemporal Characteristics of the 2019 Novel Coronavirus Disease (COVID-19) in Libya

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COVID-19 is a global pandemic that has affected all aspects of life. Understanding its geographical and epidemiological characteristics has become particularly important in controlling the spread of the pandemic. Such studies are lacking in North African countries, particularly in Libya, which has the second largest area of any country in Africa and the longest coast facing Europe. The objectives of this study are to determine the epidemiological parameters and spatiotemporal patterns of COVID-19 and outline strategies for containing the spread and consequences of the pandemic. This comprehensive study included all the confirmed cases of COVID-19 since its emergence in Libya on March 24, 2020 until July 31, 2020. The epidemiological characteristics of COVID-19 were analyzed and the spatial dynamic trends were explored. Regional counts of weekly reported cases were used to characterize the spatial dynamics of COVID-19. A total of 3,695 confirmed cases of COVID-19 were recorded: 2,515 men (68.1%) and 1,180 women (31.9%), with a male-to-female ratio of 2.1:1. Ages ranged between 2 and 78 years. Older patients infected with COVID-19 were at a risk of higher disease severity and mortality. Broad geographic variability and spatiotemporal spread variation of the COVID-19 pandemic in Libya was observed, indicating a significant increase of COVID-19 spread starting in the middle of July 2020, particularly in the western and southern regions, although it was consistently reported in the central and eastern regions as well. Assessing the spatiotemporal dynamics of COVID-19 in the early stages of the epidemic is particularly important in understanding the pandemic spread. Such assessments are essential for designing effective prevention and control programs aimed at reducing the impact of the COVID- 19 pandemic, particularly in countries with limited resources.

Keywords: Libya, COVID-19, epidemiology, spatiotemporal analysis, dynamics, geography

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BACKGROUND

The ongoing coronavirus disease (COVID-19) pandemic has reached each country in the world and no one can be considered safe. The pandemic affects all aspects of life, socially, economically, politically, and even morally. Since its emergence, countries and health authorities have responded comprehensively but differently (1). However, the impact

may vary between and within countries, partly because of the degree to which control strategies are adopted and executed. Countries such as Sweden and Germany responded early and successfully. Others, such as Italy, Spain, and France, acted differently and thus it resulted in a high number of deaths (2–4). The impact was even worse in developing countries such as Iran and Brazil, as the early action was inappropriate and was influenced by local understanding (5, 6). Hence, the epidemiology and impact of COVID-19 varies greatly from one country to another. Understanding these epidemiological parameters has become particularly important for each country.

In Africa, the incidence of COVID-19 has varied considerably between countries, possibly reflecting variations in the volume of air travel and differences in SARS-CoV-2 testing (7). Tackling COVID-19 has become increasingly difficult in northern and sub-Saharan countries, where the effects of internal armed conflicts and the emergence of other viral epidemics on the economy and health structures are still being felt (8, 9). Only a few African states have been successful in implementing detection, prevention, and control measures. Yet the COVID-19 pandemic poses a challenge not only for fragile African countries but also for those with well-functioning health systems. Until now, studies evaluating the epidemiological and spatial spread of COVID-19 in Africa have been limited. Understanding the spread of the pandemic is critical for predicting local outbreaks and developing public health policies during the early stages of COVID-19 (10, 11).

Libya, the second largest country in Africa and with the longest coast on the Mediterranean, facing Europe, has been involved in an armed conflict since 2011. The country is considered vulnerable to the spread of infectious diseases, including COVID-19. Armed conflicts and internal instability challenge disease control and have a very deleterious effect on the provision of health services (12, 13). Due to the low levels of international commerce and travel in the country, the seeding of COVID-19 came later than in other North African countries. The first few cases of COVID-19 identified in Libya arrived in March 2020 (14). Now that COVID-19 has taken a strong hold in the country, displaced people and immigrants can help spread it from one city to another (15, 16). Accordingly, COVID-19 is likely spreading rapidly in Libya but is to a large extent undetected by the health authorities. Understanding the epidemiological manifestations and local variation in the dynamics of the pandemic is a crucial step for developing more effective strategies for mitigating the risk of infection in vulnerable communities. Unfortunately, to date, there is no global standard response to the pandemic and each country is facing the crisis based on its own possibilities, expertise, and hypotheses (17).

Different studies have analyzed the epidemiological manifestations and geographic mapping of COVID-19 (10, 18). Such information is particularly important not only for controlling COVID-19 but also for planning to ameliorate the consequences of the epidemic. However, there is a lack of information on the epidemiology and clinical features of COVID-19 patients in North Africa and particularly in Libya. The objectives of this study were to evaluate the epidemiological and spatiotemporal distribution of COVID-19 in Libya and to highlight strategies for appropriate allocation of the healthcare resources to combat the spread of the pandemic.

MATERIALS AND METHODS

Patient Information and Data Collection

The National Center for Disease Control in Libya performs laboratory tests for SARS-CoV-2, investigations, contact tracing, and quarantine at the regional or district level. We collected information provided by the Center on the demographics, epidemiological information, clinical symptoms, and outcomes from all laboratory-confirmed cases of COVID-19 initially suspected/identified by symptoms or through contact tracing all over the country between March 24, 2020 and July 31, 2020. The data of all the registered patients were collected, extracted from the hospital records, and checked by a clinical epidemiologist. Furthermore, we collected the countrywide, daily-updated number of laboratory tests for SARS-CoV-2 and their results, which were done at a rate of about 2,000 samples per day.

Case Definitions

The definitions of the confirmed cases of COVID-19 were based on our previous publication (14, 19–21): a patient with evident clinical symptoms of COVID-19 and with a positive Nucleic Acid Amplification Test. The clinical severity of the disease was categorized as follows: (1) mild (only mild symptoms without evidence of pneumonia and not requiring oxygen therapy); (2) moderate (fever, respiratory tract symptoms, and imaging evidence of pneumonia); (3) severe (respiratory distress and respiratory rate of 30 per min in the resting state, finger oxygen saturation of 93%, and arterial blood oxygen partial pressure [PaO_2/oxygen concentration (FiO_2) of 300 mmHg (1 mmHg = 0.133 kPa)]. Critical cases were defined as those exhibiting respiratory failure and requiring mechanical ventilation, with the occurrence of septic shock, and admission to an intensive care unit with multiple organ dysfunction/failures. The pandemic spread was traced weekly (epi-weeks), which is a standard method for comparison of data during epidemic spread.

Statistical and Geographic Analysis

The epidemiological characteristics of confirmed cases of COVID-19 were analyzed descriptively using computer software (StataCorp. 2013 version 11.0. Stata Statistical Software Release 13. College Station, TX: StataCorp LP). Spatiotemporal analysis and geographic mapping of COVID-19 cases was carried out using GraphPad Software as previously described (22–24). Briefly, the geographic coordinates were recorded at the centers of the enumeration areas based on the geo-referenced information of the patients. The corresponding national standard geo-codes at the provincial, city, and county levels were included in the analysis to identify the location of the reported cases.

RESULTS

The study population consisted of all confirmed cases of COVID-19 reported in Libya by July 31, 2020 (12:00 a.m.) 2020. A total of

3,695 cases were reported, and their epidemiological and clinical characteristics are illustrated in **Table 1**. Of these cases, 2,515 were men (68.1%), with a male-to-female ratio of 2.1:1. Ages ranged from 2 to 78 years.

Only 74 patients (2%) died. The case fatality rate was higher among men (53; 2.1%) than women (21, 1.2%). Of the deceased patients, 39 (52.7%) were aged ≥ 55 years (particularly >66 years). Only 5 patients (6.6%) were under 40 years of age, and 21 patients (28.4%) were 41–55 years old. Of all the cases, 782 (21.2%) were imported, and 2,913 (78.8%) cases were acquired locally ($p \leq 0.001$). The imported cases were mainly from Egypt (257, 32.9%), Turkey (219, 28%), Tunisia (209, 26.7%), and Saudi Arabia (96, 12.3%).

The western region contributed the largest fraction of infections (1,755, 47.5%), followed by the southern region (1,133, 30.7%), the eastern region (738, 20%), and the central region (429, 11.1%). However, mortality rates were highest in the southern and central regions (2.7 and 2.6%, respectively) and lowest in the western and eastern regions (1.3 and 1.2%, respectively).

Of all the confirmed cases, 2,368 (64.0%) were mild, 1,108 (30%) were moderate, 128 (3.5%) were severe, and 91 (2.5%) were critical. The highest mortality rates were observed among the critical and severe cases: 38 (51.4%) and 26 (35.1%), respectively ($p < 0.001$). It was much lower among the moderate (7, 9.5%) and mild cases (3, 4.1%).

The age distribution of men and women is shown in **Figure 1**. The median age of the infected individuals was 55 years; 26.7% were aged ≥ 60 years and only 2.4% were <15 years. The occurrence of infection increased progressively with age, with men showing higher rates except for the oldest age group (>65 years) ($p \leq 0.001$). This difference was also significant among patients above 50 years of age. The number of infected cases was higher among male patients (68%), indicating that COVID-19 tended to be more serious in men according to the clinical classification of severity. The association between illness severity and age is shown in **Figure 2**: illness severity increased with age. Most mild cases were among those aged below 45 years, followed by moderate cases. Most severe and critical cases were among older patients, particularly those aged ≥ 60 years ($p < 0.001$),

TABLE 1 | Epidemiologic and demographic characteristics of 3,695 confirmed cases of COVID-19 infection in Libya.

		Survived	Died	Total	*P* value
Demographic characteristics		**3,621**	**74**	**3,695**	
			***n* (%)**		
	Male	2,462 (68)	53 (71.6)	2,515 (68.1)	<0.001
	Female	1,159 (32)	21 (28.4)	1,180 (31.9)	0.01
Age group					
	≤ 15	86 (2.38)	0 (0)	86 (2.3)	0.01
	16–20	142 (4)	0 (0)	142 (3.8)	0.01
	21–25	161 (4.5)	1 (1.4)	162 (4.5)	0.02
	26–30	241 (7.5)	0 (0)	241 (6.5)	0.01
	31–35	271 (7.5)	0 (0)	271 (7.3)	0.01
	36–40	307 (9)	3 (4.1)	310 (8.4)	0.01
	41–45	327 (9)	5 (6.8)	332 (9)	0.01
	46–50	350 (9.5)	7 (9.5)	367 (10)	0.01
	51–55	379 (10.5)	9 (12.2)	388 (10.5)	0.01
	56–60	398 (11)	13 (17.6)	409 (11.1)	<0.001
	61–65	427 (11.8)	15 (20.3)	442 (12)	<0.001
	≥ 66	541 (14.9)	21 (24.4)	562 (12.2)	<0.001
Source of infection					
	Imported	751 (20.2)	31 (42)	782 (21.2)	0.01
	Local	2,870 (79.3)	43 (58)	2,913 (78.8)	<0.001
Clinical severity					
	Mild	2,327 (64.3)	3 (4.1)	2,330 (36.1)	0.01
	Moderate	1,101 (30.4)	7 (9.5)	1,108 (30)	0.01
	Severe	102 (2.8)	26 (35.1)	128 (3.5)	<0.001
	Critical	91 (2.5)	38 (51.4)	129 (3.5)	<0.001
Geographic region					
	Western region	1,732 (47.8)	23 (31.1)	1,755 (47.5)	<0.001
	Middle region	418 (11.5)	11 (14.9)	429 (11.1)	0.01
	Southern region	1,102 (30.4)	31 (41.9)	1,133 (30.7)	<0.001
	Eastern region	729 (20.1)	9 (12.2)	738 (20)	0.01

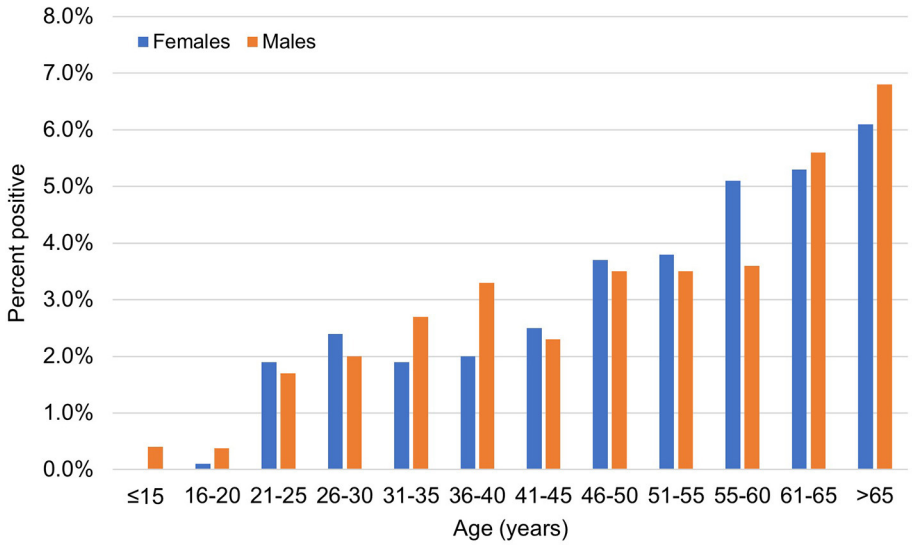

FIGURE 1 | The age and sex distribution of confirmed cases of COVID-19 infections during the study period. Men (blue bars) and women (red bars).

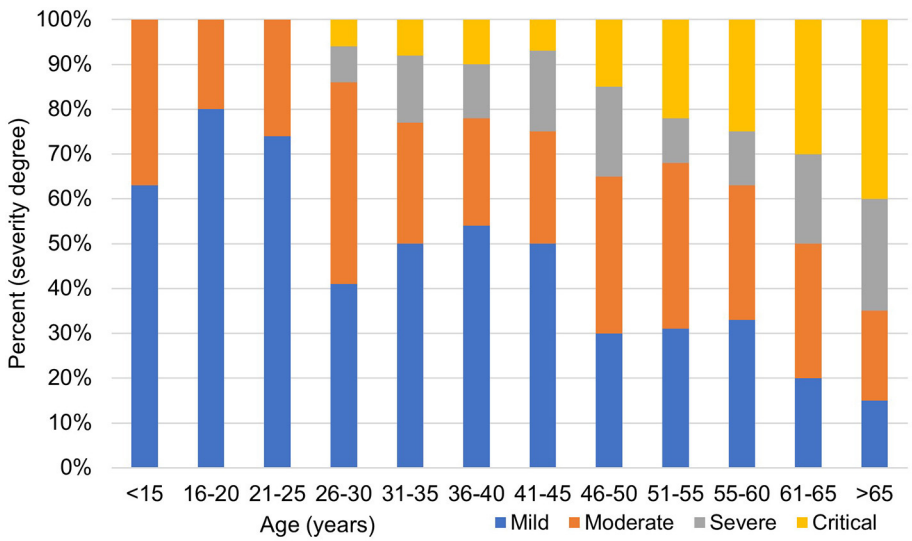

FIGURE 2 | The clinical severity of confirmed cases of COVID-19 infection.

who accounted for 45 deaths of mild cases (44.1%) and 51 severe cases (56%).

Figure 3 shows the overall temporal trend of weekly counts of newly confirmed COVID-19 cases in the four Libyan regions. Infections were sporadic until early May (first 9 epi-weeks); 120 confirmed cases were reported, mainly in the western region (97). The number of weekly confirmed COVID-19 cases subexponentially increased across the country from the 10th to the 17th epi-week, followed by a slow decrease. During the entire observation period, the highest number of cases was reported in the western region (47.8%), followed by the southern region (30.4%), middle region (30.4%), and eastern region (20.1%) (**Figure 4**). However, during epi-weeks 9–16, the proportion of confirmed cases decreased in the eastern and central regions, but increased substantially in the southern region (**Figure 4**).

Spatiotemporal analysis and geographic mapping showed a marked geographic and temporal variation of COVID-19 cases during the pandemic period, as shown in **Figure 5**. In the first eight epi-weeks, the emergence of the pandemic was detected in five counties in the western region, including Tripoli, which hosted the largest number of cases, followed by Zawia, Surman, Aljalaet, and Nalut. Clustering analysis showed that new clusters

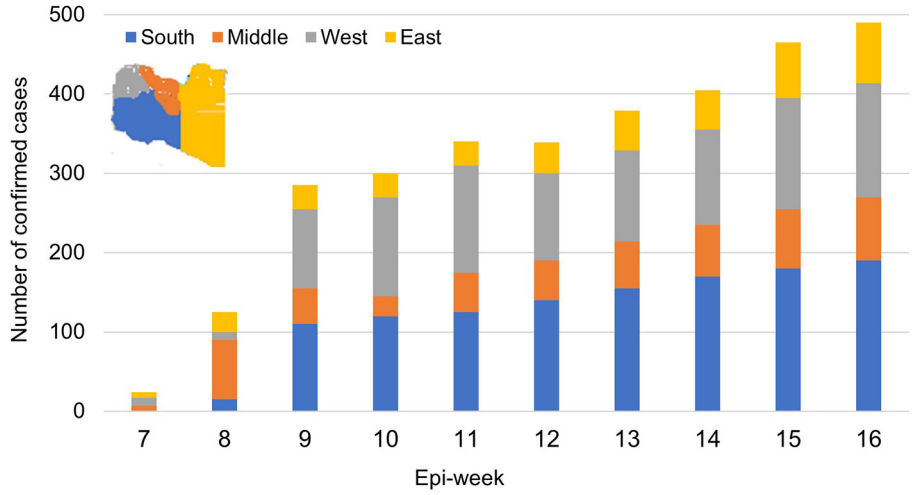

FIGURE 3 | The weekly incidence trends of confirmed cases of COVID-19 in each Libyan region during the study period.

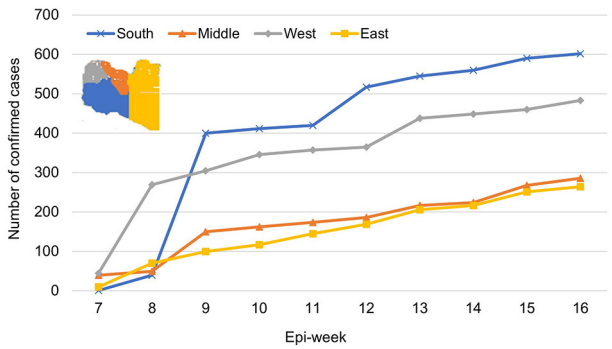

FIGURE 4 | The prevalence of COVID-19 infections among the Libyan population in the four geographic regions over 16 epi-weeks (April 03–July 31, 2020).

emerged in the 9th epi-week, largely in the southern and western regions ($p \leq 0.001$). In the following epi-weeks, the epidemic spread all over the country and new cases were reported in each of the 22 counties (**Figure 5B**). The cases were spatially distributed with agglomeration characteristics (**Figure 5**). The increase in the number of infections in one city will inevitably lead to increases in adjacent cities, which means that a positive spillover effect occurs.

DISCUSSION

The epidemiological and clinical features of the COVID19 pandemic in Libya are characterized in this study. By July 31, 2020, 3,695 cases were reported, representing 6.2/10,000 of the population, and the overall death rate of infected individuals was 1.3%. Median age was 55 years and the male to female ratio was 2.1:1. Our data show that COVID-19 infected men more than women; these findings are in concordance with other studies reported from China and Iran (25, 26). This indicates that sex and gender disparities are involved, or even sociocultural factors, particularly in the Middle East and Africa, and points to the need to gain a better understanding of the impact of sex and gender on the incidence and case fatality of the disease and to tailor treatment accordingly. However, other studies have shown that susceptibility to SARS-CoV-2 does not differ between men and women (27). In Libya, geographic differences were obvious. In particular, the southern region, which has the smallest population, contributed 30.7% of all infections.

Taking patient age into consideration, children (<15 years) accounted for only 2.4% and those aged 20–50 years accounted for 9.0% of all infections. Those aged over 60 years represented the largest fraction of infected cases, accounting for 20%. Clearly, COVID-19 among Libyans corresponds with higher age. In Italy, Spain, and France, most deaths in infected individuals occurred in elderly people suffering from severe conditions, particularly in the early phases of the epidemic (28, 29).

Based on our data, most of the reported cases were mild (2,368, 64.0%) or moderate (1,108, 30.0%). Only 128 patients (3.5%) had severe illness, and 91 (2.5%) were critical. The association between illness severity and age was evident. This study has shown that men tend to have more serious illness than women. This is in agreement with other studies carried out by Jin et al. (19) and Li et al. (30). Therefore, male sex may be considered as a risk factor for higher severity and mortality in patients with COVID-19, independently of age.

In this study, we evaluated the spatial and temporal patterns of the COVID-19 pandemic in Libya during the first 16 epi-weeks of the epidemic. In the early stage of the COVID-19 outbreak, a few sporadic cases were reported in first six epi-weeks in Tripoli in the western region. By the end of the 8th epi-week, the infections had spread to cities neighboring Tripoli, such as Musrata and Zawia. Since then, the number of weekly confirmed

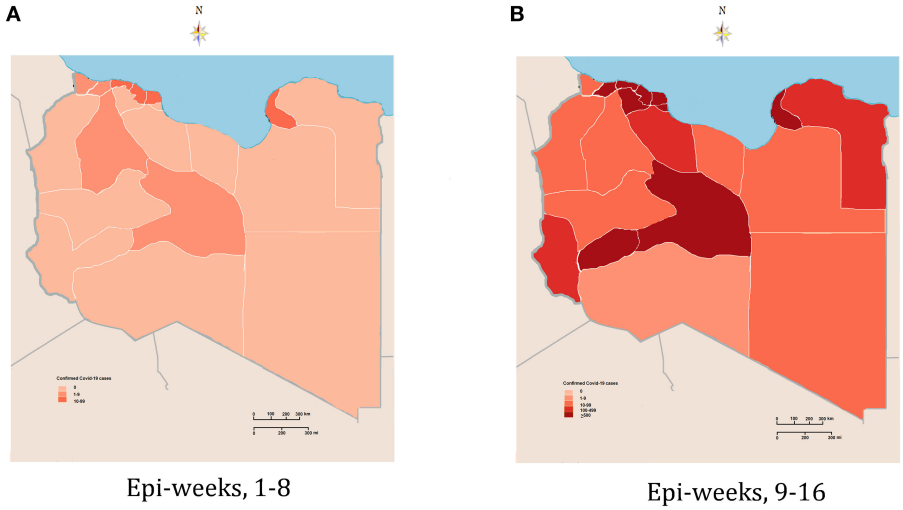

FIGURE 5 | Geographic and spatiotemporal distribution of the confirmed cases of COVID-19 in Libya. **(A)** Early stage of the epidemic (epi-weeks 1–8). **(B)** Second stage of the epidemic (epi-weeks 9–16).

COVID-19 cases exponentially increased across the country from the 9th to the 16th epi-week. During the entire study period, the prevalence of COVID-19 in the Libyan regions showed striking geographic differences, with many more infections in the western and southern regions. This likely depended on the arrival of the first wave and the population movements in the regions (31, 32).

It is clear in this study that the dynamics of the epidemic in Libya followed a geographical differentiation, with a strong western to southern gradient. This indicates that an increase in the number of infections in one city may lead to increases in adjacent cities. Further, studies are needed to shed light on this speculation. However, similar trends have been observed in the early stages of the spread of COVID-19 in Italy, Spain, and France. Hence, specific strategies should be implemented to contain the expansion of the pandemic (33, 34).

Though the study gives detailed information on the epidemiology of COVID-19 in Libya, there might be some uncertainties about how well the reported data represent reality because many asymptomatic or mild cases go undetected. Another limitation is that the study may not highlight the impact of the armed conflict, which has stopped only recently, on the spread of COVID-19 in Libya and the ability to trace and identify infections in some cities and towns (35–38).

CONCLUSION

This study is the first to provide information on the epidemiological characterization and spatial and temporal patterns of the COVID-19 outbreak during the first wave of the pandemic, which started in Libya in March 2020. The epidemic has involved the whole country, with infection rates varying from one region to another. Meanwhile, the prevention and control of COVID-19 in Libya still face an uphill struggle. The study demonstrated the spatiotemporal characteristics and trends of COVID-19 in Libya, which is essential for focusing preventive efforts. Hence, swift action to control further spread of the virus and to improve the response capabilities is urgently needed.

DATA AVAILABILITY STATEMENT

The data presented in this paper are freely available upon request.

ETHICS STATEMENT

The Medical Ethics Committee at the Faculty of Medicine, University of Tripoli approved the study and waived the need for approval by the Libyan National Ethics Committee. As the study was an analysis of epidemiological data obtained at a national population level, it needed no consent from the participants.

AUTHOR CONTRIBUTIONS

MD conceived and designed the study, wrote the paper, designed the analysis, analyzed the data, and performed cartography. MD and AE-B contributed to the analysis tools. AE-B and MA made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. MD, MA, and AE-B provided advice and critically reviewed the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Association of Gender With Outcomes in Hospitalized Patients With 2019-nCoV Infection in Wuhan

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Aim: The aim of this study was to analyze the association of gender with psychological status and clinical outcomes among patients with 2019-nCoV infection to provide new directions for the prevention and control of the pandemic.

Methods: One hundred and thirty-eight patients with confirmed 2019-nCoV infection at Wuhan Union Hospital, between February 8 and March 31, 2020, were included in the study analysis. General information and data on clinical characteristics were collected from patients' medical records. Participants' responses to self-report measures of psychological status were also collected.

Results: Anxiety levels, depression levels, and recovery rates were significantly higher among women compared to men. Conversely, chronic disease history and smoking rates, dry cough incidence, C-reactive protein levels, and disease severity were significantly higher among men than women ($p < 0.05$).

Conclusion: Female patients experienced more severe psychological issues, due to higher levels of anxiety and stress, than male patients; indicating that more attention should be paid to the psychological care of female patients. In contrast, the general condition of male patients was more severe, particularly among elderly male patients with a history of chronic disease and smoking, suggesting that, to prevent and control 2019-nCoV infection, male patients should be encouraged to quit smoking as soon as possible to reduce the risk of severe pneumonia.

Keywords: 2019-nCoV, gender, disease outcome, psychological situation, smoking

INTRODUCTION

On December 8, 2019, several cases of unexplained pneumonia were reported among patients with a history of contact with the Huanan seafood market, in the city of Wuhan, China. These patients further presented symptoms of severe acute respiratory infection, quickly developing into acute respiratory distress syndrome (ARDS) and acute respiratory failure, which were later confirmed to be caused by a novel corona-virus (1). On January 7, 2020, this novel corona-virus was isolated and

identified in a sample from a patient's throat swab by the Chinese Center for Disease Control and Prevention (China CDC). The World Health Organization subsequently named it as corona-virus disease 2019 (2019-nCoV).

2019-nCoV was first identified in China; however, countries in North and South America and Europe have been most affected by the virus. The virus spread quickly and became a global public health emergency. At present, there are more than 153 million confirmed cases of 2019-nCoV around the world, and more than 3.2 million related deaths have been recorded. In the current public health emergency, it is imperative to understand the epidemiological and clinical features of the 2019-nCoV infection. Catastrophic events and their devastating consequences are unforeseeable and unavoidable. The psychological impact of such events on the population includes fear, anxiety, depression, stress, and sleep problems, among other issues (2). High levels of fear and anxiety have significant impact on patients diagnosed with 2019-nCoV, leading to psychological complications and influencing the effectiveness of treatment. Therefore, attention to patients' psychological status is an important part of effective treatment.

Gender differences in the severity and psychological impact of the 2019-nCoV infection have not been well-researched thus far. Understanding the gender differences associated with susceptibility and vulnerability toward 2019-nCoV infection is important to respond effectively to the public health emergency and minimize the health, economic, and social effects of the pandemic. This study explored the epidemiological and clinical features of 138 hospitalized patients with confirmed 2019-nCoV infection. Patient health questionnaire-9 (PHQ-9) and generalized anxiety disorder scale-7 (GAD-7) were used to the assess the patients' depression level and anxiety level, respectively. The clinical classification of 2019-nCoV infection and MulBSTA score were used to identify the severity of 2019-nCoV infection. The associations among psychological status, clinical outcomes, and patient gender were analyzed to inform psychological and therapeutic intervention for the prevention and control of the 2019-nCoV pandemic.

MATERIALS AND METHODS

Study Design and Subjects

A retrospective, single-center study was conducted. One hundred sixty patients with confirmed 2019-nCoV infection were recruited from the three specialist wards of "2019-nCoV" in the west campus of Wuhan Union Hospital, a local hospital in Wuhan, between February 8, 2020 and March 31, 2020. Inclusion criteria was: (1) age $\geq$18 years; (2) with a positive 2019-nCoV nucleic acid test result by the reverse transcription polymerase chain reaction (RT-PCR) method; (3) met the diagnostic criteria of the Guidelines for the Diagnosis and Treatment of Novel Corona-virus (2019-nCoV) Infection (Trial Version 5) released by the Chinese National Health Commission (3); (4) hospital stay duration $\geq$ 2 weeks. Exclusion criteria was: communication barriers or the consciousness disorder due to disease severity. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Xiangya Hospital of Central South University (Approval No. 202003049; Date:04/22/2020). All the participants in the study were informed and signed the relevant consent forms.

Data Collection

General information and data on the clinical characteristics of the participating 2019-nCoV infection patients were collected from the patients' medical record. General information included gender, age, education level, smoking history, chronic disease history and infectious disease history were collected after admitted to the hospital. Data on the clinical characteristics of the 2019-nCoV infection included incubation period, pulse oxygen saturation (SPO2), clinical symptoms, related complications, laboratory reports, radiologic features, clinical classification of 2019-nCoV infection and MulBSTA score were also collected after admitted to the hospital, and used the first data after hospitalization. Patients' depression levels were assessed using PHQ-9, and their anxiety levels were assessed using GAD-7 within 3 days after hospitalization, used the first data after hospitalization. The treatment effect (discharge, death, and continued hospitalization) and hospital stay were collected at discharge.

The PHQ-9 is a self-report scale of depression consisting of nine items (4). Subjects were asked to rate each item on a scale of 0–3 on the basis of how much a symptom has bothered them during the last 2 weeks (0 = not at all, 1 = several days, 2 = more than half the days, 3 = nearly every day). A total score ranging from 0 to 27 by summing up the scores from each item. The higher the score, the more severe the depression level. A cut-off score of 5 or above on the summed-item score was recommended as depressive disorder. The total score $\geq$ 10 was believed as moderate depression, and $\geq$ 15 was considered as severe depression. The Cornbach' α coefficient of PHQ-9 scale is 0.832, the test-retest reliability is 0.934, the sensitivity is 88%, and the specificity is 99% (5).

The GAD-7, consisting of 7 items, is a self-rated scale used for screening anxiety disorder. Items are rated on a 4-point scale (0 = not at all, 1 = several days, 2 = more than half the days, 3 = nearly every day). The total score ranges from 0–21 with higher scores presenting more severe anxiety disorder. A cut-off score of 5 or above on the summed-item score was recommended as anxiety disorder. The total score $\geq$ 10 was believed as moderate anxiety, and $\geq$ 15 was considered as severe anxiety. The Cornbach' α coefficient of GAD-7 scale is 0.898, the test-retest reliability is 0.856, the sensitivity is 86%, the specificity is 96%, and Kappa value is 0.825 (6).

The clinical classification of 2019-nCoV infection was divided to three types: mild, severe and critical cases, in accordance with "Guidelines for the Diagnosis and Treatment of Novel Corona-virus (2019-nCoV) Infection (Trial Version 5)." MulBSTA scale (7) was used in the study to assess the illness severity of 2019-nCoV pneumonia: imaging multilobe infiltration (5 scores); lymphocyte $\leq 0.8*10^9$/L (4 scores); bacterial infection (4 scores); acute-smoker (3 scores); former-smoker (2 scores); hypertension (2 scores); age $\geq$ 60 years (2 scores). The higher score, the more severe the pneumonia.

Statistical Analysis

SPSS25.0 statistical software was applied for data analyzing. Measurement data conforming to a normal distribution was expressed as mean ± standard deviation ($\bar{x}\pm SD$), while *t*-test and Mann-Whitney U-test were used to compare the difference between the groups. Enumeration data was described as case numbers and percentages, and χ^2 test was employed to compare the differences between gender groups. A two-sided $p < 0.05$ was considered statistically significant.

RESULTS

Subjects Characteristics

In the study, a total of 160 patients with confirmed 2019-nCoV infection were recruited, and 22 cases were excluded from the analysis due to missing data. Among the 138 valid subjects, 59 were female (42.8%). The mean age of the subjects was 62.32 ± 11.19 years old, and 93 cases (67.4%) were ≥ 60 years old. Overall, 27 cases (19.6%) had a history of smoking, 46 cases (33.3%) had hypertension, 28 cases (20.3%) had coronary artery disease (CAD), and 26 cases (18.8%) had diabetes. Regarding the transmission route, a large cohort (61.6%) were unclear of the source of transmission of their infection. The most common clinical symptoms among patients were fever (64.5%), dry cough (58.7%), and dyspnea (26.8%). Complications, such as respiratory distress, bacterial infection, and respiratory failure, occurred in 50.7, 10.9, and 2.2% of the subjects, respectively. Most subjects (91.3%) were classified as severe and critical cases. By the end of this study, 110 subjects (79.7%) had recovered and were discharged from the hospital. The average duration of hospital stay was 28.08 ± 13.03 days (see **Tables 1**–**3**).

Gender Differences in General Characteristics

Results indicate that smoking rates were significantly higher among men (32.9%) than women (1.7%; $p < 0.05$). No significant differences were found between male and female participants in their age, education level, chronic disease history (i.e., hypertension, CAD, and diabetes, among others), and source of transmission (see **Table 1**).

Gender Differences in Clinical Characteristics

Results show that dry cough incidence was significantly higher in male patients (67.1%) than in their female counterparts (47.5%). The rate of increased C-reactive protein levels was also significantly higher in male patients (50.6%) compared to female patients (28.8%; $p < 0.05$) (see **Table 2**).

Gender Differences in Psychological Status and Clinical Outcomes

Results of the analysis of gender differences in participants' psychological status (based on PHQ-9 and GAD-7 scores) and clinical outcomes (based on 2019-nCoV infection classification, MulBSTA score, treatment effect, and hospital stay duration) show that the anxiety levels, depression levels, and recovery rates of female patients were significantly higher than male patients. Conversely, disease severity scores (based clinical classification and MulBSTA score) was significantly higher in male patients than in the female patients ($p < 0.05$) (see **Table 3**). Results of multivariate logistic regression analysis on the clinical classification of 2019-nCoV further confirm that there was significant difference between genders in the participants with severe 2019-nCoV infection (see **Table 4**).

DISCUSSION

The aim of this study was to analyze gender differences in clinical outcomes and psychological status of patients with 2019-nCoV infection. The results demonstrate that the smoking rates of male patients was significantly higher than female patients, which is consistent with smoking demographics in China. Smoking is considered a critical factor in the progression of 2019-nCoV infection (8). In addition, the participants' laboratory results showed that the rate of increased C-reactive protein levels was significantly higher in male patients than in female patients. C-reactive protein is an important indicator of inflammation and is currently believed to be a crucial factor in the prognosis of 2019-nCoV infection (8). As pneumonia progresses, C-reactive protein levels are relatively higher in the most severe cases (9, 10). However, no statistically significant differences were discovered in white blood cell counts, lymphocyte counts, and procalcitonin levels between genders in the present study. Based on the above findings, it was indicated that smoking might be the cause of the higher rate of dry cough among male patients in this study and related to the progression to critical illness of more male cases than female cases. As such, smoking cessation is an effective measure in the treatment of 2019-nCoV infection.

The most salient finding in this study is that anxiety and depression were more prevalent in the female cohort compared to the male cohort, while the overall clinical classification and MuLBSTA score of male patients was higher than female patients. When faced with a sudden public health emergency, people are generally prone to develop psychological issues, such as tension, anxiety, panic, and pessimism, among others. Research on the SARS pandemic found that, while it was initially ignored, people's emotional responses to the event included fear, annoyance, complaints, and anxiety; these responses progressively developed into depression, loneliness, helplessness, hopelessness, and sadness (11, 12). Anxiety and depression may decline over time; however, some of the symptoms may persist throughout the disease process, affecting the effectiveness of treatment and follow-up recovery (13). Results of an analysis of gender differences in the mental health of patients during the SARS pandemic show that the severity of psychological problems among female patients was significantly higher than in male patients (14). Moreover, female gender was identified as the most potent indicator of post-traumatic stress symptoms after the 2019-nCoV outbreak (15). The results of this study are consistent with previous studies, which show that females have experienced greater psychological problems than males during the 2019-nCoV pandemic. Thus, greater attention should be paid to the mental health of the female patients during the pandemic.

TABLE 1 | Gender differences in general characteristics of 2019-nCoV infection.

Variables	Total (n = 138)	Male (n = 79)	Female (n = 59)	t/X^2 value	p-value
Age (years)	62.32 ± 11.19	62.26 ± 9.72	62.14 ± 12.98	0.166	0.869
Academic degree *n* (%)				0.599	0.439
≤Junior school	79 (57.2)	43 (54.4)	36 (45.6)		
≥High school	59 (42.8)	23 (39.0)	36 (61.0)		
Smoking *n* (%)				20.914	0.000
Yes	27 (19.6)	26 (32.9)	1 (1.7)		
No	111 (80.4)	53 (67.1)	58 (98.3)		
Chronic disease history *n* (%)					
HBP	46 (33.3)	30 (38.0)	16 (27.1)	1.791	0.181
CAD	28 (20.3)	14 (17.7)	14 (23.7)	0.754	0.385
DM	26 (18.8)	15 (19.0)	11 (18.6)	0.003	0.959
Transmission route *n* (%)				0.225	0.635
Exposure history	53 (38.4)	29 (36.7)	24 (40.7)		
Unknown cause	85 (61.6)	50 (63.3)	35 (59.3)		

HBP, hypertension; CAD, coronary artery disease; DM, diabetic mellitus.

TABLE 2 | Gender differences in clinical characteristics of 2019-nCoV infection.

Variables	Total (n = 138)	Male (n = 79)	Female (n = 59)	Z/X^2 value	p-value
Incubation period (days)	9.19 ± 5.18	9.73 ± 5.32	8.44 ± 4.94	−1.380	0.168
Symptom *n* (%)					
Fever	89 (64.5)	52 (65.8)	37 (62.7)	0.143	0.706
Dry cough	81 (58.7)	53 (67.1)	28 (47.5)	5.369	0.020
Dyspnea	37 (26.8)	21 (26.6)	16 (27.1)	0.005	0.944
Asthenia	36 (26.1)	22 (27.8)	14 (23.7)	0.297	0.586
SPO_2(%)	95.06 ± 4.59	94.85 ± 3.97	95.34 ± 5.34	−0.870	0.384
Complication *n* (%)					
Respiratory distress	70 (50.7)	45 (57.0)	25 (42.5)	2.876	0.090
Bacterial infection	15 (10.9)	9 (11.4)	6 (10.2)	0.052	0.819
Respiratory failure	3 (2.2)	2 (2.5)	1 (1.7)	0.111	0.739
Laboratory result *n* (%)					
Normal or decreased WBC count	134 (97.1)	77 (97.5)	57 (96.6)	0.088	0.766
Decreased lymphocyte count	55 (39.9)	32 (40.5)	23 (39.0)	0.033	0.857
Increased C-reactive protein level	57 (41.3)	40 (50.6)	17 (28.8)	6.633	0.010
Increased procalcitonin	48 (34.8)	32 (40.5)	17 (28.8)	2.770	0.250
Imaging multilobe infiltration *n* (%)	135 (97.8)	79 (100)	56 (94.9)	4.106	0.128

SPO_2, oxygen saturation; WBC, white blood cell.

Previous studies have shown conflicting results regarding gender differences in the severity of 2019-nCoV infection. A recent meta-analysis proposes that male gender may be a predictor of more severe 2019-nCoV infection but not of mortality (16). An analysis of 78 patients with 2019-nCoV infection in Anhui Province found that male patients accounted for more severe and critical cases compared to female patients (9), which may be closely associated with the higher rate of smoking among Chinese men. Additionally, the MuLBSTA score has been reported to be a strong predictor of the risk of death in patients with viral pneumonia (7). Some studies have revealed that male 2019-nCoV infection cases, especially among elderly patients with underlying health problems, have a higher mortality rate compared with female cases (17). In the present study, male patients had higher MuLBSTA scores compared with female patients, and all three deaths that occurred during the study were male. These findings confirm the suggestion that male gender plays a critical role in the severity and mortality of 2019-nCoV infection.

It was not unexpected to find that the mental health status of the study patients was not parallel to the severity of their infection and or risk of mortality, since the mental health

TABLE 3 | Gender differences in psychological status and clinical outcomes of 2019-nCoV infection.

Variables	Total (n = 138)	Male (n = 79)	Female (n = 59)	Z/X^2 value	p-value
PHQ-9 score (± SD)	5.74 ± 4.95	4.52 ± 4.40	7.31 ± 5.20	−3.165	0.002
GAD-7 score (± SD)	4.23 ± 4.35	3.32 ± 3.93	5.40 ± 4.62	−2.899	0.004
Clinical classification *n* (%)				6.605	0.037
Mild	12 (8.7)	4 (5.0)	8 (13.6)		
Severe	110 (79.7)	62 (78.5)	48 (81.4)		
Critical	16 (11.6)	13 (16.5)	3 (5.0)		
MuLBSTA score (± SD)	8.04 ± 3.25	8.87 ± 3.48	6.91 ± 2.50	−3.360	0.001
Treatment effect *n* (%)				7.157	0.028
Discharge	110 (79.7)	57 (72.2)	53 (89.8)		
Death	3 (2.2)	3 (3.8)	0 (0.0)		
Continued hospitalization	25 (18.1)	19 (24.0)	6 (10.2)		
Hospital stay (days)	28.08 ± 13.03	28.78 ± 12.45	27.31 ± 13.72	0.661	0.508

TABLE 4 | Multivariate logistic regression analysis on the clinical classification of 2019-nCoV.

Variables	Severe			Critical		
	β	OR (95%CI)	p	β	OR (95%CI)	P
Gender (0.female,1.male)	1.577	4.842 (1.066–22.006)	0.041	1.701	5.478 (0.536–56.027)	0.152
Smoking (0.no,1.yes)	−0.470	0.625 (0.095–4.135)	0.626	1.366	3.921 (0.386–39.844)	0.248
PHQ-9 score	0.096	1.100 (0.888–1.364)	0.382	0.131	1.140 (0.847–1.534)	0.387
GAD-7 score	0.120	1.128 (0.875–1.452)	0.352	0.004	1.004 (0.702–1.437)	0.981

OR, odds ratio. The clinical classification of 2019-nCoV infection was divided to three types: mild, severe and critical cases, in accordance with "Guidelines for the Diagnosis and Treatment of Novel Corona-virus (2019-nCoV) Infection (Trial Version 5)".

of the less severe patients is usually ignored by front-line clinical staff. Therefore, aside from the treatment of pneumonia, more psychological interventions should be provided for female patients, and smoking cessation interventions and advanced therapeutic treatments should be provided to male patients in clinical care.

There are not without some limitations in this study. First, the data was taken from the inpatients of 2019-nCoV infection in a single hospital, and 2019-nCoV infection presenting with mild symptoms was not included as this population was either quarantine at home or in the mobile field hospitals, therefore some finding might not by fully representative the whole cohort of 2019-nCoV infection. Secondly, due to the termination of the researchers' support work in Wuhan, some few patients were not fully tracked till the end, so the disease outcomes of these patients were not completely accurate. In a follow-up study, the research team plans to collect more data from more hospitals, and extend the investigation content. Multivariate logistic regression analysis in the single gender group will also be applied to provide a stronger evidence base for the prevention and control of 2019-nCoV infection.

CONCLUSION

Results of this study indicate the need for the implementation of different interventions and nursing measures based on gender in the treatment of 2019-nCoV infection in hospitals. Generally, female patients experienced more severe psychological issues due to higher levels of anxiety and stress; thus more attention might be paid to the psychological counseling and care of these female patients. Comparatively, the psychological status of male patients appeared to be less intense, but their general condition was more severe, particularly in elderly patients with a history of chronic disease and smoking. This population is, therefore, the focus of clinical care and is likely to require increased monitoring and respiratory support. Male patients should be encouraged to quit smoking as soon as possible in order to reduce the risk of severe pneumonia during the 2019-nCoV infection.

RECOMMENDATION

1. This study sheds light on the gender differences in psychological status and reveled that mental health level was not parallel to the severity degree of disease.
2. The study discovered that female experienced sever psychological issues than male, thus more attention should be paid to the psychological counseling and care of these female during the 2019-nCoV infection.
3. The study revealed that male patients had a higher level of mortality rate and disease severity degree than those of female patients, particularly in the elderly patients with a history of chronic disease and smoking.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Xiangya Hospital of Central South University (Approval No. 202003049). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HH, FZ, XP, GD, XC, and LP contributed in conception, study design, coordination of data collection, and acquisition in data. HH, FZ, XP, and GD were responsible for interpretation of data, drafting, writing, and finishing the manuscript. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Clinical Profile, Pharmacological Treatment, and Predictors of Death Among Hospitalized COVID-19 Patients With Acute Kidney Injury: A Population-Based Registry Analysis

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Introduction: One of the worst clinical outcomes of the coronavirus disease 2019 (COVID-19) pandemic was acute kidney injury (AKI).

Methods: This manuscript presents results from a population-based registry study assessing treatment, comorbidities, and predictors of hospital death among COVID-19 patients with AKI from March 1st to May 31th, 2020. Death, oxygen delivery and ventilation, acute dialysis need, use of medications, and various clinical outcomes, in addition to the length of stay in the hospital and intensive care unit (ICU), were evaluated.

Results: In Castile and Leon, the largest region of Spain, 10.87% of the patients admitted for COVID-19 ($n = 7{,}307$) developed AKI. These patients were known by having hypertension (57.93%), cardiovascular disease (48.99%), diabetes (26.7%) and chronic kidney disease (14.36%), and they used antibiotics (90.43%), antimalarials (60.45%), steroids (48.61%), antivirals (33.38%), anti-systemic inflammatory response syndrome (SIRS) drugs (9.45%), and tocilizumab (8.31%). Mortality among patients with AKI doubled that observed in patients without AKI (46.1 vs. 21.79%). Predictors of hospital death in COVID-19 patients with AKI were ventilation needs (OR = 5.9), treatment with steroids (OR = 1.7) or anti-SIRS (OR = 2.4), severe acute respiratory syndrome (SARS) occurrence (OR = 2.8), and SIRS occurrence (OR = 2.5).

Conclusions: Acute kidney injury is a frequent and serious complication among COVID-19 patients, with a very high mortality, that requires more attention by treating physicians, when prescribing medications, by looking for manifestations particular to the disease, such as SARS or SIRS.

Keywords: SARS-CoV-2, COVID-19, acute kidney injury, chronic kidney disease, treatment, mortality

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INTRODUCTION

Acute kidney injury (AKI) continues to affect between 10 and 40% of in-hospital coronavirus disease 2019 (COVID-19) patients (1–4). Since the beginning of the COVID-19 pandemic, mostly elderly individuals with many comorbidities have developed AKI and died (5, 6). The adaptation of mechanisms to the kidneys that respond to hemodynamic changes, inflammation, and other stress-inducing situations, perform worse in cases of previous kidney affectation, diabetes, heart failure, etc. Furthermore, in COVID-19 patients, systemic inflammatory response syndrome (SIRS) and severe acute respiratory syndrome (SARS) may exhaust kidney function capacities, leading to the appearance of AKI (4).

Acute kidney injury is a direct result of COVID-19 infection (7) and is common in critically ill patients, being one of the poor clinical outcomes with a negative prognosis for survival (4, 8).

In addition, AKI incidence and death rates are changing throughout the regions of world, probably in relation to the characteristics of the individuals in those regions. In this sense, we report our pharmacological, clinical, and epidemiological findings related to the in-hospital COVID-19 patients with AKI.

The main aim of this study was to describe the pharmacological treatment and the clinical baselines of the in-hospital COVID-19 patients affected by AKI (March 1st to May 31th, 2020), in Castile and Leon, the largest region of Spain. Furthermore, we have analyzed the risk factors associated with deaths of COVID-19 patients with AKI. Finally, the influence of AKI on the survival of the in-hospital COVID-19 patients was analyzed.

METHODS AND MATERIALS

Real-World Study Details

This article presents findings from an epidemiological analysis carried out following a population-based registry study design, with the collection of clinical and administrative data. The Reporting of studies Conducted using Observational Routinely-collected health Data (RECORD) recommendations (9) and the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) standards (10) were adhered to. Ethics committee approval reference and date: PI 20-1863, June 11th, 2020.

This study evaluated cross-sectionally clinical findings, treatment, and outcomes from a population of COVID-19 patients with AKI. These study participants were selected from the total COVID-19 patient population with a recorded stay in public Castile and Leon hospitals between March 1st and May 31th, 2020. COVID-19 was diagnosed by in-hospital treating physicians who decided on hospitalizing the patients on the basis of clinical or radiological findings defining SARS (**Supplementary Table 1**). A positive result on the COVID-19 real-time reverse transcription polymerase chain reaction (rRT-PCR) test for qualitative detection of the nucleic acid from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was not required to formally diagnose the disease. SIRS was diagnosed following the proper criteria (**Supplementary Table 1**) (11).

Definitions and Data Sources

Acute kidney injury was diagnosed in the hospital by treating physicians using the Kidney Disease Improving Global Outcomes (KDIGO) criteria (12) and, following these recommendations, by calculating glomerular filtration rate (GFR) with the Modification of Diet in Renal Disease (MDRD) study equation, or the Chronic Kidney Disease EPIdemiology Collaboration (CKD-EPI) equation (**Supplementary Table 1**): briefly, an increase in serum creatinine (SCr) by $\geq$0.3 mg/dL within 48 h or $\geq$1.5 times within the prior 7 days, in addition to an urine volume $<$0.5 ml/kg/h for 6 h, was required.

These patients may have chronic kidney disease (CKD), categories 3–5, defined by an estimated GFR of 60 ml/min or lower (13): the MDRD or CKD-EPI equations were used for following up the kidney function of patients with visits to Nephrology units, and the Cockcroft-Gault formula was used for patients with visits to primary healthcare centers depending on public Castile and Leon hospitals. Dialysis patients were excluded.

Cardiovascular disease was defined by the occurrence of major adverse cardiovascular events (MACE), which included all non-fatal coronary events, including revascularization procedures, and all cerebrovascular events, including transient ischemic attacks (TIA). Peripheral artery disease and decompensated heart failure (HF) were considered indicative of cardiovascular disease. Diabetes, hypertension, and other well-known cardiovascular risk factors were also considered.

Access to registries containing EHR information from the Castile and Leon hospitals and associated primary healthcare centers (Jimena and Medora, https://www.saludcastillayleon.es/sanidad/cm), hospital pharmaceutical care information in our region (Concylia, http://www.saludcastillayleon.es/portalmedicamento/es/indicadoresinformes/concylia) and hospital discharges information in Castile and Leon (Pestadistico, https://pestadistico.inteligenciadegestion.mscbs.es/publicoSNS/N/rae-cmbd/rae-cmbd), was obtained.

Variables

The main outcome during the study period was death (March 1st to May 31th, 2020). Other outcomes were stays in the hospital and intensive care unit (ICU) (length in days), the need for acute dialysis, SARS, SIRS, disseminated intravascular coagulation (DIC), cardiomyopathy, and bacterial and fungal superinfection.

The use of medications to treat COVID-19 according to the Spanish national recommendations (14, 15) (**Supplementary Table 2**) during the study period (i.e., antibiotics, antimalarials, steroids, antivirals, tocilizumab, and other anti-SIRS), was assessed through dispensaries in public hospitals in Castile and Leon. Anatomical Therapeutic Chemical (ATC) classification was used to evaluate medication consumption (**Supplementary Table 3**). Data on the use of oxygen delivery using low-flow systems (nasal cannula and simple face masks) and high-flow systems (high-flow nasal cannula, venturi masks, and rebreather masks), non-invasive pressure positive ventilation (NIPPV), and invasive ventilation (IV), was also assessed.

TABLE 1 | Baseline characteristics, treatment, and clinical outcomes of in-hospital COVID-19 patients with acute kidney injury in Castile and Leon (Spain) (March 1st–May 3th 2020).

	Total	AKI	No. of AKI	*p*
N	7,307	794	6,513	
Age (median and IQR)	76 (63–86)	84 (75–89)	75 (62–85)	0.001
Age < 65 (95% CI)	27.23 (26.21–28.25)	8.19 (6.28–10.09)	29.56 (28.45–30.67)	0.001
Age ≥ 65 (95% CI)	72.77 (71.75–73.79)	91.81 (89.91–93.72)	70.44 (69.33–71.55)	0.001
Chronic diseases (95% CI)				
Hypertension	43.74 (42.6–44.88)	57.93 (54.5–61.37)	42.01 (40.81–43.21)	0.001
Cardiovascular disease	35.83 (34.73–36.93)	48.99 (45.52–52.47)	34.22 (33.07–35.38)	0.001
Diabetes	18.9 (18–19.8)	26.7 (23.62–29.78)	17.95 (17.02–18.88)	0.001
Chronic kidney disease	5.9 (5.36–6.44)	14.36 (11.92–16.8)	4.87 (4.34–5.39)	0.001
Treatment				
Oxygen delivery and ventilation (95% CI)				
IV	3.5 (3.08–3.93)	10.2 (8.1–12.31)	2.69 (2.29–3.08)	0.001
Oxygen delivery	2.52 (2.16–2.88)	2.52 (1.43–3.61)	2.52 (2.14–2.9)	0.999
NIPPV	1.63 (1.34–1.92)	1.76 (0.85–2.68)	1.61 (1.31–1.92)	0.751
Drugs (95% CI)				
Antibiotics	90.83 (90.17–91.49)	90.43 (88.38–92.47)	90.88 (90.18–91.58)	0.677
Antimalarial	69.74 (68.69–70.79)	60.45 (57.05–63.85)	70.87 (69.77–71.98)	0.001
Steroids	44.37 (43.23–45.51)	48.61 (45.14–52.09)	43.85 (42.65–45.06)	0.011
Antivirals	42.63 (41.52–43.93)	33.38 (30.1–36.66)	43.79 (42.58–44.99)	0.001
Tocilizumab	9.37 (8.71–10.04)	8.31 (6.39–10.23)	9.5 (8.79–10.22)	0.277
Other anti-SIRS*	7.34 (6.74–7.93)	9.45 (7.41–11.48)	7.05 (6.43–7.67)	0.007
Clinical outcomes				
Hospital LoS (median and IQR)	9 (5–15)	10 (5–18)	9 (5–14)	0.001
ICU LoS (median and IQR)	15 (7–30)	13 (7–22)	16 (7–33)	0.271
N	491	99	392	
Death (95% CI)	24.43 (23.44–25.41)	46.1 (42.63–49.56)	21.79 (20.79–22.79)	0.001
Acute dialysis (95% CI)	0.95 (0.73–1.17)	3.9 (2.56–5.25)	0.6 (0.41–0.79)	0.001
SARS (95% CI)	14.03 (13.23–14.82)	24.18 (21.2–27.16)	12.79 (11.98–13.6)	0.001
Bacterial superinfection (95% CI)	3.59 (3.16–4.01)	13.85 (11.45–16.26)	2.33 (1.97–2.7)	0.001
Fungal superinfection (95% CI)	2.23 (1.89–2.57)	5.92 (4.28–7.56)	1.78 (1.46–2.1)	0.001
SIRS (95% CI)	2.22 (1.88–2.56)	10.71 (8.55–12.86)	1.77 (1.45–2.09)	0.001
Carodiomyopathy (95% CI)	1.15 (0.91–1.39)	2.9 (1.73–4.06)	0.94 (0.7–1.17)	0.001
DIC (95% CI)	0.18 (0.08–0.27)	1.01 (0.31–1.7)	0.08 (0.01–0.14)	0.001

**Anakinra, baricitinib, interferon, ruxolitinib, siltuximab.*

CI, confidence interval; IQR, interquartile range; AKI, acute kidney injury; IV, invasive ventilation; NIPPV, Non-invasive positive pressure ventilation; SIRS, systemic inflammatory response syndrome; ICU, intensive care unit; LoS, length of stay; SARS, severe acute respiratory syndrome; DIC, disseminated intravascular coagulation.

Statistical Analysis

Statistical analysis was carried out considering age and gender distributions and an age cut-off at 65 years of age. A 15-day period analysis was performed for all measurements and consideration of all clinical outcomes (March 1–14 to May 15–31, 2020). Frequencies (in percentages) and their corresponding 95% confidence intervals (95% CI) and means or medians with, respectively, their standard deviations (SD) or interquartile ranges (IQR), are presented, as appropriate.

Comparisons were performed using the Student *t*-test or the Mann-Whitney *U*-test (for continuous variables), after confirmation of normal distribution of data in a given variable (Kolmogorov-Smirnov-test), and using Pearson's chi-square-test or Fisher's exact-test (categorical variables).

Multiple logistic regression, with a forward selection approach, was performed for in-hospital COVID-19 patients with or without AKI, who died, as opposed to those who did not die. The odds ratio (OR) and a 95% CI were presented. The following variables were included in the analysis: age (>65 years), gender, comorbidities (hypertension, diabetes, cardiovascular disease, CKD), obesity (BMI ≥ 30 kg/m^2), need for ventilation, acute dialysis need (only in patients developing AKI), medications used (antibiotics, antimalarials, steroids, antivirals, tocilizumab, or anti-SIRS), occurrence of SARS, SIRS, DIC, cardiomyopathy, and bacterial and fungal superinfections.

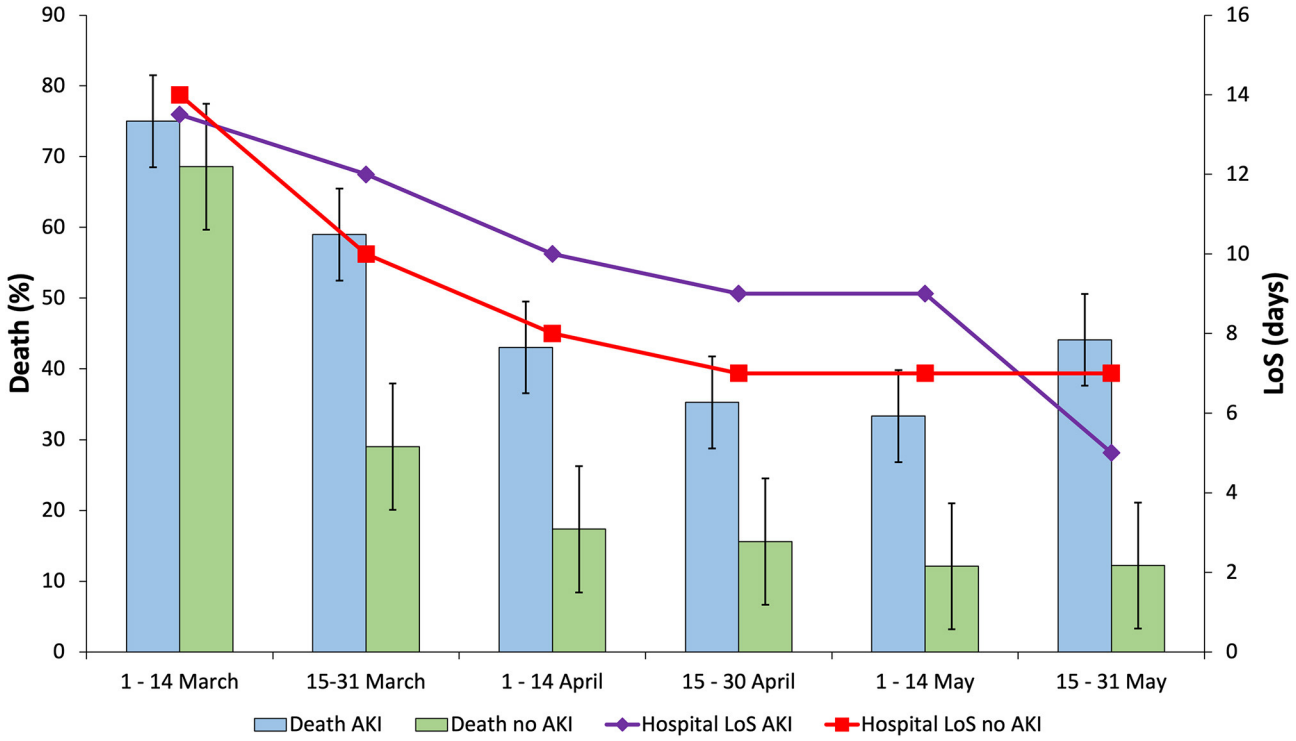

FIGURE 1 | Death and hospital length of stay (LoS) corresponding to the in-hospital COVID-19 patients with or without acute kidney injury (AKI) in Castile and Leon (Spain) (March 1st–May 31th, 2020).

The survival of COVID-19 patients with and without AKI was performed using the Kaplan-Meier approach and the log-rank-test for comparison between groups.

The level of significance was set at $p < 0.05$. All statistical analyses were performed by using the Statistical Package for the Social Sciences (SPSS) software version 24.0. (SPSS Inc., Chicago, IL).

RESULTS

Clinical Findings

From a total population of 7,307 in-hospital COVID-19 patients for which data were available, the findings presented here describe 794 patients with AKI (10.87%), most of them males aged 65 years or more. Around half of the patients had hypertension or cardiovascular disease (**Table 1**). Many of these patients also had diabetes mellitus (26.7%) and CKD categories 3–5 (14.36%).

Into the group of COVID-19 patients presenting AKI, there were no differences in comorbidities between the two gender groups. Nevertheless, compared with females, twice as many male patients needed IV and three times as many male patients needed at least one session of acute dialysis, even if NIPPV and oxygen delivery were used similarly by both genders (**Table 1**). Males also had SARS and cardiomyopathy more commonly, and their hospital length of stay (median 10 days) was more prolonged, compared with that of females. In addition to antibiotics, male patients also used more antimalarials, steroids, antivirals, other anti-SIRS, and tocilizumab, compared with females (**Table 1**).

In addition, within the first 15-day period the mortality rate was very high (75%), while decreasing to 44.12% in the last 15-day period. The same trend applies to the length of hospital stay (**Figure 1** and **Supplementary Table 4**). The peak of AKI incidence was reached between April 15th and May 14th (about 14%). COVID-19 patients with AKI needed to stay in the ICU more frequently in the second half of March 2020 (27.2%) (**Supplementary Table 4**).

Pharmacological Treatment

While the use of antibiotics and steroids, to some extent, remains stable throughout the time, the use of rest of the medications decreased during the study period (**Figure 2** and **Supplementary Table 4**).

Table 2 shows the medication received by COVID-19 patients with AKI who died or survived: significantly lower use of antibiotics and greater use of beta interferon (another anti-SIRS drug category) were noted in those who died compared with those patients who did not die.

Survival and Risk Factors for Clinical Outcomes and Medication Prescribed

No impact on survival in patients with or without AKI was observed (median survival: 12 vs. 12 days; $p = 0.55$)

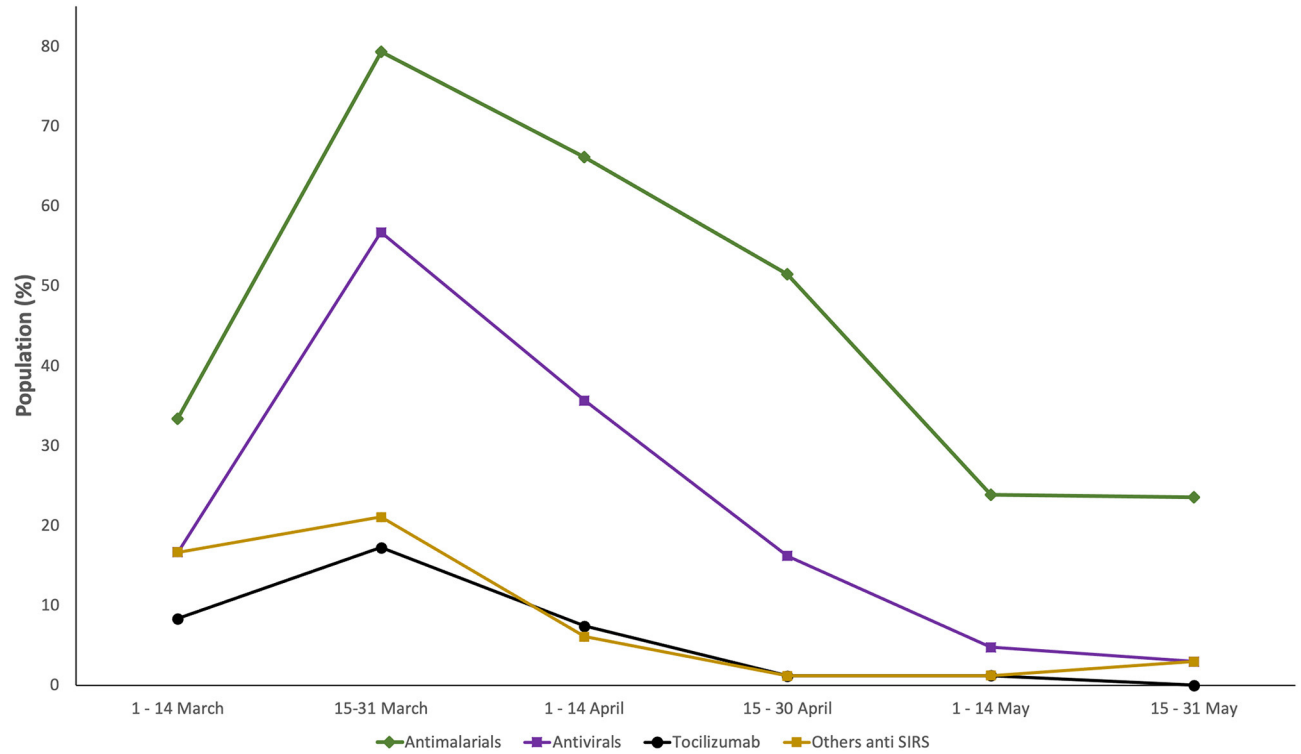

FIGURE 2 | Trends in the use of the medications used by in-hospital COVID-19 patients with acute kidney injury (AKI) in Castile and Leon (Spain) (March 1st–May 31th, 2020).

(**Supplementary Figure 1**). However, multiple logistic regression analysis for all in-hospital COVID-19 patients shows the impact of AKI on death (OR: 2.6; 95% CI: 1.73–2.45), as well as comorbidities, such as cardiovascular disease (1.56, 1.37–1.76), diabetes mellitus (1.18, 1.01–1.36), and particularly having an age of 65 years or more (7.34, 5.93–9.08). Furthermore, among the COVID-19 patients with AKI, death was more likely to occur in those requiring ventilation, without distinguishing between invasive and non-invasive modalities (OR: 5.89; 95% CI: 3.13–11.06), in those treated with steroids (1.73, 1.24–2.41) or anti-SIRS drugs (2.38, 1.27–4.44), and in those who developed SARS (2.75, 1.83–4.14) or SIRS (2.52, 1.46–4.34).

DISCUSSION

This study shows that AKI was present in 1 out of 10 in-hospital COVID-19 patients (March 1st to May 31th, 2020) and that 9 out of 10 cases occurred in people aged more than 65 years, with a mortality around 50%. Ventilation, the use of some medications (steroids, anti-SIRS), and SARS and SIRS incidence may be present in COVID-19 patients with AKI who have an increased risk of death. In addition, AKI had no impact on median survival (in days) compared with in-hospital COVID-19 patients who did not develop AKI. However, mortality among patients with AKI was twice that observed in patients without AKI (46.1 vs. 21.79%) (16), and it is higher than in other studies (17).

Surprisingly, obesity had no influence on death of the patients both with AKI and without AKI, contrary to other studies (18, 19). Probably, lower rates of obesity (in patients with or without AKI, 21.16 and 18.58%, respectively) compared with other cohorts, may be an explanation (20).

Meta-analyses assessing COVID-19 patients from all over the world with AKI confirm that the incidence of AKI was greater in our region compared with Asia (4.3%) but lower than in North America (22.6%) and similar to that of other European countries (11.6%) (3, 8, 21, 22). Surprisingly, the mortality rate in Castile and Leon, Spain, was higher than in all of them. It is difficult to establish which factors are associated with this high mortality rate, which probably is related to the aging of the population in Castile and Leon, but also to other factors, such as the "collapse" of the Spanish public health system, the limited expertise of physicians in treating COVID-19 patients at such a moment, professional extenuation, etc. In this sense, there were differences with respect to other cohorts having an elevated percentage of patients aged 65 years or older, as in our region, which obliging to define the prognosis of the COVID-19 patient with AKI (21): AKI itself seems to have an impact on death, as demonstrated by our analysis and according to findings in other studies (8, 22); however, the impact from other factors should be characterized and considered for different world regions. However, it seems

TABLE 2 | Medications used by in-hospital COVID-19 patients with acute kidney injury (AKI) in Castile and Leon (Spain) (March 1st–May 31th 2020).

Medications	Total *N* = 794	No. of deaths *N* = 428	No. of deaths *N* = 366	*p*
Antibiotics	**90.43 (88.38–92.47)**	**92.76 (90.3–95.21)**	**87.7 (84.34–91.07)**	**0.016**
Ceftriaxone	70.28 (67.1–73.46)	71.5 (67.22–75.77)	68.85 (64.11–73.6)	0.417
Azithromycin	60.71 (57.31–64.1)	62.62 (58.03–67.2)	58.47 (53.42–63.52)	0.233
Levofloxacin	19.14 (16.41–21.88)	19.86 (16.08–23.64)	18.31 (14.34–22.27)	0.579
Teicoplanin	2.27 (1.23–3.3)	2.8 (1.24–4.37)	1.64 (0.34–2.94)	0.272
Cefditoren	1.89 (0.94–2.84)	3.04 (1.41–4.66)	0.55 (0.01–1.08)	0.010
Clarithromycin	0.76 (0.15–1.36)	1.17 (0.15–2.19)	0.27 (0.02–0.53)	0.147
Moxifloxacin	0.25 (0.1–0.4)	0.23 (0.02–0.45)	0.27 (0.02–0.53)	0.912
Cefotaxime	0.25 (0.1–0.4)	0.23 (0.02–0.45)	0.27 (0.02–0.53)	0.912
Ceftaroline	0.13 (0.02–0.23)	0.23 (0.02–0.45)	-	0.355
Antimalarials	**60.45 (57.05–63.85)**	**59.58 (54.93–64.23)**	**61.48 (56.49–66.46)**	**0.586**
Hydroxycloroquine	55.54 (52.09–59)	55.37 (50.66–60.08)	55.74 (50.65–60.83)	0.918
Cloroquine	6.55 (4.83–8.27)	5.84 (3.62–8.06)	7.38 (4.7–10.06)	0.383
Steroids	**48.61 (45.14–52.09)**	**47.2 (42.47–51.93)**	**50.27 (45.15–55.4)**	**0.387**
Methylprednisolone	45.84 (42.38–49.31)	43.69 (38.99–48.39)	48.36 (43.24–53.48)	0.188
Prednisone	10.33 (8.21–12.44)	13.08 (9.89–16.28)	7.1 (4.47–9.74)	0.006
Antivirals	**33.38 (30.1–36.66)**	**31.31 (26.91–35.7)**	**35.79 (30.88–40.7)**	**0.182**
Lopinavir-Ritonavir	33.25 (29.97–36.53)	31.31 (26.91–35.7)	35.52 (30.62–40.42)	0.209
Remdesevir	0.13 (0.02–0.23)	–	0.27 (0.02–0.53)	0.279
Other anti-SIRS	**9.45 (7.41–11.48)**	**6.54 (4.2–8.88)**	**12.84 (9.41–16.27)**	**0.010**
Interfereon Beta	8.06 (6.17–9.95)	5.37 (3.24–7.51)	11.2 (7.97–14.43)	0.003
Anakinra	1.26 (0.48–2.04)	0.93 (0.02–1.85)	1.64 (0.34–2.94)	0.375
Ruxolitinib	0.38 (0.05–0.7)	0.23 (0.02–0.45)	0.55 (0.01–1.08)	0.474
Baricitinib	0.25 (0.05–0.44)	0.46 (0.12–0.81)	–	0.190
Tocilizumab	**8.31 (6.39–10.23)**	**7.24 (4.79–9.7)**	**9.56 (6.55–12.58)**	**0.238**

CI, confidence interval; SIRS, systemic inflammatory response syndrome.
Values in bold correspond to medicines groups.

clear that mortality is higher in patients with chronic kidney disease who develop AKI (23).

Our study shows that ventilation, the use of some medicines (steroids, anti-SIRS), the occurrence of SARS or SIRS may depict a patient with a poor prognosis: Physicians should understand that such an individual has an elevated risk of dying, and they should consider these factors in order to assist COVID-19 patients with AKI now and in the future. Importantly, previous clinical conditions, such as age and gender, as well as the need for acute dialysis, seem to have no influence on the change in patients, but the use of some medications (steroids, anti-SIRS) and the severity of COVID-19 (SARS occurrence, signs of SIRS), should lead to more intensive interventions.

This study provides real-world evidence of risk factors associated with the deaths of COVID-19 patients with AKI in the largest region of Spain. As in other healthcare settings (24), assessing data that come from the actual clinical practice has contributed to the characterization of a population suffering bad outcomes. Nevertheless, questions about the quality of the evidence may arise. The findings presented here may be considered to belong to "emerging sources" from outside classic research environments (25).

This real-world data study presents a comprehensive analysis from the COVID-19 pandemic in Castile and Leon, the largest region of Spain, with a population of 2,323,770 inhabitants and a network of public hospitals with a total capacity of 7,141 beds (14 hospitals). Our findings thus cover all in-hospital COVID-19 patients ($n = 7{,}307$), of whom those presenting with AKI are presented here ($n = 794$).

This study has limitations that should be mentioned. First, although all extracted COVID-19 cases were recorded in the health administration registries accessed as COVID-19 patients, in one third of the cases, COVID-19 diagnosis was clinical or radiological, without microbiological confirmation, as tests were not available for all and because clinical judgment was the only tool at that time. Therefore, risk of selection bias should invite prudence in interpreting the results of this article. Second, due to the collapse of the health system, errors in the clinical data register during the first COVID-19 wave have been observed, which may explain study attrition. Furthermore, selection bias is suspected in the figures of CKD prevalence as not all CKD patients in KDIGO GFR categories 3–5 return to general practitioners after the first consultation in nephrology departments, and not all patients in those CKD categories

are sent by general practitioners to the nephrologist. The Angiotensin-Converting Enzyme Inhibitors (ACE)/Angiotensin II Receptor Antagonists (ARB) ratio could be related to the COVID patients' survival; unfortunately, that information was not available, which may be a limitation. Over 8 months have elapsed since the collection of the data, which may be a limitation; however, AKI is still one of the COVID-19 outcomes with the worst prognosis. Finally, other medications not included in Spanish guidelines (14, 15) were not taken into account in this study.

In conclusion, AKI was observed in one 1 out of 10 COVID-19 patients, and almost half of them died before discharge, which demonstrates a mortality rate higher than that observed in other regions including Spain (3, 4, 26–29).

With respect to the pharmacological treatment of these patients, with the exception of antibiotics and steroids, the use of the medications analyzed decreased throughout the study period, either due to their availability or that of other medications not used in our studio. Our study has characterized the subjects hospitalized with COVID-19 and highlighted the use of medicines to treat systemic inflammation, and this situation has not changed up to now.

Lastly, AKI is a serious complication of COVID-19, and it must be taken into account by physicians in order to pay better attention to patients' treatment and to the occurrence of manifestations such as SARS or SIRS associated with a poor prognosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by East Valladolid Health Area Ethics Committee (reference: PI 20–1863).

AUTHOR CONTRIBUTIONS

EG-A, ET, and FÁ: conceptualization. EG-A, FH-G, DM-G, and FÁ: methodology, validation, and investigation. EG-A and ET: software. EG-A, FH-G, ET, and FÁ: formal analysis. FÁ: resources, supervision, project administration, and funding acquisition. EG-A: data curation and visualization. EG-A, FH-G, and FÁ: writing—original draft preparation. EG-A, FH-G, ET, DM-G, and FÁ: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.657977/full#supplementary-material

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SYSTEMATIC REVIEW
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The Risk of COVID-19 Related Hospitalsation, Intensive Care Unit Admission and Mortality in People With Underlying Asthma or COPD: A Systematic Review and Meta-Analysis

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Background: Several underlying diseases have been associated with unfavorable COVID-19 related outcomes including asthma and Chronic Obstructive Pulmonary Disease (COPD), however few studies have reported risks that are adjusted for confounding variables. This study aimed to examine the adjusted risk of COVID-19 related hospitalsation, intensive care unit (ICU) admission, and mortality in patients with vs. without asthma or COPD.

Methods: A systematic review of major databases was undertaken for studies published between 1/12/2019 and 19/4/2021. Studies reporting the adjusted (for one or more confounder) risks of either hospitalsation, ICU admission, or mortality in asthmatics or COPD patients (control group = no asthma or no COPD) were identified. Risk of bias was determined via the QUIPS tool. A random effect meta-analysis was undertaken.

Findings: 37 studies were eligible for analysis, with a total of 1,678,992 participants. The pooled ORs of COVID-19 hospitalsation in subjects with asthma and COPD was 0.91 (95% CI 0.76–1.09) and 1.37 (95% CI 1.29–1.46), respectively. For ICU admission, OR in subjects with asthma and COPD was 0.89 (95% CI 0.74–1.07) and 1.22 (95% CI 1.04–1.42), respectively. For mortality, ORs were 0.88 (95% CI 0.77–1.01) and 1.25 (95% CI 1.08–1.34) for asthma and COPD, respectively. Further, the pooled risk of mortality as measured via Cox regression was 0.93 (95% CI 0.87–1.00) for asthma and 1.30 (95% CI 1.17–1.44) for COPD. All of these findings were of a moderate level of certainty.

Interpretation: COPD was significantly associated with COVID-19 related hospital admission, ICU admission, and mortality. Asthma was not associated with negative COVID-19 related health outcomes. Individuals with COPD should take precautions to limit the risk of COVID-19 exposure to negate these potential outcomes. Limitations include differing population types and adjustment for differing cofounding

variables. Practitioners should note these findings when dealing with patients with these comorbidities.

Review Protocol Registration: https://www.crd.york.ac.uk/prospero/.

Keywords: COVID-19, COPD, asthma, mortality, hospitalsation, meta-analysis, ICU, intensive care

INTRODUCTION

In March 2020, the World Health Organization (WHO) declared the COVID-19 outbreak a global pandemic, and as of 3rd February 2021, over 103,000,000 confirmed cases have been diagnosed in more than 130 countries and areas, resulting in ∼2,238,000 deaths to date (1). Several risk factors associated with increasing severity of the disease have been reported, including age (2), obesity (3), and underlying conditions such as hypertension (4), and diabetes (5).

An important risk factor for unfavorable COVID-19 outcomes is Chronic Obstructive Pulmonary Disease (COPD); a group of lung conditions including emphysema and chronic bronchitis (6), primarily caused by tobacco smoking, with air pollution, genetic factors, diet and tuberculosis also contributing to the disease (7).

COPD has been associated with increased risks of unfavorable outcomes in non-COVID-19 related pneumonia (8). For COVID-19, some primary studies have questioned whether COPD is associated with worse outcomes (9), whilst the majority of reviews conclude that COPD patients yield significantly worse outcomes than those without (10–13) and others report no effects (14).

An additional risk factor for COVID-19 related complications is the presence of asthma, a common allergy that can cause breathing difficulties including coughing, wheezing, breathlessness and a tight chest (15). Asthma exacerbations have been shown to be strongly associated with other respiratory viral infections, including previous coronaviruses (16, 17). Although some primary studies have reported associations between asthma and negative COVID-19 outcomes, the majority of reviews that have examined associations of COVID-19 outcomes and asthma have concluded a lack of association between asthma and negative COVID-19 outcomes (18, 19).

One limitation of all of the systematic reviews, to date, on COVID-19 outcomes and asthma or COPD is that they report on risk that has not been adjusted for any potential confounding factors, making the true risks of these comorbidities, and subsequent clinical implications, difficult (20)—indeed, of the 16 similar meta-analyses that were published in 2021 (as of April 2021), none of them reported exclusively on adjusted risks; they either report unadjusted risks or the inclusion of adjusted or unadjusted risks is unclear. Several primary studies report on adjusted risks that are lower than the unadjusted risks in several COVID-19 related outcomes, including in asthma (21) and COPD (22). Furthermore, several studies advocate the use of pooling adjusted effect sizes (23, 24), especially in the case of determining COVID-19 related risks (20, 25).

The aim of this review was to examine the risks of negative COVID-19 outcomes in subjects with asthma or COPD, that have been adjusted for one or more COVID-19 related risk factor, including age, sex, smoking status (20, 25), or comorbid disease. Specifically our aims were to assess:

1. Adjusted risk of COVID-19 related hospitalsation in subjects with vs. without asthma or COPD.
2. Adjusted risk of COVID-19 related intensive care unit (ICU) admission in subjects with vs. without asthma or COPD.
3. Adjusted risk of COVID-19 related overall mortality in subjects with vs. without asthma or COPD.

This review has the potential to inform clinicians regarding the true risks of unfavorable COVID-19 outcomes in patients with asthma and COPD, increase awareness in people of the potential risks should they contract COVID-19 and to inform healthcare and public health policies.

METHODS

Study Registration

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (26), and was registered on 29th June 2020 with the international prospective register of systematic reviews (PROSPERO: protocol ID CRD42020194155)—note that the full PRIMSA checklist can be found in **Supplementary Table 1** and justifications of any deviations from the registered protocol can be found in **Supplementary Table 2**.

Search Strategy

Databases were searched from 1/12/2019 to 19/4/2021 including Embase, MEDLINE, Pubmed, Scopus, Web of Science, CINAHL, The Cochrane library UK clinical Research Network: Portfolio database, and the International Standard Registered Clinical/soCial sTudy Number (ISRCTN) registry, using the following search terms:

(SARSCoV-2 OR 2019-nCoV OR COVID-19 OR coronavirus OR "Wuhan Coronavirus")
AND
(2019 or 2020)
AND
(asthma* OR COPD OR "chronic obstructive pulmonary disease")

No other limiters were applied.

Study Selection

Two researchers (MV,SW) independently screened titles and abstracts of all identified studies after duplicates were removed. Discrepancies between reviewers were resolved by discussion before screening full texts independently against the inclusion criteria. If it was not possible to determine whether a study met the inclusion criteria from the title and/or abstract, it was marked for a full paper review. Where necessary, the reviewers contacted corresponding authors to request missing information or clarification. All references were imported to Mendeley.

Study Inclusion and Exclusion

Two reviewers (MV & SW) independently screened all titles and abstracts. The relevance of each study was assessed according to the inclusion and exclusion criteria. Studies were included if they met the following criteria.

Population

Studies including humans with COPD and/or asthma and a confirmed case (via polymerase chain reaction or antibody test) of COVID-19 were included in this review. Children <18 yrs and animal studies were excluded from this review. We also excluded studies on previous human coronaviruses: 229E, NL63, OC43, HKU1, MERS-CoV, and SARS-CoV.

Intervention

Observational studies, including case-control and cohort studies were included. Randomized studies that reported the prognostic role of asthma/COPD in *post-hoc* analyses (e.g., Cox regression models) were also included.

Comparison

Comparator groups include humans with confirmed COVID-19 and no evidence of COPD and/or asthma.

Outcomes

Studies had to report one or more of the following:

1. Number of COVID-19 cases hospitalised *vs.* COVID-19 cases non-hospitalised cases.
2. Number of hospitalised COVID-19 cases treated in intensive care unit (ICU) *vs.* hospitalsation but not admitted for ICU care.
3. Number of COVID-19 related deaths *vs.* survival.

Furthermore, studies were excluded if they were:

1. Not written in English.
2. Not peer reviewed (e.g., preprints).
3. Studies in a non-adult (<18 years) population.
4. Had insufficient data to calculate an adjusted odds ratio (aOR; adjusted for more than one COVID-19 related covariate) related to the stated outcomes.

Data Extraction

Data was extracted by two reviewers (MT & MV) and included: first author, study title, date of study, dates in which study data were collected, country, aim/objective, study type, number of participants, disease investigated, method of disease diagnoses, method of COVID-19 diagnosis, outcome type, sample size, participant characteristics, adjusted OR and 95% confidence intervals (CIs) (or raw data in which an adjusted odds ratio could be calculated), details of confounding variables the OR was adjusted for. Where data was missing, required clarification or particular variables of interest were not reported in the paper, corresponding authors were contacted to enable inclusion in the meta-analysis, and given 2 weeks to respond. If no response was received within 2 weeks, or the data was unavailable, these studies were excluded.

Quality Assessment

Risk of bias was assessed by two independent researchers (MT & MV) using the Quality In Prognosis Studies (QUIPS) tool (27). The QUIPS is a non-scoring appraisal tool for assessing the scientific validity of articles, which requires the identification of whether or not relevant information is present in each article using a yes, no or not applicable rating, with an overall verdict of "low," "medium," or "high" risk of bias. Any discrepancies over the final risk of bias verdict was made by consensus, with involvement of a third review author (SP) where necessary.

Statistical Analysis

Due to anticipated heterogeneity, a random-effects model was conducted using the DerSimonian and Laird method, with studies weighted according the inverse variance, using Comprehensive Meta-Analysis (28). The meta-analysis was conducted using the following steps:

(1) Adjusted odds ratios (aORs), or adjusted Hazard Ratios (aHRs) and 95% CIs were inputted (with significance set as $p = 0.05$). Note that if the raw data were available, a binary logistic regression was conducted.
(2) Heterogeneity between studies was assessed using the I^2 statistic (29). If high (>50%) heterogeneity was found, subgroup analyses were conducted based on total participants (>10 vs. <10k participants).
(3) Publication bias was assessed with a visual inspection of funnel plots and with the Egger bias test (30). As per the recommendations by Fu et al. (31) and Sterne et al. (32), these tests were only conducted if the number of studies in each analysis exceeded ten.
(4) Sensitivity analyses were conducted to assess the robustness of the pooled effect sizes through the one study removed method.

Certainty of Evidence

To ascertain the certainty of the evidence, the Grading of Recommendations, Assessment, Development and Evaluations (33) (GRADE) framework was used.

RESULTS

The literature search yielded 3,701 results, of which 780 were duplicates and were automatically removed, leaving 2,921 studies to be screened using the title and abstract. Of these studies, 416 full-texts were screened, where five extra studies were

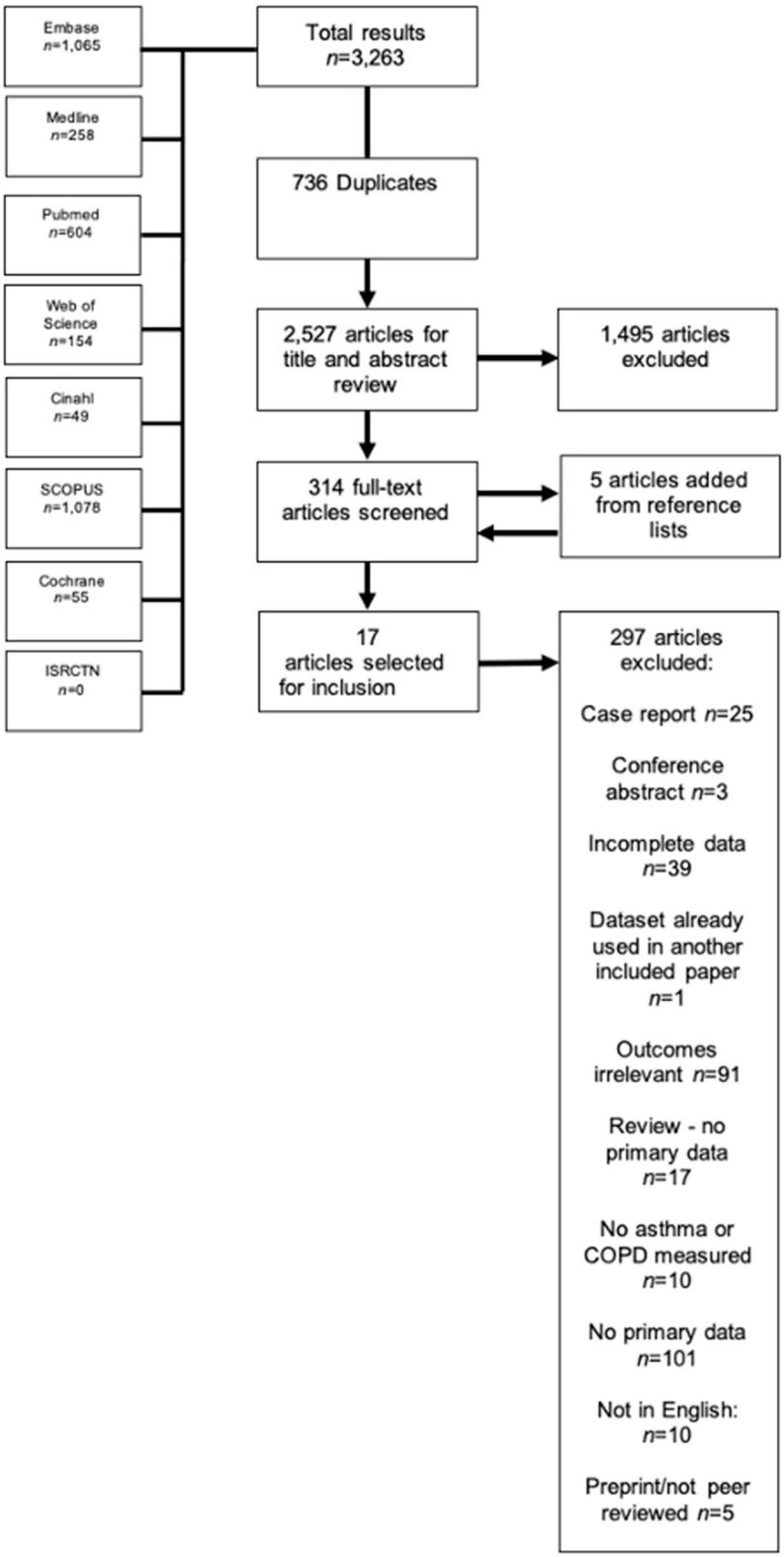

FIGURE 1 | PRISMA flowchart of included studies.

TABLE 1 | Descriptive characteristics of included studies.

Authors	Study design	Country	Total *n*	Age (mean)	Percentage female	Type of outcome(s) measured	Disease	Method of asthma diagnosis	Method of COPD diagnosis	Confounding/adjusted variables	Conflict of interest	Risk of bias
Atkins et al. (38)	Cohort	UK	268,704	73.1	NR	Hospitalsation risk; mortality risk	Asthma or COPD	"existing diagnoses were available from baseline questionnaires (2006–2010) eliciting participant reports of doctor-diagnosed disease. New disease diagnoses since baseline were from linked electronic medical records to hospital inpatient routine data (to March 2017), coded according to the International Classification of Diseases 10th revision (ICD-10)"		Age group, sex, ethnicity, education, baseline assessment centre, CHD, Atrial fibrillation, stroke, hypertension, T2D, CKD, depression, dementia, asthma, COPD, Osteoporosis, previous delirium, previous pneumonia, previous falls/fragility fractures.	Reported—none declared	Low
Attaway et al. (39)	Cohort	USA	2527	NR	NR	Hospitalsation risk; ICU admission risk; mortality risk	COPD	–	"Patients were asked if they had a diagnosis of COPD, and the diagnosis was confirmed if it was also included in the patient's medical chart"	Age, race, sex, BMI, asmoking status (current vs. former), hypertension, cancer, diabetes mellitus, coronary artery disease, immunosuppressive therapy.	Reported—none declared	Low
Aveyard et al. (55)	Retrospective cohort	UK	811	NR	NR	Mortality risk	Asthma and COPD	NR	NR	Age, sex, ethnicity, socioeconomic status, region of England, body-mass index (categorical variable), and smoking status (with current intensity of smoking as categorical variables), on-smoking-related illness (hypertension, type 1 diabetes, chronic liver disease, chronic neurological disease) and smoking-related illness (coronary heart disease, stroke, atrial fibrillation, type 2 diabetes, chronic kidney disease).	Reported—several potential conflicts declared	Low
Azoulay et al. (59)	Retrospective cohort	France	376	NR	NR	Mortality risk	COPD	–	NR	Age, comorbidities (asthma, diabetes, COPD, hypertension, immunosuppression), time from viral symptom onset to ICU admission, acute kidney injury, and troponin	Reported—none declared	Low

(Continued)

TABLE 1 | Continued

Authors	Study design	Country	Total *n*	Age (mean)	Percentage female	Type of outcome(s) measured	Disease	Method of asthma diagnosis	Method of COPD diagnosis	Confounding/adjusted variables	Conflict of interest	Risk of bias
Bloom et al. (69)	Retrospective cohort	UK	47,398	NR	NR	Mortality risk	Asthma and COPD	NR	NR	Age, sex, ethnicity, smoking, obesity, malignancy, chronic cardiac disease, CKD, and centre	Reported—several potential conflicts declared	Low
Cellina et al. (40)	Retrospective observational	Italy	246	63.0	31.0%	Mortality risk	COPD	–	NR	Age, diabetes, and radiological outcomes	Reported—none declared	Low
Choi et al. (21)	Cohort	Korea	7,590	NR	NR	ICU admission risk; mortality risk	Asthma	"An asthma diagnosis was determined when patients visited the hospital (at least once) due to asthma symptoms from January 2019 to December 2019. Furthermore, only patients who met the following criteria during the assessment period were regarded as having asthma: (1) ICD- 10 codes for asthma (J45 and J46) as primary diagnosis or first sub-diagnosis; and (2) prescription of asthma medications on at least 2 occasions during outpatient visits or prescription of asthma medication following an outpatient visit and admission with treatment using systemic corticosteroids during the assessment period."	–	Age, sex, and underlying conditions	Reported—none declared	Low
Choi et al. (54)	Retrospective cohort	South Korea	4,057	NR	60.4%	Mortality risk	Asthma	NR	–	Age, sex, obesity, systolic blood pressure, diastolic blood pressure, heart rate, temperature, diabetes, hypertension, heart failure, chronic heart disease, chronic obstructive pulmonary disease, chronic kidney disease, cancer, chronic liver disease, rheumatic or autoimmune disease, and dementia.	Reported—none declared	Low

(Continued)

TABLE 1 | Continued

Authors	Study design	Country	Total *n*	Age (mean)	Percentage female	Type of outcome(s) measured	Disease	Method of asthma diagnosis	Method of COPD diagnosis	Confounding/adjusted variables	Conflict of interest	Risk of bias
De Vito et al. (41)	Retrospective observational	Italy	87	72 (median)	35.6%	Mortality risk	COPD	–	NR	Age >72 years, Hypertension, > 3 comorbidities, >5 comorbidities, non-compliance, moderate ARDS, lymphocyte <900/mm^3	Reported—none declared	Low
De Vito et al. (57)	Retrospective cohort	Italy	264	81.9 (10.1)	62.5%	Mortality risk	COPD	–	NR	Age, sex, hypertension, diabetes, neurological syndrome, hypokinetic disease, autonomy, fever + dyspnoea, LMWH	Reported—none declared	Low
Giannouchos et al. (42)	Cross-sectional	Mexico	89,756	46.2	43.6%	Hospitalsation risk; ICU admission risk	Asthma and COPD	NR	NR	Age, gender, smoking, CKD, diabetes, immunosuppression, obesity, hypertension, CVD, asthma or COPD	Reported—none declared	Low
Girardin et al. (56)	Retrospective cohort	USA	4,446	NR	NR	Mortality risk	Asthma and COPD	NR	COPD was defined as presence of chronic bronchitis or emphysema.	Age, sex, PAD, low income, asthma, ethnicity, obesity, CAD, cancer, smoking, diabetes, auto-immune disease, hyperlipidaemia, sleep apnoea, hypertension	Reported—none declared	Low
Grandbastien et al. (43)	Cross-sectional	France	106	63.5 (median)	37.7%	ICU admission ris	Asthma	"clinical diagnosis of asthma– based on the clinical history recorded by medical staff"		Age, sex, hypertension, diabetes, body mass index <30, and heart failure	Reported—one author reports conflict of interest with pharmaceutical companies	Low
Grasselli et al. (60)	Retrospective cohort	Italy	3,988	NR	20.1%	Mortality risk	COPD	- -	NR	Age, sex, respiratory support type, HTN, hypercholesterolemia, heart disease, T2D, malignancy, ACE inhibitor therapy, ARB therapy, statin, diuretic, PEEP at admission, Fio2 at admission, Pao2/Fio2 at admission	Reported—several potential conflicts declared	Low

(Continued)

TABLE 1 | Continued

Authors	Study design	Country	Total *n*	Age (mean)	Percentage female	Type of outcome(s) measured	Disease	Method of asthma diagnosis	Method of COPD diagnosis	Confounding/adjusted variables	Conflict of interest	Risk of bias
Guan et al. (66)	Retrospective cohort	China	39,420	55.7 (NR)	NR	Mortality risk	Asthma and COPD	NR	NR	Age, sex, other systemic comorbidities	Reported—none declared	Low
Gupta et al. (44)	Cohort	USA	2,215	60.5	35.2%	Mortality risk	COPD	–	"Per chart review"	Age, sex, race, hypertension, diabetes, body mass index, coronary artery disease, congestive heart failure, current smoking status, active cancer, duration of symptoms before ICU admission, and covariates assessed at ICU admission (lymphocyte count, ratio of the PaO2 to the fraction of inspired oxygen [FIO2], shock, and the kidney, liver, and coagulation components of the sequential organ failure assessment score).	Reported—several authors report conflict of interest	Low
Harrison et al. (45)	Retrospective cohort	USA	31,461	50 (median)	54.5%	Mortality risk	COPD	–	NR	Age, sex, ethnicity, myocardial infarction, congestive heart failure, peripheral vascular disease, cerebrovascular disease, dementia, rheumatic disease, peptic ulcer disease, mild liver disease, moderate/severe liver disease, diabetes, hemiplegia or paraplegia, renal disease, any malignancy, metastatic solid tumor, AIDS/HIV	Reported—several authors report conflict of interest	Low
Hernandez-Galdamez et al. (46)	Cross-sectional	Mexico	211,003	45.7	45.3%	Hospitalsation risk; ICU admission risk; mortality risk	Asthma and COPD	"The information is obtained through a dichotomous questionnaire that the physician fills with the information provided by the patient."		Age, sex, CKD, immunosuppression, diabetes, hypertension, cardiovascular disease, COPD or asthma, obesity and smoking.	Reported—none declared	Low

(Continued)

TABLE 1 | Continued

Authors	Study design	Country	Total *n*	Age (mean)	Percentage female	Type of outcome(s) measured	Disease	Method of asthma diagnosis	Method of COPD diagnosis	Confounding/adjusted variables	Conflict of interest	Risk of bias
Ho et al. (64)	Retrospective cohort	USA	10,523	58.35 (18.81)	45.8%	Hospitalsation risk; ICU admission risk; mortality risk	Asthma	NR		Age, sex, BMI, race, COVID-19 disease severity, Charleston Comorbidity Index, COPD, C-reactive protein (>150 mg/L), interleukin-6 (>80 mg/L), ferritin (>2,000 ng/L), D-dimer (>2.0 mg/L), use of anticoagulation, use of corticosteroids, and smoking (current and former).	Reported—none declared	Low
Hu et al. (47)	Cohort	China	821	NR	NR	Mortality risk	COPD	–	"COPD patients diagnosed by lung function"	Age, sex, hypertension, diabetes, CAD, CVD, Malignancy, CKD, chronic liver disease	Reported—none declared	Low
Hu et al. (72)	Retrospective cohort	China	213	44 (median)	NR	ICU admission risk	COPD	–	NR	Age, Dyspnoea, Poor appetite, WBC>10 × 10-9/l, D-dimer>0.5 mg/l, Albumin <35 g/L, ALT, AST, LDH.	Reported—none declared	Low
Jiang et al. (68)	Retrospective cohort	China	281	NR	NR	Mortality risk	COPD	–	NR	Age, sex, anorexia, comorbidities, CD8+ count, lymphocyte count, CRP, D-dimer, LDH, high sensitivity troponin I, osmotic pressure, PCT, and SOFA score on ICU admission	Reported—none declared	Low
Kammar-Garcia et al. (51)	Cohort	Mexico	13,842	NR	NR	Hospitalsation risk; ICU admission risk; mortality risk	Asthma and COPD	"Self-report and defined as present or absent"	Age, sex, pneumonia, diabetes, asthma or COPD, immunosuppression, hypertension, CVD, obesity, CKD, other comorbidities	Not reported	Medium	Low
Lee et al. (67)	Retrospective cohort	South Korea	4,610	NR	NR	Mortality risk	COPD	–	Medical records—Identification of COPD patients with ICD-10 codes (J43 and J44 except J43.0)	Age, sex, and Charleston Comorbidity Index score	Reported—none declared	Low

(Continued)

TABLE 1 | Continued

Authors	Study design	Country	Total *n*	Age (mean)	Percentage female	Type of outcome(s) measured	Disease	Method of asthma diagnosis	Method of COPD diagnosis	Confounding/adjusted variables	Conflict of interest	Risk of bias
Li et al. (53)	Case-series	China	204	68 (median)	51%	Mortality risk	COPD	–	NR	None	Reported—none declared	Low
Mahdavinia et al. (52)	Case-series	USA	1,003	NR	NR	Hospitalsation risk; mortality risk	Asthma	"asthma diagnosis based on-Global Initiative for Asthma (GINA) guidelines"		None	Reported—none declared	Low
Martos-Benitez et al. (37)	Retrospective cohort	Mexico	38,324	46.9 (15.7)	41.7%	ICU admission risk; mortality risk	COPD	–	NR	Age, sex, smoking habit, time from symptoms onset to medical contact, and all the comorbidities	Reported—none declared	Low
Murillo-Zamora et al. (58)	Retrospective cohort	Mexico	66,123	NR	NR	Mortality risk	Asthma and COPD	NR	NR	Age, sex, diagnosed pneumonia at admission, tobacco use, obesity, COPD, diabetes, arterial hypertension, immunosuppression, CKD	Reported—none declared	Low
Parra-Bracamonte et al. (48)	Cohort	Mexico	331,298	44 (median)	46.2%	Mortality risk	Asthma and COPD	As confirmed by dataset used—no specific method reported		Age, sex, smoking status, hospitalsation, pneumonia, hypertension, obesity, diabetes, cardiopathy, COPD or asthma, immunosuppressed, CKD, other complications.	Not reported	Low
Rosenthal et al. (63)	Retrospective cohort	USA	727	49.46 (17.93)	NR	Hospitalsation risk	Asthma	NR	–	Age, BMI, race, and a number of comorbidities (chronic kidney disease, coronary artery disease or congestive heart failure, diabetes, and hypertension)	Reported—none declared	Low
Timerlake et al. (65)	Retrospective cohort	USA	274	NR	NR	ICU admission risk; mortality risk	COPD	–	NR	Age, sex, race, admission diagnosis (COVID-19 vs. other), CAD, and obesity	Reported—several potential conflicts declared	Low
Wang et al. (61)	Case-series	China	339	69 (median)	51.0%	Mortality risk	COPD	–	NR	Age, CVD, cerebrovascular disease	Reported—none declared	Low
Wang et al. (62)	Retrospective cohort	China	141	64 (median)	30.0%	Mortality risk	COPD	–	NR	Ventilation status, creatinine ?104 umol/; vs. <104 umol/l and chronic renal diseases	Reported—none declared	Low
Wang et al. (70)	Case-series	USA	1,827	54 (median)	67.4%	Hospitalsation risk; ICU admission risk; mortality risk	COPD	-	NR	Age, sex, race, marital status, educational level, insurance type, smoking history, BMI, diabetes, CKD, CLD, CVD, HTN, allergic rhinitis	Reported—several potential conflicts declared	Low

(Continued)

TABLE 1 | Continued

Authors	Study design	Country	Total *n*	Age (mean)	Percentage female	Type of outcome(s) measured	Disease	Method of asthma diagnosis	Method of COPD diagnosis	Confounding/adjusted variables	Conflict of interest	Risk of bias
Wu et al. (49)	Retrospective observational	China	443	NR	NR	ICU admission risk	COPD	–	NR	Age, sex, smoking status, diabetes, hypertension, coronary heart disease, cerebrovascular diseases, hepatitis B infection, cancer, chronic renal diseases, immunodeficiency.	Reported—none declared	Low
Yoshida et al. (71)	Case-series	USA	776	60.5 (16.1)	NR	ICU admission risk; mortality risk	COPD	–	NR	Age, sex, hospital site, and the Charleston Comorbidity Index	Reported—none declared	Low
Zhu et al. (50)	Cohort	UK	492,768	NR	NR	Hospitalsation risk	Asthma	Measurement of genetic asthma phenotypes	-	Age, sex, race/ethnicity, and BMI	Reported—none declared	Low

obtained by way of reference lists, resulting in 421 full texts that were finally screened. Thirty-eight studies appeared to be eligible for inclusion, however one (34) was excluded because the reported 95% CIs were not symmetrical, and therefore could not be pooled, leaving 37 finally eligible for inclusion (21, 35–69). The full PRISMA flowchart is shown in **Figure 1**, and a full list of excluded studies with reasons for exclusion can be found in **Supplementary Table 2**. There were a total of 1,678,992 participants across the included studies, with a mean age range of 45.7–81.9 years. Of the included studies, 10 (38, 42, 46, 48, 51, 55, 56, 58, 66, 69) examined outcomes in both asthma and COPD, seven (21, 43, 50, 52, 63, 64) examined outcomes exclusively in asthma, and the remaining 20 studies (37, 39–41, 44, 45, 47, 49, 53, 57, 59–62, 65, 67, 68, 70, 71) reported on outcomes exclusively regarding COPD. All but one study was classified as having low risk of bias (see **Supplementary Table 4** for full QUIPS scoring). Full descriptive characteristics of included studies are shown in **Table 1**.

Meta-Analysis

Risk of COVID-19 Related Hospitalsation

When adjusted for one or more comorbidity, the pooled aOR was 0.87 (95% CI 0.73–1.05; $p = 0.15$; $I^2 = 85.36$) for asthma and 1.39 (95% CI 1.31–1.48; $p = <0.001$; $I^2 = 4.24$) for COPD (see **Table 2** and **Figure 2**). The sensitivity analysis found that the removal of any one study did not significantly change the direction of results for either asthma or COPD (see **Supplementary Figures 1**, **2** for full details).

Risk of COVID-19 Related ICU Admission

When adjusted for one or more comorbidity, the pooled aOR was 0.75 (95% CI 0.55–1.02; $p = 0.07$; $I^2 = 87.20$) for asthma and 1.34 (95% CI 1.14–1.57; $p =< 0.001$; $I^2 = 66.64$) for COPD (see **Table 2** and **Figure 3**). The sensitivity analysis found that for asthma the aOR became significant with the removal of one study (46) (OR = 0.65 95% CI 0.44–0.97 $p = 0.04$). The removal of any one study did not significantly change the direction of results for COPD (see **Supplementary Figures 3**, **4** for full details).

Risk of COVID-19 Related Mortality

When adjusted for one or more comorbidity, the pooled aOR was 0.83 (95% CI 0.71–0.96; $p = 0.01$; $I^2 = 61.48$) for asthma and 1.28 (95% CI 1.18–1.39; $p = <0.001$; $I^2 = 34.51$) for COPD (see **Table 2** and **Figure 4**). The sensitivity analysis found that for asthma the aOR became non-significant with the removal of one study (46) (OR = 0.83 95% CI 0.66–1.05 $p = 0.118$), and the results did not significantly change for COPD when any one study was removed (see **Supplementary Figures 5**, **6** for full details).

Regarding studies that reported aHRs in the form of Cox regression models, the pooled risk of mortality was 0.93 (95% CI 0.87–1.00; $p = 0.049$; $I^2 = 64.18$) for asthma and 1.30 (95% CI 1.17–1.44; $p =< 0.001$; $I^2 = 88.39$) for COPD (see **Table 2** and **Figure 5**). The sensitivity analysis found that the removal of any one study did not significantly change the direction of results for COPD, and the removal of any one of three studies

TABLE 2 | Meta-analysis showing the pooled adjusted risk of unfavorable COVID-19 outcomes in subjects with asthma or COPD.

Study details			Meta-analysis		Heterogeneity	Publication bias	GRADE rating
Lung disease	**Number of studies**	**Number of participants**	**Odds ratio (95% CI)**	***p*-value**	I^2	**Egger bias and *p*-value**	
Hospitalisation							
Asthma	7	1,087,689	0.873 (0.726–1.049)	0.148	85.355	0.747 $p = 0.678$	Moderate (downgraded due to high heterogeneity)
COPD	6	588,025	1.390 (1.307–1.478)	<0.001	4.236	1.453 $p = 0.050$	Moderate (downgraded due to possible publication bias)
ICU admission							
Asthma	4	167,849	0.746 (0.545–1.020)	0.067	87.198	−1.979 $p = 0.653$	Moderate (downgraded due to high heterogeneity)
COPD	9	197,108	1.336 (1.139–1.566)	<0.001	66.643	1.537 $p = 0.075$	Moderate (downgraded due to high heterogeneity)
Mortality (aORs)							
Asthma	7	876,759	0.827 (0.711–0.961)	0.013	61.481	0.007 $p = 0.996$	Moderate (downgraded due to high heterogeneity)
COPD	17	950,502	1.276 (1.176–1.385)	<0.001	34.508	0.881 $p = 0.038$	Moderate (downgraded due to possible publication bias)
Mortality (aHRs from Cox regression models)							
Asthma	4 (5 outcomes)	122,786	0.930 (0.865–1.000)	0.049	64.176	1.400 $p = 0.414$	Moderate (downgraded due to high heterogeneity)
COPD	8 (9 outcomes)	123,886	1.296 (1.170–1.436)	<0.001	88.386	2.179 $p = 0.093$	Moderate (downgraded due to high heterogeneity)

GRADE, Grading of Recommendations, Assessment, Development, and Evaluations; COPD, Chronic Obstructive Pulmonary Disease; aOR, adjusted odds ratio; aHR, adjusted hazard ratio.

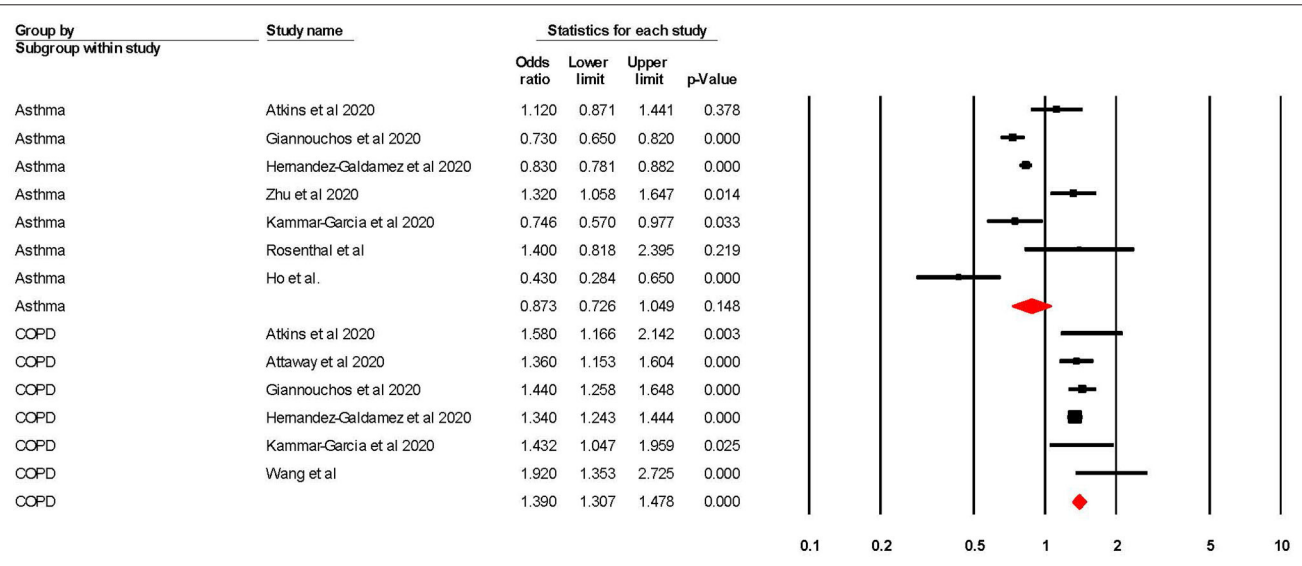

FIGURE 2 | Forest plot showing odds ratios (adjusted for at least one confounder) for COVID-19 related hospitalisation in subjects with asthma or Chronic Obstructive Pulmonary Disease (COPD).

(56, 58, 69) changed the significance of results in asthma (see **Supplementary Figures 7**, **8** for full details).

Certainty of Evidence Using the GRADE Approach

Using the GRADE (33) approach, all of the results were rated as being a "moderate" level of certainty. The two reasons why the level of evidence was not rated as "high" was because of either (1) high heterogeneity, or (2) the presence of publication bias.

Sub-Group Analyses

When sub-grouped between studies with >10 vs. <10k participants, no significant changes were found, except for in

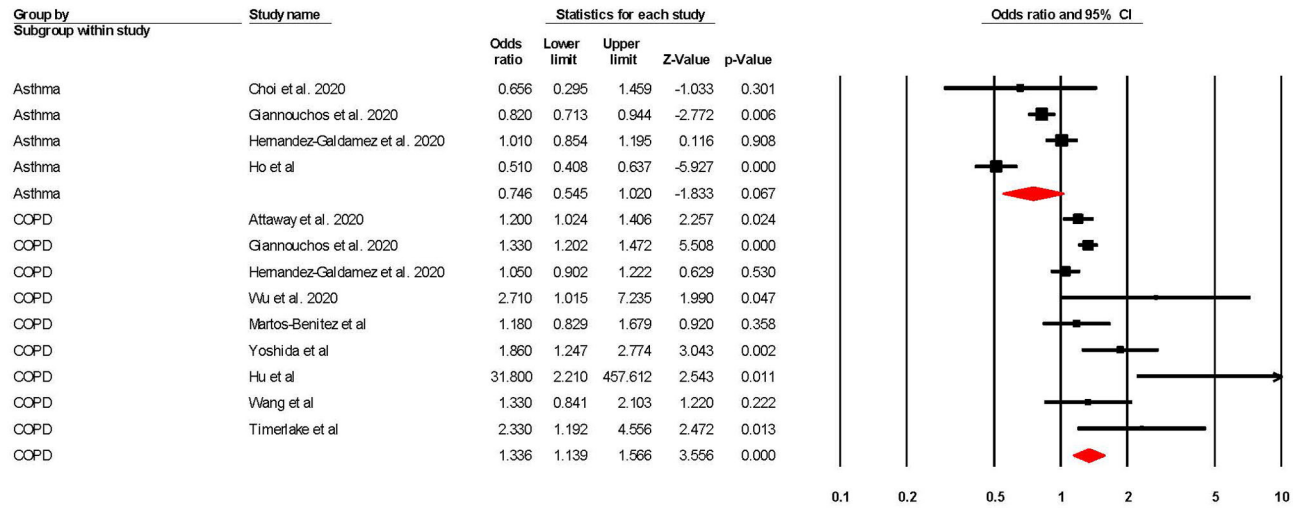

FIGURE 3 | Forest plot showing odds ratios (adjusted for at least one comorbidity) for COVID-19 related intensive care admission in subjects with asthma or Chronic Obstructive Pulmonary Disease (COPD).

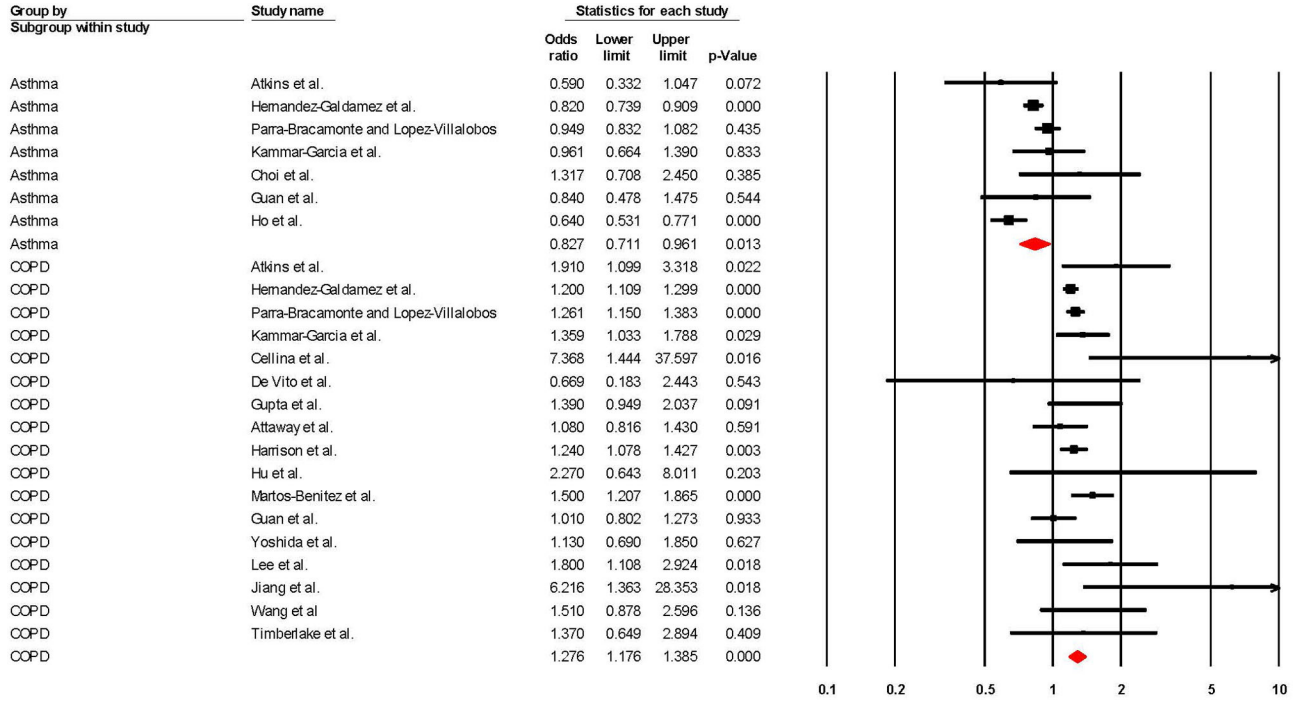

FIGURE 4 | Forest plot showing odds ratios (adjusted for at least one comorbidity) for COVID-19 related overall mortality in subjects with asthma or Chronic Obstructive Pulmonary Disease (COPD).

risk of mortality (as measured by Cox regression) in participants with COPD. It was found that studies with >10k participants yielded significantly lower ($p = 0.001$) risk of mortality (aHR = 1.13 95% CI 1.10–1.17) when compared to studies that had <10k participants (aHR = 1.59 95% CI 1.31–1.94), and also yielded lower heterogeneity in this subgroup (>10k = 36.19%; <10k = 58.32%). Although the differences between sub-groups were significant, both pooled aHRs were still, respectively, statistically significant. Full information can be found in **Table 3** and in **Supplementary Figures 9–16**.

DISCUSSION

This meta-analysis included 37 studies examined the adjusted risks of COVID-19 related hospitalsation, ICU-admission, and mortality in populations with and without either asthma or

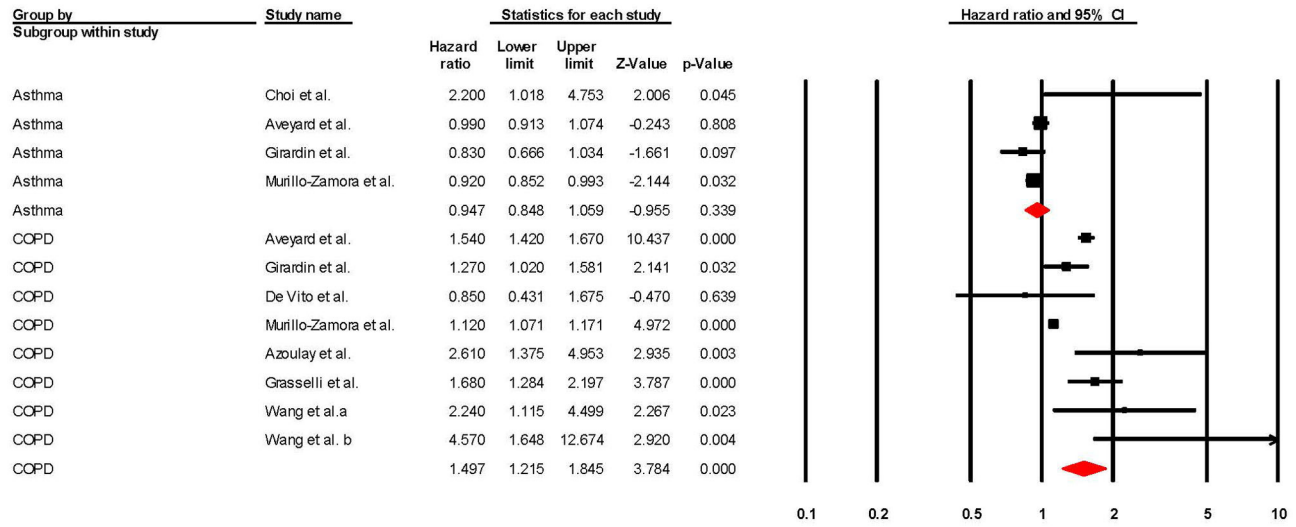

FIGURE 5 | Forest plot showing Cox regression hazard ratios (adjusted for at least one comorbidity) for COVID-19 related overall mortality in subjects with asthma or Chronic Obstructive Pulmonary Disease (COPD).

COPD. The analysis suggests with a moderate level of certainty that COPD is a significant risk factor for COVID-19 related hospitalsation, ICU admission, or mortality when the risks were adjusted for at least one comorbidity. Furthermore, with a moderate level of certainty, asthma was not shown to be a significant risk factor for COVID-19 related hospitalsation, ICU admission, or mortality when adjusted for at least one comorbidity.

COPD was shown to be a significant risk factor in all three outcomes, with the sensitivity analysis reporting robustness in all outcomes. These results broadly agree with previous meta-analyses exploring similar outcomes in this population (10–14). When directly comparing reported risks, this study shows a marked decrease in mortality risk (5.69 vs. 1.25) when compared to Lippi and Henry (10), which would be expected. Although the mechanisms that underpin this risk are not clear, several hypotheses, including the increased expression of the angiotensin-converting enzyme 2 (ACE-2) in COPD patients, have been reported as COVID-19's route of entry into susceptible cells (73). Furthermore, it has been reported that morbidity and mortality in COPD patients are frequently related to acute exacerbation (12), and severe respiratory failure (67) which may add to already compromised respiratory capacity among COVID-19 patients (12, 74, 75). Moreover, the effect of smoking could be a reason why people with COPD appear to have increased COVID-19 risks; indeed, a recent systematic review and meta-analysis (76) reported that both current and former smokers have increased risks of COVID-19 related deaths, although these risks do not appear to have been adjusted for any co-variates. Further exploration into adjusted smoking risk, in particular adjusted for COPD and/or asthma presence, would be beneficial.

Other comorbidities have also been shown to be significant risk factors for unfavorable COVID-19 related outcomes including (but not limited to), hypertension (4), diabetes (5), and obesity (3). It is difficult to directly compare our results with previous data as these previous estimates report unadjusted data making true risks of each comorbidity hard to compare. We agree with Jordan et al. (20) and recommend that future studies aim to report risks based on adjustments for, at the very least, age, sex, and smoking status so that true risks can be determined. It is recommended that clinicians continue to consider COPD patients to be at greater risk of COVID-19 related morbid outcomes. Individuals with COPD should take extra precautions to ensure that exposure to COVID-19 is minimal.

Although asthma has been related to worse outcomes in other viral infections, including other forms of coronavirus (16, 17), our analysis did not suggest asthma as a significant risk factor for any of the outcomes measured in this review, apart from mortality (measured as a non-time dependent OR), however sensitivity analysis suggested that the significance of this outcome was subject to the influence of one large study. These results broadly agree with previous meta-analyses that concluded that asthma was not a significant risk factor for either mortality or "severe" health outcomes (14, 18, 35, 77). When directly comparing reported risks across these meta-analyses, this study's mortality risk is lower (0.83 and 0.93 vs. 0.96 and 1.03) (35, 77), which is an expected result given we pooled adjusted ORs and the other meta-analyses were not adjusted for any other covariates. These results, however, need to be interpreted with caution as the included studies have used asthma as an umbrella term and did not differentiate between different types or severities of the disease. The National Health Service (NHS) in the UK has severe asthma listed "high risk of severe outcomes," and other severities at "moderate risk" of COVID-19 (78), and although this study does not support this, more data is required to differentiate between different severity of asthma, and, as such, individuals with asthma should still aim to minimize their risk of COVID-19 exposure.

TABLE 3 | Sub-group analyses showing the pooled adjusted risk of unfavorable COVID-19 outcomes in participants with asthma or COPD stratified >10 vs. <10k participants.

Study details			Meta-analysis			Heterogeneity
Lung disease	**Sub-group**	**Number of studies**	**Odds ratio (95% CI)**	***p*-value**	**Differences between groups**	I^2
Hospitalisation						
Asthma	>10k	1	1.400 (0.818–2.395)	0.219	$p = 0.079$	0.000
	<10k	6	0.841 (0.697–1.014)	0.070		86.609
COPD	>10k	4	1.374 (1.291–1.463)	<0.001	$p = 0.463$	0.000
	<10k	2	1.559 (1.120–2.169)	0.008		67.174
ICU admission						
Asthma	>10k	3	0.757 (0.537–1.065)	0.110	$p = 0.748$	91.376
	<10k	1	0.656 (0.295–1.459)	0.301		0.000
COPD	>10k	3	1.191 (0.994–1.426)	0.058	$p = 0.077$	69.159
	<10k	6	1.708 (1.196–2.441)	0.003		65.159
Mortality (aORs)						
Asthma	>10k	6	0.808 (0.695–0.938)	0.013	$p = 0.133$	62.813
	<10k	1	1.317 (0.708–2.450)	0.005		0.000
COPD	>10k	7	1.251 (1.160–1.349)	<0.001	$p = 0.320$	37.046
	<10k	10	1.425 (1.115–1.821)	0.005		36.935
Mortality (aHRs from Cox regression models)						
Asthma	>10k	2 (3 outcomes)	0.913 (0.852–0.978)	0.009	$p = 0.529$	59.036
	<10k	3	0.993 (0.772–1.275)	0.954		69.146
COPD	>10k	2 (3 outcomes)	1.132 (1.097–1.168)	<0.001	$p = 0.001$	36.191
	<10k	7	1.590 (1.305–1.937)	<0.001		58.320

COPD, Chronic Obstructive Pulmonary Disease; aOR, adjusted odds ratio; aHR, adjusted hazard ratio.

Although this is the first review to systematically examine risks of unfavorable COVID-19 outcomes in populations with asthma or COPD with effect sizes adjusted for at least one covariate, our results should be considered within its limitations. Firstly, although the majority were deemed as low risk of bias, the effect of methodological bias cannot be ruled out. Secondly, the pooling of adjusted ORs (with different studies adjusting for different covariates) inherently creates a degree of inconsistency, meaning that the results should be treated only as indicative. Thirdly, there was considerable heterogeneity in some of the reported analyses, especially in the asthmatic populations, which could not be explained by the presence of large studies vs. smaller ones. One probable reason for this is the different asthma diagnosis methods, in particular regarding the type and severity of asthma. Furthermore, there was some evidence of publication bias, which could not be explained. Lastly, meta-analyses have inherent limitations: their findings are dependent on estimates selected from each primary study and thus are dependent on the accuracy of primary studies (79).

CONCLUSIONS

COPD is significantly associated with worse COVID-19 related, hospital admission, ICU admission and mortality, even when adjusted for at least one comorbidity. Asthma, when pooling risks were adjusted for other comorbidities, was not associated with a higher risk of COVID-19 related hospitalsation, ICU admission

and mortality. Clinicians should note these findings when dealing with patients with these comorbidities. Furthermore, individuals with COPD should take special precautions to limit the risk of COVID-19 exposure to negate these potential outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MT and MV acquisition and analysis. MT, MV, and SP drafted the work. MT and MV verified the underlying data. All authors made substantial contributions to the conception, design of the work, interpretation of data for the work, revising it critically for important intellectual content and final approval of the version to be published, and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.668808/full#supplementary-material

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Abnormal Coagulation Function of Patients With COVID-19 Is Significantly Related to Hypocalcemia and Severe Inflammation

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This study aimed to detect, analyze, and correlate the clinical characteristics, blood coagulation functions, blood calcium levels, and inflammatory factors in patients with mild and severe COVID-19 infections. The enrolled COVID-19 infected patients were from Wuhan Jin Yin-tan Hospital (17 cases, Wuhan, China), Suzhou Infectious Disease Hospital (87 cases, Suzhou, China), and Xuzhou Infectious Disease Hospital (14 cases, Xuzhou, China). After admission, basic information was collected; X-ray and chest CT images were obtained; and data from routine blood tests, liver and kidney function, myocardial enzymes, electrolytes, blood coagulation function, (erythrocyte sedimentation rate) ESR, C-reactive protein (CRP), IL-6, procalcitonin (PCT), calcitonin, and other laboratory tests were obtained. The patients were grouped according to the clinical classification method based on the pneumonia diagnosis and treatment plan for new coronavirus infection (trial version 7) in China. The measurements from mild (56 cases) and severe cases (51 cases) were compared and analyzed. Most COVID-19 patients presented with fever. Chest X-ray and CT images showed multiple patchy and ground glass opacities in the lungs of COVID 19 infected patients, especially in patients with severe cases. Compared with patients with mild infection, patients with severe infection were older ($p = 0.023$) and had a significant increase in AST and BUN. The levels of CK, LDH, CK-MB, proBNP, and Myo in patients with severe COVID-19 infection were also increased significantly compared to those in patients with mild cases. Patients with severe COVID-19 infections presented coagulation dysfunction and increased D-dimer and fibrin degradation product (FDP) levels. Severe COVID-19 patients had low serum calcium ion (Ca^{2+}) concentrations and high calcitonin and PCT levels and exhibited serious systemic inflammation. Ca^{2+} in COVID-19 patients was significantly negatively correlated with PCT, calcitonin, D-dimer, PFDP, ESR, CRP and IL-6. D-dimer in COVID-19 patients was a significantly positively correlated with CRP and IL-6. In conclusion,

patients with severe COVID-19 infection presented significant metabolic dysfunction and abnormal blood coagulation, a sharp increase in inflammatory factors and calcitonin and procalcitonin levels, and a significant decrease in Ca^{2+}. Decreased Ca^{2+} and coagulation dysfunction in COVID-19 patients were significantly correlated with each other and with inflammatory factors.

Keywords: COVID-19, metabolic disorder, coagulation function, hypocalcemia, inflammation

BACKGROUND

It has been nearly a year since the discovery of the novel coronavirus—COVID-19, which has become a global-scale disaster event. COVID-19 infected patients can develop serious pneumonia and metabolic disorders (1), acute respiratory distress syndrome (ARDS), multiple organ dysfunction (MODS), and even septic shock and death (2–4).

Dysfunction of the coagulation/fibrinolysis system is an important pathophysiological feature of COVID-19 patients, and it is related to the inflammatory cascade induced by viral infection (5–7). The diffuse intravascular coagulation (DIC) induced by severe infections often becomes a decisive factor in the death of patients with severe infection (8–11).

To date, many studies have revealed dysfunction of the coagulation/fibrinolysis system in COVID-19 infected patients. Although a review titled "Coagulation and anticoagulation in COVID-19" was recently published (12), the specific relationship between the coagulation function and inflammation in patients with mild and severe COVID-19 infection, as well as the correlation between coagulation function and serum Ca^{2+} [which was abnormally decreased in patients with severe COVID-19 infection (13)], has not yet been determined.

Therefore, we analyzed basic information, X-ray and chest CT images, routine blood tests, liver and kidney function, myocardial enzymes, electrolytes, blood coagulation function, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), IL-6, procalcitonin (PCT), and calcitonin in mild and severe cases of COVID-19. We focused on the coagulation function of these cases and compared and analyzed the correlations among decreased Ca^{2+}, coagulation disorder and other test results, including systemic inflammation and metabolic disorders, in mild and severe COVID-19 infected patients.

In this study, the above indicators and correlations of COVID-19 patients with different severity were analyzed to further explore the role of coagulation function in COVID-19 infection, and in especial, to reveal the role of hypocalcemia in severe COVID-19 infection. Our present study suggests that coagulation function and serum Ca^{2+} concentration should be closely supervised when treating patients with COVID-19 infection.

MATERIALS AND METHODS

COVID-19 Infection Diagnosis

All cases met the diagnostic criteria of "The Pneumonia diagnosis and treatment plan for new coronavirus infection (trial version 7)" of China (14) (http://www.nhc.gov.cn/yzygj/s7653p/202003/46c9294a7dfe4cef80dc7f5912eb1989.shtml). All cases had the corresponding clinical manifestations: fever, imaging features of pneumonia, normal or decreased white blood cell count or decreased lymphocyte count in the early stage of COVID-19 infection, and pathogenic evidence of a positive SARS-CoV-2 gene.

For SARS-CoV-2 gene detection, throat swab samples were collected from patients and immediately tested by using a transcription polymerase chain reaction (PCR) system to detect the SARS-CoV-2 gene. A reverse transcription polymerase chain reaction (RT-PCR) kit (Da'an Gene, Shenzhen, China) was used to detect SARS-CoV-2 conserved genes through the ABI7500 system (Roche). All samples that were positive for SARS-CoV-2 or highly homologous with known SARS-CoV-2 (15) were confirmed to be positive for COVID-19.

COVID-19 Patients

The data for 107 cases were collected from three hospitals that received and cured COVID-19 patients from February 2 to June 25, 2020: Wuhan Jin Yin-tan Hospital (17 cases), Suzhou Infectious Disease Hospital (76 cases), and Xuzhou Infectious Diseases Hospital (14 cases). These data include basic clinical records, laboratory results, and X-ray and computed tomography (CT) scan images of the chest. The basic clinical records included the general information of the patients, such as the age, sex, contact history, admission temperature, disease course, and comorbidities (hypertension or diabetes). The laboratory test results included the blood cell count, liver and kidney functions, electrolytes, myocardial enzymes, coagulation function, ESR, CRP, IL-6, PCT, and calcitonin levels. Death cases were excluded, and all patients were eventually discharged from the hospital. Patients were grouped according to the clinical classification method of "The Pneumonia diagnosis and treatment plan for new coronavirus infection (trial version 7)." A total of 107 patients were divided into two groups according to the severity of the disease: the mild group (56 cases) and the severe group (51 cases). The study design was approved by the hospital ethics committee of the three hospitals.

Laboratory Data Analysis

Blood samples from patients were used for laboratory tests. A DxH 800 Coulter blood cell analyzer (Beckmann, America) was used to detect the patient's blood cell classification and ESR. Serum biochemical tests, which reflect the patient's liver and kidney function, electrolytes, and myocardial enzymes, including ALT, AST, ALP, ALB, CRP, TBIL, DBIL, BUN, Cr, TnT, CK, LDH, CK-MB, proBNP, Myo, Na^{+}, K^{+}, Cl^{-}, and Ca^{2+} were carried out by an automatic biochemistry analyzer system (Roche, Germany). IL-6, PCT and calcitonin levels

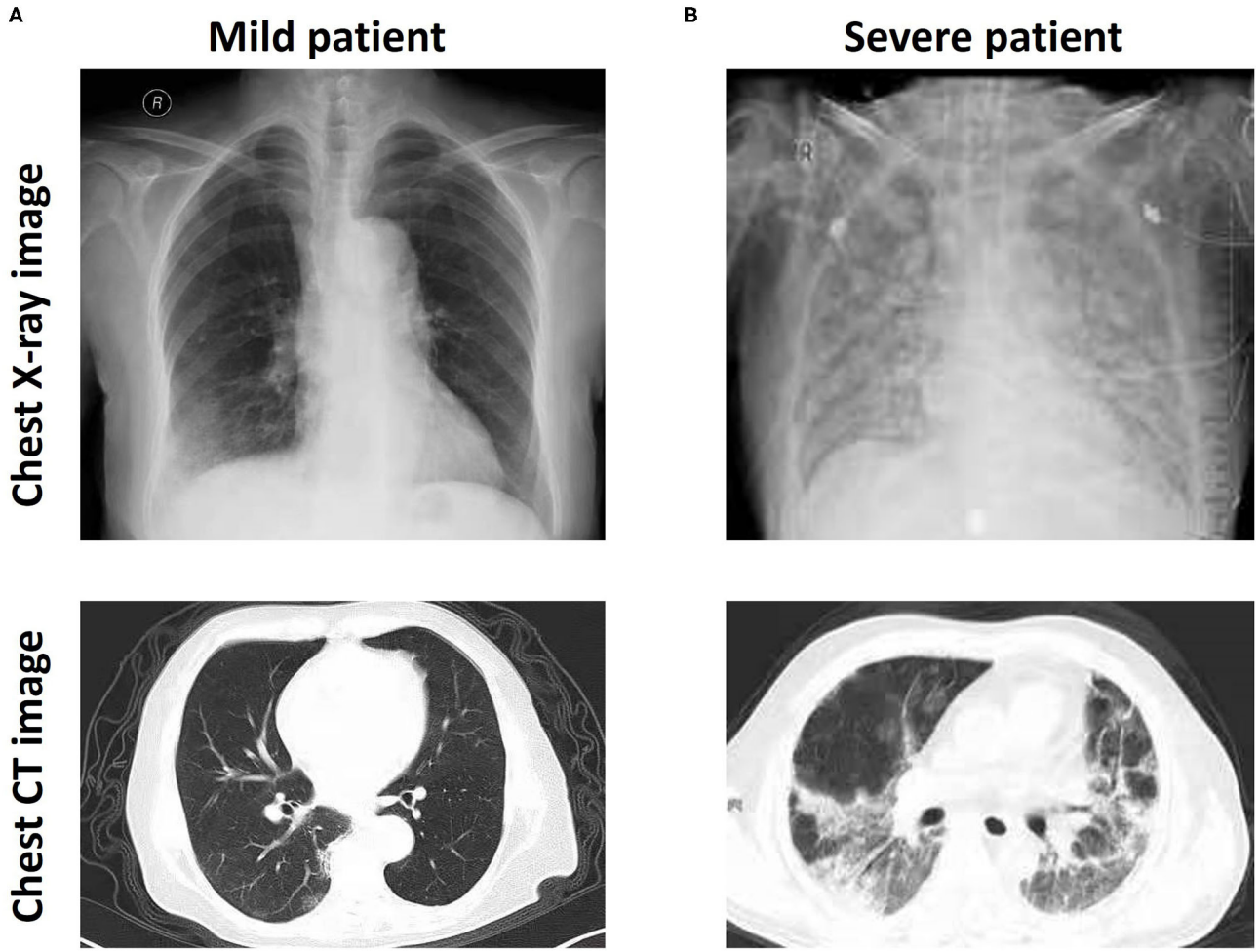

FIGURE 1 | CT and bedside chest X-ray images of patients with mild and severe COVID-19. Bedside chest X-ray and chest CT images showing multiple patchy shadows and ground glass opacity in the lungs of patients with mild **(A)** and severe **(B)** COVID-19.

were analyzed by a CL-2000i chemiluminescence immunoassay system (Mindray, Shenzhen, China). An ACL TOP 700 hemostasis test system (Wolfen, USA) was used to detect coagulation indicators.

Statistical Analysis

The SPSS 23.0 statistical software was used to analyze the data. Firstly, the Levene variance equality test was used to determine whether the variance within the groups was equality according to the *F*-value. After that, an independent sample *T*-test was performed, and the *p*-value under the assumption of equal variance or unequal variance were all analyzed. The reasonable statistical results of *p*-value were selected according to the *F*-value of Levene variance equality test. The measurement data are expressed as the mean ± standard deviation. A value of $p < 0.05$ was considered statistically significant. Pearson correlation analysis between different indexes was carried out using SPSS 23.0 statistical software. Some indexes were regarded as a same variable data set and Canonical correlation was used to analyze the correlation between these data sets using SPSS 23.0 statistical software. GraphPad Prism 7.0 (San Diego, CA, USA) was used to perform the correlation analysis of the data. The correlation coefficient and linear regression were jointly used to analyze the correlation of the indicated data.

RESULTS

Changes in Clinical Characteristics and Blood Cell Counts of Patients With Mild and Severe COVID-19 Infection

All patients were diagnosed with positive SARS-CoV-2 gene expression by RT-PCR. Most patients presented with fever (the average body temperature of all patients was 37.79 ± 1.08°C). Chest X-ray and CT images showed multiple patchy and ground glass opacities in the lungs (**Figure 1A**), thus revealing a wide range of lesions and pulmonary damage, especially in patients with severe COVID-19 infection (**Figure 1B**). The average age of 56 patients with mild disease was 46.16, with

TABLE 1 | Baseline characteristics in patients with mild and severe COVID-19 infection.

	All patients ($n = 107$)	Patients with mild disease ($n = 56$)	Patients with severe disease ($n = 51$)	p-value	F
Age	48.86 ± 10.75	46.16 ± 10.48	51.82 ± 10.14	0.006	0.294
Gender					
Male	54 (50.46%)	31 (55.35%)	23 (45.09%)		
Female	53 (49.53%)	25 (44.62%)	28 (54.90)		
Height (cm)	166.73 ± 8.08	165.73 ± 7.48	167.82 ± 8.47	0.182	1.409
Weight (kg)	66.47 ± 11.43	66.95 ± 11.65	65.94 ± 11.05	0.652	0.964
Admission Temperature (°C)	37.79 ± 1.08	37.88 ± 1.12	37.69 ± 1.01	0.363	1.481
Contact history					
Yes	105 (98.13%)	54 (96.42)	51 (100%)		
No	2 (1.86%)	2 (3.57%)	0 (0%)		
Disease course (day)	11.11 ± 6.05	11.2 ± 6.09	11.02 ± 5.95	0.881	0.265
Complication					
Diabetes	6 (5.60%)	4 (7.14%)	2 (3.92%)		
Hypertension	7 (6.54%)	2 (3.57%)	5 (9.80%)		

31 males and 25 females in the group, and the average age of 51 patients with severe infection was 51.82, with 23 males and 28 females in the group. More than 98% of the patients (105/107) had a contact history. In total, 12 patients had other diseases, such as hypertension and diabetes. Patients with severe disease were older on average than those with mild disease, but there were no significant differences observed in the other characteristics, including height, weight, admission temperature, and the duration of infection (**Table 1**).

Compared with the normal range, the number of white blood cells in patients with severe COVID-19 infection increased significantly ($7.69 \pm 1.61 \times 10^9$/L, Normal range 4.3–5.8 $\times 10^9$/L), while the white blood cells of patients with mild infection ($4.98 \pm 0.55 \times 10^9$/L) were still within the normal range. The increase in the level of white blood cells in the severe group was attributed to the increase in the level of neutrophils. The level of neutrophils was significantly increased in the blood of patients with severe cases ($3.59 \pm 2.53 \times 10^9$/L vs. $5.72 \pm 2.76 \times 10^9$/L) compared with mild cases. The level of lymphocytes in patients with severe infection was lower than the normal range ($1.03 \pm 0.74 \times 10^9$/L, normal range 1.1–3.2 $\times 10^9$/L) and was also significantly lower than the levels in patients with mild infection ($1.32 \pm 0.57 \times 10^9$/L vs. $1.03 \pm 0.74 \times 10^9$/L; **Table 2**).

Patients With Mild and Severe COVID-19 Infection Present Varying Degrees of Metabolic Disorders and Abnormal Biochemical Tests

We observed a sharp increase in neutrophil levels in patients with severe COVID-19 infection, which may suggest that a very pronounced inflammatory response has occurred and disrupted the metabolism and function of multiple organs in the body (16). Therefore, we next evaluated the liver and kidney functions, myocardial enzymes, and electrolytes of the two groups of patients by using laboratory biochemical tests.

The liver and kidney function indexes of patients with severe disease were compared with those of patients with mild disease. For liver function, patients with severe disease had significantly increased levels of ALT, AST, ALP, TBIL, and DBIL ($p < 0.001$) and a higher level of BUN ($p < 0.001$). However, a considerable proportion of these liver and kidney function indicators were still within the normal range, including the levels of ALT, ALP, TBIL, DBIL, and BUN in patients with severe cases, which were significantly higher than those in patients with mild cases. It is worth noting that AST (46.06 ± 11.59 U/L, normal range 0.0–37.0 U/L) was extremely elevated in patients with severe disease, while ALB (28.61 ± 5.83 g/L, normal range 40–55 g/L) was extremely decreased, and both values were far from the normal range (**Table 3**).

More attention should be given to the heart function of patients with severe COVID-19 infection. Regarding myocardial enzymes, although the difference in TnT levels between mild and severe cases was not significant (11.52 ± 5.68 ng/ml vs. 13.69 ± 6.02 ng/ml, $p = 0.06$), the TnT levels of all patients were sharply increased and far exceeded the normal range (0–0.15 ng/ml). The levels of CK, LDH, CK-MB, proBNP, and Myo in severe cases were significantly increased compared with the levels of mild cases ($p < 0.001$). Among these indicators, the levels of LDH (520.78 ± 121.38 U/L, normal range 90–245 U/L) and proBNP (399.22 ± 184 pg/ml, normal range 0–125 pg/ml) increased tremendously in patients with severe disease and far exceeded the normal range (**Table 3**). This result suggested that patients with severe COVID-19 infection have abnormal heart function and may even have heart failure.

For serum electrolytes, the Na^+, K^+, and Cl^- levels in the mild and severe groups were within the normal range, but the level of Ca^{2+} in the severe group was significantly lower than that in the mild group (2.22 ± 0.07 mmol/L vs. 1.91 ± 0.06 mmol/L, $p < 0.001$), and it was also below the normal range (2.5–2.7 mmol/L) (**Table 3**). The decrease in Ca^{2+} may be related to the changes in myocardial enzymes in patients with severe COVID-19 infection.

TABLE 2 | Comparison of blood cell counts between patients with mild and severe COVID-19 infection.

	Normal range	Patients with mild disease ($n = 30$)	Patients with severe disease ($n = 30$)	*p*-value	*F*
White blood cells (10^9/L)	4.3–5.8	4.98 ± 0.55	7.69 ± 1.61	<0.001	58.146
Red blood cells (10^{12}/L)	3.5–9.5	5.42 ± 2.49	5.95 ± 3.13	0.337	2.529
Hemoglobin (g/L)	130–175	152.34 ± 19.91	154.45 ± 20.64	0.595	0.015
Platelets (10^9/L)	125–350	184.36 ± 62.22	193.67 ± 64.4	0.453	1.279
Neutrophils (10^9/L)	1.8–6.3	3.59 ± 2.53	5.72 ± 2.76	<0.001	0.771
Lymphocytes (10^9/L)	1.1–3.2	1.32 ± 0.57	1.03 ± 0.74	0.026	1.707
Monocytes (10^9/L)	0.1–0.6	0.42 ± 0.16	0.38 ± 0.14	0.251	1.479
Eosnophils (10^9/L)	0.02–0.52	0.02 ± 0.03	0.02 ± 0.06	0.914	0.120

TABLE 3 | Comparison of blood biochemical tests and serum electrolytes between mild patients and patients with severe COVID-19 infection.

	Normal range	Patients with mild disease ($n = 30$)	Patients with severe disease ($n = 30$)	*p*-value	*F*
Liver related					
ALT (U/L)	0.0–40.0	22.2 ± 5.8	28.37 ± 9.18	<0.001	20.995
AST (U/L)	0.0–37.0	20.45 ± 3.33	46.06 ± 11.59	<0.001	69.83
ALP (U/L)	45–125	62.48 ± 11.19	77.29 ± 14.93	<0.001	9.462
ALB (g/L)	40–55	44.64 ± 3.51	28.61 ± 5.83	<0.001	17.214
TBIL (μmol/L)	5.13–22.24	10.49 ± 2.47	13.01 ± 3.28	<0.001	6.064
DBIL (μmol/L)	1.70–8.55	3.67 ± 0.81	5.33 ± 1.39	<0.001	17.239
Kidney related					
BUN (mmol/L)	1.7–8.3	5.23 ± 1.48	6.32 ± 1.84	0.001	2.81
Cr (μmol/L)	36–132	58.75 ± 8.27	61.31 ± 7.95	0.109	0.073
Heart related					
TnT (ng/mL)	0–0.15	11.52 ± 5.68	13.69 ± 6.02	0.06	0.106
CK (U/L)	30–170	61.09 ± 19.52	94.69 ± 30.91	<0.001	16.535
LDH (U/L)	90–245	163.93 ± 34.48	520.78 ± 121.38	<0.001	53.866
CK-MB (ng/mL)	0–5	0.72 ± 0.12	1.88 ± 0.51	<0.001	68.471
proBNP (pg/mL)	0–125	52.95 ± 22.66	399.22 ± 184	<0.001	119.995
Myo (ng/mL)	0–70	26.77 ± 3.34	48.55 ± 7.7	<0.001	25.186
Serum electrolytes					
Na^+ (mmol/L)	135–155	147.16 ± 10.23	145.46 ± 8.47	0.359	4.488
K^+ (mmol/L)	3.5–5.5	4.3 ± 0.63	4.09 ± 0.61	0.079	0.033
Cl^- (mmol/L)	95–115	108.07 ± 4.94	107.8 ± 5.14	0.786	0.46
Ca^{2+} (mmol/L)	2.25–2.7	2.22 ± 0.07	1.91 ± 0.06	<0.001	0.149

Patients With Severe COVID-19 Infection Present More Severe Coagulation Dysfunction

Ca^{2+} is an important factor in coagulation function (17), and it was significantly decreased in patients with severe COVID-19 infection. Thus, we examined indicators related to coagulation function in COVID-19 patients. Compared with patients with mild disease, patients with severe disease showed increased PT, including PT (s) (12.05 ± 0.63 s vs. 13.3 ± 1.36 s) and PT (%) (74.23 ± 9.19% vs. 95.59 ± 12.08%), but they also showed decreased fibrinogen (3.35 ± 0.75 g/L vs. 2.60 ± 0.59 g/L) and AT-3 (88.14 ± 4.29% vs. 73.73 ± 5.77%) activity reduction ($p < 0.001$). It is worth noting that the D-dimer and fibrin degradation products (FDPs) were within the normal range for patients with mild disease, while the D-dimer (5.54 ± 2.36 mg/L, normal range 0–1.5 mg/L) and FDP (70.15 ± 30.47 μg/ml, normal range 0–5 μg/ml) in patients with severe disease presented enormous abnormal increases that were several times or even dozens of times higher than the normal range (**Table 4**).

Patients With Severe COVID-19 Infection Present More Severe Systemic Inflammation and Overaugmented Ca^{2+} Reducing Function

For inflammation indicators, including ESR, CRP and IL-6, COVID-19 all patients with COVID-19 infection showed a significantly elevated state (the IL-6 level in patients with mild cases was within the normal range). Compared with the levels in the mild group, ESR (31.96 ± 11.24 vs. 84.14 ± 34.08 mm/h), CRP (44.61 ± 13.99 vs. 68.17 ± 15.98 mg/L), and IL-6 (3.77 ±

TABLE 4 | Comparison of coagulation indicators between patients with mild and severe patients with COVID-19 infection.

	Normal range	Patients with mild disease ($n = 30$)	Patients with severe disease ($n = 30$)	p-value	F
PT (s)	10.5–13.5	12.05 ± 0.63	13.3 ± 1.36	<0.001	59.902
PT (%)	75–125	74.23 ± 9.19	95.59 ± 12.08	<0.001	4.433
APTT (s)	21–37	26.96 ± 3.57	27.82 ± 3.72	0.228	0.141
TT (s)	13–21	16.62 ± 3.7	16.6 ± 3.24	0.967	1.793
INR	0.8–1.2	1 ± 0.12	1.02 ± 0.14	0.603	4.048
Fibrinogen (g/L)	2–4	3.35 ± 0.75	2.60 ± 0.59	<0.001	5.788
AT-3 (%)	75–125	88.14 ± 4.29	73.73 ± 5.77	<0.001	6.226
D-dimer (mg/L)	0–1.5	2.43 ± 0.65	5.54 ± 2.36	<0.001	99.096
FDP (μg/ml)	0–5	2.37 ± 0.66	70.15 ± 30.47	<0.001	117.06

1.69) vs. 8.66 ± 2.16 pg/mL) were all significantly increased in the severe group ($p < 0.001$; **Table 5**). These results suggest that patients with severe COVID-19 infection present more severe systemic inflammation.

Because of the presence of coagulation dysfunction and the significant decrease in Ca^{2+} concentration in patients with severe disease, we then examined the levels of PCT and calcitonin in the patients. The results showed that the PCT and calcitonin levels of all patients with COVID-19 infection were significantly higher than the normal range, which may explain why the serum Ca^{2+} levels of COVID-19 patients were significantly reduced. Compared with the mild group, the severe group had higher levels of PCT (0.04 ± 0.02 vs. 1.17 ± 0.56 ng/mL) and calcitonin (0.04 ± 0.02 vs. 1.07 ± 0.58 ng/mL; $p < 0.001$; **Table 5**), which may explain why patients with severe disease have lower Ca^{2+} and more severe coagulation dysfunction than those with mild disease.

Decreased Ca^{2+}, Coagulation Dysfunction, and Inflammation Indicators in Patients With COVID-19 Infection Are Significantly Correlated

Finally, we analyzed the correlation between the indicators related to the reduction of Ca^{2+} (including serum Ca^{2+}, PCT and calcitonin), coagulation function-related indicators (including D-dimer and PFDP), and inflammation indicators (ESR, CRP, and IL-6) in COVID-19 patients. The analysis results showed that the serum Ca^{2+} of COVID-19 patients was significantly negatively correlated with PCT, calcitonin, D-dimer, PFDP, ESR, CRP, and IL-6 (**Figures 2A–G** and **Supplementary Table 1**). The coagulation function-related indicator D-dimer had a significant positive correlation with CRP and IL-6 in COVID-19 patients (**Figures 2H,I** and **Supplementary Table 1**).

On the other hand, PT (s), PT (%), APTT (s), TT (s), INR, Fibrinogen (g/L), AT-3 (%), D-dimer (mg/L), and FDP (μg/ml) were regarded as a same variable data set and canonical correlation analysis was performed between this variable data set with other data sets, including a data set of Ca^{2+} and calcitonin and another data set of IL6, PCT, ESR, and CRP. The analysis results provided direct evidence that coagulation dysfunction was significantly related to decreased blood calcium and increased inflammation (**Supplementary Tables 2**, **3**).

These results revealed systemic pathological changes in COVID-19 patients, and they were all correlated, including decreased Ca^{2+}, coagulation dysfunction, and systemic inflammation. Disorders of metabolic function, abnormal biochemical tests, and changes in blood white blood cells present in COVID-19 patients may be caused by the decreased blood calcium and coagulation dysfunction.

DISCUSSION

In the present study, we first analyzed the difference between the clinical characteristics and blood cell classification of patients with mild and severe COVID-19 infection. We ruled out death cases and all the patients in the present study recovered from the infection. The data from the test results of COVID-19-infected patients were used to determine when severe symptoms appeared in the severe group. The mild group did not have severe symptoms throughout the entire course of infection.

Our data show that patients with severe disease tend to be older than patients with mild COVID-19 infection. Patients with severe disease have abnormally increased levels of white blood cells, which is consistent with previous studies (18, 19). A previous report showed that the percentage and count of monocytes in patients with mild infection are higher than those in healthy adults (20). However, our study shows that the level of monocytes in the blood is not significantly different between patients with mild and severe COVID-19 infection, and both are within the normal range. Our results also show that patients with severe disease exhibit a remarkable increase in neutrophils and a decrease in lymphocytes, while those with mild disease present neutrophils and lymphocytes in the normal range. These changes indicate that a very pronounced inflammatory response has occurred in the body of patients with severe COVID-19 infection and that the immune system suffers damage (21).

COVID-19 infection causes systemic responses in the body, including the liver (22, 23), kidney (22, 24, 25), heart (24, 26), and even the brain (27, 28). Consistent with previous reports (29, 30), we found that patients with severe COVID-19 infection have significantly increased ALT, AST, ALP, TBIL, and DBIL levels, especially AST levels, which are much higher than the normal

TABLE 5 | Comparison of inflammatory factors, calcitonin and PCT in patients with mild and severe patients with COVID-19 infection.

	Normal range	Patients with mild disease ($n = 30$)	Patients with severe disease ($n = 30$)	*p*-value	*F*
ESR (mm/h)	0–20	31.96 ± 11.24	84.14 ± 34.08	<0.001	70.769
CRP (mg/L)	0–10	44.61 ± 13.99	68.17 ± 15.98	<0.001	1.433
IL-6 (pg/mL)	0–7	3.77 ± 1.69	8.66 ± 2.16	<0.001	6.231
PCT (ng/mL)	0–0.1	0.04 ± 0.02	1.17 ± 0.56	<0.001	208.606
Calcitonin (ng/mL)	0–0.028	0.04 ± 0.02	1.07 ± 0.58	<0.001	160.418

range. The level of BUN is also significantly increased in patients with severe COVID-19 infection. We need to pay more attention to heart damage in patients with COVID-19 infection because the levels of myocardial enzymes, including CK, LDH, CK-MB, proBNP, and Myo (especially LDH and proBNP), are extremely high in patients with severe COVID-19 infection. These changes are signs of cardiac dysfunction and even heart failure (31, 32). Overall, these data reflect how patients with severe COVID-19 infection have a phenotype of systemic metabolic dysfunction, and may have damage to the liver, kidney, and heart.

Some metabolic diseases, including hypertension and diabetes mellitus, significantly affect the prognosis of COVID-19 infected patients. Hypertensive patients may have a higher risk of COVID-19 infection (33). Evidence showed that different blood type of COVID-19 hypertensive patients has different inflammatory and thrombosis status (34), which seems to be attributed to the ABO blood type may affect the coagulation process (35). The homeostasis of glucose influences the prognosis of COVID-19 infected patients with diabetes (36, 37), and hyperglycemia leads to severe inflammatory response in COVID-19 infected patients (38). The incidence of severe COVID-19 infection was significantly higher in diabetic patients compared with non-diabetic patients (39). In the present study, the data of patients with mild or severe COVID-19 infection accompanied by diabetes or hypertension were also collected. However, due to the small number of cases in the current study, the influence of hypertension or diabetes on abnormal coagulation function in COVID-19 infected patients was not further explored, but this aspect is worthy of close attention.

Ca^{2+} plays an important role in maintaining heart function and coagulation function (17, 40, 41). When testing serum electrolytes, we found that the levels of Na^{+}, K^{+}, and Cl^{-} were all normal in COVID-19 infected patients, although the level of Ca^{2+} in patients with severe COVID-19 infection was significantly reduced. Coagulation dysfunction may continue to alternate during COVID-19 infection. The comparison of coagulation function-related indicators of patients with mild and severe COVID-19 infection revealed that patients with severe disease had prolonged PT, lowered fibrinogen, and decreased AT-3 activity as well as extremely high D-dimer and FDP.

The above changes in coagulation function-related indicators indicate that as the severity of the disease increases, the microthrombotic load caused by the activation of the coagulation system gradually increases, while the activation and consumption of the anticoagulation system are more serious. Primary and secondary hyperfibrinolysis and bleeding tendency occur. The above characteristics indicate that the coagulation system of patients with severe COVID-19 infection presents a hypercoagulable state and microthrombosis, accompanied by activation of the anticoagulation system and consumption of anticoagulants. These features are clearly in line with the pathophysiological process of DIC (42, 43). As reported, COVID-19 patients often experience embolization and bleeding of vital organs and die of multiple organ dysfunction (3).

Coagulation dysfunction may be the reason why patients with severe COVID-19 infection have phenotypes that present systemic metabolic dysfunction and damage to other vital organs in addition to the lungs. We observed that the levels of inflammation indicators in severe COVID-19-infected patients, including ESR, CRP, and IL-6, were significantly increased. These changes in inflammation indicators have also been reported by other studies (44). Coagulation dysfunction may be the reason why damage to the liver, kidney, and heart occurs.

This and other reports (13, 45) confirmed that the Ca^{2+} concentration was reduced in patients with severe COVID-19 infection, so we detected the levels of PCT and calcitonin in the patients. In patients with severe COVID-19 infection, the decreased Ca^{2+} level corresponded to significantly increased levels of PCT and calcitonin in the blood.

At the end of this study, we conducted correlation analysis of the indicators related to the reduction of Ca^{2+}, coagulation function, and inflammation in COVID-19 patients. These analyses include correlation analysis between Ca^{2+} and other indicators, and correlation analysis between D-dimer and CRP and IL-6. As we speculated, Ca^{2+} was significantly negatively correlated with PCT, calcitonin, D-dimer, PFDP, ESR, CRP and IL-6, while D-dimer was positively correlated with CRP and IL-6. We speculate that the abnormality of blood coagulation function may be caused by metabolic function disorders and abnormal biochemical tests in patients with severe COVID-19 infection. Coagulation dysfunction may also be an important factor leading to the death of COVID-19 patients because DIC often leads to embolism and bleeding in vital organs, and multiple organ dysfunction (46, 47).

Our study is not exempt from limitations. Out sample size was not large enough. Many studies have suggested the disorder of coagulation function and decreased calcium ions in COVID-19 patients; we further analyzed and discussed this theme but did not carry out the basic research for further exploration. Thereby, this aspect requires further study in order to be confirmed. Again, association between hypertension or diabetes and coagulation

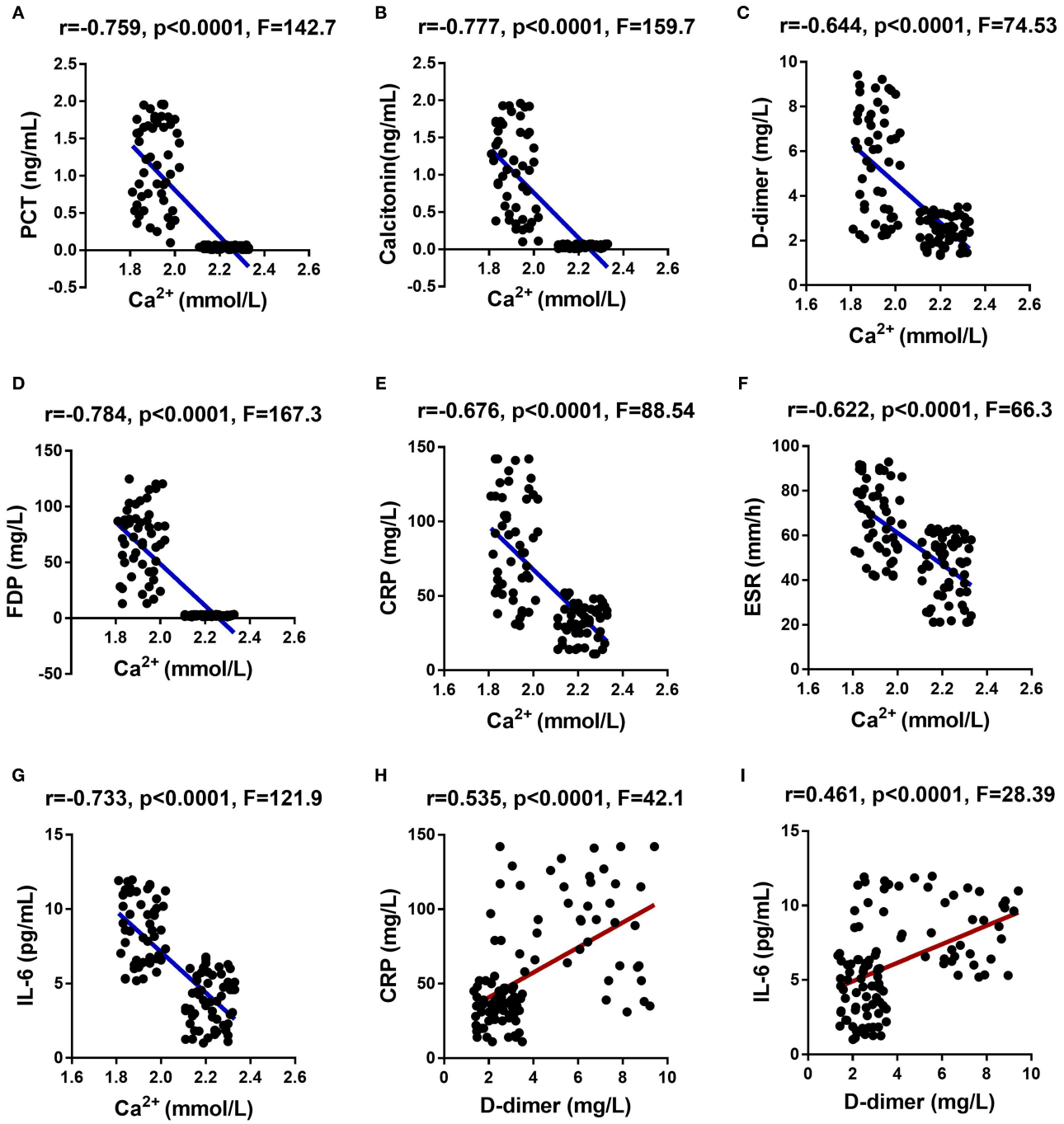

FIGURE 2 | Correlation analysis among the indicators related to the reduction of Ca^{2+}, coagulation function-related indicators, and inflammation indicators in COVID-19 patients. Ca^{2+} showed a significant negative correlation with PCT **(A)**, calcitonin **(B)**, D-dimer **(C)**, FDP **(D)**, ESR **(E)**, CRP **(F)**, and IL-6 **(G)**. D-dimer showed a significant positive correlation with CRP **(H)** and IL-6 **(I)**.

function in COVID-19 patients is worth exploring. On the other hand, all the patients we selected were eventually cured and discharged from the hospital, so it is worthwhile to perform further studies in COVID-19 patients without excluding the patients who eventually died.

CONCLUSIONS

Our data confirmed that patients with severe COVID-19 infection present significant metabolic and coagulation dysfunctions, a sharp increase in serum inflammatory factors,

calcitonin and PCT levels, and a significant decrease in Ca^{2+} concentration. Decreased Ca^{2+} and coagulation dysfunction in COVID-19 patients have significant correlations with inflammation. The evidence from our study may provide a better understanding of COVID-19 infection. In the treatment and monitoring of COVID-19 infected patients, attention should be devoted to the changes in the coagulation function and Ca^{2+} levels.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Wuhan Jin Yin-tan Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XQ, MW, HK, and WD drafted the manuscript. XQ and MW collected all the data. CW conducted the correlation analysis of the data. NJ, MH, TL, XW, and JW respectively, analyzed the data of the five tables in this study. WW and CH applied for the ethics of Wuhan Jin Yin-tan Hospital. YLi provided a lot of advice on this study, guided the manuscript writing, and revised the manuscript. YLiu and JT conducted the laboratory detection and applied for data disclosure from General Project of Jiangsu Provincial Health Commission. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.638194/full#supplementary-material

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Persisting Antibody Response to SARS-CoV-2 in a Local Austrian Population

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused a global pandemic recently. The prevalence and persistence of antibodies following a peak SARS-CoV-2 infection provides insights into the potential for some level of population immunity. In June 2020, we succeeded in testing almost half of the population of an Austrian town with a higher incidence of COVID-19 infection. We performed a follow-up study to reassess the prevalence of SARS-CoV-2-specific IgA and IgG antibodies with 68 participants of the previous study. We found that the prevalence of IgG or IgA antibodies remained remarkably stable, with 84% of our cohort prevailing SARS-CoV-2-specific antibodies (only a slight decrease from 93% 4 months before). In most patients with confirmed COVID-19 seroconversion potentially provides immunity to reinfection. Our results suggest a stable antibody response observed for at least 6 months post-infection with implications for developing strategies for testing and protecting the population.

Keywords: COVID-19, SARS-CoV-2, antibody, population, serotype

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The world is still challenged by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic with the second wave culminating in autumn 2020 all over Europe, including Austria. It is still controversial, as to what extent and for how long previously affected people are immune to a recurring infection. During an infectious disease, B-lymphocytes produce immunoglobulin M (IgM) antibodies, which are later replaced by immunoglobulin A (IgA) and immunoglobulin G (IgG) antibodies. Persisting IgG antibodies are essential for developing a long-lasting immune response. In fact, more than 90% of people with known SARS-CoV-2 infections robustly develop antibodies to the SARS-CoV-2 spike protein, which comprises the receptor binding domain (RBD), enabling the virus to access human target cells (1–4). Thus, the antibody-based immune response is likely to play a decisive role in immunity toward SARS-CoV-2 infection.

In June 2020 (06/20/2020), we tested 835 participants, comprising 47% of the population of the Austrian town of Weißenkirchen in the Wachau, with a reported higher incidence of COVID-19 infection during the first wave in early spring 2020, and participants of less affected neighboring communities. In this pilot study (5), we used a sensitive enzyme-linked immunosorbent assay

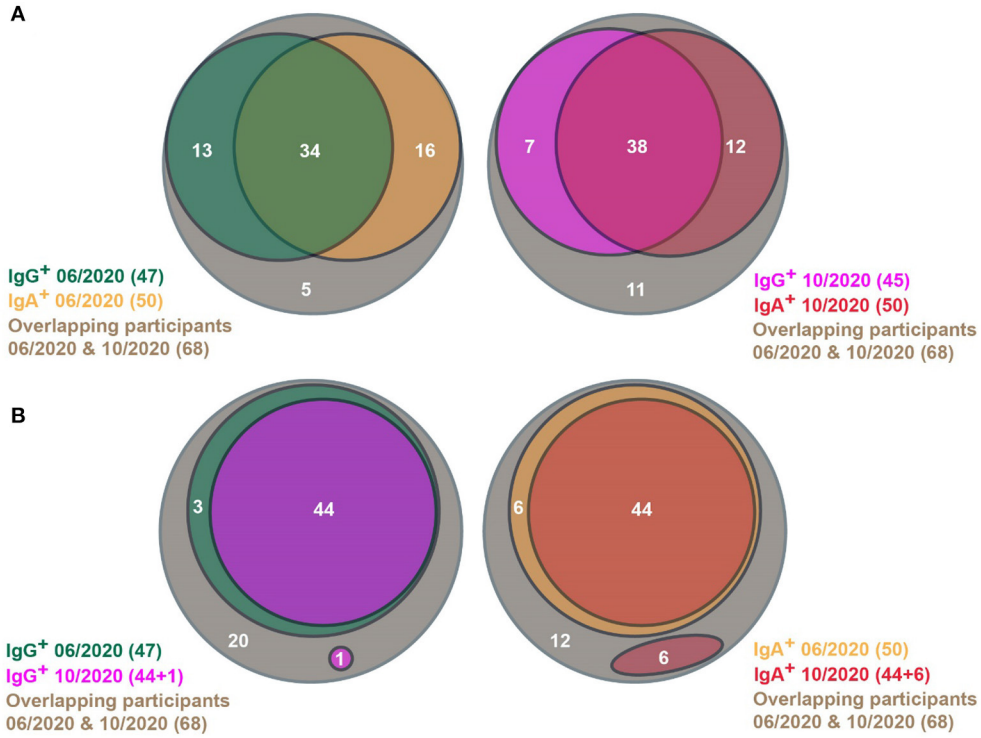

FIGURE 1 | Venn diagrams showing SARS-CoV-2-specific antibody prevalence in the pilot (06/2020) and the follow-up (10/2020) studies. **(A)** SARS-CoV-2-specific antibody status of participants in the pilot (left) and the follow-up studies (right), respectively. **(B)** Persistence of SARS-CoV-2-specific IgG (left) and IgA antibodies (right), respectively, between the pilot and the follow-up studies. **(A,B)** Only people were considered, who participated in both studies.

(ELISA), enabling the semi-quantitative measurement of serum levels of IgG and IgA antibodies, specific for the RBD of the SARS-CoV-2 spike protein. We observed that 12% (98/835) of the tested were infected and consequently, developed SARS-CoV-2-specific IgG or IgA antibodies (5). Almost 9% (71/835) were positive for IgG antibodies and 9% (75/835) contained IgA antibodies. In June 2020, 6% (48/835) of our test population were serum-positive for both SARS-CoV-2-specific IgG and IgA antibodies (5).

In October 2020 (10/17/2020), we performed a follow-up study to reassess the prevalence of SARS-CoV-2-specific IgA and IgG antibodies in Weißenkirchen and neighboring communities. Blood samples were obtained to detect IgA and IgG antibodies specific for the RBD of the SARS-CoV-2 spike protein with a CE-certified laboratory-based ELISA method (Euroimmun Anti-SARS-CoV-2-ELISA IgG and IgA) performed in a certified diagnostic laboratory (Bioscientia, Ingelheim, Germany), as described in the pilot study (5). The study was conducted in accordance with the guidelines of the Local Ethics Committee and in approval of the local and national authorities. We specifically invited the 98 seropositive participants of the pilot study, but seronegative participants of the previous study were not excluded. In total we tested a group of 68 participants who had already participated in the pilot study.

Among the 68 participants, 93% (63/68) already tested positive in June 2020 (**Figure 1A**, left panel). Thus, our follow-up study comprised 64% (63/98) of the seropositive participants of the pilot study. In June 2020, 69% (47/68) of the patients were positive for IgG antibodies and 74% (50/68) contained IgA antibodies. Fifty percent (34/68) contained both IgG and IgA antibodies. In October 2020, we found in 84% (57/68) SARS-CoV-2-specific IgG or IgA antibodies (**Figure 1B**, right panel). Sixty-six percent (45/68) contained IgG antibodies and 74% (50/68) contained IgA antibodies. In 56% (38/68) of cases, both classes of antibodies were found. Thus, the prevalence of SARS-CoV-2-specific IgG and IgA antibodies remained extremely stable in the re-tested participants (**Figure 1A**, c.f. left and right panels). After four months, we found that 84% of our cohort still had SARS-CoV-2-specific antibodies, which is only a slight decrease from 93% in the previous test in June 2020.

This could be due to the high persistence of individual antibody responses. However, the antibody responses could wane in some individuals, which is superimposed by novel infections in other participants of the same subpopulation. Therefore, we analyzed the changes in antibody prevalence on an individual basis. Ninety-Four percentage (44/47) of people with SARS-CoV-2-specific IgG antibodies in June 2020 were still positive for IgG in October 2020 (**Figure 1B**, left panel). In one person, SARS-CoV-2-specific IgG antibodies could be found the first time in October 2020. Eighty-Eight percentage (44/50) of participants with SARS-CoV-2-specific IgA antibodies

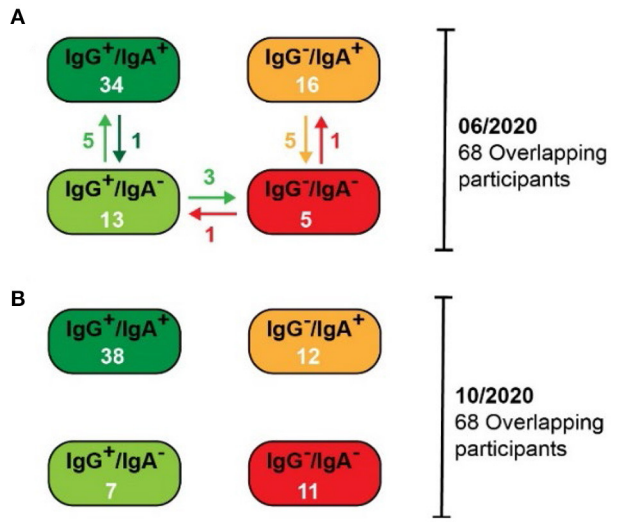

FIGURE 2 | Alterations in the SARS-CoV-2-specific antibody prevalence between the pilot (06/2020) and the follow-up (10/2020) studies. **(A)** Antibody prevalence in the pilot study. Specific changes are indicated with arrows. **(B)** Antibody prevalence in the follow-up study.

in June 2020 still contained marked IgA levels in October 2020 (**Figure 1B**, right panel). IgA antibody responses wer detected in October 2020 in six participants. Therefore, the continuance of antibody levels is only marginally influenced by novel infections.

When considering the alterations of antibody prevalence on an individual basis, the persistence of antibody responses remained very robust. Consequently, 97% (33/34) of participants with both SARS-CoV-2-specific IgG and IgA antibodies by June 2020, still contained significant levels of both classes of antibodies in October 2020 (**Figure 2**). Notably, the IgA antibody levels waned only in one of these participants, whereas the IgG antibody level remained significantly high in most. Only three persons with IgG (but lacking IgA) by June 2020 lost their IgG antibodies by October 2020. Surprisingly, five persons that lack IgA in June 2020 developed IgA by October 2020, then having both SARS-CoV-2-specific IgG and IgA antibodies. In five persons with IgA (but without IgG) in June 2020, their IgA antibodies waned by October 2020. Thus, the IgG antibody responses persisted very efficiently from June to October 2020, and the waning of the IgA antibody response was surprisingly low. One would expect a significant loss of the IgA antibodies because they are described as rather early and transient responders to an infection prior to the production of long-lasting IgG antibodies (6, 7). In contrast, in our study, a robust immune response with high levels of both SARS-CoV-2-specific IgG and IgA antibodies guaranteed the most efficient persistence of human antibody response, at least within the first 6 months after infection.

The SARS-CoV-2-specific serum antibody levels may decrease over time in most individuals, but if the signals are above the threshold of the applied ELISA test system, this waning could be missed in our analysis so far. Therefore, we compared the relative IgG and IgA antibody levels from June 2020 to October 2020 for every participant (**Figure 3**). Using a semi-quantitative ELISA system, both IgG and IgA antibody levels hardly waned (on average 10% for IgG and 14% for IgA). Indeed, in some cases, we observed increased IgG and IgA antibody levels over time. Thus, these results support our notion that the antibody-based immune responses were very stable in the tested population between June and October 2020. Since most known COVID-19 cases in Weißenkirchen were noted in March 2020, our results suggest that the antibody-based immune responses last for more than 6 months. This may also have implications for the efficiency of SARS-CoV-2 vaccination. A strong antibody-based immune response involving both IgG and IgA antibodies upon vaccination may be predictive of immunity for more than 6 months after.

The duration of SARS-CoV-2-specific antibodies persistence to provide immunity is still an open debate. Several studies suggest that the immune response persists for at least several months (6–12), whereas others propose rapid waning of the SARS-CoV-2-specific antibodies in the blood serum of previously infected individuals (2, 13). Although our study is limited by the small population size of our follow-up study, our findings support the idea of a prolonged immune response.

So far, studies determining antibody-based immune responses have been performed with either corona antibody rapid tests (which are less sensitive), or semi-quantitative ELISA tests (as in our study). Currently, ELISA methods for the quantitative assessment of SARS-CoV-2-specific IgG and IgA antibodies are emerging, allowing for a much more precise determination of antibody waning post-infection. In this study, samples were measured with both test systems in parallel for comparison of the semi-quantitative (see **Figure 3**) and quantitative analyses (data not shown and to be published later) in order to set a common base for subsequent studies.

In light of these technological advancements and the insufficient knowledge about the stability of SARS-CoV-2-triggered antibody-based immune responses, we will continue to test our cohort for SARS-CoV-2-specific IgG and IgA antibodies with both semi-quantitative and quantitative ELISA and combine these with novel tests for SARS-CoV-2-specific T-cell immunity. Waning of immune responses are expected, and we will test whether waning is influenced by age, sex, behavior (smoking, alcohol intake), weight, pre-existing conditions. We will also consider the role of the previous COVID-19 disease severity, as this has been proposed to influence the persistence of immunity with COVID-19 (14). To date, we have not detected any significant correlation between the persistence of antibody responses and these hallmarks. However, this may change when antibody waning becomes more relevant.

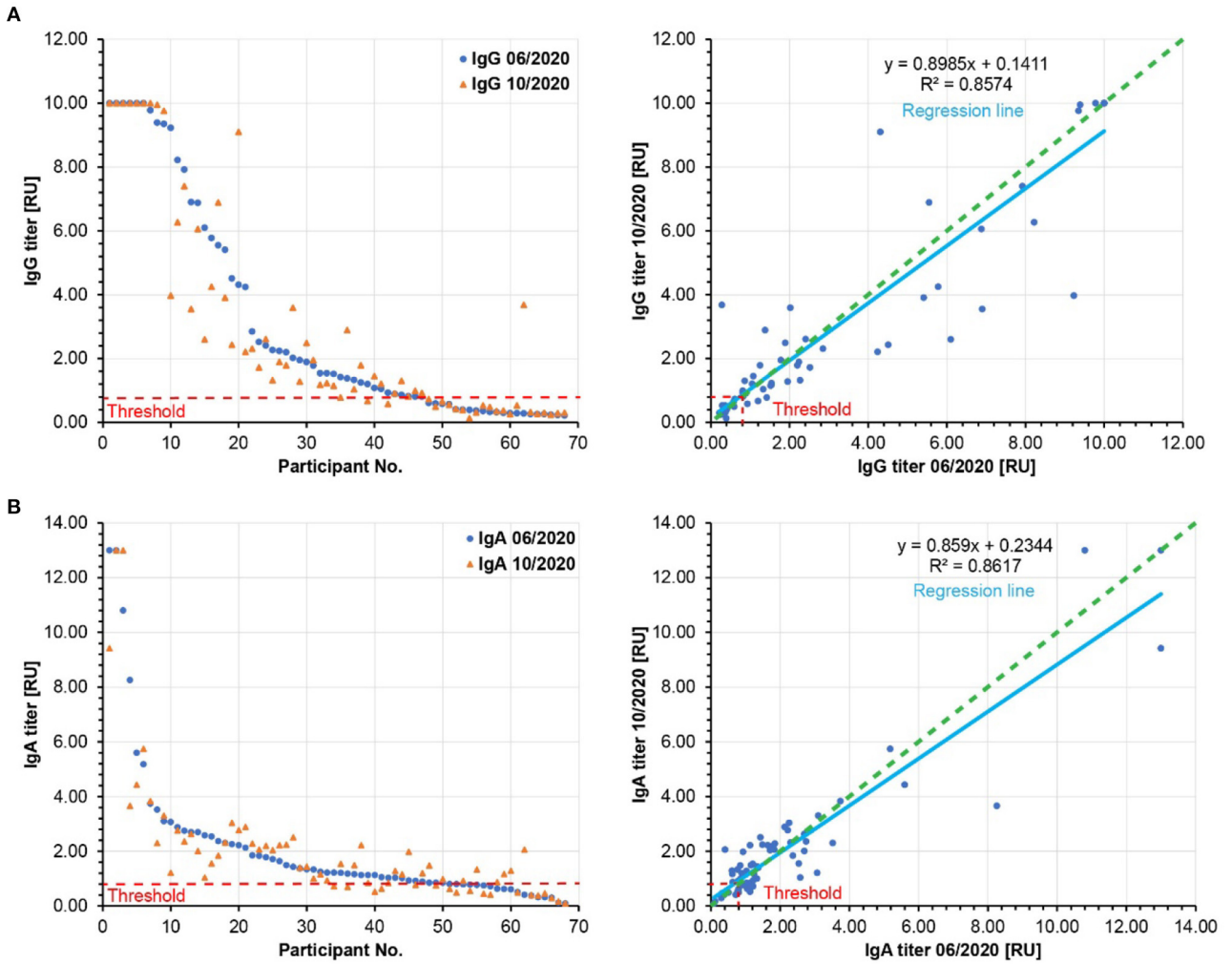

FIGURE 3 | Relative SARS-CoV-2 specific IgG **(A)** and IgA **(B)** antibody titers. Left panels: The participants are ordered according to decreasing relative antibody titers of the pilot study (blue, 06/2020). The respective relative antibody titers of the follow-up study (10/2020) were plotted in orange. Right panels: The relative antibody titers of the follow-up study (10/2020) are plotted against the relative antibody titers of the pilot study (06/2020). The slopes of the regression lines (light blue) are below 1.0, showing a moderate waning to the relative antibody titers by 10% for IgG and by 14% for IgA. Green dashed lines: hypothetical regression lines in the case of 100% antibody persistence. Red dashed lines: Threshold of significant antibody detection (0.8 for both IgG and IgA). Only the data of the 68 participants are shown here, whose sera were analyzed in both pilot and follow-up studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Kommission für wissenschaftliche Integrität und Ethik (Ethikkommission), Danube Private University, Krems an der Donau, Austria. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DeL and RB analyzed the data and wrote the manuscript. OH, DR, CS, and DoL contributed to data analysis. OH and CA provided intellectual input. All authors contributed to the article and approved the submitted version.

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HYPOTHESIS AND THEORY
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Severe Clinical Worsening in COVID-19 and Potential Mechanisms of Immune-Enhanced Disease

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Infection by the novel SARS-CoV-2 coronavirus produces a range of outcomes, with the majority of cases producing mild or asymptomatic effects, and a smaller subset progressing to critical or fatal COVID-19 disease featuring severe acute respiratory distress. Although the mechanisms driving severe disease progression remain unknown, it is possible that the abrupt clinical deterioration observed in patients with critical disease corresponds to a discrete underlying expansion of viral tropism, from infection of cells comprising respiratory linings and alveolar epithelia to direct infection and activation of inflammatory monocytes and macrophages. Dysregulated immune responses could then contribute to disease severity. This article discusses the potential role of monocyte/macrophage (Mo/Mϕ) infection by SARS-CoV-2 in mediating the immune response in severe COVID-19. Additional mechanisms of immune-enhanced disease, comprising maladaptive immune responses that may aggravate rather than alleviate severity, are also discussed. Severe acute clinical worsening in COVID-19 patients may be influenced by the emergence of antibodies that participate in hyperinflammatory monocyte response, release of neutrophil extracellular traps (NETs), thrombosis, platelet apoptosis, viral entry into Fc gamma receptor (FcγR)-expressing immune cells, and induction of autoantibodies with cross-reactivity against host proteins. While the potential roles of Mo/Mϕ infection and immune-enhanced pathology in COVID-19 are consistent with a broad range of clinical and laboratory findings, their prominence remains tentative pending further validation. In the interim, these proposed mechanisms present immediate avenues of inquiry that may help to evaluate the safety of candidate vaccines and antibody-based therapeutics, and to support consideration of pathway-informed, well-tolerated therapeutic candidates targeting the dysregulated immune response.

Keywords: COVID-19, clinical deterioration, monocytes/macrophages, antibodies, vaccines

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INTRODUCTION

The SARS-CoV-2 coronavirus emerged in late-2019 in Wuhan, China, presenting as pneumonia of unknown etiology. The virus was isolated on January 7, 2020, and its genetic sequence was published 5 days later. Within 10 weeks, the associated disease, COVID-19, was declared a global pandemic by the World Health Organization (WHO). As of December 2020, 65 million confirmed or probable cases of SARS-CoV-2 infection have been identified, with over 1.5 million fatalities (1). Severe clinical outcomes and fatalities among a subset of symptomatic COVID-19 patients have created an urgent need for the development of safe and effective vaccines and therapeutics.

Infection by the novel SARS-CoV-2 coronavirus results in multi-modal outcomes, with the majority of cases producing mild or asymptomatic effects, and a smaller subset progressing to critical or fatal COVID-19 disease featuring severe acute respiratory distress. While the mechanisms driving severe disease progression remain unknown, it is possible that the abrupt clinical deterioration observed in patients with critical disease corresponds to a discrete underlying expansion of viral tropism, from infection of cells comprising respiratory linings and alveolar epithelia to direct infection and activation of inflammatory monocytes and macrophages. Direct viral infection of these cells can promote a transcriptional shift toward invasive and inflammatory phenotypes, consistent with those observed in severe COVID-19. This shift may coincide with the induction of antibodies that participate in immune-enhanced disease severity.

We begin by describing several immune hallmarks of mild vs. severe COVID-19, with an emphasis on the contribution of inflammatory monocyte/macrophage (Mo/Mϕ) subsets to features observed in patients with severe disease. Potential mechanisms of Mo/Mϕ infection and immune-enhanced disease progression are discussed. "Immune enhancement" in this context refers to maladaptive immune responses that may aggravate rather than alleviate disease severity, beyond cytopathic effects of the virus. Attention is given to the emergence of neutralizing IgG antibodies directed against the SARS-CoV-2 spike, and their potential contribution to hyperinflammatory monocyte response, release of neutrophil extracellular traps (NETs), thrombosis, platelet apoptosis, and antibody-dependent enhancement (ADE) of viral entry into Fc gamma receptor (FcγR)-expressing immune cells. Features of antibody response that may be relevant in the evaluation of vaccine safety are described, including IgG fucosylation, potential generation of autoantibodies with cross-reactivity to host proteins, and interactions of antigen–antibody immune-complexes with Fcγ receptors and components of the complement pathway. Signal transduction pathways, particularly downstream of viral pattern-recognition receptors (PRRs) and FcγRs, are also discussed in the context of immune-enhanced pathology and possible therapeutic modulation.

The potential roles of monocyte/macrophage infection and immune-enhanced pathology in COVID-19 are consistent with a broad range of evidence, but their prominence remains tentative pending further validation. In the interim, these proposed mechanisms present specific points of investigation that may be of immediate benefit in the testing of candidate biological interventions. The development of safe and effective vaccines and antibody-based therapeutics relies on evaluation to limit the possibility of immune-enhanced disease. Clinical treatment of active cases may also benefit from the consideration of pathway-informed, well-tolerated therapeutic candidates targeting mediators of the maladaptive immune response.

IMMUNE CORRELATES OF DISEASE PROGRESSION IN COVID-19

Immune defense against viral pathogens involves the coordination of immediate innate and later pathogen-specific adaptive responses that promote viral recognition, containment, clearance, and host immunological memory. Entry of enveloped viruses such as coronaviruses into host target cells is achieved by binding of a viral surface protein to a receptor protein on the host cell membrane, followed by membrane fusion or endocytosis, and introduction of the viral genome into the host cell. Viral components and genetic material are sensed by host innate PRRs such as Toll-like (TLR), NOD-like (NLR), C-type lectin (CLR), and RIG-I-like (RLR) receptors. Downstream signaling cascades promote the transcription of interleukin (IL)-1, IL-18, Type-I (α/β), -II (γ), and -III (λ) interferons (IFNs), a large set of IFN-stimulated genes (ISGs), inflammatory cytokines such as TNF-α, IL-12, and IL-6, and leukocyte chemoattractants such as CCL2/MCP-1 and CCL3/MIP-1a. These molecules act to impede viral replication, and to recruit cytolytic immune cells such as natural killer (NK) cells and neutrophils, as well as phagocytes such as monocyte-derived macrophages and dendritic cells (DC). Complement proteins contribute to the inactivation of viruses, and phagocytes ingest and present viral antigens, via major histocompatibility complex (MHC) molecules, to cells of the adaptive immune system. These interactions promote expanded populations of pathogen-specific CD4+ T-helper cells, CD8+ cytotoxic T-lymphocytes, antibody-producing B-cells, and T and B memory cells (2).

COVID-19 produces varied levels of disease severity in infected individuals. The majority of infections with SARS-CoV-2 produce mild or asymptomatic outcomes. Based on large-scale, unbiased testing, it is estimated that 40–45% of individuals infected with SARS-CoV-2 are asymptomatic (3). Close to half of these cases are reported to show lung abnormalities such as ground-glass opacities and consolidation based on CT imaging. Some individuals who are asymptomatic at the time of a positive test may become symptomatic later; these individuals can potentially be distinguished by elevated levels of lactate dehydrogenase (LDH) during the pre-symptomatic phase (4). While viral shedding by asymptomatic or pre-symptomatic individuals may account for close to half of SARS-CoV-2 transmission (5), the prevalence of mild outcomes among individuals in low-risk groups may confound containment efforts. Among symptomatic cases, approximately 81% are classified as having mild disease featuring mild or absent pneumonia, 14% having severe disease featuring respiratory distress, lung infiltrates, or low oxygen saturation, and 5% having critical disease including acute respiratory failure, septic shock, or multi-organ failure (6).

Progression to severe COVID-19 disease is associated with a variety of alterations in immune cell populations and inflammatory response. Asymptomatic presentation in COVID-19 is reported to be associated with a high prevalence of NK cells, with severely diseased patients not requiring ICU treatment having significantly higher NK cell counts than ICU patients (7). The onset of symptoms in COVID-19 is accompanied by a rapid increase in "classical" CD14+CD16– monocytes expressing the sialic acid-binding immunoglobulin-like lectin CD169/Siglec-1. These monocytes are observed with significantly higher frequency in patients with mild disease, relative to those with severe disease, and express IFN-γ and monocyte chemoattractant protein CCL8/MCP-2 (8). The functional roles

of CD169 include sialic-acid based pattern recognition and maintenance of immunological tolerance. CD169 also helps to bridge the innate and adaptive immune response by facilitating the capture and presentation of viral particles to invariant NK T-cells, CD8+ T-cells and B-cells (9).

In COVID-19 patients stratified by disease course, longitudinal profiling of peripheral blood samples identifies the most significant changes to be in the function and proliferation of monocytes (10). Inflammatory monocytes and monocyte-derived macrophages are abundant in the lungs of patients with severe COVID-19 (11). Increased proliferation of "intermediate" CD14+CD16+ monocytes during severe disease progression is associated with a corresponding reduction in NK cell frequency (7). Elevated levels of inflammatory CD14+CD16+ monocytes expressing costimulatory protein CD80/B7, the hemoglobin-haptoglobin scavenger receptor CD163, and lysosomal proteins CD68/LAMP-2 and CD208/LAMP-3 are predictive of severe disease and ICU admission (12). Increased proliferation of CD14+CD16 monocytes is also coupled with a near-universal reduction of antigen-presentation molecules CD86 and HLA-DR in monocytes of patients with severe disease (13). These changes suggest that severe COVID-19 may be mediated by transcriptional changes in Mo/Mϕ subsets that favor pro-inflammatory and cytotoxic functions.

In patients with severe COVID-19, a marked shift in monocyte populations toward cells expressing the Fc-gamma III receptor CD16/FcγRIII is accompanied by expression of chemoattractants including macrophage inflammatory proteins CCL3/MIP-1a, CCL4/MIP-1b, and CCL23/MIP-3 (8). Increased monocyte expression of the proliferation marker Ki-67 is observed, and strongly correlates with levels of C-reactive protein (CRP) (10). Increased levels of the chemokine CXCL10/IP-10 are also observed in nearly all COVID-19 patients, but unlike moderately diseased patients, in which high IP-10 levels are transient, severely diseased patients maintain elevated levels that are proportional to disease progression (13).

Severe/critical COVID-19 pneumonia is associated with significant elevation of inflammatory cytokine release, including IL-6, IL-2R, IL-8, and TNF, as well as the anti-inflammatory cytokine IL-10, and chemoattractants that mediate leukocyte recruitment, particularly CXCL10/IP-10, CCL2/MCP-1, and CCL3/MIP-1a. Hallmarks of severe infection include elevated acute phase markers such as CRP, serum ferritin, LDH, D-dimer, and procalcitonin. Severe cases feature elevated neutrophil counts and depressed lymphocyte counts, resulting in a significantly higher neutrophil-to-lymphocyte ratio (NLR) relative to non-severe cases. Age is a significant risk factor, with a low incidence of symptomatic cases in children and young adults (< age 24). Chronic diseases such as diabetes, hypertension, cardiovascular disease, and chronic obstructive pulmonary disease confer additional risk, although only half of severe cases feature these predisposing factors (14, 15).

Taken together, increasing severity in COVID-19 appears to be associated with a reduction in NK cells, profound lymphopenia, increased proliferation and activation of CD14+CD16+ inflammatory monocytes with reduced antigen-presentation markers, increased cytokine release, elevated acute-phase reactants, and expression of chemokines that mediate the recruitment of inflammatory monocytes, macrophages, and neutrophils to infected tissue.

Notably, severe disease cases resulting from infection by the related SARS-CoV (Severe Acute Respiratory Syndrome) and MERS-CoV (Middle East Respiratory Syndrome) coronaviruses feature similar inflammatory infiltrates, elevated cytokine and chemokine release, and respiratory distress marked by diffuse alveolar damage (DAD) (16).

SEVERE CLINICAL WORSENING IN COVID-19

A commonly reported feature of severe COVID-19 is an abrupt deterioration in clinical condition characterized by rapid progression to respiratory distress, with elevation of acute phase reactants and inflammatory mediators resembling those observed in cytokine release syndrome (CRS) and macrophage activation syndrome (MAS)/secondary hemophagocytic lymphohistiocytosis (HLH) (17). Several features of progression to severe disease, including hyperinflammation, cytokine release, and dysregulated coagulation suggest that macrophage activation may contribute to COVID-19 pathology (18, 19).

It is possible that the abrupt clinical deterioration observed in patients with critical disease corresponds to an expansion of viral tropism, from infection of cells comprising respiratory linings and alveolar epithelia to direct infection and activation of inflammatory monocytes and macrophages (Mo/Mϕ). In the present context, infection of Mo/Mϕ describes viral entry characterized by escape from lysosomal degradation, with a resulting capacity to amplify, inhibit, or otherwise reprogram cellular activities. These potential responses include IFN-independent cytokine/chemokine release triggered by endosomal or intracellular PRRs, transcription of the viral genome, and even support of productive viral replication.

Monocytes and macrophages have been demonstrated to mediate the persistence or spread of viruses belonging to 13 different families, including coronaviruses. Mo/Mϕ have high phagocytic activity, and provide an early line of immune surveillance and defense by ingesting and degrading viruses, releasing cytokines in response to PRR signaling, and bridging the innate and adaptive immune system as professional antigen-presenting cells. Viral infection of Mo/Mϕ themselves can provoke alterations in cytokine and chemokine expression, transcriptional response, cell motility, and differentiation to distinct or even simultaneous inflammatory and immunosuppressive polarization. Nikitina et al. provide an excellent review of these mechanisms (20).

Notably, SARS-CoV-2 is capable of directly infecting Mo/Mϕ without cytopathic effect, resulting in significant release of both pro- and anti-inflammatory cytokines including IL-6 and IL-10, with induction of CD163 in CD16-expressing monocytes, and diminished HLA-DR expression (21). Indeed, in SARS-CoV infected macaques, CD163+ macrophages are productively infected, and may act as viral reservoirs (22). Post-mortem lung tissues from human COVID-19 patients show extensive

infiltration of immune cells, including abundant monocytes and macrophages. In these fatal cases, broad tropism to respiratory epithelia and immune cells is reported, with 90% of infiltrating immune cells showing positive staining for SARS-CoV-2 viral proteins. The number of infected cells is also correlated with the extent of tissue damage (23).

Alterations in IFN signaling in response to SARS-CoV-2 infection may contribute to delayed control of viral replication, coupled with dysregulated inflammatory pathology. Infection of respiratory epithelia and immune cells by SARS-CoV-2 induces expression of a subset of ISGs in an IFN-independent manner, contributing to recruitment of inflammatory Mo/Mϕ into infected tissue. In naïve *ex vivo* human lung tissue, SARS-CoV-2 infects type I and type II alveolar pneumocytes as well as alveolar macrophages, with rapid viral replication and significant expression of IL-6, CCL2/MCP-1, and CXLC10/IP-10, yet without significant induction of Type I, II, or III IFNs (24). Respiratory epithelial cells infected *in vitro* by SARS-CoV-2 show exuberant inflammatory cytokine production, coupled with weak or delayed induction of IFN-I and -III, suggesting that impaired innate defense against early viral replication and epithelial infection contributes to COVID-19 pathology. Post-mortem COVID-19 lung samples also display strong induction of a subset of ISGs, particularly monocyte associated chemokines such as CCL2/MCP-1 and CCL8/MCP-2, yet without detectable expression of IFN-I or IFN-III (25).

Human monocytes and respiratory epithelial cells, but not lymphocytes, express ACE2, which is used as a viral entry receptor by both SARS-CoV-2 and SARS-CoV. In human patients with SARS-CoV infection, increased CXCL10/IP-10 levels in immune cells and lung epithelia are induced in an IFN-independent manner, and correlate with recruitment of CD68+ monocytes into interstitial lung tissue, accompanied by progressive lymphopenia and elevated LDH, consistent with rapid recruitment and apoptosis of T-lymphocytes (26).

Similarly, infection of monocyte-derived macrophages by SARS-CoV *in vitro* induces expression of CCL2/MCP-1 and CXCL10/IP-10 in an IFN-independent manner (27). Delayed IFN-I signaling in SARS-CoV-infected mice promotes inflammatory Mo/Mϕ accumulation and impaired virus-specific T-cell responses. Exogenous IFN-I delivery prior to peak virus titer ameliorates severity, yet later IFN-I delivery exacerbates Mo/Mϕ-associated inflammation. Depletion of inflammatory Mo/Mϕ by inhibiting CCR2 (the receptor for CCL2) confers protection against lethal disease (28).

Interaction between viral glycoproteins and host lectin receptors may contribute to Mo/Mϕ infection. The SARS-CoV-2 virus is heavily glycosylated, and the S protein is recognized by several CLRs including mannose receptor CD206/MR, CD209/DC-SIGN, CD209L/L-SIGN, and CD301/CLEC10A, which are highly expressed in Mo/Mϕ. Significant co-expression of CLRs including CD206/MR, CD209/DC-SIGN, and CD301/CLEC10A, along with inflammatory cytokine and chemokine production, is observed in activated macrophages and DCs from patients with COVID-19 (29).

In addition to mediating viral recognition and downstream signaling pathways, membrane-bound receptors such as CLRs can enhance viral adhesion to target cells and may also serve as viral receptors. For example, CD209L/L-SIGN binds to SARS-CoV spike, and may serve as an alternate receptor independent of ACE2, while viral binding to cells bearing CD209/DC-SIGN allows dissemination of SARS-CoV to cells that are permissive for viral entry (30). Viral attachment to host cells may also be facilitated by binding interactions between viral envelope proteins and sialic-acid binding lectins expressed on host cells (e.g., CD169, FCN1), potentially activating endocytic and immune response pathways (31).

The "cytokine storm" associated with MAS/secondary HLH generally features sustained fever, hyperferritinemia, coagulopathy, and elevated release of inflammatory cytokines such as IL-1, IL-6, and IL-18. Macrophage activation syndrome can emerge as a severe complication in a variety of inflammatory conditions, including systemic lupus, Kawasaki Disease, and systemic juvenile idiopathic arthritis. Elevated expression of CD163 is also observed in monocytes and macrophages, which can be upregulated by IL-10, suggesting that this expression may have a compensatory role (32). These inflammatory features are consistent with those observed in COVID-19. In COVID-19 patients experiencing respiratory failure, immune responses are reported to be universally classified by either MAS (based on ferritin $>$ 4,420 ng/ml) or immune dysregulation similar to septic immunoparalysis (based on HLA-DR on CD14 monocytes $<$5,000), representing about 25 and 75% of patients, respectively. In the latter group, overproduction of cytokines is combined with reduced lymphocyte count, and a decrease in HLA-DR on CD14 monocytes that is inversely correlated with IL-6 (33).

The immune response of Mo/Mϕ in severe COVID-19 shares notable characteristics with other inflammatory conditions. Zhang et al. examined single-cell RNA-seq profiles of monocytes and macrophages obtained from analysis of COVID-19 bronchoalveolar lavage fluid (BALF) and tissues from multiple inflammatory diseases including ulcerative colitis, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, and interstitial lung disease. COVID-19 was reported to share two CD14+CD16+ inflammatory macrophage phenotypes with these diseases: one characterized by a CXCL10+CCL2+ cytokine signature, as well as a population expressing the pathogen recognition and complement lectin pathway receptor FCN1/Ficolin-1 (34). Enrichment of FCN1-expressing macrophages is observed in BALF of COVID-19 patients with severe/critical disease relative to patients with moderate infection and controls (11). FCN1 shows elevated expression in both Kawasaki Disease and rheumatoid arthritis, binds both IgG and sialic acid, and correlates with levels of autoantibodies in these conditions. Blockade of FCN1 reduces inflammation in a murine model of arthritis, suggesting that downregulation of FCN1 may be a mechanism of therapeutic intravenous immunoglobulin (35).

ANTIBODY RESPONSE AND IMMUNE-ENHANCED PATHOLOGY

Disease severity in COVID-19 is positively correlated with antibody response. Emphatically, this relationship may reflect extensive host–virus interactions in patients with severe disease

without implying a pathological role for antibodies. Still, severe disease progression is not reliably curtailed by high production of neutralizing antibodies (NAbs). Rather, severe disease is strongly associated with high levels of NAbs targeting the SARS-CoV-2 receptor binding domain (RBD) located on the trimeric S1 spike (S-RBD). NAbs targeting the S1 spike are reported to be dependent on RBD-specificity for neutralization capacity. Neutralizing antibodies specific to the S2 subunit are also observed in the majority of patient sera. Patients with severe disease generate high NAb titers, while asymptomatic patients may mount little or potentially no NAb response (36).

Based on serial blood sampling, rapid exacerbation of symptoms and progressive lymphopenia among patients with severe and critical disease are observed to coincide with IgG seroconversion, and cannot be explained by uncontrolled viral replication. In contrast, symptoms such as fever, cough, and general malaise are observed to improve in patients with mild and moderate disease prior to or independent of seroconversion (37).

Antibodies with the IgG isotype exhibit the strongest positive association with COVID-19 disease severity. Age and gender (male) are also significantly correlated with antibody levels (38). Asymptomatic individuals infected by SARS-CoV-2 are reported to develop significantly lower virus-specific IgG levels than symptomatic patients, and express significantly lower levels of pro- and anti-inflammatory cytokines (39). In contrast, patients with severe COVID-19 disease produce significantly higher IgG virus-specific NAb titers, and may also seroconvert earlier than patients with mild symptoms (40). In one study, IgG levels targeting S-RBD were reported to increase early following infection only in patients with severe disease (41).

The relationship between neutralizing IgG and severity in COVID-19 mirrors that observed in SARS. In a large-scale prospective study of SARS patients, progression to critical disease generally followed a three-phase pattern. Rapid viral replication during the first week was accompanied by systemic symptoms that gradually receded. During the second week, recurrence of symptoms and severe clinical worsening occurred simultaneously with IgG seroconversion, followed by a third phase of disease progression to acute respiratory distress syndrome (ARDS) and lymphopenia in a subset of critical patients, despite a declining viral load (42). Virus-specific IgG levels in SARS-CoV infected patients were positively correlated with disease severity, including the need for ICU admission and supplemental oxygen. Moreover, early seroconversion (day 4–15 after fever onset) was observed more frequently among patients requiring admission to ICU, compared with patients who remained seronegative. Notably, the emergence of NAbs did not confer protection against disease severe progression (43). SARS patients with a short duration of illness were more likely to be seronegative, while longer duration of illness was associated with higher patient NAb levels. Patients with early seroconversion had a markedly higher fatality rate, a shorter survival time, and greater likelihood of being over 60 years of age (44). Evidently, once the host response has shifted to a hyperinflammatory state in the presence of a high viral load, neither the emergence of antibodies nor a subsequent decline in viral load appears sufficient to halt disease progression.

Although the correlation between antibody response and disease severity in COVID-19 does not establish a causative relationship, several findings suggest that antibodies to SARS-CoV-2 can participate in maladaptive immune responses. For example, anti-spike IgG from critical COVID-19 patients induces a hyperinflammatory response in monocytes cultured to resemble primary human lung macrophages. In the presence of the synthetic RNA analog poly(I:C), anti-spike IgG triggers exuberant release of IL-1β, IL-6, TNF, and IL-10 from these cells, similar to that observed in COVID-19 patients. Moreover, in the presence of human vascular endothelial cells and platelets under flow conditions, these macrophages disrupt endothelial barrier integrity and provoke microvascular thrombosis (45). Likewise, sera from COVID-19 patients show elevated levels of products indicative of NETs which can amplify inflammation and thrombosis. Patient sera also strongly trigger healthy neutrophils to undergo NETosis (46), and IgG fractions isolated from severe COVID-19 patients induce apoptosis of platelets from healthy donors via cross-linking of FcγRIIa receptors (47), possibly contributing to immune-enhanced severity in COVID-19.

ANTIBODY-DEPENDENT ENHANCEMENT OF MO/Mϕ INFECTION

As monocytes are susceptible to receptor-mediated infection via surface expression of ACE2 (26), the potential mechanisms of monocyte infection and immune-enhanced disease in COVID-19 are not reliant on ADE of infection. However, several viruses, including coronaviruses such as SARS-CoV, MERS-CoV, and feline infectious peritonitis virus (FIPV), are able to exploit antibodies to increase infection and expand tropism to immune cells expressing Fc receptors that recognize and bind to antibody Fc domains. For example, although vaccine-induced immune serum directed against the SARS-CoV spike neutralizes viral entry via ACE2 receptors in permissive cell lines, it also enables viral entry into Fcγ receptor-bearing human monocytes and B-lymphocyte derived cells. Infection by replication-competent SARS-CoV virus is accompanied by viral gene transcription and protein synthesis, which may alter immune cell function even though it does not proceed to viral replication. Blockade of FcγR abrogates antibody-mediated infection (48). Notably, genetic variations in CD14 and the Fcγ receptor CD32/FcγRIIa confer risk of severe SARS-CoV infection (49).

In a recent study of circulating monocytes isolated from SARS-CoV-2 infected patients presenting for emergency hospital care, 10% of monocytes were infected by the virus, including double-stranded RNA (dsRNA) staining indicative of viral replication, and markers of NLRP3 and AIM2 inflammasome activation. While monocytes derived from healthy donors were inefficiently infected by SARS-CoV-2, pre-incubation with anti-spike antibody or patient plasma was reported to enhance productive infection of monocytes. This effect was abrogated by Ig depletion of patient plasma (50). Moreover, positive- and negative-strand SARS-CoV-2 RNA is detected in alveolar macrophages recovered from BALF of intubated patients with

severe COVID-19, and in monocyte-derived macrophage and DC subsets that do not express ACE2 (51).

Monocyte lineages are the primary target of ADE of SARS-CoV infection in the presence of anti-S IgG, and human macrophages are also infected. Antibody-dependent enhancement is dependent on intact intracellular signaling of domains of FcγR, but productive replication of SARS-CoV in infected macrophages is not observed (52). The close relationship between the neutralizing capacity of antibodies and their capacity for ADE of coronavirus entry is notable. A similar relationship between neutralization and enhancement is observed in FIPV, which preferentially infects Mo/Mϕ. Moreover, ADE of infection can be induced by exposure to the same viral serotype to which vaccine-induced antibodies are directed (53).

A central consideration in the evaluation of vaccine safety is the immune response to subsequent viral challenge. SARS-CoV spike-based vaccination of macaques, followed by challenge by live virus, was reported to produce fever 1–5 days after challenge, with 6 of 8 vaccinated macaques developing acute DAD within 1–5 weeks. Control animals demonstrated only mild or moderate inflammation in response to viral challenge. When purified anti-spike S-IgG from vaccinated macaques was administered to healthy animals and followed by viral challenge, all recipients showed acute DAD, with features including hyaline membrane formation, hemorrhage, and infiltration of inflammatory monocytes and macrophages. While SARS-CoV infection of control macaques induced macrophages expressing CD163 and mannose receptor CD206/MR, administration of anti-S-IgG triggered a loss of CD206/MR expression and wound-healing function of macrophages, accompanied by tissue damage and uncontrolled inflammation. Notably, the effects of vaccine-induced immunity can vary markedly depending on the animal model and vaccine design under study, and may depend partly on differences in CD8+ T cell participation and vaccine-induced Th1 response (54).

The molecular mechanism underlying ADE of coronavirus entry was recently described. Wan et al. (55) demonstrate that both MERS-CoV and SARS-CoV S-RBD-specific antibodies can effectively neutralize viral entry via DDP4 and ACE2, respectively, while also mediating viral entry into IgG Fc receptor-bearing cells.

Structurally, the coronavirus spike is comprised of three S1 subunits, each containing a RBD, connected to a trimeric S2 stalk that carries the membrane fusion mechanism. Infection of the host cell first requires cleavage of the viral S1/S2 site by host proteases such as TMPRSS2, followed by cleavage of the S2' site, which liberates viral membrane fusion mechanisms. Binding of the viral RBD to its receptor (DPP4 for MERS-CoV, ACE2 for SARS-CoV) stabilizes the bound S1 trimer in a "standing up" position, which is required in order to expose the S2' site to proteasomal cleavage. In antibody-mediated entry, the NAb binds to the tip of the viral spike where the RBD is located, and stabilizes the conformation of the S1 trimer to expose the otherwise inaccessible S2' site to cleavage (55). Binding of the Fc domain of the antibody-virus complex to membrane-bound Fc receptors on host immune cells thus allows a shift in the tropism of viral infection to FcγR-expressing cells such as monocytes and macrophages.

In ADE of SARS-CoV, enhanced entry is pH-independent and minimally affected by inhibition of endosomal proteases such as cathepsin-L, suggesting that entry may occur at the cell membrane, independent of the endocytic pathway (48). Host cell expression of the transmembrane serine protease TMPRSS2 can mediate release of the viral fusion protein to enable virus-host membrane fusion, and can also induce the formation of syncytia (large multi-nucleated cells) driven by further membrane fusion with neighboring cells (56). In contrast, ADE of MERS-CoV is reliant on lysosomal acidification and endosomal protease activity, suggesting that infection of host cells is achieved by endosomal escape. As is observed in ADE of dengue virus (DENV), ADE is strongest at intermediate levels of NAb, as low antibody levels blocked receptor-based entry to a greater degree than they encouraged ADE, and high antibody levels saturated Fc-receptor molecules (55).

Antibody-mediated entry of SARS-CoV-2 into human monocyte-derived cells and B-lymphocytes has recently been reported. Antibody-enhanced infection of these cells is mediated by engagement of FcγRII (CD32), with viral fusion occurring at the cell membrane without dependence on endocytosis. Antibody-dependent enhancement is most strongly induced by patient sera derived from elderly donors with severe disease, and is mediated by virus-specific IgG directed against S-RBD (57). SARS-CoV-2 is also reported to infect CD4+ T-helper cells, resulting in functional impairment and increased expression of IL-10. However, in contrast to ADE, infection of CD4+ cells remains dependent on the presence of ACE2. In this case, binding of the SARS-CoV-2 spike to CD4 stabilizes the virus at the cell membrane, and may help to compensate for low levels of ACE2 expression by increasing the opportunity for receptor binding (58).

The potential contribution of ADE in COVID-19 has been discussed in the context of DENV infection, for which ADE has been well-studied (59, 60). Primary dengue fever (DF) typically presents with mild symptoms, with infection of blood cells resulting in leukopenia and depressed platelet count. However, the influence of ADE in secondary DENV infections can promote severe dengue hemorrhagic fever (DHF), which is characterized by fever and often fatal vascular leakage, particularly when untreated (60). Antibody-dependent enhancement in dengue is affected by the relative abundance of FcγR isoforms having activating or inhibitory effects on immune cell activation. Activating signals are mediated by receptors carrying immunoreceptor tyrosine-based activation motifs (ITAMs) such as FcγRIIa, while inhibitory signals are mediated by receptors carrying inhibitory (ITIM) motifs such as FcγRIIb. Blockade of activating FcγRs ablates infection of cells by antibody–virus immune complexes (IC) (61).

Infants with passive immunity from DENV infected mothers typically present with DHF upon initial DENV infection. Strikingly, afucosylation of maternal anti-dengue IgG Fc domains is a highly specific predictor of symptomatic infection in infants, with afucosylated IgG in excess of 10% predictive of severe disease outcomes (61).

Core fucosylation of the IgG Fc domain modifies its binding to Fc receptors, with reduced fucosylation leading to enhanced interactions with the activating CD16a/FcγRIIIa receptor. In symptomatic adults infected with SARS-CoV-2, the Fc domains of anti S-RBD IgG antibodies are characterized by significantly reduced core fucosylation relative to IgG antibodies from healthy adults. Particularly significant reduction in fucosylation of anti-RBD IgG is observed in patients with severe disease, in comparison to patients with mild COVID-19 and asymptomatic children seropositive for SARS-CoV-2 antibodies (62).

Recombinant anti-S IgG derived from patients with severe COVID-19 characterized by low fucosylation promote increased induction of inflammatory cytokines by human macrophages (45). Critical COVID-19 ICU patients with acute respiratory distress are reported to show significantly lower levels of fucosylated anti-S IgG than mild or asymptomatic patients. Comparative analysis of immune response to multiple viruses also suggests that IgG afucosylation may be more common in response to antigens embedded in viral membranes than to non-enveloped viruses or soluble protein antigens (63).

SELF-REACTIVE ANTIBODIES AND FCγR RESPONSES IN IMMUNE-ENHANCED PATHOLOGY

Immune-enhanced pathology can include the induction of cross-reactive antibodies against human endothelial cells and molecules involved in platelet function and coagulation, possibly resulting from molecular mimicry by viral proteins having sequence similarities. Such autoantibodies are induced in DHF (64). Infection by Epstein Barr virus (EBV) can induce TLR hypersensitivity, followed by increased TLR-mediated B-cell differentiation to autoreactive antibody-secreting cells (65). In the presence of high levels of viral antigen, hypergammaglobulinemia and autoreactive antibody production can result from cooperation of infected B cells with CD4+ T-helper cells (66).

In patients infected with SARS-CoV-2, the generation of self-reactive antibodies has also been observed (67–69). The production of antiphospholipid antibodies of multiple isotypes has been reported in critically infected COVID-19 patients, in association with hypercoagulation and thrombotic events (70). Strikingly, nearly 30% of COVID-19 patients with severe disease, but fewer than 4% of non-intubated patients, are reported to produce IgM antibodies that cross-react with ACE2 and induce complement pathway activation. These autoreactive IgM antibodies emerge concurrent with clinical worsening and intubation, and appear only after anti-S IgG responses have been established. These autoantibodies may emerge in a T-independent manner from splenic marginal zone B cells, and could reflect an anti-idiotypic response to IgG antibodies directed against the SARS-CoV-2 spike (71).

Immune complexes comprised of IgG-bound antigens may further contribute to vascular leakage and cytokine storm via CD16/FcγRIII engagement (72). Cross-linking of FcγRs by IgG ICs induces macrophage activation and a switch in metabolic programming to glycolysis, accompanied by hypoxia-inducible factor HIF-1α dependent cytokine release, mirroring outcomes observed in IC-mediated autoinflammatory disease (73). In this context, it is notable that monocytes from patients with severe COVID-19 show high expression of HIF-1α and associated target genes, compared with healthy controls. In SARS-CoV-2 infected monocytes, mitochondrial ROS production and resulting HIF-1α-mediated metabolic changes contribute to impaired T-cell proliferation and expression of PD-1, a marker of T-cell exhaustion (74). Disruption of mitochondrial function by SARS-CoV-2 is further implicated in suppressed IFN response, NLRP3 inflammasome activation, and reduced oxygen sensing in patients with COVID-19 (75). Cross-linking of the CD16b/FcγRIIIb receptor isoform, which is present exclusively on neutrophils, also triggers the release of NETs that can contribute to thrombus formation (76).

Thus, as is observed in SARS-CoV and MERS-CoV infection, the production of antibodies against SARS-CoV-2 may potentially mediate several forms of immune-enhanced pathology, not limited to ADE, despite contributing to viral clearance. At the same time, these mechanisms suggest specific points of investigation such as ADE, core fucosylation of IgG Fc domains, induction of cross-reactive antibodies, FcγR mediated responses, compliment activation, and related factors that may be useful in evaluating the safety of candidate vaccines and antibody-based therapeutics.

VIRAL PATTERN RECOGNITION RECEPTORS IN DYSREGULATED CYTOKINE SIGNALING

Following viral infection of immune cells, viral components such as glycosylated membrane proteins activate signaling by innate pattern recognition receptors, resulting in downstream transcription that may include dysregulated production of inflammatory cytokines and chemokines. For example, repeated activation of the ssRNA sensor TLR7 promotes Mo/Mϕ differentiation into inflammatory hemophagocytes that drive MAS-like disease (77). Notably, CD14 acts as a co-receptor for signaling by the pattern recognition receptors TLR7/9. While CD14 is dispensable for viral uptake into endosomes, it is essential for triggering inflammatory cytokine production by macrophages and DCs (78).

In MERS-CoV infected monocyte-derived macrophages, upregulation of RLR and CLRs is followed by induction of proinflammatory molecules including IL-6, CXCL10/IP-10, and CCL3/MIP-1a. Depletion of the RLR signaling adaptor MAVS (mitochondrial antiviral signaling protein) or spleen tyrosine kinase Syk significantly reduces inflammatory cytokine induction. Induction of Syk by CLR, and downstream activation of NF-κB through the CBM complex (CARD9-BCL10-MALT1) is particularly implicated in the MERS-CoV inflammatory response of macrophages (79).

Signaling by FcγRs depends on downstream signaling by Syk. Activation of PI3K by Syk induces downstream signaling to cytoskeletal proteins that mediate phagocytosis

of IgG IC and receptor internalization, while additional signaling pathways promote the expression of pro-inflammatory cytokines and chemokines (80). The inflammatory response of human macrophages to anti-spike IgG from patients with severe COVID-19 is ablated by the Syk inhibitor fostamatinib, with downregulation of inflammatory mediators as well as expression of genes involved in platelet activation (45).

The inflammatory response of monocytes in SARS-CoV-2 infection shares numerous features with monocyte response in other viral infections, particularly following activation of innate pattern recognition receptors by heavily glycosylated viruses. For example, Ebola virus (EBOV) infection of monocytes results in strong induction of inflammatory cytokines including IL-6, IL-8, and chemokines CCL3/MIP-1a and CCL4/MIP-1b, with downregulation of class II MHC expression. Recruitment and apoptosis of lymphocytes is accompanied by a marked increase in LDH levels (81). Monocytes, macrophages, and DCs are initial targets of EBOV infection, and inflammatory cytokine release in EBOV-infected monocytes is mediated by TLR4 activation and downstream NF-κB signaling (82).

Likewise, infection with Hantaan virus (HTNV), which causes uncontrolled inflammatory response and lethal hemorrhage, is associated with a sharp increase in CD14+CD16+ intermediate monocytes, particularly in acute disease. Expression of CD163 in these monocytes is associated with severe disease, with CD206 expression observed more frequently in patients with mild/moderate disease (83). Viral recognition of HTNV by TLR3, RIG-I, and MDA5 pattern-receptors induces high expression of CXCL10 via NF-κB and IRF7 signal transduction pathways (84). Progression to severe hemorrhagic shock in DENV infection is also marked by upregulation of CD14+CD16+ monocytes expressing CD163 (85). DENV infection in monocytes is detected by TLR2/6, with CD14 acting as a co-receptor, resulting in the induction of pro-inflammatory cytokine expression via NF-κB signaling pathway (86).

DISCUSSION AND IMPLICATIONS FOR ANTIBODY-BASED THERAPEUTICS

The outcomes of SARS-CoV-2 infection can range from asymptomatic presentation to critical respiratory failure, tissue damage, organ failure, and fatality. Clinical reports suggest that these outcomes do not lie along a smooth continuum, but are often marked by abrupt severe clinical worsening. It is possible that this shift toward poor clinical outcomes corresponds to a change in viral tropism from infection of cells comprising respiratory linings and alveolar epithelia to direct viral infection of immune cells such as monocytes and alveolar macrophages. Although antibody response, inflammation, complement-dependent cytotoxicity, PRR signaling, and Fc-receptor effector functions can contribute to the clearance of viral pathogens, it is possible that these responses may be dysregulated in SARS-CoV-2 infected patients in a manner that contributes to disease severity. **Figure 1** illustrates several of these proposed mechanisms.

The potential roles of Mo/Mϕ infection and immune-enhanced pathology in COVID-19 are consistent with a broad range of evidence, but their prominence remains tentative pending further validation. In the interim, given the global health imperative for the development of safe and effective vaccines and therapeutics, the mechanisms discussed in this article suggest specific avenues of investigation that may be beneficial in the evaluation of candidate interventions.

For example, severe lung injury in SARS-CoV infection is not detected in macaques until 7 days following viral challenge (54), suggesting that evaluation of SARS-CoV-2 vaccine-induced ADE based on similar or shorter periods may be inappropriate. Screening for potentially cross-reactive antibodies may be informative, particularly where antigen selection includes S-RBD epitopes that overlap ACE2 binding sites. It may be useful to examine fucosylation of vaccine-induced IgG, particularly at intermediate titers in elderly or predisposed individuals, as antibody glycosylation and immune-enhanced effector functions may not be solely a property of a given vaccine or therapeutic, but also a property of the individual host response.

Currently, the majority of candidate vaccines against SARS-CoV-2 target the viral S protein, including S-RBD (87). Accordingly, the impact of spike- and S-RBD-directed antibodies on viral infection and inflammatory response of FcγR-bearing immune cells may be a particularly important focus in the evaluation of vaccine safety and efficacy. However, the potential for immune-enhanced pathology is not restricted to S-RBD epitopes or ADE of infection. For example, immunization of mice with the SARS-CoV full-length nucleocapsid protein can provoke pulmonary inflammation and immune cell infiltration upon viral challenge, despite reduction of viral titer to negligible levels (88).

As antibodies directed against the SARS-CoV and MERS-CoV S-RBD can functionally mimic the viral receptor and enable transition to a post-fusion conformation (89), inclusion of non-RBD epitopes may be advantageous. Antigen designs including modifications to stabilize the coronavirus spike in a pre-fusion conformation limit reliance on the S-RBD, concealing its immunodominant but poorly-conserved receptor binding motif (RBM), and are reported to increase both the breadth and potency of NAb responses (90, 91). Notably, the pre-fusion conformation does not prevent immune access to conserved epitopes at the periphery of the RBD (92) and at the S2 hinge (93), which mediate potent neutralization of both SARS-CoV and SARS-CoV-2.

Even after the development of initial vaccines against SARS-CoV-2, continued research and development efforts will remain important. While the coronavirus S-RBD is highly immunogenic, it is capable of readily generating antibody escape mutations in response to immune pressure (94). Adaptive S-RBD mutations have also been described after serially passaging the SARS-CoV-2 virus through the respiratory tract of aged mice. One recovered strain, carrying an N501Y substitution in the RBM, displayed enhanced ACE2 binding and replication in the respiratory tract of aged BALB/c mice, with subsequent tissue infiltration by inflammatory cells, including activated CD163+ macrophages

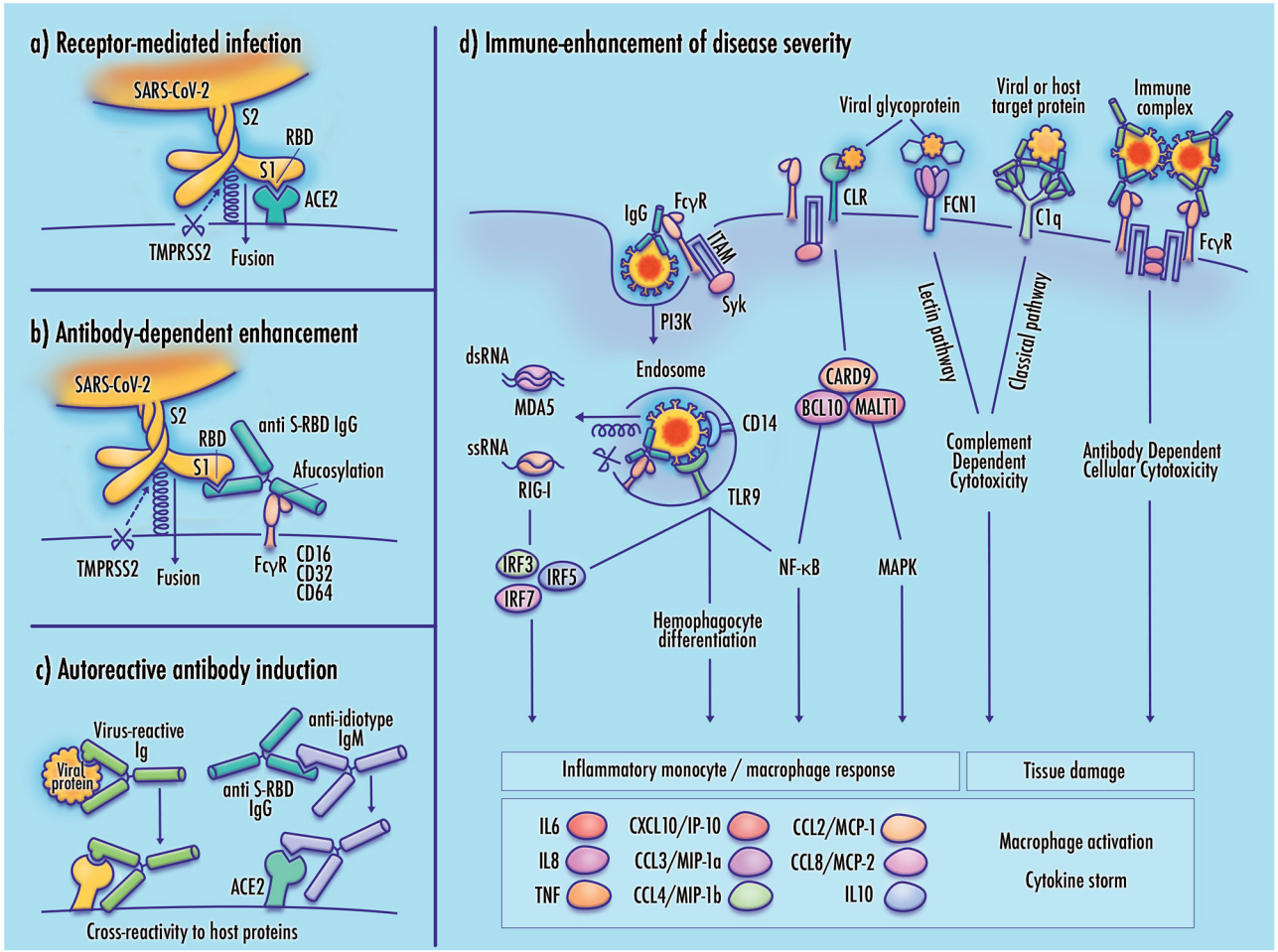

FIGURE 1 | Potential mechanisms of monocyte infection and immune-enhanced severity in COVID-19. **(a)** Receptor-mediated infection of target cells by SARS-CoV-2 is achieved by binding of the viral receptor binding domain (RBD) with host membrane-bound ACE2, which allows TMPRSS2 proteasomal cleavage of the viral spike membrane to enable virus–host membrane fusion; **(b)** In antibody-dependent enhancement (ADE), binding of the virus-antibody complex to an Fc gamma receptor stabilizes the viral spike in a manner that mimics the function of the viral receptor, enabling cleavage of the viral membrane and virus–host membrane fusion; **(c)** Induction of autoantibodies may result from molecular mimicry by viral proteins having sequence similarity to host proteins, anti-idiotype antibodies with cross-reactivity to host receptors, or direct disruption of immunological tolerance, which may be induced by TLR7 hyperactivation (not shown); **(d)** Increased disease severity may result from maladaptive immune responses to the SARS-CoV-2 virus. Viral infection of monocyte/macrophages can contribute to inflammatory pathology, activating downstream cytokine signaling, and cellular differentiation pathways. Inflammatory responses may also be induced by activation of pattern-recognition receptors including RIG-I-like receptors (RLR), Toll-like receptors (TLR), and C-type lectin receptors (CLRs). Receptors expressed at the immune cell membrane mediate adhesion of viral membrane glycoproteins, potentially contributing to infectivity by stabilizing the virus at the host cell membrane. Activation of complement pathway receptors by viral glycoproteins or antibody-bound target proteins may produce tissue damage by inducing complement-dependent cytotoxicity (CDC). Cross-linking of Fc gamma receptors by immune complexes can induce antibody-dependent cellular cytotoxicity (ADCC), and the release of neutrophil extracellular traps (not shown). Elevated cytokine and chemokine expression promotes cell recruitment, increased vascular permeability, and inflammatory damage to infected tissue.

(95). Such mutations in the RBD have recently emerged in novel strains first identified in the United Kingdom and South Africa, conferring greater resistance to neutralization by antibodies to ancestral strains (96). Prefusion spike vaccine designs and targeting of conserved epitopes may reduce this risk. Antibodies specific to a variable loop region of the betacoronavirus S2 spike subunit induce similar escape mutations in MERS-CoV, suggesting that such epitopes might best be excluded from SARS-CoV-2 vaccine designs (90).

The durability of protection conferred by vaccination against SARS-CoV-2 will likely become an active focus of research. While B-cell responses and NAbs to SARS-CoV decline significantly 1–2 years after infection, induction of memory CD4+ T-cells is suggested to confer more durable protection (97). As optimal protection against SARS-CoV-2 may rely on both antibody and T cell-mediated immunity, inclusion of highly conserved epitopes of structural or functional proteins may help to elicit a broad and durable immune response (98). Notably, much of the antibody

response induced by the ChAdOx1 vaccine, encoding the full-length spike antigen without pre-fusion stabilization, appears directed toward the RBD. Three mutations in the RBD harbored by the B.1.351 variant (K417N, E484K, and N501Y) result in a loss of antibody neutralization, with reduced protection against mild-to-moderate disease. However, ChAdOx1 vaccination also elicits expansion of CD4+ and CD8+ T cells specific to a large number of spike-specific antigens, most of which are unaffected by B.1.351 mutations (99). Considerations related to antibody escape mutations and T-cell mediated-immunity may become increasingly important over time, as the combination of intermediate NAb levels with an altered future serotype may create a potentially relevant context for ADE. Although this risk remains speculative at present, careful deliberation may nonetheless be appropriate for vaccine designs that rely heavily on presentation of the viral RBD, such as chimeric S-RBD constructs.

In the event that immune enhancement is observed in a given subgroup in response to viral exposure, such as individuals predisposed to autoimmune or inflammatory response, identification of therapeutic alternatives, and mitigation strategies for at-risk individuals may be beneficial. Such options may include the use of monoclonal antibodies, potentially with Fc-domain mutations to disrupt FcγR crosslinking (100); blockade of FcγR or PRR signaling via Syk or NF-κB pathways (45, 79); downregulation of NLRP3 inflammasome activation (101); blockade of the terminal complement pathway (102); and saturation of FcγRs (55, 103) or downregulation of the complement pathway receptor FCN1 (35) via therapeutic IVIG, possibly excluding afucosylated or activating fractions. Meanwhile, ongoing consideration and testing of pathway-informed, well-tolerated therapeutic candidates may be beneficial in active cases, including repurposed therapeutics targeting viral replication (e.g., remdesivir, ivermectin), leukocyte-mediated tissue damage (e.g., doxycycline, IFN-λ), and dysregulated inflammatory response (e.g., barcitinib, ruxolitinib, tocilizumab, dexamethasone) (104) in COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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SYSTEMATIC REVIEW
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A Systematic Review and Bibliometric Analysis of the Scientific Literature on the Early Phase of COVID-19 in Italy

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Background: Studying the scientific literature about COVID-19 and Italy, one of the first countries to be hit by the pandemic, allows an investigation into how knowledge develops during a public health emergency.

Methods: A systematic review of the literature was conducted to identify articles published on the topic between January and April 2020. Articles were classified according to type of study. Co-occurrence of terms, and geographic and temporal trends were analyzed.

Results: Of the 238 articles included in the systematic review, the majority (37%) focused on hospital and clinical management of COVID-19, while 23.9% were commentaries. Epidemiological studies constituted 45.5% of the articles published by authors with non-Italian affiliations.

Conclusion: The scientific articles on COVID-19 in Italy were varied in type of study, though with limited international impact. The lockdown and the pressure placed on hospitals during the first wave of the pandemic mainly resulted in publications on disease management and commentaries.

Keywords: coronavirus, COVID-19, Italy, bibliometric analysis, public health, systematic review

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INTRODUCTION

Since the first cases of COVID-19 were reported in December 2019 in Wuhan, China (1), the SARS-CoV-2 virus has continued to spread. The World Health Organization (WHO) declared COVID-19 to be a Public Health Emergency of International Concern (PHEIC) by January 30, 2020 (2) and a pandemic by March 11, 2020 (3). In the following months the virus has spread all over the world (4). Italy was one of the countries first affected and, with 1,770,149 confirmed cases and 61,739 COVID-19-related deaths (as of 11 December 2020) (5), it is one of the hardest hit. Italy entered a national lockdown on March 9th (6), which lasted over 2 months, but in March 2021 it is still dealing with COVID-19 and many areas across the peninsula are experiencing new lockdowns (7).

During a pandemic, circulation of information is one of the main weapons allowing the organization of a coordinated response in different countries facing the same emergency (8). This has led many journals to speed up the process of peer review and publication in order to provide large amounts of accessible information to the scientific community and the general public (9, 10).

Since much of the scientific literature on the pandemic has been produced in a short time span, it is important to describe and understand the nature of this output and the main elements that characterize it (11). A combination of qualitative analysis and quantitative bibliometric analysis is an effective approach to the analysis of the large amount of scientific literature produced and the identification of the main messages (12). For this purpose, many bibliometric tools such as VOSviewer have been used to investigate the global status and trends of the pandemic (13, 14), to make comparisons among countries (15) or to analyze the scientific output of a single country (16). Italy represents a unique case study: it was the first European country to be hit by the pandemic, and the consequences of the outbreak had a shocking impact on the population. The experience of the Italian hospitals and territories, given their arduous struggle with the pandemic, drew the attention of the entire scientific community. Analysing the scientific literature on COVID-19 and Italy in the first pandemic wave can therefore help us to understand how the scientific output evolves as a new public health threat emerges.

The aim of this systematic review is to describe the key features of the peer-reviewed scientific literature on the COVID-19 outbreak in Italy over the first 4 months of the epidemic (up to April 24, 2020) using both a qualitative and a quantitative approach.

METHODS

A systematic search of the literature was performed using Scopus and PubMed databases on the 24th of April 2020. A comprehensive search strategy was developed to identify articles published since December 2019 which included the terms ("covid" OR "SARS-CoV-2" OR "coronavirus") AND ("Italy" OR "Italian") in their title and/or abstract. In order to be included in our study, articles had to address the COVID-19 pandemic in the Italian setting, with no restriction based on language or study design. This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyzes (PRISMA) 2009 Statement (17), although we do not present the characteristics for each article included, as it is beyond the scope of the study.

Retrieved articles were then evaluated independently by three researchers to ensure only articles related to the current SARS-CoV-2 pandemic in Italy were included in the analysis. For each included item, publication date, title, journal, first author's gender and first author's nationality of affiliation (Italian or non-Italian) were extracted. For Italian publications, region of first author's affiliation was determined; for non-Italian publications, country of first author's affiliation was determined. When the first author was affiliated to a research center managed by different regions, such as IRCCS (Istituto di Ricovero e Cura a Carattere Scientifico), we assigned the region according to where the institute is based. Gender was not assigned when the author was an institution and for authors where gender could not be inferred. Region/country of affiliation was not assigned when the affiliation was a national institution, a journal or a scientific society. The impact factor for each journal was obtained from the Journal of Citations Report 2019 (18).

Articles were classified according to study type based on the classification of studies in medical research developed by Röhrig et al. (19), which was expanded and adapted for the purposes of this study. The type of study was defined according to the contents, rather than its form of publication (e.g., commentaries including case reports were classified as case reports rather than commentaries). The following categories were added by the researchers to the original classification by Röhrig et al. (19): *Modeling and prediction* included studies in which mathematical models were developed to make predictions about the pandemic; the *Management* category included *Hospital management case report* (accounts of hospital management strategies undertaken to combat the pandemic, for example, reorganization of wards, reallocation of HCWs), *Clinical management case report* (accounts of algorithms used to manage COVID-19 patients) and *Experts' recommendation* (recommendations on hospital and/or clinical management issued by scientific societies or groups of experts); the category defined as *Other* included *Ethics and Legal Medicine* (considerations on ethical or legal aspects relating to decision-making during the pandemic), *Commentary and Viewpoint* (generic considerations without original information).

VOSviewer (version 1.6.15) was used to perform co-occurrence analyzes on terms from titles and abstracts in order to visualize the main topics of the publications. Co-occurrence analysis reveals how often two words appear together in the same text as well as the connections between terms. In the resulting visual network, each sphere represents a term, and the size of the sphere is proportional to the occurrence of the term. The links between the spheres represent the association between words: the thicker the line, the stronger the association (co-occurrence). The program identifies clusters of words that are very often cited together and likely refer to the same topic. Two co-occurrences analyzes were performed: one including words from both title and abstract and one considering words from abstract only. The occurrence threshold for our study was set at five, with an automatic selection of 60% of co-occurring words based on relevance. Time trends, geographical analyzes and journal analyzes were carried out in Microsoft Excel.

RESULTS

Of the 321 studies retrieved from the search (**Figure 1**), 238 articles were included in the analysis: 205 where the first author's affiliation was with an Italian institution and 33 where the first author had a non-Italian affiliation. [From this point, studies with a first-author Italian affiliation will be called "Italian" studies, with the others being called "non-Italian" studies].

Content Analysis

Abstracts were not available for 108 out of 205 Italian and 22 out of 33 non-Italian articles. For these studies, only words included in the title were analyzed with the VOSviewer software for the co-occurrence analysis performed on title and abstract. Based on this analysis, four clusters emerged, highlighted in different colors (**Figure 2**): red cluster, labeled as "hospital

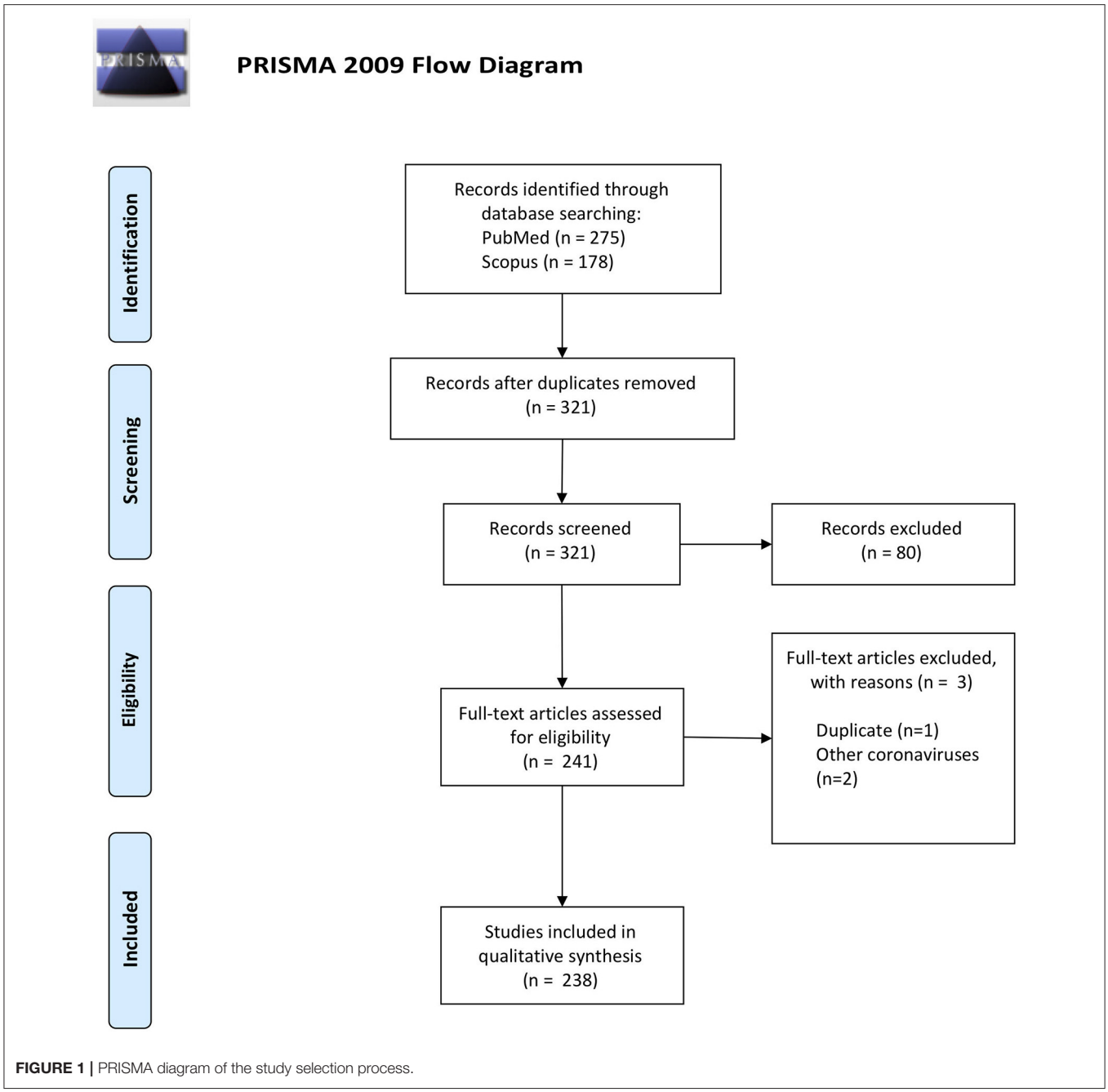

FIGURE 1 | PRISMA diagram of the study selection process.

and clinical management," containing 24 items; blue cluster, labeled as "descriptive epidemiology," containing 22 items; green cluster, labeled as "policies and public health," containing 18 items; yellow cluster, labeled as "generic," with transverse items not specific to other clusters, containing 13 items. The most cited words were: "experience" (36 occurrences), "management" (27 occurrences), "February" (25 occurrences) and "death" (25 occurrences).

A further analysis based on abstracts only was performed. The resulting network is shown in **Supplementary Figure 1** and includes four clusters: red cluster, labeled as "hospital management", containing 16 items; blue cluster, labeled as "clinical management," containing 12 items; green cluster, labeled as "epidemiology," containing 12 items; yellow cluster, labeled "generic," with transverse items not specific to other clusters, containing 12 items.

Classification of the Retrieved Articles

Articles were classified according to study type, using Röhrig's classification (19) as baseline (**Table 1**). Half of the Italian publications were classified as either *Hospital management case report* (55) or *Commentary and Viewpoint* (48) (103 out

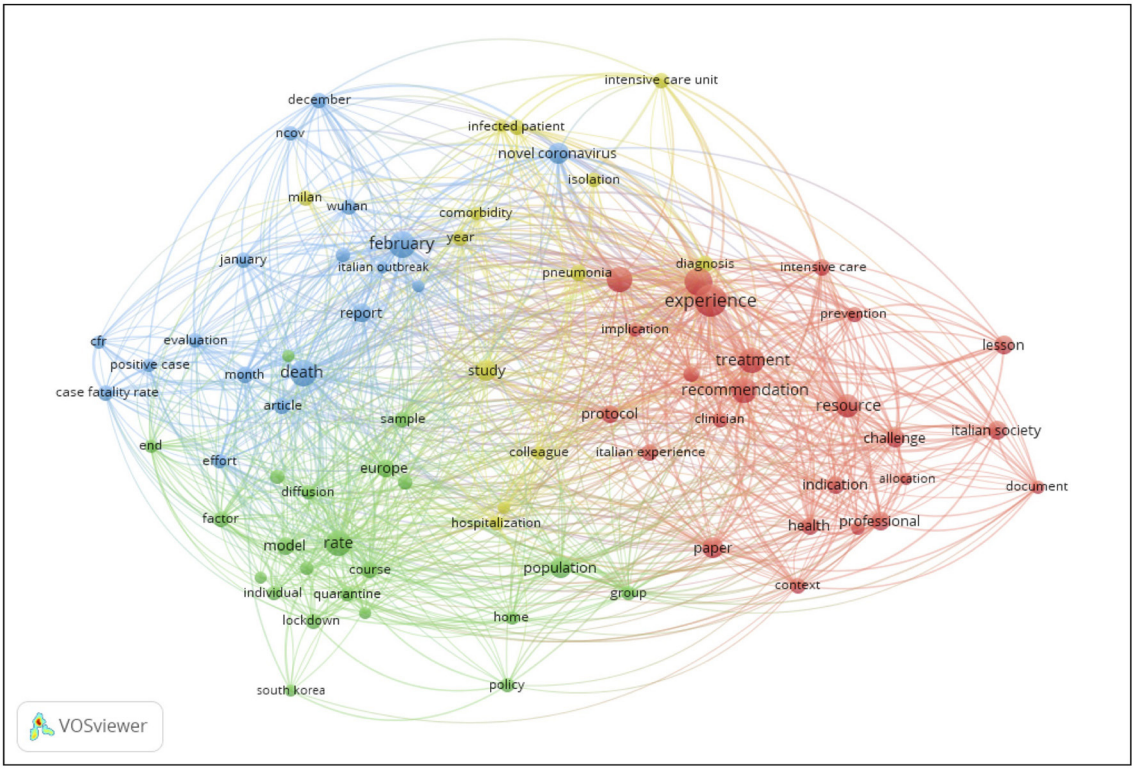

FIGURE 2 | Co-occurrence analysis using VOSviewer of terms in titles and abstracts of Italian studies.

of 205, 50.2%). Non-Italian publications were more equally distributed: 21 out of 33 (63.6%) were either *Commentary and Viewpoint* (nine), *Monitoring and Surveillance* (eight) or *Narrative review* (four). All the *Basic* research studies identified (five Italian and two non-Italian) were *Genetic engineering and Gene sequencing* articles. Italian *Clinical* research studies were mostly observational (26), and only one was experimental, while no *Clinical* studies were found among non-Italian articles. Among the observational epidemiological studies, Italian articles mainly reported results of *Monitoring and Surveillance* and *Modeling and Prediction* studies (eight and six, respectively), while the non-Italian were mostly *Monitoring and Surveillance* (four) and *Ecological study* (four) articles. Articles assigned to the *Management* category were mainly *Hospital management case report* (55), *Experts' recommendation* (24) and *Clinical management case report* (nine). No experimental *Epidemiological* studies were found. Among *Secondary* research studies, 10 Italian and four non-Italian *Narrative review* articles and *one* Italian and one non-Italian *Systematic review* articles were found.

Geographical Distribution

We compared the geographical distribution of the Italian publications with the distribution of COVID-19 density of cases at the end of the study period (**Table 2**). The region was not attributable in 13 articles (6.4%).

By April 24th Italy had accumulated 192,994 cases (20). Density of cases per region showed a clear north-south gradient (**Supplementary Figure 2**). Of the 205 Italian articles, 73 (35.6%) were published in Lombardy, which was the second region for density of cases, while 33 (16.1%) were published in Lazio, which was among the regions with the lowest density of cases (**Table 2**). The analysis of the characteristics of the type of study showed that *Management* studies were published mainly in Lombardy (42), Emilia-Romagna (nine) and Lazio (seven). *Clinical* studies followed a similar pattern, with 12 publications from Lombardy and eight from Lazio, while *Commentary and Viewpoint* articles were more equally distributed among regions, as were *Epidemiological* studies (**Supplementary Figure 3**).

The 33 non-Italian articles were published in fifteen countries. Nine were published by authors based in the United Kingdom, four in the United States and Sweden, three in China, two in Brazil and two in Iran.

Trends in Type of Publication

There was a marked increase in publications over time as the pandemic progressed, beginning with a single article published in January to 144 articles published in April. The first type of publication to appear was a *Narrative review* in January, after which various types of articles were published in February, although in small numbers: one *Basic* study, two *Clinical* studies, one *Epidemiological* study, one *Secondary* study, one *Management* study and two *Commentary and Viewpoint* articles.

TABLE 1 | Classification of the 238 retrieved articles by type of study.

				Italian		Non-Italian	
				n	%	*n*	%
Primary (157)	Basic (7)	Theoretical (method development)	Analytical measurement procedure	0	0	0	0
			Imaging procedure	0	0	0	0
			Biometric procedure	0	0	0	0
			Test development assessment procedure	0	0	0	0
		Applied	Animal study	0	0	0	0
			Cell study	0	0	0	0
			Genetic engineering and Gene sequencing	5	2.4	2	6.2
			Biochemistry	0	0	0	0
			Material development	0	0	0	0
			Genetic studies	0	0	0	0
	Clinical (27)	Experimental	Clinical study	1	0.5	0	0
		Observational	Therapy study	3	1.5	0	0
			Prognostic study	0	0	0	0
			Diagnostic study	3	1.5	0	0
			Observational study with drugs	0	0	0	0
			Secondary data analysis	0	0	0	0
			Case series	15	7.3	0	0
			Single case report	5	2.4	0	0
	Epidemiological (35)	Experimental	Intervention study	0	0	0	0
		Observational	Cohort study	0	0	0	0
			Case control study	0	0	0	0
			Cross-sectional study	2	1	0	0
			Ecological study	2	1	4	12.1
			Monitoring and Surveillance	8	3.9	8	24.2
			Modeling and Prediction	6	2.9	2	6.1
			Description with registry data	2	1	1	3
	Management (88)		Hospital management case report	55	26.8	0	0
			Clinical management case report	8	3.9	0	0
			Experts' recommendation	24	11.7	1	3
Secondary (16)	Meta-analysis			0	0	0	0
	Review (16)		Narrative	10	4.9	4	12.1
			Systematic	1	0.5	1	3
Other (65)			Ethics and Legal Medicine	7	3.4	1	3
			Commentary and Viewpoint	48	23.4	9	27.3
Total				205	100.0	33	100.0

From March onward, the number of publications in each category, especially *Management* studies, increased. *Ethics and Legal Medicine* articles started to appear in March (three) and April (four) (**Figure 3**). *Management* articles increased in absolute numbers and in percentage, making up half of the publications in March, then slightly decreased in April by percentage, but not in absolute numbers. *Commentary and Viewpoint* articles emerged relatively early and remained more or less stable though time (28.6% in February, 17.0% in March and 25.7% in April), with an increase in absolute number month by month.

Non-Italian publications started to appear in February with two articles categorized as *Commentary and Viewpoint*. In March and April, there was an increase in the number and variety of articles. The most represented category, appearing in March and increasing in April, was *Epidemiological* studies (six and nine articles, respectively). We found only one *Management* report, published in April (**Figure 4**). The proportion of *Commentary and Viewpoint* articles decreased with time, with a simultaneous increase in the other types of publication.

Journals

The Italian articles were published in 153 different journals. Among these, 30 journals published more than one article each and five journals more than three. In particular, seven articles appeared in the *Journal of Medical Virology*, five in *The Lancet* and *Giornale Italiano di Nefrologia*, and four in *Eurosurveillance*

TABLE 2 | Geographical distribution of cases of COVID-19 and articles published up until 24th April 2020. Regions are ordered by decreasing density of cases.

	Cumulative cases of COVID-19[a]	Population[b]	Density of cases (cases per 10.000 inhabitants)	N. of articles published
Valle d'Aosta	1,100	125,034	87,98	0
Lombardy	71,256	10,027,602	71,06	73
Trentino-Alto Adige	6,232	1,078,069	57,81	4
Piedmont	23,822	4,311,217	55,26	10
Emilia-Romagna	23,970	4,464,119	53,69	18
Liguria	7,173	1,524,826	47,04	2
Marche	6,028	1,512,672	39,85	5
Veneto	17,229	4,879,133	35,31	7
Tuscany	8,877	3,692,555	24,04	11
Friuli Venezia Giulia	2,882	1,206,216	23,89	6
Abruzzo	2,803	1,293,941	21,66	2
Umbria	1,363	870,165	15,66	0
Lazio	6,132	5,755,700	10,65	33
Apulia	3,881	3,953,305	9,82	4
Molise	287	30,0516	9,55	0
Sardinia	1,257	1,611,621	7,80	4
Campania	4,282	5,712,143	7,50	7
Basilicata	360	553,254	6,51	0
Sicily	2,981	4,875,290	6,11	3
Calabria	1,079	1,894,110	5,70	3

[a] *Source of data: Italian Civil Protection Department (20).*
[b] *Population at January 1, 2020. Source of data: National Institute of Statistics (ISTAT) (21).*

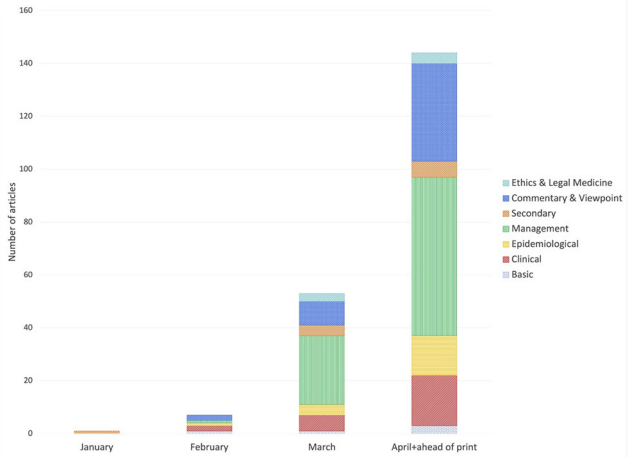

FIGURE 3 | Italian articles published each month, by type of study.

and *Recenti Progressi in Medicina*. Two of these five journals do not have an impact factor (IF) according to the Journal of Citations Report 2019. Four of the top ten journals ranked by IF published at least two articles. *The Lancet*, the journal with the highest IF (60.39), published five articles, followed by *JAMA* (45.54) with three publications. Globally, 18 articles were published in the top ten journals by IF. The median IF score for Italian publications was 3.75, with a mean of 8.16 (**Figure 5**).

Non-Italian articles were published in 25 different journals. Three journals published more than one article: *The BMJ—British Medical Journal* published five articles, *Eurosurveillance* and *Journal of Medical Virology* three articles each. Ranked by IF, the first three journals (*The New England Journal of Medicine* IF 74.699, *JAMA* IF 45.54 and *Nature* IF 42.78) had one publication each. The median IF and the mean IF of the non-Italian journals were 6.46 and 15.11, respectively (**Figure 5**).

DISCUSSION

The number of articles published globally relating to the pandemic has grown exponentially since the first cases were confirmed in China. An analysis carried out on PubMed on the 25th of April by Kambhampati and colleagues detected 6,831 articles on the pandemic and showed an exponential growth of publications relating to COVID-19 since the beginning of the year (22). Our review focused on a specific portion of the global literature on the pandemic, that is, those publications pertaining to Italy. Accordingly, our search yielded mainly articles published by authors with an Italian affiliation, and a smaller number of articles published by non-Italian authors that included Italy in wider analyzes.

Due to its early involvement in the current pandemic, Italy has scaled up its contribution to research in the field of coronaviruses (23). During the SARS and MERS outbreaks, Italy did not appear in the top 10 contributing countries (24) and, according to the literature, the Italian share of the global scientific production on COVID-19 was itself limited up to the end of February 2020 (25). However, by the end of March, Italy's contribution amounted to almost 7% of global output (26), and increased further to 7.6% by the end of April (27) and to almost 9% by the beginning of May 2020 (28).

The regional distribution of the scientific output from Italy is comparable to the distribution of COVID-19 density of cases reported in the different regions, with some exceptions: the Lazio region released a relatively high number of publications given its share of cases, but this can be explained by the presence of national research institutes in this region. On the other hand, other regions with a high density of cases, like Veneto and Piedmont, published relatively few articles. As might be expected, the hardest hit region at the time (Lombardy) published a proportionally large number of articles relating to the management of the outbreak and to clinical aspects, thus illustrating the differing impact of the pandemic across the country.

The analyzes carried out with VOSviewer showed that the main themes were the epidemiology of the disease and the management of the outbreak in hospital settings. The focus of many studies on management aspects of the pandemic was confirmed in our analysis when articles were classified by study type, which revealed that most articles with an Italian

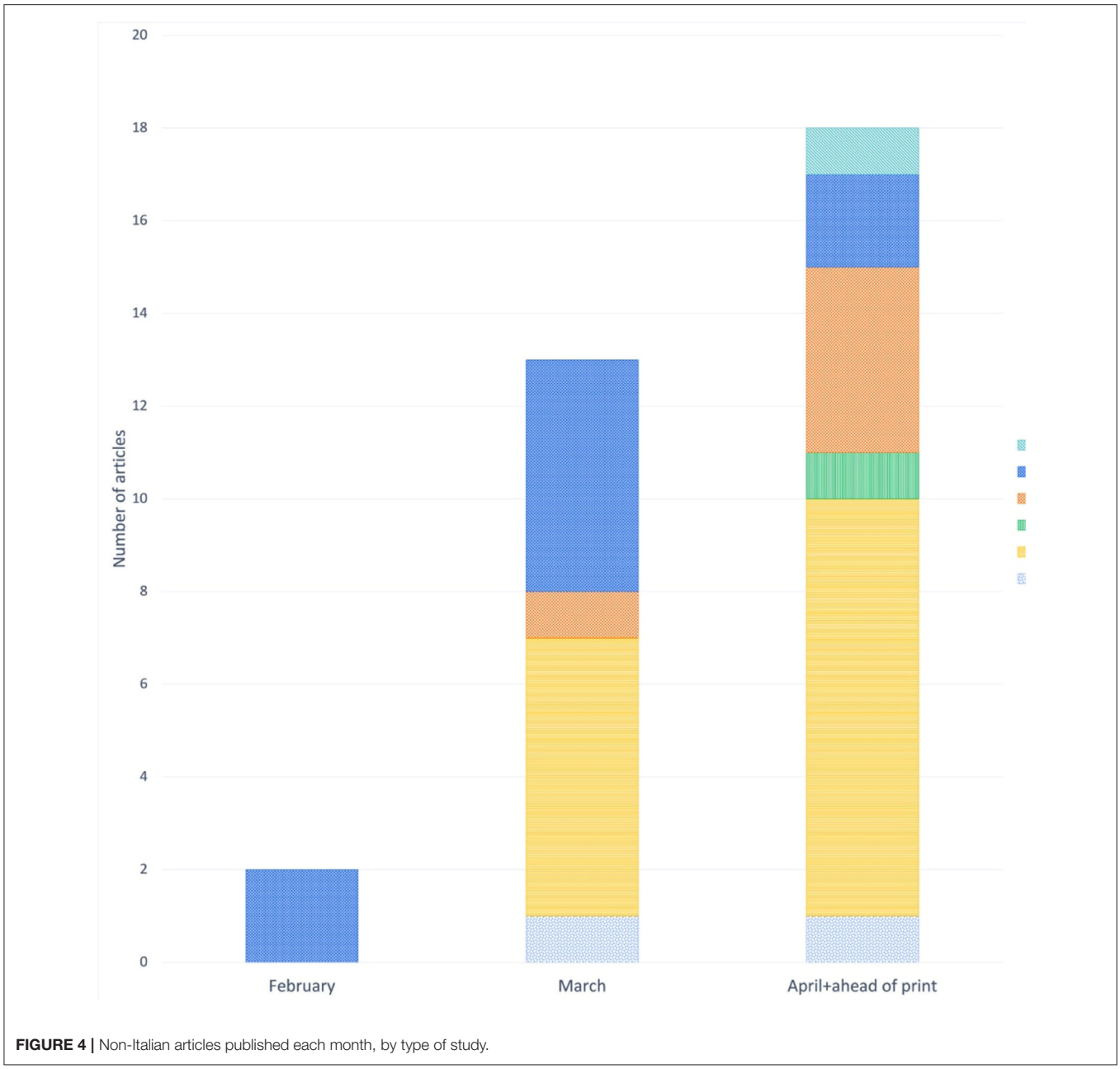

FIGURE 4 | Non-Italian articles published each month, by type of study.

affiliation consisted of hospital management case reports and commentaries. Our resulting map (**Figure 2**) differs from similar analyzes of the global literature using keywords carried out on VOSviewer, which showed a wider prevalence of clinical terms (29–31). A focus on clinical aspects related to COVID-19 was also found in an analysis of Iranian publications (16). The content analysis carried out on abstracts only did not identify a policy field. This is probably due to the fact that articles dealing with policy aspects were mainly commentaries and viewpoints, which were not always provided with an abstract.

All types of publication increased with time, with a notable increase in the share of articles relating to the management of the pandemic, which mainly comprised hospital management case reports and experts' recommendations, in March and April. This might reflect the need to share experience accumulated in the field through publication. The increase in the number of publications that aim to provide expert consensus on COVID-19 management has raised concerns with some authors, due to the lack of evidence underlying such recommendations (32). Non-Italian articles showed a different publication pattern: most were epidemiological studies, followed by commentaries and narrative reviews, while there were, unsurprisingly, relatively few management reports due to our search strategy.

With respect to original research, Chahrour et al. (33) have pointed out that until mid-March 2020 the Italian contribution was small compared to the number of cases of COVID-19 in the

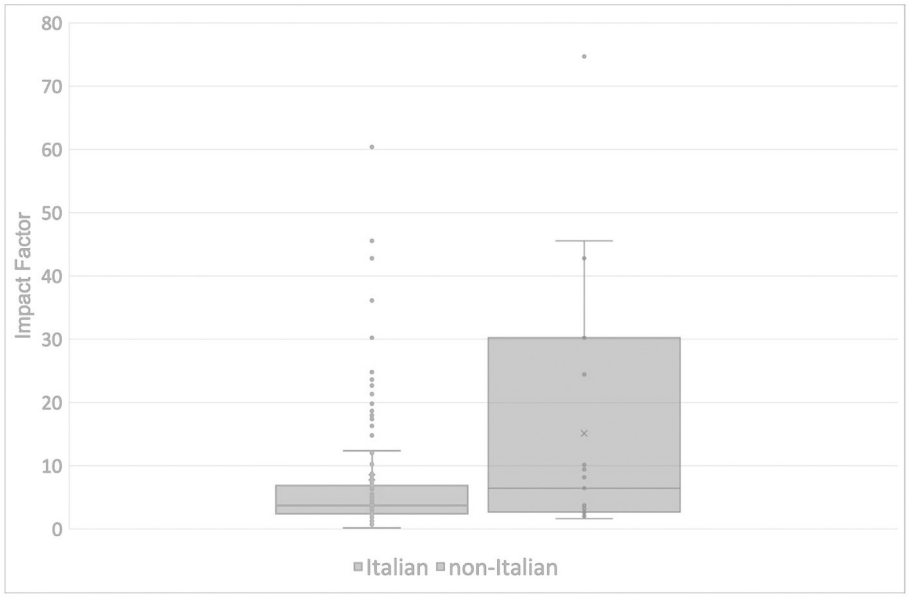

FIGURE 5 | Distribution of Impact Factor of the journals that published Italian (left) and non-Italian (right) articles.

country. This was confirmed by Nowakowska et al. (26), who quantified the Italian contribution as 3.2% of the global output of original research by the end of March 2020; Chinese authors were the most prolific, with a 57.7% share of published articles. Our analysis of Italian output shows that there was an increase in basic, epidemiological and clinical research publications in March and especially in April 2020. Part of what we observed could be due to the fact that the first cases in Italy were identified in late February 2020, 2 months after the outbreak in China. It should also be noted that in our analysis we classified the articles according to their content rather than the format of publication. Since many articles were published as letters to the editor or commentaries in order to speed up the publication process, even when they contained original information (26), classifications based on format of publication could lead to an underestimation of the contribution to original research. As Zhai et al. (23) have shown, the number of articles published as letters was also relatively high during the year of the MERS and SARS outbreaks, and then decreased in the following years.

By analyzing the journals and impact factors, we found that, overall, non-Italian articles were published in journals with higher impact factor than Italian articles. This could be due to the need for Italian authors to share knowledge with a small circle of colleagues who faced the same challenges within the country. This hypothesis is supported by the prevalence of management publications. In contrast, non-Italian articles usually included Italy in broader epidemiological analyzes and were addressed to a wider public.

It is interesting to note that women constituted only a small proportion of the first authors of the articles retrieved in our analysis. The proportion was remarkably low for Italian articles (22%) compared to non-Italian articles (48.5%). Further analyzes could clarify whether there has been a decline in the number of female first authors in Italy with the pandemic, as has been shown by Andersen and colleagues for global medical output (34).

We acknowledge some limitations to our analysis. First, we searched only the PubMed and Scopus databases, thereby potentially underestimating the number of publications. Second, since less than half of the articles included in the analysis had an abstract, VOSviewer mainly considered terms included in the titles, which could have provided a less sensitive analysis of the content of the studies. Finally, our study was limited to items published up to 24th April 2020, and therefore provides only an initial overview of the contribution of Italian publications to the growing body of scientific output on COVID-19. Indeed, a bibliometric analysis of global scientific output of COVID-19 carried out in June 2020 already showed that the Italian contribution had grown to 12.2% (31).

To our knowledge, however, this is the first study to comprehensively evaluate scientific publications on COVID-19 in Italy, the first country in Europe to be hit by the pandemic. We believe this analysis provides an important starting point for understanding the mechanisms of dissemination of knowledge in critical times such as the current COVID-19 pandemic.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FT and EM equally contributed to the literature searches, data extraction, data analysis, and drafting the manuscript. FP contributed to the data extraction. GM contributed to

the bibliometric analysis. PV and CD conceived the study. CD contributed to the planning of the work, reviewed, and edited manuscript drafts. All authors contributed to the design of the study, revised, and approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.666669/full#supplementary-material

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COMMUNITY CASE STUDY
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The Significance of Duration of Exposure and Circulation of Fresh Air in SARS-CoV-2 Transmission Among Healthcare Workers

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Background: The true risk of infection after exposure to SARS-CoV-2 of healthcare workers (HCWs) in the workplace has not yet been established. This descriptive study analyzes the exposure characteristics of HCWs to SARS-CoV-2.

Methods: In March 2020, at the beginning of the pandemic, a total of 58 HCWs in a regional hospital in Greece were exposed to three patients with symptomatic SARS-CoV-2 infection. These three index cases had taken part in an 8-day religious tour, during which 52 travelers spent 10 h every day in a tour bus. A study was made of the circumstances of the hospital exposure.

Results: Of the 52 travelers in the bus, 48 contracted SARS-CoV2. None of the 58 HCW contacts developed symptoms related to COVID-19, although, 43% were exposed to a SARS-CoV-2 infected patient for more than 15 min, and 74% were within a distance of <1 m, and half of the contacts were not wearing a surgical mask. Additional information was that 63% of the contacts were exposed in a room sized more than 15 m^2, and in more than 80% of cases, the window or the door to the room was open during their exposure. In about one-third of the exposure events, the HCW contacts were not wearing a mask and were at a distance of <1 m, and just under half of them were exposed for more than 15 min. One-fourth of the contacts underwent RT-PCR testing, and 11% IgG/IgM antibody testing for SARS-CoV-2, all of which were negative. All observed quarantine at home for 14 days.

Conclusion: This observational study was made before the extent of the SARS-CoV-2 became apparent, and before routine preventive measures were observed by all HCWs. Comparing the conditions of exposure in the two different settings (bus vs. regional health facility), it is apparent that the duration of exposure and the small, enclosed nature of the bus are the distinguishing factors. In the healthcare setting, the elimination of both factors and the implementation of additional measures protected the exposed HCWs from contracting SARS-CoV-2 infection.

Keywords: COVID-19, SARS-CoV-2 transmission, health care worker, health care facilities, safety measures

INTRODUCTION

As the spread of COVID-19 is changing rapidly, there are still many unknown factors regarding its transmissibility. Recently, details of the aerogenic transmission of SARS-CoV-2 have been documented by several researchers (1).

The combination of several factors may affect the transmissibility of SARS-CoV-2, including distance (2, 3), viral load (4), duration of exposure (5, 6), and mask wearing (1). The World Health Organization (WHO) (7) and the US Centers for Disease Control and Prevention (CDC) (8) have recommended specific protection measures for work places. In addition, mutations in spike protein cause increased infectivity (9).

Healthcare workers (HCWs) are in the front line of fighting the pandemic (10). The published findings on infections and deaths among exposed HCWs are devastating (11, 12). Personal protective equipment (PPE) including mask, gloves, and non-woolen robes are recommended by several public health authorities (7, 13, 14).

In this observational study, we analyzed the characteristics of the personnel exposed to three patients with COVID-19 in a regional hospital in Greece at the very beginning of the pandemic, when no other cases had been identified in the community, and before the policies regarding the protection of the HCWs from SARS-CoV-2 had been broadly implemented.

TABLE 1 | The clinical characteristics of the first three patients with COVID-19 diagnosed in a Greek regional hospital.

Characteristics	Patient 1	Patient 2	Patient 3
Estimated time from exposure to onset of symptoms (days)	7	7	6
Symptoms—duration (days)			
• Cough	3	6	No
• Fever	3	3	2
• Myalgia	3	No	3
• Fatigue	3	4	5
• Headache	3	No	No
• Sore throat	3	2	2
• Loss of smell	No	No	No
• Shortness of breath (days)	3	2	No
• Gastrointestinal symptoms	No	No	No
• Hospitalization	Yes	Yes	No
Duration of symptoms prior to visit to regional hospital (days)	2	6	3
Duration of hospitalization in regional hospital (days)	3	4	No hospitalization
Transfer to a tertiary hospital	Yes	Yes	No hospitalization
Lung involvement	Pneumonia	Pneumonia	No lung involvement
Outcome	Death	Cure	Cure

METHODS

The Patients

Patient No 1 was 66-year-old man, who had just returned to Greece from an organized religious group trip to holy sites in the Middle East. He became ill the day after his arrival back in Greece, with a sore throat, fever of 38°C, and myalgia. Two days later, his fever rose to 39.2°C, at which stage he attended the emergency department (ED) of the regional hospital. He was hospitalized for 2 days, then, on diagnosis of SARS-CoV-2, he was transferred to a COVID-19 unit in a tertiary hospital, where he finally died on day 15 of his illness. His wife, who had also been on the religious trip, was diagnosed with COVID-19 3 days after this patient's admission.

Patient No 2 was a 45-year-old man who had been on the same religious trip. He attended the ED with fever and cough and was admitted with pneumonia. He was transferred to the tertiary hospital and remained hospitalized for 21 days, but recovered. This patient had attended a funeral the day prior to his admission, and several other funeral attendees were subsequently diagnosed with COVID-19. The brother of patient No 2 also developed COVID-19, as they met shortly after the return from the religious tour.

Patient No 3 was 35-year-old woman, who was an administrative officer in the regional hospital, and who also had been on the religious trip. She developed fever and myalgia 3 days prior to returning to work. She was informed about the spread of SARS-CoV-2 during the religious trip after being back at work for 2 days, at which time she had minimal symptoms, with no cough. She was not hospitalized but remained in isolation at home.

Table 1 summarizes the clinical characteristics of the three patients (index cases) with COVID-19 diagnosed at the regional hospital. All three individuals had been symptomatic for a minimum of 2 days prior to their hospital visit. Two of them had cough, were diagnosed with pneumonia, and stayed hospitalized in the regional hospital for 2–4 days prior to transfer to a tertiary Medical Center due to their clinical deterioration. None of the patients had gastrointestinal symptoms.

These three patients were the index cases of an outbreak of COVID-19 among a group of 52 Greeks who had participated in an organized religious bus tour to holy sites in the Middle East. They were riding in the bus for approximately 10 h per day for a total of 8 days. Of the 52 individuals in the group, 48 tested SARS-CoV-2 positive, and 2 finally died of complications of the disease.

All contacts of the three index cases who were identified, including HCWs in the hospital, completed a questionnaire regarding their exposure to the infected person. In this way, we gathered information relating to the duration of the exposure of the HCWs, their distance from the index case, the size of the room, whether the windows/door were open or closed, the age of the exposed person, the occupation, and the PPE that was used, if any. The protective measures introduced by the healthcare facility to medical personnel at that time was in accordance to WHO interim guidelines (2/27/2020) for suspected cases. Those included, application of surgical mask and gloves,

provide adequate space to allow at least 1 m distance, limit the time of exposure, and open air ventilation. However, the index of suspicion was low, as no other COVID-19 cases were identified in Greece up that point (15). Those measures were suggested but not mandated since no COVID-19 cases were identified in Greece up to that point. RT-PCR for SARS-Co-V-2 (VIASURE, CerTest Biotec) was performed by nasopharyngeal swabs on all the HCWs who were exposed at a distance of <1 m from the symptomatic patients. All exposed hospital personnel remained on home isolation for 14 days. They were instructed to self-assess and report symptoms related to COVID-19. Eight weeks after the exposure, the contacts were questioned again about their clinical status and were tested for SARS-CoV-2 antibodies using Abbott SARS-CoV-2 IgM and IgG performed on the Abbott automated analyzer.

RESULTS

A total of 58 contacts, each with one of the three index cases, were identified among the hospital HCWs. None of the exposed personnel developed symptoms.

The median age of the HCW contacts was 47.74 years, ranging from 25 to 60 years, with a predominance of female staff (72.42%). Of the exposed personnel, 17 (29.3%) were physicians, 20 (34.5%) were registered nurses, and 12 (20.9%) were administrative staff.

Regarding the duration of exposure, 33/58 (56.9%) had remained in contact with one of the index cases for <15 min, 16 (27.6%) for 15 min to 2 h, 4 (8.6%) for 2–4 h, and 5 (8.6%) for more than 4 h. Regarding the distance from the index case, 43 (74.1%) of the contacts were within 1 m of an index case, 13 (22.4%) were at a distance of 1–3 m, and 2 contacts (3.4%) were at a distance more than 3 m.

The size of the room in which the contact had been made was evaluated. Exposure in a small room of less 15 m^2 was reported by 21/58 (36.2%) contacts, and 32/58 (55.2%) in a room of 15–30 m^2; overall, 63.8% of the contacts were exposed in a room sized more than 15 m^2.

Full PPE was not worn by any of the contacts (the events took place before the extent of the COVID-19 threat was apparent). Some of the exposed hospital staff were using surgical masks, surgical gloves, and/or a cotton robe during exposure. Almost half (44.8%) of the exposed staff wore a surgical mask during the exposure, while 10.4% did not remember. Of the 16 administrative staff members exposed to patient No 3 (their colleague), only 2 (12.5%) wore a surgical mask. They were at a distance of 1–3 m, and their exposure lasted for <15 min. **Figure 1** shows a comparison of the contact characteristics of the contacts with and without masks.

Medical and nursing care was administered by 24 of the 58 contacts (41.4%). Of these 24 contacts, 17 (70.8%) wore a surgical mask, 5 of the 24 (20.8%) did not wear a mask, and 2 did not remember. All five contacts who did not wear a mask were at a distance of <1 m from an index case, and three of them for more than 15 min, one of the three in a room sized more than 15 m^2. Of the 58 contacts, 36 (62%) wore gloves, with 24 of the 36 (66.7%) performing medical or nursing procedures. In addition, 22 of the 24 (91.7%) who performed medical or nursing procedures wore a cotton gown.

We also investigated the question of circulation of fresh air in the room occupied by the index cases, by recording whether the windows/door remained open or closed during the exposure. An open door was reported by 50/58 contacts (86.2%), and open windows by 42/58 (72.4%). Comparisons among all the contacts based on the characteristics of their exposure are shown in **Figure 2**.

RT-PCR SARS-CoV-2 tests were performed in 14/58 contacts (24.1%), antibody tests for SARS-CoV-2 in 11/58 contacts (19%), and 8/58 (13.8%) had both tests done. All the tests were reported negative.

On analyzing the data collected from the total group of 58 contacts, a highly exposed subgroup was identified, consisting of 18 contacts (31%) who did not wear a mask and had been closer than 1 m to an index case. Of these, 8/18 (44.4%) were exposed for more than 15 min, and 4/18 (22.2%) were exposed for more than 4 h. In five of the eight cases of close contact (62.5%), the windows had been open, and in two of the three with the windows closed, the door was open. In addition, 8/21 (38%) contacts who reported being in a small room with an index case said that the windows were closed, but in 4/8 (50%) cases, the doors were open. Only three of the contacts reported being in a small room at a distance <1 m from the index case with both doors and window closed, two wearing a surgical mask, but one without a mask. Two others did not remember whether they were wearing a mask or not. One physician who came in contact with patient No 1 did not wear a mask and was at a distance of <1 m from the patient for more than 15 min with windows and door closed, in spite of which she tested negative for SARS-CoV-2 antibodies and she never developed any symptoms related to COVID-19.

RT-PCR SARS-CoV-2 was performed in 6/18 (33.3%) highly exposed contacts and one had an antibody test, all of which were reported negative.

In total, 42 hospital staff (72.4%) came into contact with the symptomatic patients No 1 and No 2, of whom 37 were at a distance of <1 m, and 17/42 (40.5%) for a duration of more than 15 min. In addition, 12/42 contacts (28.6%) were not wearing a mask and were at a distance of <1 m; of these, four had a negative RT-PCR test for SARS-CoV-2 and 3 had a negative SARS-CoV-2 antibody test.

The case of patient No 3 was different from the other two, as she was working in the administration department, and her hospital contacts were mainly colleagues, 16 in number. The duration of the contact was more than 15 min in 50%, and 6/16 (37.5%) were at a distance of <1 m, four of them for more than 4 h. Most (87.5%) of these contacts did not wear a surgical mask, but in 13/16, the windows were open during the exposure, and the door was open in all cases. The size of the room was more than 15 m^2 in 8/16 cases. All the personnel exposed at a distance of <1 m said that they had the windows open, but they did not wear a surgical mask.

The characteristics of the contacts with patients No 1 and 2 with severe symptoms and patient No 3 with mild symptoms are shown in **Table 2**.

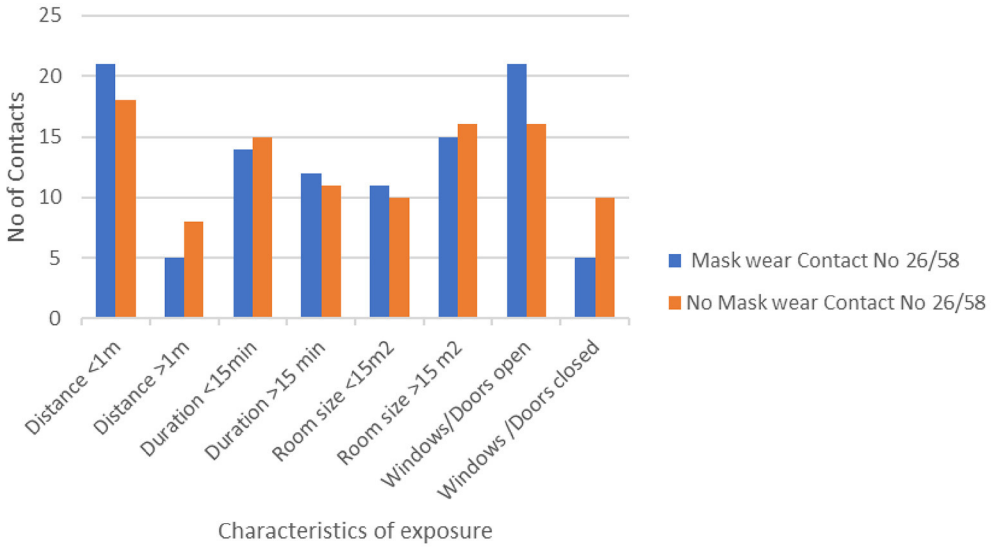

FIGURE 1 | Characteristics of the contact of healthcare workers ($N = 58$) with three patients with SARS-CoV-2, with and without masks.

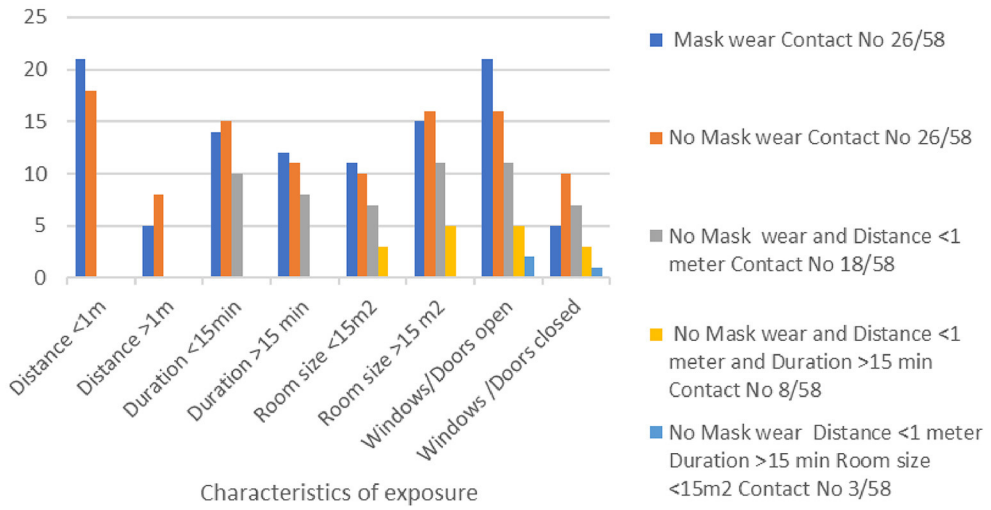

FIGURE 2 | Characteristics of the contact of healthcare workers ($N = 58$) with three patients with SARS-CoV-2 in a regional hospital in Greece at the beginning of the COVID-19 pandemic.

DISCUSSION

This study represented a unique opportunity to analyze the exposure of HCWs to patients with SARS-CoV-2 in a Greek regional healthcare setting at the beginning of the COVD-19 pandemic at a time when there were no recognized community exposures, and the relevant safety measures had not yet been fully introduced.

The three most important components of the recommended safety measures, namely, wearing of a mask, distance from the index case, and duration of exposure, were all significantly compromised. In spite of this, the final outcome of all the exposed HCWs was to remain asymptomatic during the 8-week follow-up in isolation, which was implemented when the diagnosis of SARS-CoV-2 was made (16, 17).

This group of 58 contacts with three index cases of SARS-CoV-2 recorded several specific high-risk factors; two-thirds of the contacts were at a distance of <1 m from the index case, half were not wearing a surgical mask during their exposure, and two-fifths of them remained in contact with an index case for more than 15 min. Several other factors appear to have protected them from contracting the virus.

When comparing the bus riders (48/52 got infected) and the HCWs (none got infected), it seems that the most distinguishing

TABLE 2 | Exposure characteristics of hospital staff ($N = 58$) who came into contact with three patients with SARS-CoV-2 (patients 1 and 2 with severe, and patient 3 with mild symptoms).

	Exposure to patient no 1 and 2	Exposure to patient no 3
	Total number of contacts: 42	Total number of contacts: 16
Exposure characteristics	**Number of contacts (%)**	**Number of contacts (%)**
Surgical masks		
Yes	24 (57.2%)	2 (12.5%)
No	12 (28.5%)	2 (12.5%)
Don't remember	6 (14.3 %)	12(75%)
Surgical gloves		
Yes	32 (76.2%)	6 (37.5%)
No	8 (19.0%)	9 (56.3%)
Don't remember	2 (4.8%)	1 (6.2%)
Distance		
<1 m	37 (88%)	6 (37.5%)
1–3 m	4 (9.6%)	9 (56.3%)
>3 m	1 (2.4%)	1 (6.2%)
Duration of contact		
<15 min	25 (59.5%)	8 (50%)
15 min−2 h	13 (30.9%)	3 (18.8%)
2–4 h	3 (7.1%)	1 (6.2%)
>4 h	1 (2.5%)	4 (25%)
Room size		
<15 m^2	13 (31.0%)	8 (50%)
15–30 m^2	25 (59.5%)	8 (50%)
>30 m^2	4 (9.5%)	
Windows		
Open	29 (69.0%)	13 (81.3%)
Closed	13 (31.0%)	3 (18.7%)
Doors		
Open	34 (81.0%)	16 (100%)
Closed	8 (19.0%)	
RT-PCR		
Yes	12 (28.6%)	2 (12.5%)
No	30 (71.4%)	14 (87.5%)
SARS-CoV2 antibodies		
Yes	8 (19%)	2 (12.5%)
No	34 (81%)	14 (87.5%)

differences are the length of contact and the small and enclosed nature of the bus. The high viral transmissibility in small, confined spaces has been shown in a study performed by Kasper et al. (18) in a nuclear-powered aircraft carrier.

Although, this incident took place before COVID-19 regulations were fully implemented, and there was initially no reason to suspect that patients 1 and 2 posed a special threat, a significant proportion (70.8%) of the personnel who performed a medical or nursing procedure on these patients reported wearing a mask during contact with them. Additional protective equipment such as a cotton robe and surgical gloves were also worn in some cases, but the HCWs did not perform aerosolized procedures (1). None of the exposed HCWs developed symptoms, even though 13.8% did not wear a mask and were within close distance for more than 15 min, and three were in a small room within a distance of <1 m of the index case for more than 15 min with doors and window closed, one without a mask (19, 20). Patients 1 and 2 were symptomatic on admission, with cough, and a diagnosis of pneumonia was made. They both required medical intervention, with 2–4 days of hospitalization before transfer to the tertiary center, and one subsequently died. About 25% of the HCW wore no mask and were within <1 m while examining or administering treatment, and 25% of those were exposed for more than 15 min.

Regarding the administrative staff exposed in their workplace to patient 3, their colleague, only a small number of the administrative officers wore a mask, but most were at a distance of more than 1 m and the duration of exposure was <15 min.

The three index cases presented in this study contracted SARS-CoV-2 while traveling on the same religious bus tour where 48/52 tourists in the group were infected. They were riding in the bus for a total of 8 days, in close contact with each other, for approximately 10 h per day, with breaks every 2–3 h when the bus was naturally ventilated. This implies that the transmissibility of the specific viral strain was high, at least in the contained environment of a tour bus, with lengthy exposure. In the hospital environment where the factors of the enclosed space and extended duration of contact were eliminated, the HCWs did not contact the virus.

This is among the first known reports where occupational transmission of SARS-CoV-2 has not been recorded, despite the fact that the HCWs were not using contact, droplet, or airborne precautions when in contact with an infected patient. The results of this study do not negate the need for application of PPE for protection of HCWs, as has been suggested on previous studies (12, 21), but they indicate the value of additional attention to environmental measures to augment the protection of this vulnerable group of first line workers. Those measures should be reinforced in the face of the merge of new SARS-CoV-2 variants with increased infectivity (22).

Nguyen and colleagues conducted a prospective cohort study, using the COVID Symptom Study smartphone application, and found that adequate supplies of PPE did not completely mitigate the infection rate in high-risk exposures for HCWs (21).

Our study has certain limitations, including the inadequate number of RT-PCR tests for SARS-CoV-2 in the exposed personnel, and the absence of infection in the HCW contacts was evaluated according to the reported absence of symptoms. This study was conducted in the ED and the regular hospital in-patient department of a small regional hospital, and not in an intensive care unit. No aerosolized procedures were performed on the index cases. As noted above, the HCWs were exposed to only one infected patient. The air circulation and filtration in the

bus were not evaluated in this study, which was restricted to hospital exposure.

CONCLUSION

Our findings point out the high transmissibility of the virus in lengthy exposure and in a small, enclosed place of a bus. On the other hand, in the healthcare facility where those factors were eliminated, and further measures were in place, the HCWs were protected. Additional studies are needed to be performed on the air circulation of buses where a high infection rate was seen.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital ethics and scientific committee of Amaliada Hospital Unit, General Hospital of Ilia, Amaliada, Greece. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

VV, GF, and AP: concept and design of the study. GF and AP: acquisition of data. VV, GF, and ST: analysis and interpretation of data and drafting the article. All authors have approved the final version to be submitted.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

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Real-World Evidence: The Low Validity of Temperature Screening for COVID-19 Triage

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Background: The COVID-19 pandemic forced health-related organizations to rapidly launch country-wide procedures that were easy to use and inexpensive. Body temperature measurement with non-contact infrared thermometers (NCITs) is among the most common procedures, both in hospital settings and in many other entities. However, practical hospital experiences have raised great doubts about the procedure's validity.

Aim: This study aimed to evaluate the validity of the body temperature measured using NCITs among oncological and transplant patients who took the polymerase chain reaction test for SARS-Cov-2 PCR+ and PCR- in a Romanian Hospital.

Methods: Body temperature was measured for 5,231 inpatients using NCITs. The cutoff point for fever was equal to or above 37.3°C. Patients then completed a questionnaire about their symptoms, contact, and travel history.

Findings: Fever was detected in five of 53 persons with PCR+, resulting in a sensitivity of 9.43% (95% CI, 3.13–20.66%). No fever was verified in 5,131 of 5,171 persons with PCR-, resulting in a specificity of 99.15% (95% CI, 98.86–99.38%). A defensive vision of NCIT procedure (maximum standard error only in favor) had a sensitivity of 15.09% (95% CI, 6.75–27.59%).

Conclusions: The use of NCITs in a triage provides little value for detection of COVID-19. Moreover, it provides a false sense of protection against the disease while possibly discriminating individuals that could present fever due to other reasons, such as oncologic treatments, where fever is a common therapeutical consequence. The consumption of qualified human resources should be considered, especially in the context of the shortage of healthcare professionals worldwide.

Keywords: triage, screening, non-contact temperature measurement, COVID-19, SARS-CoV-2

INTRODUCTION

The infectious disease COVID-19, caused by the SARS-CoV-2 virus, has been widely disseminated globally, with over 40 million infection cases and over 1.1 million deaths registered during the pandemic (1). In Romania, 324,094 cases and 8,389 deaths due to COVID-19 have been reported since the beginning of the pandemic, that is, from February 26 to November 11, 2020 (1). During April–August 2020, the monthly average of new cases varied from 334 in April to 230 in May and 248 in June. Romania declared a state of emergency (lockdown) on March 16, which ended on May 14 but was followed by a state of alert. The monthly average of new cases reached 477 in July and 721 in August 2020.

Although the most common symptoms of the disease (fever, dry cough, and tiredness, among others) are mild (2), it has been reported that asymptomatic people may be transmission vectors for the disease. Asymptomatic rates have a broad variability ranging from 5 to 80% (3).

In Romania, each hospital carries out procedures for screening, testing, and patient management (Appendix A in **Supplementary Material**) based on periodical recommendations issued by the National Center for Surveillance and Control of Transmittable Disease (NCSCTB) of the National Institute of Public Health from Romania (NIPH) (4, 5).

These procedures change according to the pandemic's evolution, access to new findings and evidence on symptoms (including measurement of temperature), travel history, contact history, and criteria for different categories of patients or medical personal to qualify for PCR testing. Recommendations by the Center for Disease Control and Prevention (CDC) and European Centre for Disease Prevention and Control (ECDC) (6, 7) are implemented for symptom assessment for COVID-19 in new and returning patients along with practitioners and visitors, including the daily measurement of body temperature for all patients in healthcare settings.

Globally, massive non-contact temperature screening is used in hospitals, malls, office buildings, airports, and so on, as a fundamental test in the COVID-19 triage for clinical, epidemiologic, and other public health reasons. According to the U.S. Food & Drug Administration (FDA) (8), the normal body temperature should range between 36.1 to 37.2°C with the use of non-contact infrared thermometers (NCITs). However, the actual behavior of this procedure remains to be proved in the field. It is imperative to know the extent to which it is an effective and safe method, or whether it leads to false clues or bias against patients—particularly in oncology and transplant patients where fever may occur for multiple reasons. It must be noted that various factors may influence measurement of body temperature while screening for fever (e.g., environmental conditions, antipyretics, and other respiratory diseases) (9). For example, there are treatments in oncology and immunocompromised states that may lead to fever. In these cases, a positive measurement in patients who visit oncology/transplant hospitals may be due to a priori fever associated with their condition, leading to a potentially erroneous measurement of body temperature. A study in a radiation oncology center in China, from January to April, identified 27 cases of fever in a total of 770 patients with cancer (10). A case study of four COVID-19 patients with cancer in China reported that all of them registered fever 6–36 days after hospital admission (11). Another study, with a cohort of 138 COVID-19 inpatients in a Chinese hospital, regardless of cancer status, reported that 20% of these patients had a fever below 38°C (12).

One study reported that fever was registered in ∼43% of COVID patients at the time of admission to hospital and in ∼89% by the time of hospitalization (13). The median duration of fever in these patients was between 8 and 11 days (14).

In many cancer patients with fever, the symptom could be due to febrile neutropenia (FN) or even a flu-like syndrome (15).

Cancer patients are at a higher risk of any infection due to immunosuppression, commonly caused by cancer and cancer-related treatment. Thus, they are at a higher risk of COVID-19 infection than the general population. Patients receiving active cancer treatment are at a higher risk than those in remission.

Fever is also a common clinical manifestation in patients who have had a transplant, as well as for other clinical reasons not related to COVID-19. Although the purpose of hematopoietic stem cell transplant (HSCT) is curative, and the patient could suffer damage if the procedure is delayed, treatment-related toxicities and technical difficulties would be amplified and overwhelming during this pandemic. Therefore, professional societies such as the European Society for Blood and Marrow Transplantation (EBMT) have issued guidelines to help physicians during the pandemic. These include sorting and testing patients (including those without symptoms) before being admitted to a transplant ward and allocating separate areas, distinct from the transplant units, for symptomatic patients awaiting COVID-19 test results. There are also strict rules for stem cell donors.

Highly immunocompromised pediatric and adult hematopoietic cell transplant (HCT) recipients frequently experience respiratory infections caused by viruses that are less virulent in immunocompetent individuals (16).

Respiratory viral infections in allogeneic HCT recipients contribute to significant mortality rates ranging between 10 and 50% if the infection progresses to the lower respiratory tract (17).

Another dimension that should be discussed is that fever is a non-specific, non-sensitive indicator of infection. In developing countries, infections are the major cause of a fever of unknown origin (18, 19).

In Romania, each hospital/medical center allocates resources for screening with questionnaires and body temperature. The Clinical Institute Fundeni (CIF) is one of the few hospitals in the country doing PCR tests for all inpatients, in view of its

Abbreviations: CDC, Centers for Disease Control and Prevention; CIF, Clinical Institute Fundeni; EBMT, The European Society for Blood and Marrow Transplantation; ECDC, European Centre for Disease Prevention and Control; FDA, U.S. Food & Drug Administration; FN, Febrile Neutropenia; HCT, Hematopoietic Cell Transplant; HSCT, Hematopoietic Stem Cell Transplant; CI, Confidence Interval; IVD, *In Vitro* Diagnostic; NCIT, Non-contact Infrared Thermometers; NCSCTB, National Center for Surveillance and Control of Transmittable Disease; NIPH, National Institute of Public Health; PCR, Polymerase Chain Reaction; RNA, Ribonucleic Acid; UTM, Universal Transport Medium.

specific oncology and transplants profile, and is aligned with similar hospitals in Europe.

We aim to provide evidence for the low validity of the body temperature measurement method as it has the possibility of inducing error and stigmatizing people without COVID-19. This will allow people infected with COVID-19 to pass through the triage with a false sense of safety.

The primary objective of this study was to evaluate the sensitivity of fever measurement with NCITs for positive SARS-Cov-2 test results that identified the disease in the first PCR+ testing. The secondary objective was to determine the specificity and accuracy of the fever measurement with NCITs.

METHODS

Setting

The study was conducted at the Clinical Institute Fundeni, a major referral hospital in Bucharest, the capital city of Romania, with core competencies on transplant (medullar, liver, renal) and oncology. The Clinical Institute Fundeni has had an average of 53,060 inpatients in the last 5 years, with more than 80% being oncological and transplant patients. The study was conducted in accordance with the Declaration of Helsinki, the protocol was approved by the Ethics Committee of Clinical Institute Fundeni, and the patients provided informed consent.

Sample

The sample consisted of 5,231 patients with age ranging from 6 months to 91 years ($M = 53.97$, $SD = 17.95$). The sample included both female (47%) and male (53%) individuals, and was highly representative (assuming equal probability of fever over the year), with CI of $95 \pm 1.30\%$ and CI of $99 \pm 1.71\%$.

All patients tested for SARS-CoV-2 from March 16 to August 30, 2020 were eligible for inclusion. Patients with non-conclusive PCR in the initial testing were not included (N-3 cases); see **Figure 1**. All patients with at least 1 day of hospitalization were considered. Consultations, visits, or accompanying patients were excluded from the sample.

Design

This was a consecutive observational study encompassing all patients admitted to the hospital from March to August 2020.

Procedure

Temperature was measured on the day of admission in the triage tents placed in front of the hospital entrances with NCITs held at a distance of one to five centimeters from the skin.

Along with the temperature measurements, a questionnaire about symptoms, contacts, and travel history was completed by patients. Data were collected for body temperature at the time of testing, age, sex, admission department, criteria for testing measurement, symptoms, contacts, and travel history (**Supplementary Material 1**).

In line with the FDA recommendation (8), fever was defined as a $\geq 37.3^{\circ}$C body temperature.

The triage data recording system included a handwritten paper registry completed by the triage teams, trained and designated by the hospital.

After the triage, all admitted patients were allocated to one of three zones according to the triage's findings for nose and throat swabs—green (no findings for COVID-19), yellow (suspicions for COVID-19), or red (possibly positive for COVID-19).

Testing of nose and throat swab samples was performed using a quantitative reverse-transcription polymerase chain reaction in the CIF laboratory.

Nasal and oropharyngeal samples from patients to be hospitalized at the Fundeni Clinical Institute were collected within a single tube of universal transport medium (UTM®; Copan Italia S.p.A., Brescia, Italy) to prevent viral RNA degradation and bacterial/fungal overgrowth. Briefly, 200 µl of the sample was processed with a Seegene Nimbus automated system, which performs both RNA extraction–using STARMag 96 × 4 Viral DNA/RNA 200 C Kit—and PCR assay setup. The RNA isolation procedure comprised four steps: sample lysis, binding nucleic acid to magnetic beads, washing debris, and purified nucleic acid elution. The Allplex 2019-nCoV Assay (Seegene, Seoul, South Korea) is an *in vitro* diagnostic (IVD) real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay developed for the detection of the nucleic acids in human samples from individuals with symptoms of severe acute respiratory syndrome related to coronavirus 2 (SARS-CoV-2). This assay was designed for amplifying three viral targets: the E gene (specific to the subgenus Sarbecovirus), as well as the N and the RdRP genes (both specific to SARS-CoV-2) (20). The tube PCR strips with the extracted RNA was loaded onto a real-time PCR C1000 CFX96 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Positive and negative controls were included in each run. After completion of the assay, the Seegene Viewer 2019-nCoV software allowed automated analysis and interpretation of results.

After receiving the PCR results, the patient was finally allocated to the department's red or green zone. Data from the laboratory were available in an Excel format. Two resident doctors worked for 2 months to manually match registered temperatures with PCR results in the Microsoft Excel software.

Quantitative Analysis Tools

Results were reported using proportions with 95% confidence intervals. All analyses were conducted with SPSS version 25. Calibration is not necessary for the devices mentioned, eliminating the need to include the calculation or the calibration error or to verify the calibration date and accreditation of the calibrating entity. The devices used are authorized for operation without calibration, and certified for medical or private use for measuring human or animal temperature by the FDA (Certificate of conformity number 3015697152), European Metrology (CE) (Certificate of compliance number 4M200326.SZTDD37), and ISO13485: 2016 (Certificate of compliance number TW50598U, issued on April 8, 2019).

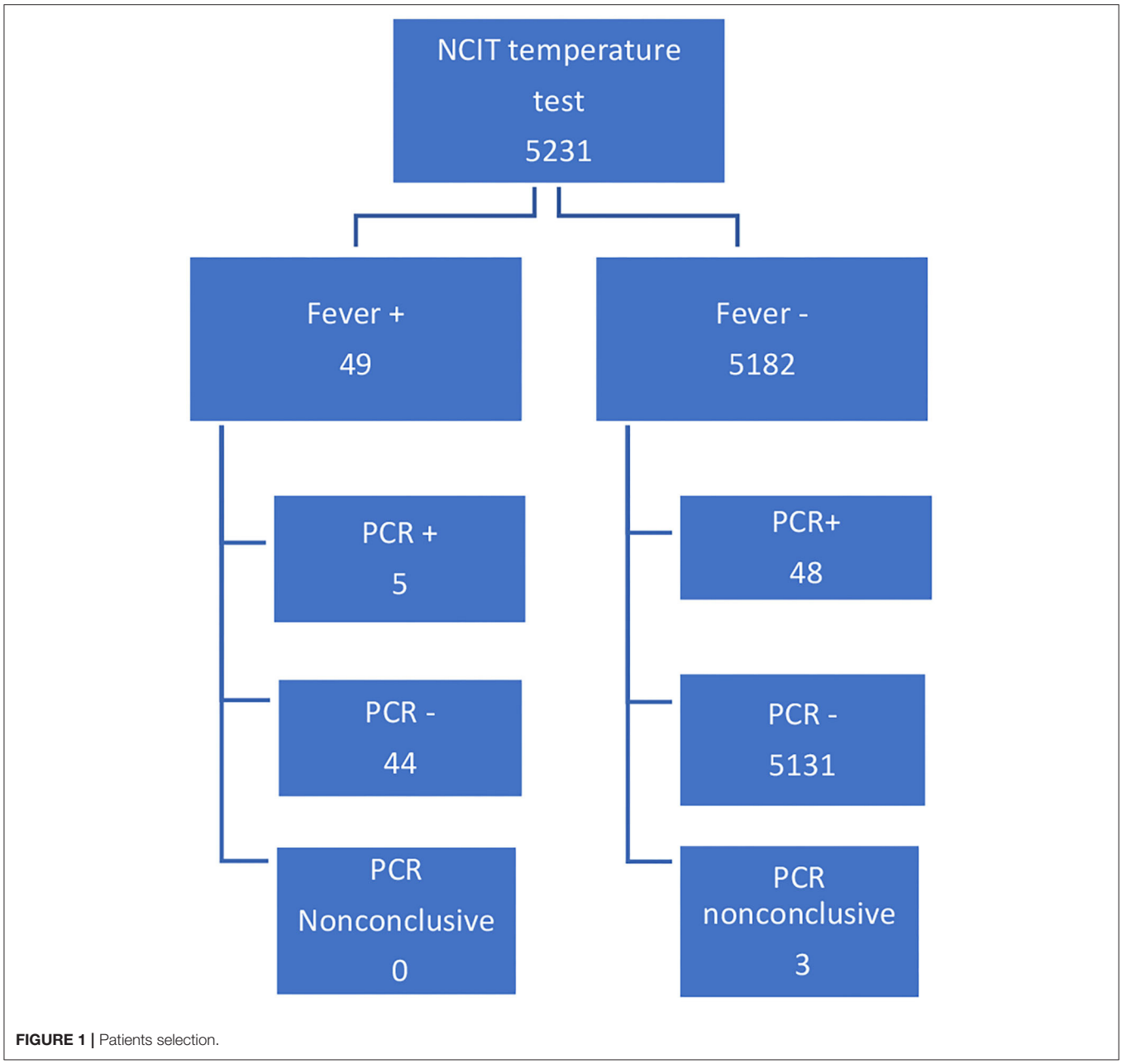

FIGURE 1 | Patients selection.

RESULTS

Of the 5,231 individuals, 49 tested positive for fever (fever +), and 53 were PCR +. Three PCR tests were inconclusive, and the remaining 5,175 were negative. For fever or PCR, there was no significant difference between males and females. Out of the 53 PCR + cases, five registered a fever, but 48 did not. Out of the 5,175 PCR—cases, 44 registered a fever (**Figure 1**). Sensitivity 9.43%, 95% CI (3.13–20.66%). Specificity 99.15% 95% CI (98.86–99.38%). Accuracy 98.25%, 95% CI (97.86–98.59%). Fisher exact <0.001. Disease prevalence 1.00%.

DISCUSSION

A sensitivity value of 9.5% at the first testing of PCR+ is considered to be extremely low. This means that a significant number of SARS-CoV-2 patients would pass through the temperature triage and be admitted to the hospital as *negative* when, in fact, they would be *positive*—suggesting the possibility of a large number of *false negatives*. Consequently, the effort and cost of mobilizing staff to measure temperatures at the entrance increase all nosocomial risks since more than 90% of people with COVID-19 end up proceeding with the clinical process. The

value of this NCIT method for the daily assessment of COVID-19 inpatients was not evaluated.

Our findings suggest that 48 patients, representing 90.5% of PCR + patients (1—sensitivity), were *false negatives*. Patients were admitted to a green, yellow, or red zone of the department, following triage findings based on symptoms, history, contact, and temperature. The internal code for this procedure was PO-MED-40. The PCR was performed within 6–24 h, depending on the status of the medical emergency. The hospital also conducted the PCR on all admitted patients, even those who did not fulfill the criteria (289 of 5,321).

Specificity value was found to be very high, reaching 99.15%. Nonetheless, 44 patients out of 5,228 were *false positive* (1—specificity). This implies delay in medical services for these patients, at least until the PCR tests were conducted.

It must be noted that no calculation was done for the whole hospital triage scheme in place, which also included questions about contact, symptoms, and travel history. The results may vary if these questions are added to the temperature triage. The cut-off point of 37.3°C considered for fever was another concern.

Our findings are consistent with the previous literature (21, 22). A study in an Australian hospital concluded that fever screening lacked sensitivity to detect patients with SARS-CoV-2 (23). In another study set in a hospital, out of 40,887 patients who attended health services, fever was detected in five patients on the outdoor triage and in 37 patients in the indoor clinic zone after being acclimatized (24). Therefore, screening for fever in Taiwan hospitals needs to be reinforced, with body temperature measured in two separate time slots and zones (24), allowing for acclimatization to each environment, which could otherwise mask the presence of fever. This was considered an important step to reduce the risk of admitting individuals with a fever that was not detected at the first screening.

Although body temperature measurement provides some help in identifying patients with COVID-19, its use involves some risk, as follows:

- The vast majority of PCR + cases are not identified. Besides providing a false sense of security, people who are not infected are set in the same space as those who are infected, possibly for hours.
- Most people who are referred to the red zone are false positives, generating stigmatization and great anxiety in most cases, which are harmful for patients who are already fragile due to their condition.
- We suggest studying screening alternatives other than NCITs in the context of COVID-19. Once the resistance to rapid testing based on the most recent bibliography (about the generation of false negatives) is overcome, use of rapid PCR is a possibility. Although its safety level is lower than that of serologic tests, it is much more sensitive than screening based on body temperature measurement.

For future research directions, it would be interesting to use the same assessment to verify whether fevers, false positives, and false negatives occur in other types of hospital services with the same density as they do in cancer and transplant hospitals. We estimate the probability of fever results directly from concrete phases of treatment, as well as with the type of cancer. However, it would be important to verify and study patients who should always be excluded from this type of procedure. Recognizing that there is no reliable method, it would be interesting to compare the validity of fever measurement, with or without insertion in a broader protocol, by using rapid PCR tests in another sample. Finally, we believe it is of the utmost importance to complement this work with an economic study involving the direct and indirect costs of this procedure. Costs include labor, use of space and equipment, and, in particular, risk of contagion outbreaks in the hospital from unidentified COVID-19 cases.

CONCLUSION

Our findings indicate that testing for fever with NCITs has a very low sensitivity to COVID-19, thus questioning NCIT use for fever screening. The issue is not about the utility of the device (for metrological reading) but its use in clinical testing procedures (patient safety reading).

Not only is the usefulness of NCIT-based testing open to question, but it might induce a false sense of security while reducing the patient's effective safety in hospital settings as it allows passage of COVID-19 patients. It also diverts attention from public health measures that are more likely to be effective uses of resources, such as self-isolation when ill, physical distancing, mask-wearing, and contact tracing.

the context of the severe shortage of qualified personnel to respond to needs of the growing number of cases in the pandemic while catering to the demands of the current healthcare setting, the fever-screening procedure increases financial costs and personnel allocation needs with no apparent gain.

Therefore, we recommend that body temperature measurement be eliminated from hospital admission procedures, especially in oncological hospitals or other hospitals with high immunological impairment.

A possible alternative solution for screening patients for admission to hospital could be rapid low-cost testing for a first triage while serological PCR results are determined. It also allows for a quick response to the circulation of people within the hospital with a greater degree of safety than currently verified.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://osf.io/grsqh/?view_only=dc293a57a99a4d21bb0fd138e75cc7d7/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Council of the Fundeni Clinical Institute. Written informed consent to participate in

this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

BP, FF, AT, and AC contributed to conceptualization of the study. BP, HL, FF, AT, and AC contributed toward the methodology. Software was organized by BP and validation was done by BP, HL, and FF. BP and HL performed the formal analysis, and investigation was carried out by HL, DF, AR, MS, AT, and AC. AT and AC gathered the resources, while BP, AR, and MS curated the data. The original draft was prepared by BP, HL, FF, DF, AT, and AC. HL, FF, DF, AT, and AC reviewed and edited the manuscript, while HL supervised. BP administered the project. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.672698/full#supplementary-material

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STUDY PROTOCOL
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Study Protocol for a Global Survey: Awareness and Preparedness of Hospital Staff Against Coronavirus Disease (COVID-19) Outbreak

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Background: The outbreak of Coronavirus disease (COVID-19) caused by a novel coronavirus (named SARS-CoV-2) has gained attention globally and has been recognized as a Public Health Emergency of International Concern (PHEIC) by the World Health Organization (WHO) due to the rapidly increasing number of deaths and confirmed cases. Health care workers (HCWs) are vulnerable to this crisis as they are the first frontline to receive and manage COVID-19 patients. In this multicenter multinational survey, we aim to assess the level of awareness and preparedness of hospital staff regarding COVID-19 all over the world.

Methods: From February to March 2020, the web-based or paper-based survey to gather information about the hospital staff's awareness and preparedness in the participants' countries will be carried out using a structured questionnaire based on the United States Centers for Disease Control and Prevention (CDC) checklist and delivered to participants by the local collaborators for each hospital. As of March 2020, we recruited 374 hospitals from 58 countries that could adhere to this protocol as approved by their Institutional Review Boards (IRB) or Ethics Committees (EC).

Discussion: The awareness and preparedness of HCWs against COVID-19 are of utmost importance not only to protect themselves from infection, but also to control the virus transmission in healthcare facilities and to manage the disease,

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especially in the context of manpower lacking and hospital overload during the pandemic. The results of this survey can be used to inform hospitals about the awareness and preparedness of their health staff regarding COVID-19, so appropriate policies and practice guidelines can be implemented to improve their capabilities of facing this crisis and other future pandemic-prone diseases.

Keywords: awareness, preparedness, COVID-19, hospital staff, global survey

INTRODUCTION

The recent outbreak of Coronavirus disease (COVID-19) caused by a novel coronavirus (named SARS-CoV-2) has gained attention globally and has been recognized as a serious public health threat by the Centers for Disease Control and Prevention (CDC). The first case was detected in Wuhan City, Hubei Province, China and since then, the disease has spread rapidly (1). As of February 28, 2020, the World Health Organization (WHO) declared that the outbreak of COVID-19 as a Public Health Emergency of International Concern (PHEIC) with 62 countries now reporting 85,176 confirmed cases (79,250 of which have been in mainland China) and 2,919 deaths to date (2).

The SARS-CoV-2 is a novel strain of coronavirus emerging in the human population in the past two decades, preceded by the SARS-CoV outbreak in 2002 and the MERS-CoV outbreak in 2012 (3). The exact origin of this novel coronavirus and its precise disease mechanism has not been fully understood. At present, no antiviral medication or vaccine is approved for SARS-CoV-2 infection and the infected patients are managed with supportive care (1). The highly contagious capacity of SARS-CoV-2 led to rapid growth in the number of COVID-19 patients (4). As a result, hospital overload occurred in several regions where the SARS-CoV-2 infection became widespread in the community (5, 6). HCWs are at the core of the combat against COVID-19. Consequently, HCWs in most settings are overworked and more vulnerable to be infected with COVID-19. In Italy, by 22 March, 4,824 healthcare workers (HCWs) had been infected (9% of total cases) and 24 had died - these figures are worse than those observed in China (3,300 infected cases and 23 deaths among HCWs) (7). The awareness and preparedness of HCWs in response to the COVID-19 outbreak are of great importance not only to prevent disease contraction from the infected patients but also to help them cope with emergency situations and prevent further transmission.

To control the virus transmission in the healthcare facilities and protect the medical staff, the CDC in the United States and the WHO have developed the preparedness and prevention checklists of SARS-CoV-2 infection to be used by healthcare professionals (8, 9). However, the awareness and preparedness of medical staff against COVID-19 outbreaks around the world have not been reported.

Abbreviations: CDC, centers for disease control and prevention; COVID-19, Coronavirus Disease 2019; EC, ethics committee; HCWs, healthcare workers; HDI, human development index; IRB, institutional review board; MCQ, multiple-choice question; PHEIC, public health emergency of international concern; WHO, world health organization.

METHODS AND ANALYSIS

A. **Objectives:** This is a multicenter multinational survey aiming to assess the level of awareness of hospital staff regarding COVID-19 all over the world. It will also measure the level of preparedness of hospital staff in response to the crisis of COVID-19 and how will they react to limit and prevent further transmission.
B. **Study design:** Cross-sectional study.
C. **Time period:** February to March 2020.
D. **Study Settings:** Any hospital in the world that can adhere to this protocol to conduct the survey as approved by its Institutional Review Board (IRB) or Ethics Committee (EC). Each hospital will have local collaborators.
E. **Study population:** Healthcare providers in the hospitals including physicians, nurses, pharmacists, and others. We will enroll staff members who are or will be handling suspected cases in settings such as Emergency Department, Intensive Care Unit, Outpatient Department, Infectious Disease Clinic, Respiratory Disease Clinic, or any department designed to treat COVID-19 patients. We will exclude participants who cannot communicate in the vernacular of the translated questionnaire. We will also exclude staff who are on leave on the day of the survey.
F. **Sample size calculation:** The survey will be conducted in a convenient selection of global hospitals. There will be no restriction on the number of hospitals per country or the number of participants per hospital.
G. **Study instrument and questionnaire design process:** The survey will be carried out using a structured questionnaire adapted from the United States' CDC checklist (8). The original questionnaire will be developed in English, consisting of 2 sections with 32 questions in total. The first section covers 6 questions about demographic and personal medical aspects. The second section includes 26 questions assessing the awareness and preparedness of hospital staff regarding COVID-19. There are different types of questions in the questionnaire including yes/no questions, open-ended questions, and multiple-choice questions (MCQ).
H. **Validation of questionnaire:** The original questionnaire will be carefully revised by a panel of healthcare professionals that includes one WHO consultant, three epidemiologists,

five physicians; three members are native English speakers. A pilot survey will be conducted by 30 international HCWs to ensure the validity of the questionnaire. This validation aims to evaluate the time needed to complete the questionnaire and assure that all the questions and sections of the questionnaire are phrased clearly and appropriately for comprehension and to avoid bias that might otherwise. After the pilot survey, the original questionnaire will be modified if needed. The local team members in each participated country are responsible for its translation into their native languages. For the translated questionnaires, forward and reverse translation will be performed to ensure their accuracy. A pre-test of the questionnaire by 5 native speakers will also be conducted for the translated version. The questionnaires will be then modified if required.

I. **Survey conduct:** To gather information about the hospital staff's awareness and preparedness in the participants' countries, we will develop an online questionnaire using SurveyMonkey© that limits one-time participation per unique IP address. However, participants can choose to use hard copies prepared by the local collaborators for each hospital.

J. **Coordination and participating sites of the survey:** Our global research team will include several medical students and doctors from many countries around the world. We will use social networks and send recruitment emails for inviting the collaborators and coordinators around the world to participate in the study.

Local Site Collaborators: Two or three collaborators are required for each local site hospital. Local collaborators will be specifically responsible for:

1. Obtaining local audit, special exemption, or research approval (IRB/EC approval).
2. Listing all departments that are or will handle the patients (Emergency Department Intensive Care Unit, Outpatient Department, Infectious Disease Clinic, Respiratory Disease Clinic, or any department designed to treat COVID-19 patients).
3. Reporting the number of doctors, nurses, other HCWs of each department. If there are only a few staff in a particular department, the collaborators will assign that department as "others."
4. Preparing the hard print of the survey questionnaire provided by our coordinator.
5. Distributing the questionnaire to the head of the department and collect it within 1 day, report the number of doctors, nurses, other workers of each department that is available on that day.
6. Scanning all collected questionnaires and sending a zip file to the corresponding coordinator via email using Google folder or via email.
7. Keeping all the hard copies of the collected questionnaires for at least 5 years and protecting the information inside those copies.

The survey questionnaire is anonymous and participant identification numbers will be used rather than any personal identifiers. The site collaborators are fully responsible for the accuracy and any misconduct of research. Data cannot be published without prior written permission from Dr. Nguyen Tien Huy. Local collaborators may request permission to publish in a local journal after the main publication.

Project coordinators: Each coordinator is responsible for 3–5 hospital sites and:

1. Recruiting 3–5 local site hospitals.
2. Supporting translation of the questionnaire (both forward and reverse translations) to the local language and conduct a pre-test with the questionnaire.
3. Assisting and communicate between the project management team and local collaborators.
4. Checking evidence of action and quality of the data scanning provided by the collaborators.

Project managing team:

1. Writing the protocol and developing the questionnaire.
2. Recruiting coordinators and follow all of their steps.
3. Importing data in an online forum and collect them in spreadsheets to prepare them for the coding process.
4. Analyzing data and writing a report.

K. **Data management:** The collected data will be organized by Google Sheets and collected in an Excel spreadsheet. The survey will be completely anonymous. Hard copies of questionnaires will be scanned and uploaded to a Google drive encrypted by a password. Only the management team will be able to access all data. Data entered Google Sheets will be quality-checked by a researcher to ensure accuracy.

L. **Data analysis:** Data collected will be exported to the Microsoft Excel sheet. Every respondent will be given an overall score for awareness and preparedness. The awareness of HCWs will be assessed using MCQ questions of 4 topics regarding COVID-19 including symptoms, diagnosis, mode of transmission, preventive measures. A score of 10 will be given for each topic. The preparedness of HCWs will be evaluated using yes/no questions, a score of "1" will be given for the option "yes," and a score of "0" will be given for the option "no" or "I don't know."

Descriptive statistics will be performed and variations among international healthcare settings will be assessed by categorizing countries with participating hospitals into lower-income, upper and lower middle-income, and higher-income groups, according to the World Bank's classification of Gross national income (GNI) per capita (10). A hierarchical logistic regression multivariate analysis will be applied to adjust the influence of GNI on the awareness and preparedness scores for confounding variables. Model coefficients will be presented as odds ratio (OR) and 95% confidence intervals. All analyses will be performed using the R Foundation Statistical Program version 3.6.3.

M. **Timetable**

Time	List of activities
31/01/2020 - 02/02/2020	• Establish the research team • Develop the survey questionnaire • Develop the survey protocol
03/02/2020 - 15/02/2020	• Revise the protocol, questionnaire (pre-test and post-test) • IRB approval by Nagasaki University • Recruit coordinators and collaborator
06/02/2020 – 03/03/2020	• Translate questionnaire to local language • IRB approval by local hospitals
16/02/2020– 16/03/2020	• Conduct the survey, import data
16/03/2020– 16/04/2020	• Data analysis and report

N. **Financial support:** Self-supported at each site.

O. **Authorship:** Each author needs to fulfill the criteria listed in this protocol, qualify as a co-author in the publication. The task must be finished before the deadline shown in the Timetable (Item L). All authors will be listed as a group of collaborators as described in previous work (**Figure 1**) (11). In addition, the author's contributions will be also recorded as presented in **Figure 2** of the previous publication (11). All data cannot be published without permission from Dr. Nguyen Tien Huy. Local collaborators may request permission to publish in a local journal after the main publication.

DISCUSSION

The awareness and preparedness of HCWs against an outbreak are crucial to public health and their issues have been raised universally. COVID-19 outbreak reached a very high transmission rate worldwide and the evaluation of front-liners dealing with such an outbreak is important. The awareness and preparedness level of HCWs play an important role in the control of a public health crisis (12). This protocol provides a way to conduct a global multicenter study regarding the level of awareness and preparedness of global HCWs in combating the crisis of COVID-19 pandemic through collaboration with participants from many hospitals around the world and can recruit medical staff to participate in the survey within a short-term framework, giving results of a global multi-center survey in a short time. As a result, the study can quickly provide a picture of global HCWs' awareness and preparedness for the spread and outbreak of the COVID-19 pandemic.

The research can cover a large number of countries in different regions, thus the overall survey provided important and useful information about the preparedness of hospitals and awareness of the staff against the country.

This survey will provide a final awareness and preparedness score that will reflect the hospital's state in regard to dealing with the COVID-19 pandemic. This score will help hospitals as a consequence to consider implementing policies and practice guidelines in case their facilities deemed to be unprepared, and will also give them information about their staff during the pandemic which will reflect their capabilities of facing other future pandemic-prone diseases.

This study was mainly conducted online among HCWs during a time when an alarming number of COVID-19 cases were being reported globally, and this might limit generalization. Also, the survey was conducted in the first few months of the pandemic where not enough information about the virus transmission and pathogenicity were available which might have an effect on participants' answers. Despite these limitations, we believe that our study is unique and the first to provide information about the awareness and preparedness of numerous HCWs during the COVID-19 pandemic.

ETHICS AND DISSEMINATION

Ethics Approval and Consent to Participate

This project protocol was approved by the Ethics Committee of Graduate School of Tropical Medicine and Global Health, Nagasaki University, Japan (NU_TMGH_2020-111-0).

Plan for getting informed consent and protecting confidentiality: All the respondents of the survey will fill a written informed consent embedded on the first page of the questionnaire. If the participant answers "YES" to the first question of the form, he/she automatically agrees to participate and will begin the survey. By using the skip-logic survey method, users who disagree with the informed consent question will be directed to the end of the survey. No respondent is forced to participate in the survey and their participation is based on their agreement that can be withdrawn at any time.

Autonomy: All participants have the right to leave a specific question unanswered or withdraw from the survey any time if they feel uncomfortable answering any question. In addition, no one even the research team will know individual answers to this questionnaire.

Risks and benefits for the participants: Data collected from this survey will play an important role in future reactions to fatal virus outbreaks. It will be used by a variety of researchers from different countries to improve the preparedness of different hospitals for outbreaks. This will play a crucial role in the early management and prevention of viral outbreaks in other areas. It will also play an important role in decreasing the response time to emergency cases at the hospital. We confirm that there are no risks associated with participating in this survey. As our study does not report individual results for each hospital, there will be no risk associated with the hospital's responsibility for their HCWs' awareness and preparedness regarding COVID-19 from our study results. Any unexpected risks that may occur during the survey will be immediately explained to both participants and the ethical committee. The responses collected from this survey are confidential and will not be revealed under any condition. In addition, the survey will be completely anonymous regarding participants and hospital names. Responses collected from this will be reported as collective combined data.

Lancet Infect Dis. 2018 May;18(5):516-525. doi: 10.1016/S1473-3099(18)30101-4. Epub 2018 Feb 13.

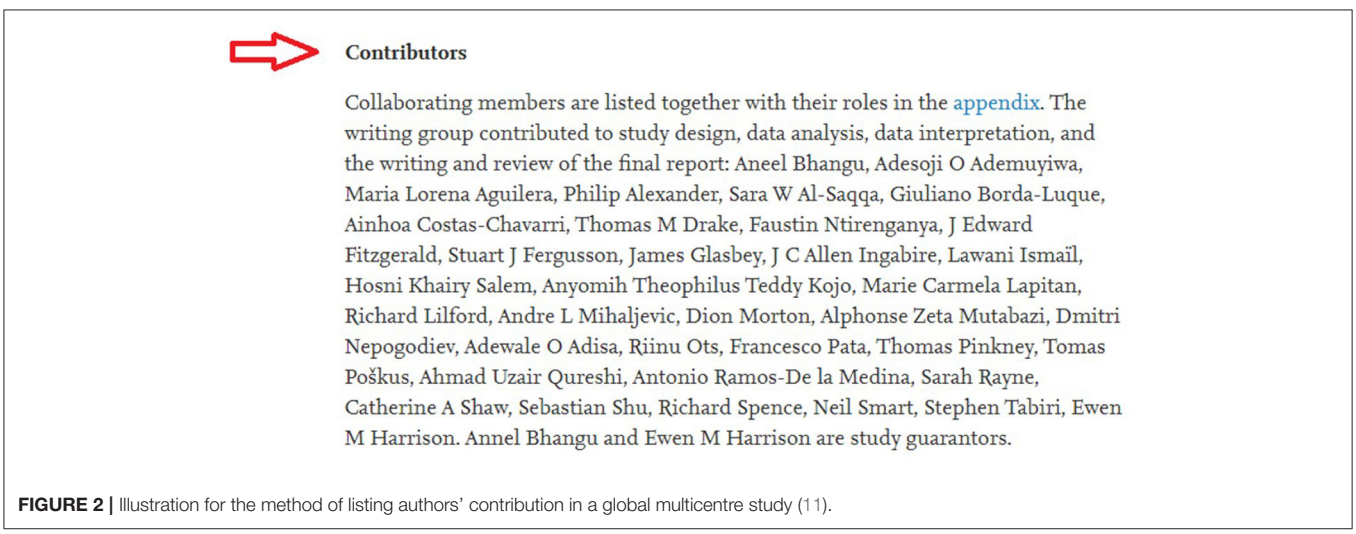
Surgical site infection after gastrointestinal surgery in high-income, middle-income, and low-income countries: a prospective, international, multicentre cohort study.

GlobalSurg Collaborative.

Collaborators (1816)

Bhangu A, Ademuyiwa AO, Aguilera ML, Alexander P, Al-Saqqa SW, Borda-Luque G, Costas-Chavarri A, Drake TM, Ntirenganya F, Fitzgerald JE, Fergusson SJ, Glasbey J, Ingabire JA, Ismaïl L, Salem HK, Kojo ATT, Lapitan MC, Lilford R, Mihaljevic AL, Morton D, Mutabazi AZ, Nepogodiev D, Adisa AO, Ots R, Pata F, Pinkney T, Poškus T, Qureshi AU, Ramos-De la Medina A, Rayne S, Shaw CA, Shu S, Spence R, Smart N, Tabiri S, Harrison EM, Khatri C, Mohan M, Jaffry Z, Altamini A, Kirby A, Søreide K, Recinos G, Cornick J, Modolo MM, Iyer D, King S, Arthur T, Nahar SN, Waterman A, Walsh M, Agarwal A, Zani A, Firdouse M, Rouse T, Liu Q, Correa JC, Talving P, Worku M, Arnaud A, Kalles V, Kumar B, Kumar S, Amandito R, Quek R, Ansaloni L, Altibi A, Venskutonis D, Zilinskas J, Poskus T, Whitaker J, Msosa V, Tew YY, Farrugia A, Borg E, Bentounsi Z, Gala T, Al-Slaibi I, Tahboub H, Alser OH, Romani D, Shu S, Major P, Mironescu A, Bratu M, Kourdouli A, Ndajiwo A, Altwijri A, Alsaggaf MU, Gudal A, Jubran AF, Seisay S, Lieske B, Ortega I, Jeyakumar J, Senanayake KJ, Abdulbagi O, Cengiz Y, Raptis D, Altinel Y, Kong C, Teasdale E, Irwin G, Stoddart M, Kabariti R, Suresh S, Gash K, Narayanan R, Maimbo M, Grizhja B, Ymeri S, Galiqi G, Klappenbach R, Antezana D, Mendoza Beleño AE, Costa C, Sanchez B, Aviles S, Fermani CG, Balmaceda R, Villalobos S, Carmona JM, Hamill D, Deutschmann P, Sandler S, Cox D, Nataraja R, Sharpin C, Ljuhar D, Gray D, Haines M, Iyer D, Niranjan N, D'Amours S, Ashtari M, Franco H, Rahman Mitul A, Karim S, Aman NF, Estee MM, Salma U, Razzaque J, Hamid Kanta T, Tori SA, Alamin S, Roy S, Al Amin S, Karim R, Haque M, Faruq A, Iftekhar F, O'Shea M, Padmore G, Jonnalagadda R, Litvin A, Filatau A, Paulouski D, Shubianok M, Shachykava T, Khokha D, Khokha V, Djivoh F, Dossou F, Seto DM, Gbessi DG, Noukpozounkou B, Imorou Souaibou Y, Keke KR, Hodonou F, Ahounou EYS, Alihonou T, Dénakpo M, Ahlonsou G, Ginbo Bedada A, Nsengiyumva C, Kwizera S, Barendegere V, Choi P, Stock S, Jamal L, Azzie G, Kushwaha S, Chen TL, Yip C, Montes I, Zapata F, Sierra S, Villegas Lanau MI, Mendoza Arango MC, Mendoza Restrepo I, Restrepo Giraldo RS, Domini E, Karlo R, Mihanovic J, Youssef M, Elfeki H, Thabet W, Sanad A, Tawfik G, Zaki A, Abdel-Hameed N, Mostafa M, Omar MFW, Ghanem A, Abdallah E, Denewer A, Emara E, Rashad E, Sakr A, Elashry R, Emile S, Khafagy T, Elhamouly S, Elfarargy A, Mamdouh Mohamed A, Saied Nagy G, Esam A, Elwy E, Hammad A, Khallaf S, Ibrahim E, Said Badr A, Moustafa A, Eldosouky Mohammed A, Elgheriany M, Abdelmageed E, Al Raouf EA, Samir Elbanby E, Elmasry M, Morsy Farahat M, Yahya Mansor E, Magdy Hegazy E, Gamal E, Gamal H, Kandil H, Maher Abdelrouf D, Moaty M, Gamal D, El-Sagheer N, Salah M, Magdy S, Salah A, Essam A, Ali A, Badawy M, Ahmed S, Mohamed M, Assal A, Sleem M, Ebidy M, Abd-Elrazek A, Zahran D, Adam N, Nazir M, Hassanein AB, Ismail A, Elsawy A, Mamdouh R, Mabrouk M, Ahmed LAM, Hassab Alnaby M, Maggy E, Abd-Elmawla M, Fahim M, Mowafy B, Ibrahim Mahmoud M, Allam M, Alkelani M, Halim El Gendy N, Saad Aboul-Naga M, Alaa El-Din R, Elgendy AH, Ismail M, Shalaby M, Adel Elsharkawy A, Elsayed Moghazy M, Hesham Elbisomy K, Abdel Gawad Shakshouk H, Hamed MF, Ebidy MM, Abdelkader M, Karkeet M, Ahmed H, Adel I, Omar ME, Ibrahim M, Ghoneim O, Hesham O, Gamal S, Hilal K, Arafa O, Adel Awad S, Salem M, Abdellatif Elsherif F, Elsabbagh N, Aboelsoud MR, Hossam Eldin Fouad Rida A, Hossameldin A, Hany E, Hosny Asar Y, Anwar N, Gadelkarim M, Abdelhady S, Mohamed Morshedy E, Saad R, Soliman N, Salama M, Ezzat E, Mohamed A, Ibrahim A, Fergany A, Mohammed S, Reda A, Allam Y, Saad HA, Abdelfatah A, Fathy AM, El-Sehily A, Abdalmageed Kasem E, Hassan ATA, Mohammed AR, Saad AG, Elfouly Y, Elfouly N, Ibrahim A, Hassaan A, Mohammed MM, Elhoseny G, Magdy M, Abd Elkhalek E, Zakaria Y, Ezzat T, Abo El Dahab A, Kelany M, Arafa S, Mokhtar Mohamed Hassan O, Mohamed Badwi N, Saber Sleem A, Ahmed H, Abdelbadeai K, Abozed Abdullah M, Lokman MAA, Bahar S, Rady Abdelazeam A, Adelshone A, Bin Hasnan M, Zulkifli A, Kamarulzamil SNA,

FIGURE 1 | Illustration for the method of listing collaborators in a global multicentre study (11).



Contributors

Collaborating members are listed together with their roles in the appendix. The writing group contributed to study design, data analysis, data interpretation, and the writing and review of the final report: Aneel Bhangu, Adesoji O Ademuyiwa, Maria Lorena Aguilera, Philip Alexander, Sara W Al-Saqqa, Giuliano Borda-Luque, Ainhoa Costas-Chavarri, Thomas M Drake, Faustin Ntirenganya, J Edward Fitzgerald, Stuart J Fergusson, James Glasbey, J C Allen Ingabire, Lawani Ismaïl, Hosni Khairy Salem, Anyomih Theophilus Teddy Kojo, Marie Carmela Lapitan, Richard Lilford, Andre L Mihaljevic, Dion Morton, Alphonse Zeta Mutabazi, Dmitri Nepogodiev, Adewale O Adisa, Riinu Ots, Francesco Pata, Thomas Pinkney, Tomas Poškus, Ahmad Uzair Qureshi, Antonio Ramos-De la Medina, Sarah Rayne, Catherine A Shaw, Sebastian Shu, Richard Spence, Neil Smart, Stephen Tabiri, Ewen M Harrison. Annel Bhangu and Ewen M Harrison are study guarantors.

FIGURE 2 | Illustration for the method of listing authors' contribution in a global multicentre study (11).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Graduate School of Tropical Medicine and Global Health, Nagasaki University, Japan. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NH raised the idea and took responsibility for the work integrity. AQ, SN, and AG designed the research study and drafted the protocol. ML, HS, SA, and NV developed and validated the questionnaire. CS, PL, RC, SC, and KH revised the questionnaire and the protocol. AS wrote the invitation letter with informed consent to recruit the coordinators and collaborators. TA-A, AS, VH, GT, MH, SD, MM, SA, NN, and SI recruited and supervised the local coordinators to collect data. SG, NV, and AS will analyze the data. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Preliminary Assessment of Chinese Strategy in Controlling Reemergent Local Outbreak of COVID-19

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Reemergent local outbreaks of coronavirus disease 2019 (COVID-19) have occurred in China, yet few Chinese response strategies and its evaluation have been reported. This study presents a preliminary assessment of Chinese strategy in controlling reemergent local outbreaks of COVID-19. Time course of accumulative and daily new cases and time-varying reproductive numbers (Rt) of outbreak areas were presented. The asymptomatic rate, days required to control the outbreaks, seeding time (ST), and doubling time (DT) of areas with over 96 reemergent cases were calculated. National and local year-on-year growth rates of gross domestic product (GDP) were presented. Accumulative numbers of 30, 8, 11, 430, 15, 139, 1,067, 382, 42, and 94 confirmed reemergent COVID-19 cases were diagnosed in Hulun Buir, Shanghai, Tianjin, Kashgar, Qingdao, Dalian, Urumchi, Beijing, Jilin, and Harbin, respectively. Among them, maximum rate of asymptomatic infections was 81.9%. Time required to control the local outbreaks in the areas given above varied from 29 to 51 days. After activation of outbreak responses, the late-stage DTs of Kashgar, Urumchi, Beijing, and Dalian were apparently lengthened compared to the early-stage DTs. Although the year-on-year GDP growth rate of Urumchi was slightly affected, the GDP growth rate of Dalian, Beijing, Jilin, and Harbin kept rising during the reemergence. Moreover, the year-on-year GDP growth rate of Mainland China turned positive regardless of the reemergent local outbreaks. In general, the Chinese strategy to maintain the status of no or minimal transmission was effective in balancing the control of COVID-19 reemergent local outbreak and the recovery of economy.

Keywords: COVID-19, SARS-CoV-2, reemergent local outbreak, asymptomatic infection, China, GDP

INTRODUCTION

According to the WHO, 80,453,105 people were diagnosed with coronavirus disease 2019 (COVID-19) and 1,775,776 of them died as of December 30, 2020 (1). According to Michael Ryan, the Executive Director of WHO Health Emergencies, China, however, has reached "extremely low levels of the virus" (2). Several reemergent local outbreaks were reported in Hulun Buir,

Shanghai, Tianjin, Kashgar, Qingdao, Dalian, Urumchi, Beijing, Jilin, and Harbin and had been later controlled. However, few Chinese strategies for tackling reemergent local outbreaks were reported. In this study, the time course of these local reemergent outbreaks was revealed, and a preliminary assessment of Chinese strategy in facing local reemergent outbreaks was presented.

METHODS

Updates of COVID-19 cases in Hulun Buir, Shanghai, Tianjin, Kashgar, Qingdao, Dalian, Urumchi, Beijing, Jilin, and Harbin were extracted from the situation reports of the official websites of local governments (3–11). Imported cases were excluded in this study. According to the "WHO COVID-19: Case Definitions" (12), a person with laboratory confirmation of COVID-19 infection, irrespective of clinical signs and symptoms, is defined as a confirmed case. Based on the Protocol for Prevention and Control of COVID-19 (Edition 6) (13), cases that resulted positive for reverse transcriptase (RT)-PCR or antibody test but without clinical syndrome were defined as cases with asymptomatic infections. People who presented with asymptomatic infection at first but later developed clinical syndrome were excluded from the asymptomatic cases. All cases were reported within 2 hours of diagnosis. Accumulated numbers of confirmed cases from the 1st day a case is reported to the 14th day with no new case of outbreaks reported in the areas mentioned above, which was regarded as the time required to control an outbreak, were presented as epidemiologic curve. Cases with identical origin as the originating site were counted together in the originating site cases. A number of daily new symptomatic and asymptomatic cases of reemergences as mentioned above were presented as a bar graph. Curves of time-varying reproduction number (Rt) were generated using the EpiEstim R package (14). The asymptomatic rate, the number of people received RT-PCR test in the first round of mass testing, and the number of people received first-round RT-PCR test per day are presented in a table.

According to the study of Zhou L (15), 30 countries meeting the following criteria were selected to structure the seeding time (ST)/doubling time (DT) model: (a) having over 5,000 cases as of March 31, 2020 or (b) having over 40 case-reporting days and 100 cases between the first case-reported day and March 31, 2020. Then, two specialists of the Chinese Center for Disease Control and Prevention independently determined the "takeoff" date of each country. When a disagreement occurred, a third specialist was involved, and discussion was conducted until the research team met a consensus. The accumulative number of cases on the day before the "takeoff" date was obtained and defined as seeding number (SN). The time interval between the date of the first reported case and the date of the a number of cases reaching the SN of an area was defined as ST. DT was the time interval required to double the accumulative number of cases in an area. For example, DT1 refers to the time interval between the confirmed cases that reached SN and 2 × SN, and DT2 refers to the time interval between the confirmed cases that reached 2 × SN and 4 × SN, etc. DT1 ∼ DT3 were defined as early-stage DTs, and DT4 ∼ DT6 were defined as late-stage DTs (15). Then, the median SN of the 30 countries mentioned above was calculated (median SN = 12) and was used to generate ST/DT model. To at least fully present the early-stage DTs, the data of four cities (i.e., Kashgar, Dalian, Urumchi, and Beijing) that reached the threshold of 96 cases were shown as a scatter diagram. Year-on-year growth rates of gross domestic product (GDP) of Dalian, Urumchi, Beijing, Jilin, Harbin, and Mainland China were extracted from the official websites of the government statistical bureau and presented as a bar graph (16–21). The statistical scopes of GDP are prefecture cities or municipalities directly under the central government where the local reemergent outbreak occurred. Data of other areas were not shown since the local GDP at the time of reemergences had not been counted.

Informed consent from an individual was not required in the presence of a public health emergency. The Clinical Research Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China, approved this study.

RESULTS

Accumulative numbers of 30, 8, 11, 430, 15, 139, 1,067, 382, 42, and 94 confirmed cases were diagnosed in Hulun Buir, Shanghai, Tianjin, Kashgar, Qingdao, Dalian, Urumchi, Beijing, Jilin, and Harbin, respectively (**Table 1**). Asymptomatic infections of local reemergent areas mentioned above, respectively, account for 6.7% (2/30), 0% (0/8), 18.2% (2/11), 81.9% (352/430), 6.7% (1/15), 29.5% (41/139), 22.5% (240/1067), 12.3% (47/382), 4.8% (2/42), and 18.1% (17/94) of all confirmed cases (**Table 1**). The first-round mass RT-PCR test of SARS-CoV-2 in Hulun Buir, Tianjin, Kashgar, Qingdao, Dalian, Urumchi, and Beijing had been carried out within 3, 4, 3, 5, 9, 10, and 21 days from the onset of the first case (**Table 1**). Data of Harbin were unavailable and thus not shown in the table.

The time required to control outbreaks in Hulun Buir, Shanghai, Tianjin, Kashgar, Qingdao, Dalian, Urumchi, Beijing, Jilin, and Harbin were 33, 29, 30, 29, 50, 29, 48, 51, 31, and 45 days, respectively (**Figures 1**, **2**). According to the previously established model of Zhou L, ST and early-stage DTs of Kashgar, Dalian, Urumchi, and Beijing revealed high escalating probability (15). After the activation of outbreak responses, the late-stage DTs were apparently lengthened compared to the early-stage DTs (**Figure 3**). Local year-on-year GDP growth rates of Dalian, Beijing, Jilin, and Harbin were continuously rising regardless of the local reemergent outbreaks. Despite the GDP growth rate of Urumchi was slightly affected during the reemergent period, a positive trend can still be recognized compared to the complete lockdown period (January 2020–March 2020). GDP of Mainland China turned positive regardless of the reemergent local outbreak (**Figure 4**).

DISCUSSION

With the rising number of cases according to the situation reports of WHO, the COVID-19 pandemic seems possible

TABLE 1 | Cases and testing data of local reemergent areas.

	Time interval	Confirmed cases[a], persons	Symptomatic cases, persons	Asymptomatic cases[b], persons (%[c])	Number of people receiving 1st round PCR test	Number of people receiving 1st round PCR test per day[d]
Hulun Buir	November 21–December 23, 2020	30	28	2 (6.7)	203,326	67,775
Shanghai[e]	November 9–December 7, 2020	8	8	0 (0)	41,852	3,219
Tianjin	November 8–December 7, 2020	11	9	2 (18.2)	2,467,411	616,852
Kashgar	October 24–November 21, 2020	430	78	352 (81.9)	4,746,500	1,582,166
Qingdao	September 24–November 12, 2020	15	14	1 (6.7)	10,430,000	2,086,000
Dalian	July 22–August 19, 2020	139	98	41 (29.5)	3,892,000	432,444
Urumchi	July 15–August 31, 2020	1,067	827	240 (22.5)	2,309,537	230,953
Beijing	June 11–July 31, 2020	382	335	47 (12.3)	10,414,000	495,904
Jilin[e]	May 7–June 6, 2020	42	40	2 (4.8)	88,303	7,358
Harbin	April 9–May 23, 2020	94	77	17 (18.1)	N/A	N/A

[a]*Cases with identical origin as the originating site were counted together in the originating site cases.*
[b]*People who presented with asymptomatic infection at first but later developed clinical syndrome were excluded from the asymptomatic cases.*
[c]*Asymptomatic rate = (Asymptomatic cases/Confirmed cases) * 100%.*
[d]*Number of people receiving first-round PCR test per day = Number of people receiving first-round PCR test/Days required for first-round mass testing.*
[e]*Shanghai and Jilin have only tested the close contacts and close contacts of close contacts due to the early detection.*

to continue for a longer period. COVID-19 pandemic has seriously affected the global economy, and according to Ms. Gopinath on the October 2020 World Economic Outlook Press Briefing, China may be the only major economy to achieve positive output growth this year (22). Although the overall level of SARS-CoV-2 transmission in China was close to zero, reemergent local outbreaks were still occurring. In this situation, how China strikes a balance between controlling outbreaks and revitalizing the economy was critical to pulling the world out of the deep economic recession. Emphases of Chinese strategy in tackling reemergent local outbreak included a rapid response to local reemergent, reasonable test range covering asymptomatic infections, precise region management according to different transmission risk, and inspection of inbound persons and imported cargoes (13). At the present stage, the goal of Chinese strategy is to maintain the status of no or minimal transmission until a safe and effective vaccine is available for everyone (23). Such strategy was able to well-manage the balance between outbreak control and economic recovery.

Firstly, a retrospective epidemiological investigation was carried out as soon as the first case was reported in a certain region. Close contacts and close contacts of close contact were found, and RT-PCR testing was immediately conducted. If the outbreak has an unclear source or a wide affected region, mass testing covering all citizens in related regions was rapidly conducted. According to the previous studies, asymptomatic infected persons can transmit SARS-CoV-2 to others and they account for approximately 40∼50% of all cases (24). In this study, the asymptomatic rate of the local reemergent outbreaks that occurred in China between April 2020 and December 2020 was up to 81.9%, indicating that the testing only for the symptomatic population was not enough. Chinese strategy of applying epidemiological investigation and a reasonable range of tests, sufficient to cover asymptomatic infections, was key to successfully control the local outbreaks.

Second, region management required flexible risk levels to be determined according to the population and epidemic status of certain districts, and a nationwide joint control strategy (JCS) was applied based on the assessment of risk level. According to the JCS (13), related regions were immediately upgraded to moderate- or high-risk areas as soon as the first case was reported. Corresponding management was applied to people in those areas and people traveled from those areas within 14 days. Such strategy ensured that no secondary outbreak was triggered outside the initial reemergent area. With gaining experience, the delineation of areas under risk was more and more fine. Local reemergent cases had also appeared in Shenyang, Dalian, Beijing, Chengdu, and Mudanjiang in December 2020. A similar strategy has been applied, and the situation has been effectively contained up to the submission date.

Finally, an inspection of imported subjects, including inbound persons and cargoes, has always been one of the important measures for outbreak control in China. Chinese researchers extrapolated that the source of the local outbreak in Harbin in April was a student returning from overseas (25). Reemergence in Jilin in May was suspected to be associated with a possible importation event, and the result of viral genome sequencing strongly suggested that the first case is related to the COVID-19 virus imported from Europe (26). Sources of early-stage local reemergence were mostly associated with people who traveled from outside China. However, subsequent local reemergences in Beijing, Urumchi, Dalian, Qingdao, and Tianjin may be related to imported cold-chain packages to various degrees. Among them, environmental swab samples of imported cold-chain packages in Beijing and Dalian reemergences were tested nucleic acid positive for SARS-CoV-2 (27, 28). Furthermore,

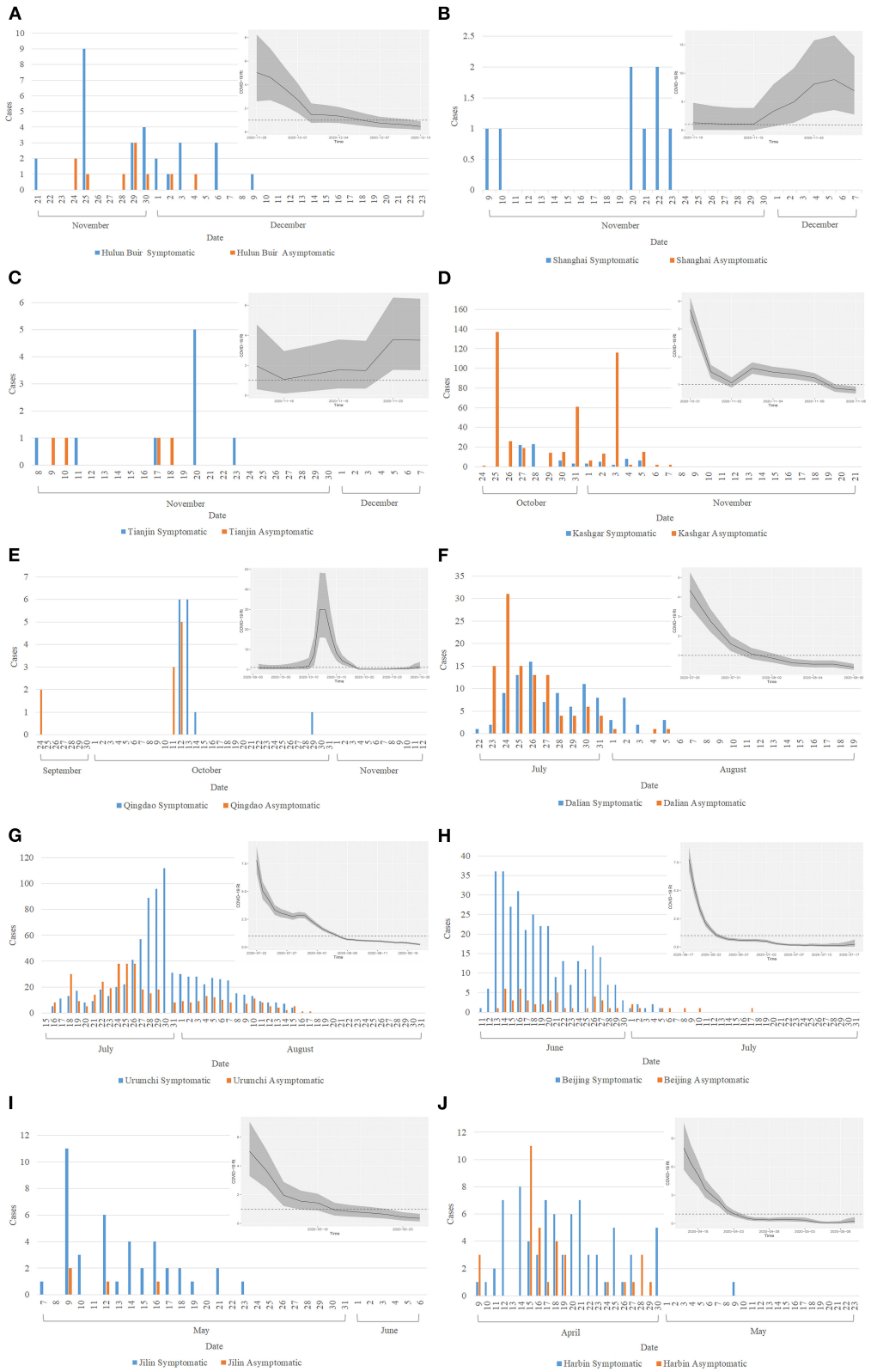

FIGURE 1 | Daily new symptomatic and asymptomatic cases and time-varying reproductive numbers (Rt) curve in reemergent areas. Bar graphs present daily new cases of the local reemergent outbreaks in Hulun Buir **(A)**, Shanghai **(B)**, Tianjin **(C)**, Kashgar **(D)**, Qingdao **(E)**, Dalian **(F)**, Urumchi **(G)**, Beijing **(H)**, Jilin **(I)**, and Harbin **(J)**. Rt curves of reemergent areas are shown on the upper right part of each bar graph, respectively. Cases with identical origin as the originating site were counted together in the originating site cases. Rt stands for time-varying reproductive number.

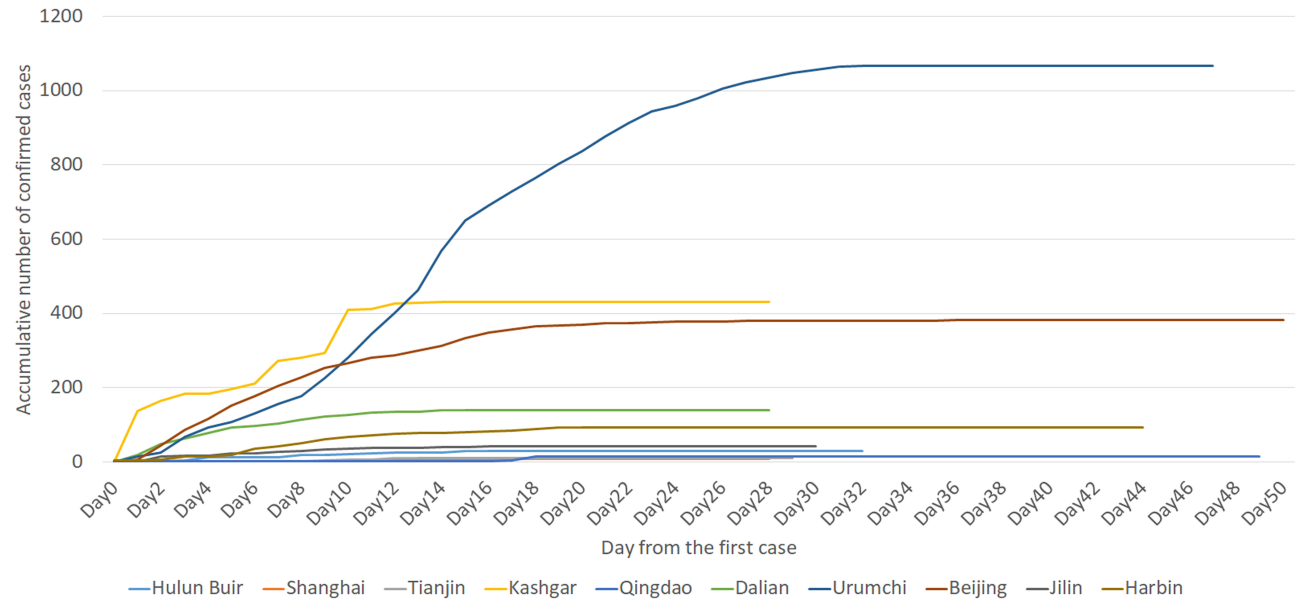

FIGURE 2 | Time course of the accumulated number of confirmed cases in reemergent areas. This epidemiological curve shows an accumulative number of confirmed cases of Hulun Buir, Shanghai, Tianjin, Kashgar, Qingdao, Dalian, Urumchi, Beijing, Jilin, and Harbin. The curve was presented by day, from the day the 1st case was reported to the 14th day with no new case reported. People who presented with asymptomatic infection at first but later developed clinical syndrome were excluded from the asymptomatic cases.

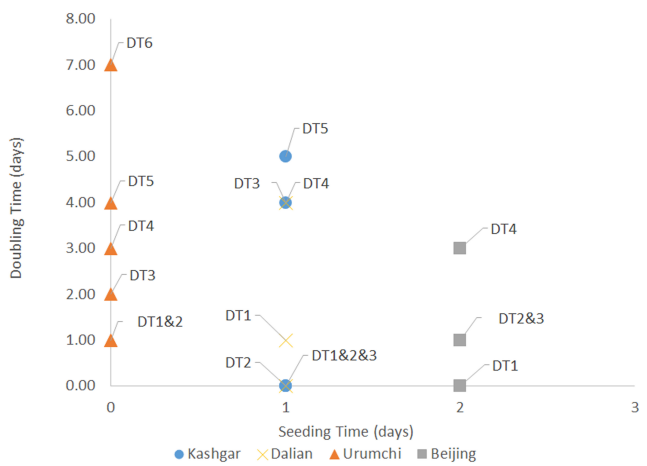

FIGURE 3 | Seeding time (ST) and doubling time (DT) of Kashgar, Dalian, Urumchi, and Beijing reemergent outbreaks. This scatter diagram shows ST and DT1∼6 of Kashgar, Dalian, Urumchi, and Beijing reemergent outbreaks. The time interval between the date of the first reported case and the date of a number of cases reaching the median seeding number (SN) of an area (median SN = 12 according to the study of Zhou L) was defined as ST. DT was the time interval required to double the accumulative number of cases of an area. DT1 ∼ DT6 were calculated according to the time interval between the 12 and 24, 24 and 48, 48 and 96, 96 and 192, 192 and 384, and 384 and 768 confirmed cases, respectively. ST stands for seeding time. DT stands for doubling time.

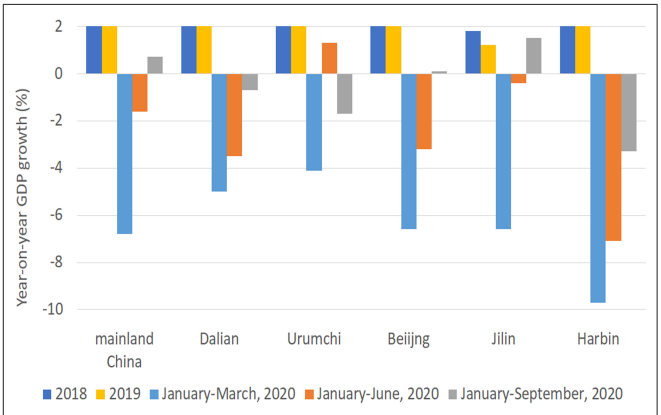

FIGURE 4 | National and local year-on-year GDP growth rate. This bar graph presents the year-on-year GDP growth rate of Mainland China and the local year-on-year GDP growth rates of Dalian, Urumchi, Beijing, Jilin, and Harbin of 2018, 2019, and the first three quarters of 2020. GDP stands for gross domestic product.

the SARS-CoV-2 virus was directly isolated from the surface of imported cold-chain cargoes in Qingdao (29). Similarly, the source of the local reemergence in Tianjin may be frozen pig heads from North America (30). These results were highly suggestive that SARS-CoV-2 may survive for a long time on cold-chain cargoes and trigger transmission. Nevertheless, research studies have shown that environmental temperature is related to the stability of SARS-CoV-2 (31). In the local reemergences in Kashgar in October and Shanghai in November, the transmission source may be both cold-chain and non-cold-chain containers imported from overseas (32, 33). These two local

reemergences suggested that with the arrival of winter, SARS-CoV-2 may survive longer on non-cold-chain-transported goods and may also cause virus transmission. Considering the severe situation in the countries outside of China, the management of incoming items requires special attention. China has set up a technical guide aiming at prevention, control, and disinfection of cold chain (34). Non-cold-chain cargoes have also gained the attention of the government, and Zhejiang Province was the first to publish an emergency plan (35). Chinese prevention and control strategy has gradually become more comprehensive from the management of inbound people from outside China to the management of imported cold-chain and even non-cold-chain cargoes.

Regarding the effect of such comprehensive strategy, late-stage DTs of Kashgar, Urumchi, Beijing, and Dalian were lengthened compared to early-stage DTs, indicating the effectiveness of local emergency control. Time course also revealed that such a strategy was able to control reemergent outbreaks within 51 days. The success of such a strategy has also been proven by the national and local GDP. According to the year-on-year GDP growth rate data of the past 2 years, the national and local GDP of China has been in a state of relatively stable growth. Therefore, the COVID-19 pandemic and reemergent local outbreak may be regarded as the biggest variable during the first three quarters of 2020 that may affect GDP. As of the first three quarters of 2020, the year-on-year growth rate of local GDP in reemergent areas turned positive in spite of the local reemergences under such a containing strategy. Despite the GDP of Urumchi was slightly affected during the reemergence period, a positive trend can still be recognized. GDP of Mainland China turned positive regardless of the reemergent local outbreak (**Figure 4**). Comprehensive means were able to efficiently suppress the transmission, and therefore, basic service and production can return to normal. Moreover, it is worth mentioning that GDP may be affected by small-scale sporadic cases that appeared in a district or block when a certain area enters the relatively stable stage. Although there may be few confirmed cases, the cost of preventing contagion may still be enormous. Taking Ruili as an example, only two cases were confirmed in September 2020, but the city paid the price of a 7-day lockdown. Such measures are necessary but may exert greater influence on the local economy.

This research study has several limitations. Firstly, in our study, further analysis involving demographic features, social relationships, and traveling history of the confirmed cases remained difficult to implement due to the limited open-access data, which hindered further epidemiologic analysis such as the reconstruction of transmission pairs (36). Secondly, the GDP of a certain place is regulated by various factors such as the local economic structure, economic development level, and economic policies. It is almost impossible to completely remove the confounders that affect GDP. Finally, the year-on-year GDP growth rate alone may not be a comprehensive assessment of the influence on the economy. Other evaluation indexes with respect to social and/or economic influence were not discussed due to the limitation of data resources. Further research studies are needed to better understand the factors that influence the containment of the pandemic and local reemergent outbreaks and their relationship with the economy and society.

CONCLUSIONS

Chinese strategy of rapid response to local reemergent, reasonable test range covering asymptomatic infections, precise region management according to different transmission risk, and inspection of inbound persons and imported cargoes was effective in balancing the control of reemergent local outbreak and the recovery of economy. The possibility of object–human transmission requires more attention in controlling local outbreaks, especially in winter.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Clinical Research Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

ZW, YJ, and XJ wrote the manuscript. XY, YL, YZ, and LL read and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Risk Factors for SARS-CoV-2 Infection, Pneumonia, Intubation, and Death in Northeast Mexico

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Despite the social distancing and mobility restriction measures implemented for susceptible people around the world, infections and deaths due to COVID-19 continued to increase, even more so in the first months of 2021 in Mexico. Thus, it is necessary to find risk groups that can benefit from more aggressive preventive measures in a high-density population. This is a case-control study of suspected COVID-19 patients from Nuevo León, Mexico. Cases were: (1) COVID-19-positive patients and COVID-19-positive patients who (2) developed pneumonia, (3) were intubated and (4) died. Controls were: (1) COVID-19-negative patients, (2) COVID-19-positive patients without pneumonia, (3) non-intubated COVID-19-positive patients and (4) surviving COVID-19-positive patients. ≥ 18 years of age, not pregnant, were included. The pre-existing conditions analysed as risk factors were age (years), sex (male), diabetes mellitus, hypertension, chronic obstructive pulmonary disease, asthma, immunosuppression, obesity, cardiovascular disease, chronic kidney disease and smoking. The Mann-Whitney *U* tests, Chi square and binary logistic regression were used. A total of 56,715 suspected patients were analysed in Nuevo León, México, with 62.6% being positive for COVID-19 and, of those infected, 14% developed pneumonia, 2.9% were intubated and 8.1% died. The mean age of those infected was 44.7 years, while of those complicated it was around 60 years. Older age, male sex, diabetes, hypertension, and obesity were risk factors for infection, complications, and death from COVID-19. This study highlights the importance of timely recognition of the population exposed to pre-existing conditions to prioritise preventive measures against the virus.

Keywords: COVID-19, SARS-CoV-2, coronavirus, pneumonia, intubation endotracheal, death, Mexico

INTRODUCTION

Currently, coronavirus disease 2019 (COVID-19) is a global public health problem. As of May 10, 2021, 158,551,526 people have been infected and 3,296,855 have died from this disease worldwide (1). The situation is no less dire in Mexico, where 2,366,496 infected people and 219,089 deaths have been documented (2). Since the World Health Organization (WHO) first declared COVID-19 a pandemic (3), and given the absence of a vaccine and effective treatment at that time, several studies have been conducted to determine the factors associated with severity and mortality. These studies demonstrated that older age and certain chronic conditions such as diabetes, hypertension and obesity, among others, were associated with higher risks of complications and death (4–9). Given the above findings, many countries, including Mexico, implemented preventive strategies aimed at reducing the exposure of vulnerable people, such as social distancing of the elderly and people with chronic diseases, closure of non-essential companies and schools, limits on the number of people in stores and essential establishments, educational reinforcement of handwashing and the use of face masks. These measures aimed to reduce the rate of infections and subsequently the mortality associated with the causal virus, however, they had significant growth in January and February of 2021 (1). Furthermore, a recently published North American study was unable to identify reductions in mortality during restricted mobility days in either the United States or Europe (10). The lack of control of the rates of infection and death is a clear example that there is much we still do not understand about the transmissibility of COVID-19 since many people around the world continue becoming infected and infecting others.

Nuevo León is a state of Mexico with a population density greater than the national average (11). It is a highly industrialised region that includes approximately 2.54 million economically active people (12). The closure of many productive activities due to the pandemic caused the loss of thousands of jobs, which triggered a growth in informal economic activities (13). In many cases, these circumstances prevented home office work, which could cause greater exposure to infected people and a greater spread of COVID-19 in this population (14). Moreover, this state has a high prevalence of chronic diseases such as obesity, diabetes and hypertension (15), which have consistently been associated with infection (16, 17) and worse outcomes in the course of this disease (4–9, 18, 19).

Therefore, it is imperative to determine not only the factors associated with a worse outcome of this disease but also those related to infection. This knowledge is necessary in a high-density population to establish risk groups that could benefit from more aggressive preventive measures, including vaccines.

The aim of this work was to determine the risk factors associated with COVID-19 infection and to determine the risk factors for pneumonia, intubation and death among those infected with COVID-19.

MATERIALS AND METHODS

Study Population

The data from patients in Nuevo León, Mexico, with suspected COVID-19 were obtained from the open access database of the General Directorate of Epidemiology of the Ministry of Health. The database contains data from all health institutions in Mexico. For this study, data collected from February 11, 2020 to September 24, 2020 were analysed (20).

Groups and Study Goals

Cases were defined as follows: (1) COVID-19-positive patients and COVID-19-positive patients who (2) developed pneumonia, (3) were intubated and (4) died. The respective controls were (1) COVID-19-negative patients, (2) COVID-19-positive patients without pneumonia, (3) non-intubated COVID-19-positive patients and (4) surviving COVID-19-positive patients. The sample size for each study objective was sufficient to identify odds ratios of 1.14–1.47 with a confidence level of 95% and a statistical power of 100% (see **Supplementary Figure 1** for a more detailed description of the size of each study group).

Procedures

Patients aged ≥18 years who were treated in Nuevo León, Mexico, were included; pregnant patients were excluded ($n = 513$). Pre-existing conditions analysed included those previously identified as risk factors for COVID-19 and its complications: age (years), sex (male), diabetes mellitus, hypertension, chronic obstructive pulmonary disease, asthma, immunosuppression, obesity, cardiovascular disease, chronic kidney disease and smoking (4–9, 18, 19). The following outcomes were evaluated: COVID-19 infection [severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) polymerase chain reaction-positive], COVID-19 pneumonia, COVID-19 intubation and COVID-19 death. Except for age, all variables were coded in a categorical binary method (Yes vs. No). The following data were also analysed: medical care institution (public or private), origin from the metropolitan area and hospitalisation.

Ethical Considerations

This protocol was subject to the institutional, national and international norms and regulations on research and ethics in health and was approved by the Local Committee on Ethics and Health Research of the Mexican Institute of Social Security No. 1909 and registered as R-2020-1909-027. In this study, an open access database available on the internet was managed, and non-personal information of the study patients was obtained or recorded.

Statistical Analysis

The measures of central tendency and proportions, as well as 95% confidence intervals, were estimated. The Mann–Whitney *U* test was used to compare age between independent groups after the Kolmogorov–Smirnov test verified the non-normal distribution of variables. Univariate and multivariate odds ratios were

TABLE 1 | General data, comorbidities and outcomes of patients analysed for SARS-CoV-2 in Nuevo León (February to September 2020).

	N = 56,715	%
Live in metropolitan area	48,274	85.1%
Gender		
Female	27,608	48.7%
Male	29,107	51.3%
Institution		
Public	53,709	94.7%
Private	3,006	5.3%
Comorbidities		
Hypertension	9,489	16.7%
Obesity	8,504	15.0%
Diabetes mellitus	7,874	13.9%
Smoking	4,627	8.2%
Asthma	1,536	2.7%
Another comorbidity	1,405	2.5%
Cardiovascular disease	1,165	2.1%
Chronic kidney disease	897	1.6%
Immunosuppression	700	1.2%
COPD	491	0.9%
COVID-19 positive patients	35,476	62.6%
Pneumonia	4,974	14.0%*
Hospitalised	7,351	20.7%*
Intubated	1,021	2.9%*
Dead	2,860	8.1%*

*COPD, chronic obstructive pulmonary disease. *Proportions of all COVID-19 positive patients.*

TABLE 2 | Comparison of patient age related to the main output variables.

	Mean age (years)	CI 95%	*P*-value
COVID-19 positive	44.7	44.6–44.9	<0.0001
COVID-19 negative	42.2	42.0–42.4	
Pneumonia	57.5	57.1–57.9	<0.0001
No Pneumonia	42.1	41.9–42.2	
Intubated	60.1	59.3–60.9	<0.0001
No Intubated	57.2	56.8–57.5	
Dead	63.6	63.1–64.0	<0.0001
Survivors	42.6	42.4–42.7	

CI, confidence interval.

estimated using Chi-square tests and binary logistic regressions, respectively. A p-value < 0.05 was considered significant.

RESULTS

From the beginning of the pandemic until September 24, 2020, 56,715 patients with suspected COVID-19 were registered in Nuevo León, of whom 85.1% lived in the metropolitan area. The most frequent comorbidity was hypertension, followed by obesity. Of the total population analysed, 62.6% were positive for COVID-19, and of these, 14% developed pneumonia, 20.7% were hospitalised, 2.9% were intubated and 8.1% died (**Table 1**).

The mean age of the infected patients was 44.7 years, whereas the mean ages of those who developed pneumonia, were intubated, or died were 57.5, 60.1, and 63.6 years, respectively. In all comparisons, the cases were older than the controls (**Table 2**).

At the univariate level, male sex, diabetes, hypertension, and obesity were shown to be significantly associated with the risk of COVID-19 infection, pneumonia, intubation and death. Conversely, chronic obstructive pulmonary disease, immunosuppression, cardiovascular disease and chronic kidney disease showed a protective effect against COVID-19 infection, but were risk factors for pneumonia and death, while smoking was a protective factor against infection and those complications. Asthma showed a protective effect against infection but did not show any association with complications (**Table 3**).

The multivariate level showed similar trends to those of the univariate analysis: male sex, diabetes mellitus, hypertension and obesity were risk factors for COVID-19 infection, whereas older age also showed an association, but this association was only marginal (odds ratio, 1.01; 95% confidence interval, 1.00–1.02). These factors also showed a consistent risk association for complications, that is, pneumonia, intubation (except for diabetes) and death. Conversely, factors that were protective against COVID-19 infection did not show an association with complications (such as chronic obstructive pulmonary disease, asthma, and cardiovascular disease) or were risk factors for pneumonia and death (immunosuppression and chronic kidney disease); smoking was also protective against death (**Table 4**).

DISCUSSION

This study, which is based on a large representative database, examines pre-existing factors associated with COVID-19 infection as well as pneumonia, intubation, and death in northern Mexico. The main risk factors for infection and its complications were older age, male sex, hypertension, and obesity. Thus, it is possible to stratify patients exposed to these conditions to reinforce preventive and therapeutic strategies.

The population analysed in this study primarily corresponds to the metropolitan area of Nuevo León, a region with a high population density (11), which is a determining factor in the risk of contagion (21). Compared with another study performed in the total Mexican population, Nuevo León registered a higher proportion of positive cases of COVID-19 (62.6 vs. 52.6%) (6). However, the rate of hospitalisations in this state was lower than that reported in the previous study (35.3%) (6), which may be due to the fact that many outpatient diagnostic tests were performed in this state.

An important finding of this study is the mean age of the infected patients, which was younger than that of those who developed complications. This agrees with the estimates of Xiong et al. (14) in California, who found that the highest infection rate was in the population aged 18–59 years, that is, the economically active population, but there was a higher fatality rate in the

TABLE 3 | Univariate analysis of pre-existing conditions associated with confirmed COVID-19 infection, pneumonia, intubation, and death.

Output variable	COVID-19 positive	Pneumonia	Intubation	Death
Pre-existing conditions	OR (CI 95%)	OR (CI 95%)	OR (CI 95%)	OR (CI 95%)
Male	1.17 (1.13–1.21)**	1.45 (1.36–1.54)**	1.14 (0.99–1.31)	1.47 (1.36–1.59)**
Diabetes mellitus	1.59 (1.51–1.68)**	3.75 (3.50–4.00)**	1.21 (1.05–1.38)*	4.17 (3.85–4.53)**
COPD	0.63 (0.53–0.75)**	4.09 (3.18–5.28)**	1.01 (0.64–1.60)	5.74 (4.40–7.48)**
Asthma	0.64 (0.58–0.71)**	1.02 (0.83–1.24)	1.10 (0.71–1.70)	0.97 (0.75–1.26)
Immunosuppression	0.51 (0.44–0.60)**	2.26 (1.77–2.89)**	1.41 (0.93–2.13)	2.89 (2.20–3.80)**
Hypertension	1.40 (1.34–1.47)**	3.68 (3.44–3.92)**	1.40 (1.22–1.60)**	4.85 (4.48–5.25)**
Cardiovascular disease	0.74 (0.66–0.83)**	3.48 (2.95–4.10)**	1.13 (0.83–1.53)	4.16 (3.47–4.98)**
Obesity	1.69 (1.61–1.78)**	1.71 (1.60–1.84)**	1.35 (1.17–1.56)**	1.74 (1.59–1.90)**
Chronic kidney disease	0.83 (0.72–0.94)*	5.49 (4.61–6.54)**	1.30 (0.97–1.75)	7.43 (6.20–8.91)**
Smoking	0.78 (0.73–0.83)**	0.81 (0.72–0.92)*	1.17 (0.90–1.52)	0.82 (0.70–0.96)*

*OR, odds ratio; CI, confidence interval; COPD, chronic obstructive pulmonary disease. *p < 0.05; **p < 0.0001.*

TABLE 4 | Multivariate analysis of pre-existing conditions associated with confirmed COVID-19 infection, pneumonia, intubation, and death.

Outcome variable	COVID-19 positive	Pneumonia	Intubated	Death
Pre-existing conditions	OR (CI 95%)	OR (CI 95%)	OR (CI 95%)	OR (CI 95%)
Age (risk for each year added)	1.01 (1.00–1.02)**	1.06 (1.05–1.07)**	1.01 (1.00–1.02)**	1.08 (1.07–1.09)**
Male	1.21 (1.17–1.25)**	1.65 (1.54–1.76)**	1.23 (1.07–1.41)*	1.74 (1.59–1.89)**
Diabetes mellitus	1.38 (1.30–1.46)**	1.65 (1.53–1.79)**	1.04 (0.89–1.20)	1.52 (1.38–1.68)**
COPD	0.53 (0.44–0.63)**	1.13 (0.85–1.49)	0.83 (0.51–1.33)	1.29 (0.95–1.74)
Asthma	0.66 (0.59–0.73)**	1.18 (0.95–1.47)	1.10 (0.70–1.72)	1.17 (0.88–1.57)
Immunosuppression	0.48 (0.41–0.55)**	1.48 (1.13–1.94)*	1.46 (0.96–2.22)	1.92 (1.41–2.61)**
Hypertension	1.11 (1.05–1.17)**	1.27 (1.17–1.38)**	1.20 (1.03–1.39)*	1.43 (1.30–1.57)**
Cardiovascular disease	0.59 (0.52–0.67)**	1.11 (0.92–1.33)	0.90 (0.66–1.24)	1.02 (0.83–1.26)
Obesity	1.67 (1.58–1.76)**	1.56 (1.44–1.70)**	1.40 (1.20–1.63)**	1.63 (1.48–1.81)**
Chronic kidney disease	0.65 (0.57–0.75)**	2.23 (1.83–2.72)**	1.14 (0.84–1.55)	2.86 (2.32–3.53)**
Smoke	0.76 (0.71–0.81)**	0.81 (0.71–0.93)*	1.08 (0.82–1.42)	0.81 (0.68–0.97)*

*OR, odds ratio; CI, confidence interval; COPD, chronic obstructive pulmonary disease. *p < 0.05; **p < 0.0001.*

population aged ≥60 years. In accordance with this finding, the higher infection rate in younger individuals seems to be related to the need to work outside home (14), whereas the higher severity and lethality seems to be explained by immunosenescence, a chronic low-grade systemic inflammation that has been proposed as a common factor resulting in greater susceptibility to COVID-19 severity (22).

Another factor consistently associated with infection and severity was male sex. In this regard, men have been described as having biological and behavioural differences that place them at a higher risk than women, such as their higher level of testosterone that inhibits antibody production, the higher presence of receptors of angiotensin-converting enzyme 2 that facilitate viral anchorage and replication, insufficient handwashing practices, non-adherence to health services and reluctance to follow public health measures (17, 23–25).

In this population, pre-existing conditions did not equally affect the risks of infection, pneumonia, intubation, and death. Notably, chronic obstructive pulmonary disease, asthma, cardiovascular disease and even smoking showed protective effects against infection but did not show any association with complications or, as in the case of smoking, this was a "protective" factor against death. More remarkable still, factors such as immunosuppression and chronic kidney disease, which acted as protectors against infection, increased the risk of pneumonia and death. This contradiction could be explained because, as mentioned before, the Mexican health authorities implemented quarantine measures for the vulnerable population, which most likely made these groups under-represented in this database. In other words, it is not necessarily true that having such comorbidities offers any protection against COVID-19 but rather that the susceptible population was less exposed to the virus. In fact, diabetes, immunosuppression and chronic kidney disease, three serious medical conditions, have been reported to increase the risk of severity and death from COVID-19 (6, 9, 26). Therefore, preventive measures should be continued and reinforced in patients with these comorbidities.

An increased risk of infection became apparent among people with diabetes, hypertension, and obesity. Additionally, these comorbidities were risk factors for pneumonia, intubation (not

diabetes) and death. This triad of comorbidities has already been described as a risk factor for adverse outcomes from COVID-19 (6, 9, 27). At this point, it is necessary to emphasise the role of these chronic diseases in susceptibility to contagion. Yadav et al. had already pointed out this relationship in the Mumbai population and noted that the concurrence of these chronic diseases was strongly associated with COVID-19 infection (16). The biological explanation is that these comorbidities can lead to the overexpression of SARS-CoV-2 receptor molecules, such as angiotensin-converting enzyme 2 and CD147 (17). The practical implication of this relationship is that in a high-density population, with a high prevalence of these comorbidities, the risk of spreading the virus, even with social distancing measures, is extremely high. These findings emphasise the need to prioritise preventive measures such as vaccination in the potentially more vulnerable groups.

A limitation of this study is its use of a secondary data source. Thus, it is possible that some data were under-represented; therefore, the results shown should be viewed with caution. However, a strength of this study is that it included almost 100% of the data in a highly industrialised area with a high population density.

In conclusion, older age, male sex, hypertension, and obesity were factors consistently associated with COVID-19 infection as well as its complications, including pneumonia, intubation and death. Additionally, diabetes mellitus, immunosuppression and chronic kidney disease were identified as risk factors for pneumonia and death from COVID-19. This study highlights the importance of timely recognition of the population exposed to pre-existing conditions to prioritise preventive measures against the virus, such as mass vaccination.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: COVID-19 open access database of the General Directorate of Epidemiology of the Ministry of Health.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by 1909-Local Committee of ethics on research, Mexican Institute of Social Security, Monterrey, Nuevo Leon, Mexico. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HC-F: data analysis, elaboration, and review of manuscript. FG-S: wrote protocol, wrote manuscript manage data, traduction, edition, and review final version. JV-V: design protocol, registry, and review final version. JM-C: design protocol, discussion, and review manuscript final. SG-G: get data, manage variables, and review final version manuscript. LD: registry protocol, get data, and review of final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.645739/full#supplementary-material

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ORIGINAL RESEARCH
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Elevation of Serum Cytokine Profiles and Liver Metabolomic Normalization in Early Convalescence of COVID-19 Patients

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Coronavirus disease 2019 (COVID-19) has become a global public health concern. We aimed to study the cytokine profile during the convalescent phase and its association with liver functions. We performed a retrospective study to investigate the longitudinal dynamic serum cytokine, liver function, and metabolomic profiles, as well as their potential correlations, from the viral replication phase to early convalescence. Our results demonstrated that liver injury was common. Liver injury was significantly associated with higher levels of interleukin (IL)-6 and IL-10 ($p < 0.05$). However, alanine aminotransferase levels decreased during the first week after hospital discharge ($p < 0.01$). In parallel, T-cell and B-cell immune response-stimulating cytokine IL-4, but not IL-2, was significantly elevated ($p < 0.05$). Furthermore, interferon-γ (IFN-γ) and tumor necrosis factor-α (TFN-α) levels increased, in contrast to the decrease in IL-6 and IL-10 levels; liver function returned to normal. The metabolomic analysis supported active recovery during early convalescence of COVID-19 patients that had distinct metabolic profiles associated with the hepatic tricarboxylic acid cycle, amino acid metabolism, and lipid metabolism. In addition, we identified a metabolomic association of IL-4 with liver repair. Our findings suggest that discharged patients continue to recover from the physiological effects of COVID-19, and the association of IL-4, IL-6, and IL-10 levels with metabolic changes and liver function repair may have important implications for clinical manifestations and treatment of COVID-19.

Keywords: SARS-CoV-2, cytokines, metabolomics, UPLC/Q-TOF-MS, liver repair

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) has rapidly spread worldwide and has become a significant public health challenge. As of June 30, 2020, over 10,000,000 cases have been confirmed, with more than 500,000 deaths in 213 countries, and the numbers continue to rise rapidly.

Numerous studies have reported that COVID-19 severity correlates with serum inflammatory cytokine concentrations (1), and mortality often results from cytokine storm (2). Uncontrolled cytokine storm has also been implicated as a central factor contributing to severe acute respiratory syndrome coronavirus (SARS-CoV), and other severe viral infections (3, 4). Several cytokines including interleukin (IL)-6, IL-10, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α)

have been observed to increase dramatically during acute infection in COVID-19 patients (1, 5, 6). However, previous serum cytokine profiling has been focused on acute infection of SARS-CoV-2; thus, very limited information has been reported on cytokine levels and kinetics during the convalescent phase of COVID-19.

Liver enzyme abnormality has also been observed in COVID-19 patients, and it appears to be correlated with disease severity (7). Cytokine storm and direct infection of the liver have been suggested to contribute to liver injury, and in some cases, liver failure in patients with COVID-19 (8, 9). To date, no studies have reported on the repair of liver damage in convalescent patients. However, in response to helminthic infections, the type 2 immune response (10), specifically IL-4-dependent macrophage proliferation and activation, is required to promote repair of both the liver and lung.

Furthermore, there is an immense metabolic demand during liver repair and regeneration. Cytokines have been shown to mediate several metabolic changes *via* a pathway that is commonly initiated through their regulation of the immune system (11). On the other hand, metabolites are required to regulate the homeostasis of cellular activities in hosts (12), such as resolving inflammation from viral infections (13). Metabolomics of H1N1 influenza virus-infected murine lungs identified that metabolic pathways and association clusters were related to inflammatory cytokines (14). Metabolomics has also been successfully used to identify severe drug-induced liver injury, as well as answer important biological questions (15–17).

Therefore, we have performed a retrospective study designed to investigate the longitudinal dynamic serum cytokine profile, liver functions, and metabolomic profiles in infected patients from the viral replication phase through to convalescence.

MATERIALS AND METHODS

Patients

From January 19 to March 29, 2020, 102 SARS-CoV-2-infection-diagnosed patients were admitted to the First Affiliated Hospital, Zhejiang University School of Medicine. All patients were confirmed to be SARS-CoV-2 nucleic acid positive by real-time fluorescent RT-PCR. Patients were diagnosed in accordance with the World Health Organization's interim guidelines for COVID-19. Data were collected at our hospital. Our study was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (2020 llT-7). The categorization of mild vs. severe COVID-19 patients was conducted according to COVID-19 Diagnosis and Treatment Guideline (Trial 5th version) (**Supplementary Material**).

Blood Sampling

Venous whole blood samples were collected from patients during early morning, prior to breakfast. All samples were immediately shipped to the biosafety level 3 laboratory in our hospital. The convalescent blood samples were collected from discharged patients who had met the official hospital discharge criteria, specifically a negative result from two consecutive COVID-19 nucleic acid tests and the disappearance of major clinical signs.

Serum Cytokine Measurement

Serum was separated and stored at −80°C for cytokine detection. The IL-2/IL-4/IL-6/IL-10/TNF-α/IFN-γ cytokine assay kits (Cat No#: 8930960) were provided by Agilent Biosciences (Agilent Technologies, California, USA). The cytokines were detected using flow cytometry (ACEA NovoCyte, Agilent Technologies, California, USA) and analyzed using Novocyte kit software, according to the manufacturer's instructions.

Metabolomic Analysis

A total of 123 blood samples were included in our final analysis, covering both the viral replication (72 samples) and convalescent (41 samples) phases, and 10 plasma samples were collected from healthy participants to serve as controls. The collected samples were centrifuged at 3,000 rpm for 5 min and the supernatant was stored at −80°C before analysis. Metabolites were extracted from plasma, and UPLC-MS/MS analysis was performed using a Waters Acquity UPLC coupled with a Xevo G2-Q-Tof (Waters, Milford, MA, USA) in both positive and negative modes. The obtained raw data were pre-processed using Progenesis QI ver. 2.2 (Nonlinear Dynamic). Metabolites were identified by searching the HMDB library (https://hmdb.ca/spectra/ms/search). Pathway analysis was performed using the MetaboAnalyst 4.0 online tool (http://www.metaboanalyst.ca/). Detailed information is outlined in supporting documents.

Statistical Analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, v. 18.0; SPSS, Inc., Chicago, IL, USA). All tests were two-tailed, and p-values < 0.05 were considered indicative of statistical significance. Student's t-test or the nonparametric Mann–Whitney U-test, as appropriate, was used for comparisons of continuous data. Categorical data were compared by Fisher's exact test and correlation analysis was calculated by Pearson correlation or Spearman correlation test.

RESULTS

Demographic and Clinical Characteristics of COVID-19 Patients

We conducted a retrospective study of all patients (102) with confirmed SARS-CoV-2 infection in our hospital. Two patients were subsequently excluded from the final analysis because they were discharged from the hospital the next day. The remaining 100 patients were included in the analysis. In total, 123 blood samples was involved in metabolomics analysis including both discovery group ($N = 110$) and validation group ($N = 13$) (**Figure 1**). The median age of the patients was 54.28 years. Most of the infected patients were men (61; 61%); less than half had underlying diseases (48; 48%), including diabetes (15; 15%), hypertension (35; 35%), cardiovascular disease (8; 8%), pulmonary disease (4; 4%), fatty liver (4; 4%), chronic kidney disease (5; 5%), and HBV (4; 4%). On admission, most patients had fever (83; 83%), cough (69; 69%), and other common symptoms including phlegm (40; 40%), chest distress (25; 25%), myalgia (18; 18%), and fatigue (14; 14%). There were only a

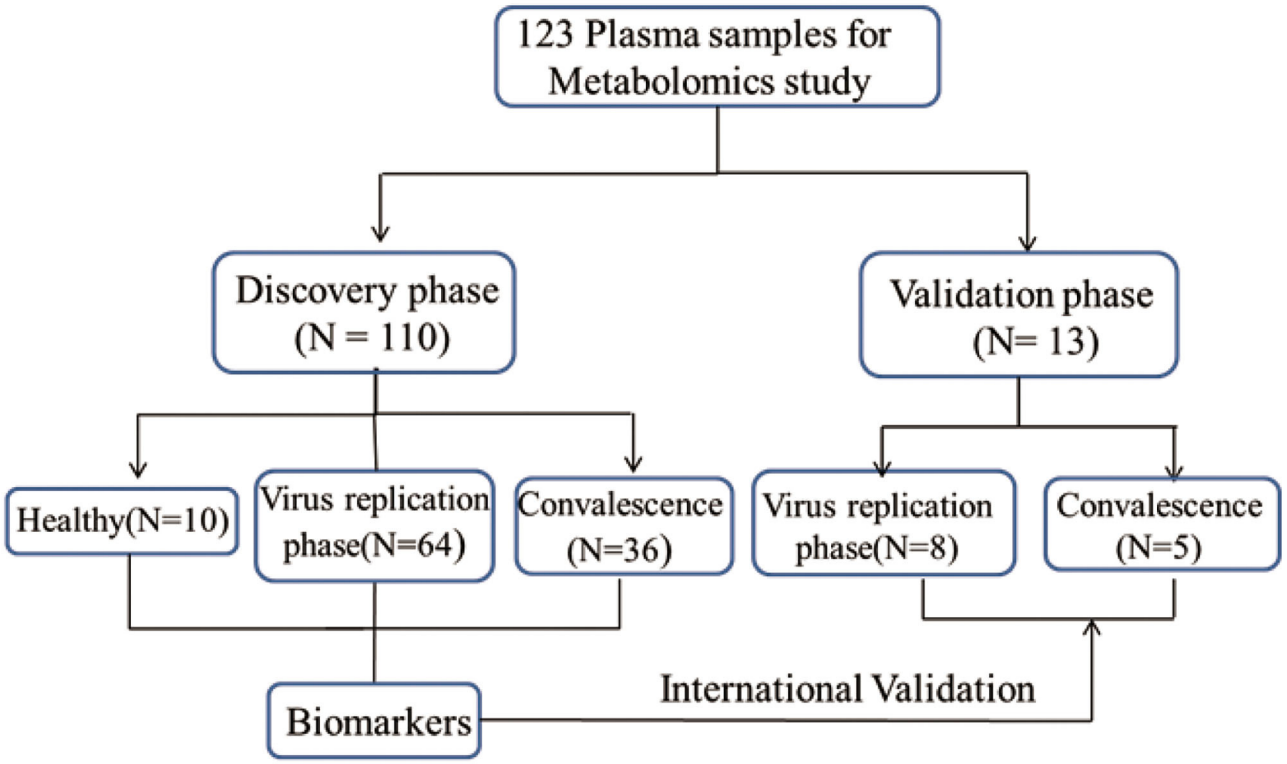

FIGURE 1 | Design of the metabolomics study. This study, involving 123 plasma samples, included both discovery group and validation group. Through the analysis of the data from the discovery set, we screened and identified metabolites that were closely related to COVID-19. To validate the reliability of results from discovery set, an independent set of 13 plasma samples was collected and all samples were correctly identified by a random forest machine learning model based on the identified metabolites (**Supplementary Table 9**).

small number of patients presenting with headache (9; 9%), dizziness (7; 7%), diarrhea (8; 8%), and nausea or vomiting (7; 7%) (**Table 1**).

Liver Injury and Recovery in COVID-19 Patients

To investigate whether liver enzyme abnormality occurred in this COVID-19 patient cohort, multiple liver function indicators, including serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total bilirubin (TBiL), were collected from the onset of the disease. Among the 100 COVID-19 patients in our hospital, 62 (62%) had increased levels of ALT, AST, ALP, and TBiL (**Table 1** and **Supplementary Table 3**). These results suggest that liver injury is a common clinical feature of SARS-CoV-2 infection. To determine the course of liver recovery, liver function indicators were measured weekly after patients were discharged from hospital. Serum ALT levels decreased within the first week after discharge ($p < 0.01$) (**Figure 2**). Levels of ALT continued to decline significantly through the second ($p < 0.05$) and fourth weeks ($p < 0.001$) (**Figure 2**). Significant changes to AST, ALP, and TBiL levels also occurred toward the normal range (**Figure 2**), indicating the active recovery of liver functions during early convalescence of COVID-19.

Association of Elevated IL-6 and IL-10 With Liver Injury

Cytokine analysis revealed that cytokines IL-6 and IL-10 were elevated in COVID-19 patients on admission and continued to increase significantly as the disease progressed (**Figure 3**), consistent with previous reports (1). IL-6 and IL-10 levels gradually declined into normal ranges as the viral RNA became undetectable ($p < 0.05$) (**Figure 3**). Furthermore, as shown in **Supplementary Table 5**, IL-6 showed sustained higher levels in the liver impaired group, compared with the normal liver function group. Elevated levels of IL-6 became significant 7–9 days after disease onset ($p = 0.034$) and became even more pronounced as the disease progressed ($p = 0.001$ at days 13–15 and $\geq$16) (**Supplementary Table 5**). These results suggest a possible correlation between liver damage and the inflammatory responses induced by SARS-CoV-2 infection. In addition, increased levels of IL-6 and IL-10 were also significantly correlated with disease severity and patient age (**Supplementary Table 5**).

Elevation of Serum IL-4 Level in Early Convalescence

Cytokine profiles in convalescent patients remain less characterized; thus, we performed serum cytokine analysis weekly after patients were discharged from hospital. Significantly,

TABLE 1 | Demographics and baseline characteristics of patients infected with COVID-19.

Variables	All patients (*N* = 100)	Liver function abnormality (*N* = 62)	Liver function normality (*N* = 38)	*P*
Demographics				
Age (years), mean ± SD	54.28 ± 16.00	55.73 ± 16.74	51.92 ± 14.62	0.250
Male sex	61 (61.00)	38 (61.29)	23 (60.53)	0.939
Chronic medical illness				
Diabetes	15 (15.00)	11 (17.74)	4 (10.53)	0.327
Hypertension	35 (35.00)	23 (37.10)	12 (31.58)	0.574
Cardiovascular disease	8 (8.00)	4 (6.45)	4 (10.53)	0.474
Pulmonary disease	4 (4.00)	3 (4.84)	1 (2.62)	1.000
Fatty liver	4 (4.00)	4 (6.45)	0	0.294
Chronic kidney disease	5 (5.00)	3 (4.84)	2 (5.26)	1.000
HBV	4 (4.00)	3 (4.84)	1 (2.62)	1.000
Symptoms				
Fever	83 (83.00)	52 (83.87)	31 (81.58)	0.767
Cough	69 (69.00)	44 (70.97)	25 (65.79)	0.587
Phlegm	40 (40.00)	28 (45.16)	12 (31.58)	0.178
Chest distress	25 (25.00)	19 (30.65)	6 (15.79)	0.096
Dizziness	7 (7.00)	4 (6.45)	3 (7.89)	1.000
Myalgia	18 (18.00)	9 (14.52)	9 (23.68)	0.247
Headache	9 (9.00)	6 (9.68)	3 (7.89)	1.000
Diarrhea	8 (8.00)	6 (9.68)	2 (5.26)	0.707
Nausea or vomiting	7 (7.00)	5 (8.06)	2 (5.26)	0.706
Fatigue	14 (14.00)	12 (19.35)	2 (5.26)	0.049
Laboratory parameters (mean ± SD)				
Leucocytes ($\times 10^9$/L)	7.44 ± 4.63	7.82 ± 4.92	6.82 ± 4.11	0.295
Neutrophils (%)	66.93 ± 28.07	68.05 ± 29.36	65.09 ± 26.08	0.153
Lymphocytes ($\times 10^9$/L)	1.29 ± 2.64	1.48 ± 3.33	0.98 ± 0.45	0.301
Hemoglobin (g/L)	134.60 ± 16.69	135.56 ± 17.26	133.03 ± 15.81	0.463
Platelets ($\times 10^9$/L)	202.40 ± 83.57	188.89 ± 70.71	224.45 ± 98.18	0.127
Total bilirubin (μmol/L)	12.98 ± 9.80	14.82 ± 11.65	9.97 ± 4.29	0.040
Direct bilirubin (μmol/L)	6.34 ± 6.31	7.39 ± 7.67	4.63 ± 2.18	0.027
ALT (μmol/L)	29.55 ± 33.70	35.32 ± 40.97	20.13 ± 11.17	0.005
AST (μmol/L)	28.10 ± 17.89	32.77 ± 20.77	20.47 ± 7.01	<0.001
ALP (U/L)	76.06 ± 69.30	79.59 ± 5.29	70.26 ± 34.14	0.867
Albumin (g/L)	38.44 ± 5.68	37.59 ± 5.29	39.83 ± 6.08	0.055
Serum creatinine (μmol/L)	87.50 ± 96.72	80.65 ± 38.98	98.68 ± 149.38	0.887
INR	1.10 ± 1.14	0.99 ± 0.07	1.29 ± 1.82	0.496
D-dimer (ng/ml)	1049.34 ± 4,406.81	1384.48 ± 5,577.97	502.53 ± 412.87	0.204
Infection-related biomarkers				
Procalcitonin (ng/ml)	0.12 ± 0.24	0.16 ± 0.29	0.06 ± 0.10	<0.001
Erythrocyte sedimentation rate (mm/h)	43.76 ± 27.95	45.42 ± 25.82	41.05 ± 31.30	0.323
Serum ferritin (ng/ml)	606.49 ± 754.97	641.64 ± 745.06	549.15 ± 777.46	0.478
C-reactive protein (mg/L)	32.00 ± 35.94	33.95 ± 33.44	28.81 ± 39.95	0.053

Data expressed as n (%) and mean ± SD. HBV, hepatitis B virus; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; INR, International normalized ratio.

IL-4 was elevated in convalescent patients within the first week after discharge ($p = 0.036$) and continued to stay high at week 2 ($p = 0.022$) (**Figure 3**), even though IL-4 had no significant fluctuation through the viral replication phase as previously reported (1). IL-4 plays a key role in promoting naïve T cells to develop into Th2-like cells (18) and immunoglobulin class-switching from IgM to IgE and IgG (19). The observed elevation of IL-4 might suggest that there is a differential set of regulatory T cell and B cell immune responses occurring in early convalescence of COVID-19 patients.

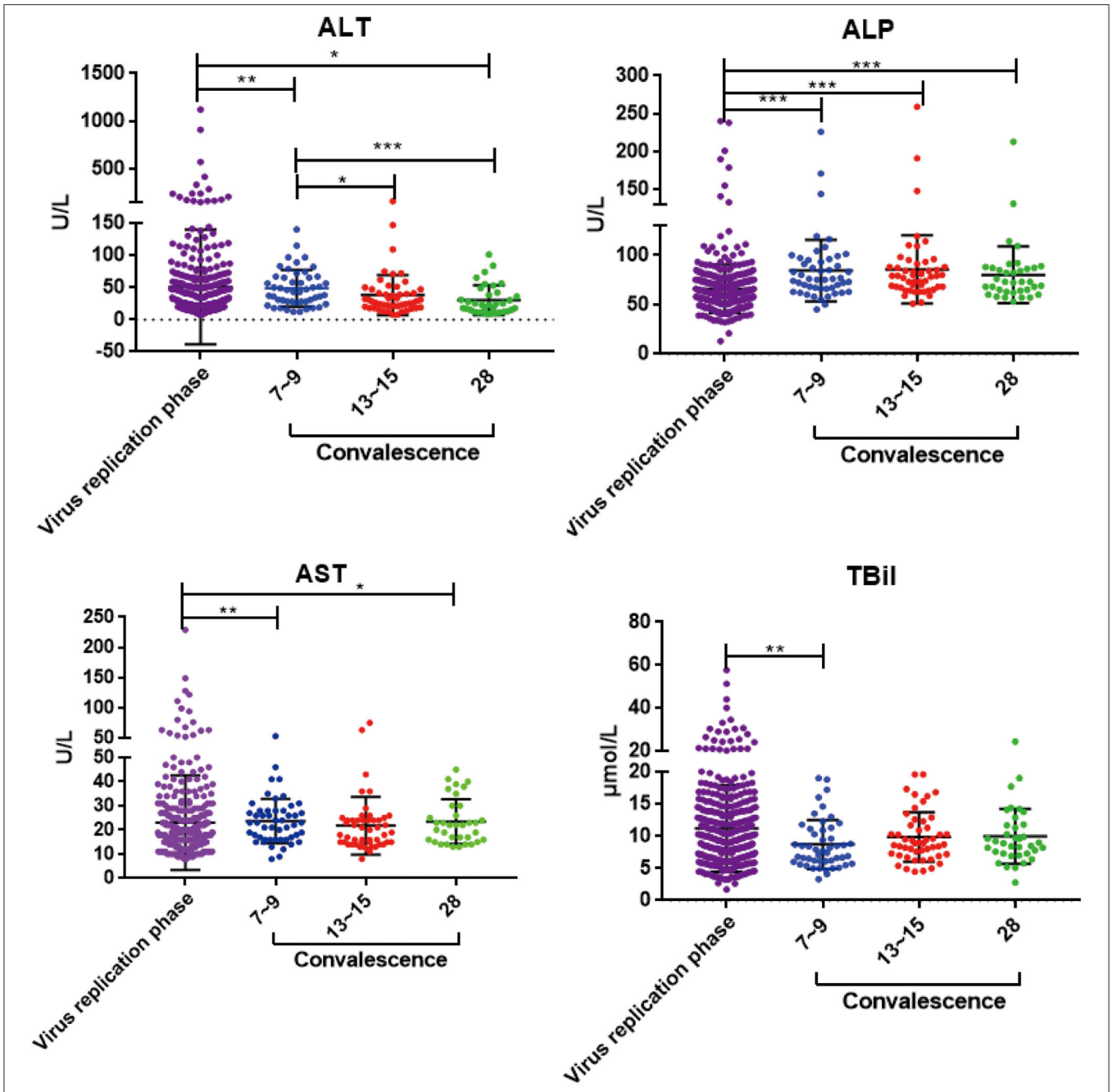

FIGURE 2 | The dynamic changes of ALT, AST, ALP, and TBiL in virus replication phase and early convalescence. Levels of significance: *p*-value: ***, <0.001; **, <0.01; *, <0.05.

Antiviral cytokines IFN-γ and TNF-α continued to stay at high levels as patients transitioned from the viral replication phase to convalescence, whereas IL-6 and IL-10 gradually declined and normalized (**Figure 3**). In particular, the serum levels of both IFN-γ and TNF-α showed meaningful increases above normal ranges from weeks 1 to 2 after hospital discharge, suggesting an additional boost of T cell immune responses (IFN-γ: 10.98 to 14.33 pg/ml; TNF-α: 23.27 to 37.96 pg/ml; **Supplementary Table 4**). In contrast, IL-2 levels remained within normal ranges throughout the course of the study (**Figure 3**).

Plasma Metabolomic Alternations Associated With Viral Replication and Convalescent Phases

Metabolites were extracted from plasma, and UPLC-QTOF MS analysis was performed for metabolomic analysis. From a total of

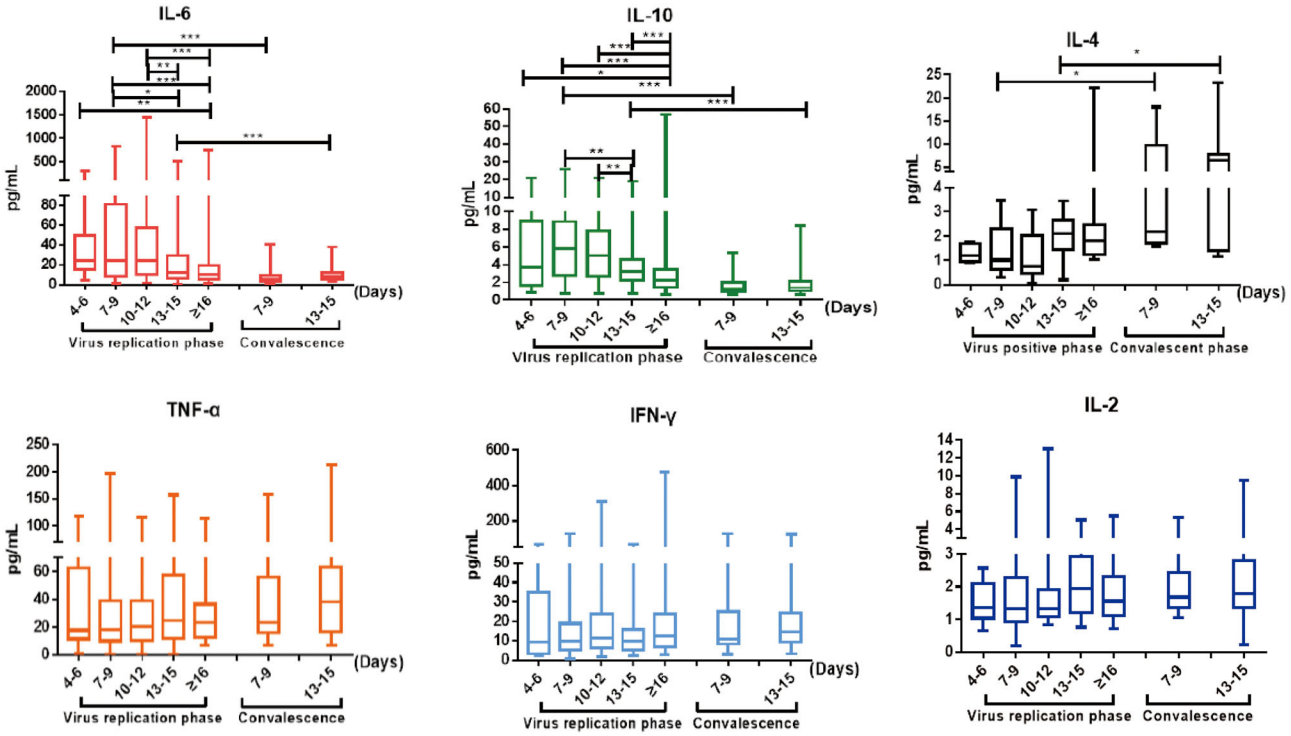

FIGURE 3 | The dynamic changes of IL-6, IL-10, IL-4, TNF-α, IFN-γ, and IL-2 in virus replication phase and early convalescence. Levels of significance: *p*-value: ***, <0.001; **, <0.01; *, <0.05.

2,077 metabolic features detected in metabolomic analysis, 243 were selected based on the following selection criteria: absolute log2 FC > 1; *p*-value < 0.05; projection variable VIP > 1; the area of ROC curve > 0.8. Among them, in comparison with replication phase, 50 metabolites showed differential profiles in convalescent patients, mostly with upregulation back toward the normal levels (**Figures 4A–C**), demonstrating that there was a significant change in metabolites during recovery from COVID-19. Using the results obtained in the viral replication phase/convalescence comparison as a reference, we ranked metabolites from the highest fold change value to the lowest value and drew a Cleveland plot showing fold changes of each metabolite across the three pairwise comparisons (**Figure 4D**). Among the three groups, the metabolites in the viral replication phase notably decreased.

As shown in **Figure 4E**, pathway analysis identified changes in 18 pathways during convalescence (**Supplementary Table 8**). Three pathways, namely, linoleic acid metabolism, biosynthesis of unsaturated fatty acids, and sphingolipid metabolism, were significantly altered. They included 32 downregulated metabolites, such as triglycerides, decanoylcarnitine, and diglycerides, and 43 upregulated metabolites, such as sphingosine 1-phosphate and cholic acid (**Supplementary Figure 7**). Notably, many metabolites returned to the levels observed in healthy controls (**Supplementary Figure 6**). Together, the metabolomic analysis indicated that discharged patients continued to recover from the physiological impacts of COVID-19.

Metabolomic Association of Inflammatory Cytokines With Liver Functions

Since liver injury from SARS-CoV-2 was associated with the extent of cytokine expression (**Supplementary Table 6**), we examined the interaction between metabolic changes of metabolites and cytokines on liver functions. As shown in **Figure 5** and **Supplementary Figures 4**, **5**, most of the upregulated metabolites in the convalescent stage were negatively correlated with IL-6, IL-10, ALT, and TBiL, while downregulated metabolites were positively correlated with IL-4. There were 28 metabolites, including amino acids, glycerophospholipids, ceramides, and unsaturated fatty acids, which were significantly correlated with cytokines (IL-4, IL-6, and IL-10) ($p < 0.05$). In addition, 10 metabolites, including amino acids, glycerophospholipids, steroids, and steroid derivatives, were significantly correlated with liver function indicators (ALT and TBiL) ($p < 0.05$) (**Supplementary Figures 4**, **5**).

DISCUSSION

In this study, we aimed to analyze the liver function repair and cytokine profiles during convalescence of COVID-19, which has barely been characterized. Cytokine storm and direct infection of the liver has been suggested to contribute to liver injury, and in some cases, liver failure in patients with COVID-19 (8, 9). Our results supported the notion that liver injury is a common clinical

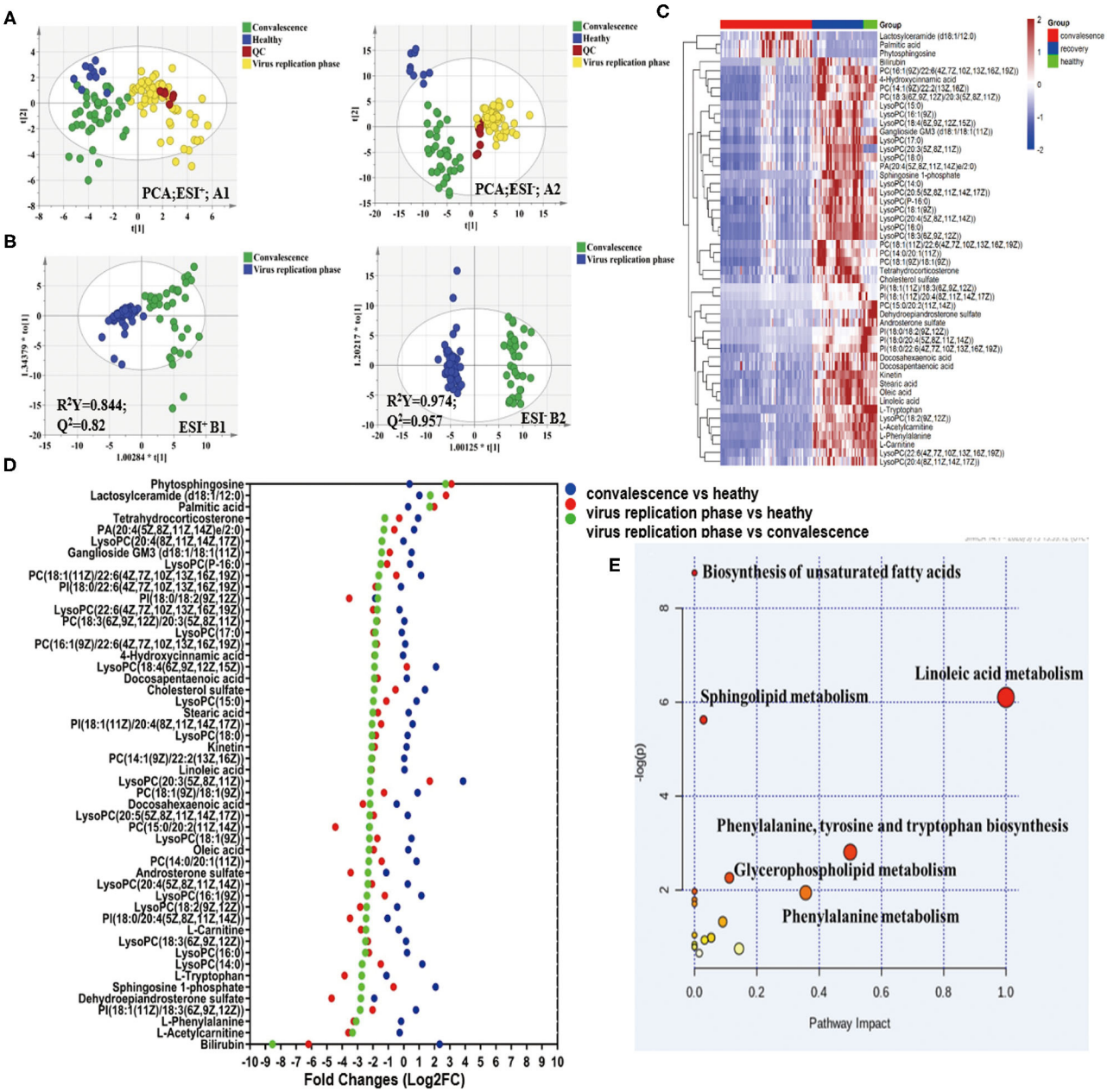

FIGURE 4 | Metabolomic profiling of COVID-19 patient plasma. **(A)** Principal components analysis of the metabolites across the three study groups based on the data of ESI+ and ESI– modes. Colors display the subjects from different groups (blue, normal controls; yellow, virus replication group; green, convalescent group; and red, QC samples). **(B)** OPLS-DA plot of COVID-19 patients in virus replication phase and early convalescence. **(C)** Heatmap of 50 selected metabolites, the colors changing from red (upregulated metabolites) to blue (downregulated metabolites). **(D)** Cleveland plots. Fifty metabolites are ranked according to their fold changes in the virus replication group vs. convalescent group (the highest fold changes on the top; the lowest on the bottom). Fold changes in the virus replication group vs. normal controls and the convalescent group vs. normal controls are also shown. **(E)** MetPA. Pathway analysis was performed using MetaboAnalyst 4.0 for the 50 differential metabolites. The color and size of each circle are based on its *p*-value and pathway impact value, respectively.

feature of SARS-CoV-2 infection, and we observed the active recovery of liver functions during the early convalescent stage of COVID-19. Importantly, serum ALT level decreased within the first week after discharge ($p < 0.01$) and continued to significantly decline through the second ($p < 0.05$) and fourth weeks ($p < 0.001$) (**Figure 2**). Significant shifts of AST, ALP, and TBiL levels toward the normal range also occurred (**Figure 2**), indicating the active recovery of liver functions during early convalescence.

Pro-inflammatory cytokines, such as TNF-α and IL-6, are primarily involved in the promotion of inflammatory processes

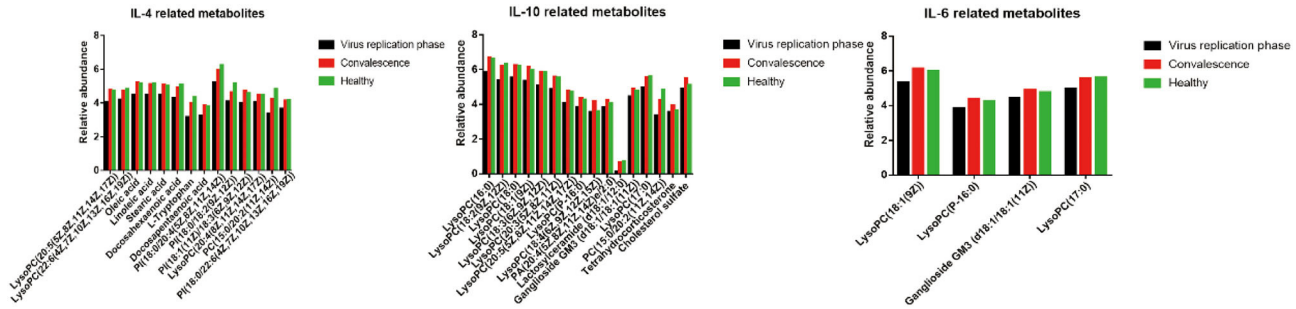

FIGURE 5 | Change of expression level metabolites related to the cytokines.

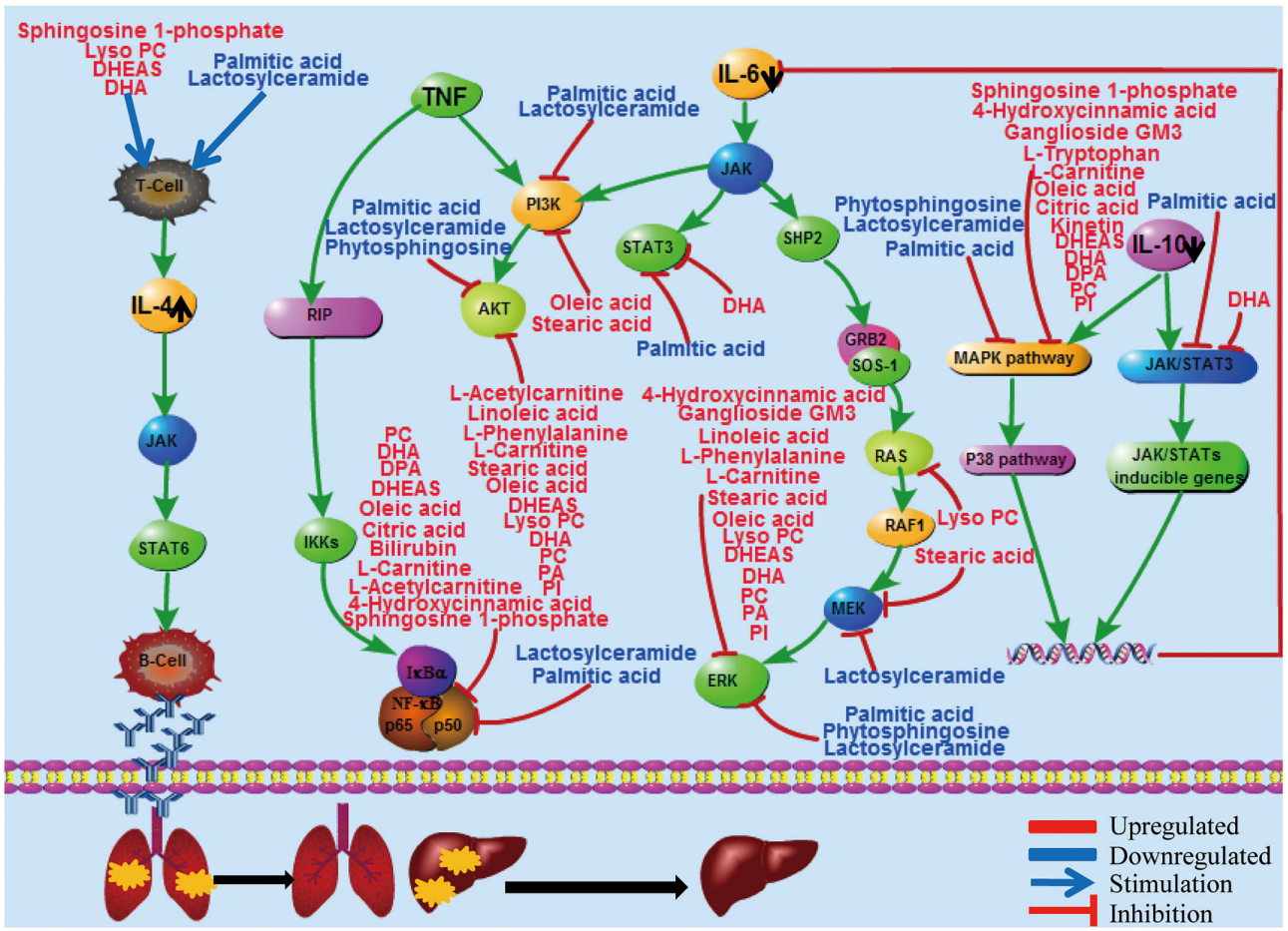

FIGURE 6 | Diagram to illustrate potential relationships among liver injury repair, inflammatory cytokine reactions, and metabolic changes in early convalescence. The red color represents upregulated metabolites and blue represents downregulated metabolites. The black arrow represents the changes of cytokines in early convalescence compared to virus replication phase. Lyso PC, lysophosphatidylcholines; PC, phosphatidylcholine; DHEAS, dehydroepiandrosterone sulfate; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; PA, phosphatidic acid; PI: phosphatidylinositol.

and have an important role in liver injury (20). Cytokine profile analysis confirmed that both IL-6 and IL-10 became significantly elevated ($p < 0.05$) during the early viral replication phase and then gradually declined into the normal range as viral RNA became undetectable (**Figure 3**). IL-6 and IL-10 levels were higher in patients with abnormal liver function than in those with normal liver function (**Supplementary Table 5**). Furthermore, after discharge from hospital, liver function

normalized during the first 2 weeks of convalescence, and both inflammation-regulating cytokines continued to decline significantly ($p < 0.05$), indicating clinical improvement.

We found that immune response-stimulating cytokine IL-4, but not IL-2, was significantly elevated during the first 2 weeks of convalescence. This was a surprising observation, as no significant fluctuation of IL-4 was seen during the viral replication phase (1), which was also confirmed in our study. IL-4 is known to regulate a variety of immune responses, including differentiation of naïve T cell into Th2 cells, and immunoglobulin class switching to IgG1 and IgE in B cells. For example, IL-4 could signal through IL-4Rα to trigger specialized macrophage activation, promoting the mitigation of helminthic infection and tissue repair in the liver and lung (21), or reduce the production of C-reactive protein (CRP) by human primary hepatocytes (22). Furthermore, IL-4 polymorphism has also been associated with an increased risk of liver disease (23) and severe respiratory syncytial virus (RSV) infection (24). All things considered, it is reasonable to postulate that an increase of IL-4 levels may play an important role in the convalescence of COVID-19 through either T cells, B cells, or other type 2 immunity-associated cells, such as macrophages.

Furthermore, abnormal liver function became normalized during the first 2 weeks of convalescence (**Figure 2**). Additionally, the antiviral cytokines IFN-γ and TNF-α, in contrast to the decline of IL-6 and IL-10, were observed to significantly increase during convalescence of those patients (**Figure 3** and **Supplementary Table 4**). Both IL-4 and IFN-γ were shown to downregulate the expression of SARS coronavirus receptor angiotensin-converting enzyme 2 (ACE2), in order to inhibit viral infection (25). Together, these clinical features further support the notion that type 2 immunity may contribute to liver and lung repair following injury by SARS-CoV-2. Finally, IL-13 has already been shown to share many biological functions with IL-4 and can inhibit IL-6 production through peripheral blood mononuclear cells (26). Therefore, it is recommended to include IL-13 in future cytokine profile analysis (27).

To further corroborate the association of liver injury and repair with cytokine profile changes, metabolic changes were determined in comparison with healthy controls. Metabolomic analysis shows significantly distinct profiles of metabolites and cytokines between the viral replication and the convalescent phases, including the liver-associated amino acid, TCA cycle, steroid hormone biosynthesis, and lipid metabolism (**Figure 6**). Remarkably, all of these metabolic changes appear to support liver repair. For example, during convalescence, the saturated fatty acid palmitic acid decreased, potentially mitigating the apoptosis of hepatocytes (28). In contrast, the unsaturated fatty acids docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) increased, possibly promoting liver repair by inhibiting ALT (29). An increase of tryptophan may reverse liver injury by maintaining protein synthesis activity (30). Importantly, during the preparation of our manuscript, part of the metabolomic profile we identified in the viral replication phase was also observed in other studies on SARS-CoV-2 (31, 32). These results are consistent with the general notion that cellular metabolites play key roles in resolving inflammation resulting from various viral infections, including H1N1 influenza (13, 14). Together, these metabolic changes strongly indicate that the liver is undergoing active recovery.

The immune system is particularly sensitive to metabolite availability (21). On the other hand, cytokines have been shown to mediate several metabolic changes *via* a pathway that is commonly initiated through their regulation of the immune system (11). Correlation analysis identified that many metabolites were associated with proinflammatory IL-6 and anti-inflammatory IL-10 cytokine levels, suggesting a potential cytokine-mediated metabolic dysfunction from COVID-19 pathogenesis (**Figure 6**). IL-6 can signal through three main pathways: JAK-STAT3, SHP-2-MAPK, and PI3K-AKT. At least 14 upregulated metabolites, such as oleic acid and DHA, and 3 downregulated metabolites, such as palmitic acid and lactosylceramide, were revealed, each of which are known to inhibit these three pathways (**Figure 6** and **Supplementary Material**). IL-10 is a major anti-inflammatory cytokine secreted by macrophages, and it exerts its effects *via* the JAK-STAT3 pathway (33). Palmitic acid and DHA levels were downregulated and upregulated, respectively, both of which may inhibit STAT3 signaling to promote liver repair. IL-4 is one of the best-known anti-inflammatory cytokines, mediating its biological roles predominantly *via* the JAK-STAT6 pathway (34). Our metabolic analysis identified four upregulated metabolites, including sphingosine 1-phosphate and DHA, and two downregulated metabolites, palmitic acid and lactosylceramide, which could stimulate T cells to produce IL-4 in early convalescence (**Figure 6**). The diagram in **Figure 6** summarizes the potential interactions among liver injury repair, inflammatory cytokine reactions, and metabolic changes.

CONCLUSION

In conclusion, by analyzing liver function repair and cytokine profiles in early convalescence of COVID-19, in tandem with the viral replication phase, we have identified that liver injury is a common clinical feature in COVID-19 patients, and it is associated with the increase of cytokine IL-6 and IL-10 levels. Importantly, serum levels of IL-4 were significantly elevated during early convalescence, suggesting a potentially important role of Th2 immune response in liver injury repair. The correlation of liver injury and repair with cytokines was further corroborated by metabolomic analysis, which identified a series of related biomarkers for the recovery of COVID-19 patients. Collectively, our new findings may have important implications in analysis of clinical manifestations and the potential therapeutic treatment of COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (2020 llT-7). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL wrote the manuscript and designed the overall scheme. XHe analyzed the medical record information of these patients. MD and LL were responsible for the collection and interpretation of data. XHu, XY, and LH were mainly responsible for the metabolomic analysis by UPLC-MS/MS. YH and QZ participated in cytokine measurement and analysis. JW and LZ were responsible for the collection of the blood samples and shipped the samples to the biosafety level 3 laboratory. XL designed the retrospective study. YQ was the general manager of the project and designed the research. All authors have read and approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.626633/full#supplementary-material

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Efficacy of Integrated Traditional Chinese and Western Medicine for Treating COVID-19: A Systematic Review and Meta-Analysis of RCTs

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Background: Integrated Chinese and Western medicine (integrated medicine) is routinely used in the treatment of coronavirus disease 2019 (COVID-19) in China. In this study, we undertook a systematic review and meta-analysis of published randomized controlled trials (RCTs) to evaluate the efficacy of integrated medicine therapy for patients with COVID-19.

Methods: In this meta-analysis, we searched PubMed, Embase, Web of Science, SinoMed, China National Knowledge Infrastructure (CNKI), Chongqing VIP (CQVIP), and Wanfang databases from inception to April 12, 2021, to identify RCTs of integrated medicine in the treatment of COVID-19. The quality of RCTs was assessed by the Cochrane risk of bias tool. RevMan v5.3 and Stata software packages were used for statistical analysis.

Results: Nineteen RCTs involving 1,853 patients met our inclusion criteria. Compared with patients treated by conventional Western medicine (CWM), patients treated by integrated medicine have a higher overall effective rate [$RR = 1.17$, 95% CI: (1.10, 1.26), $p < 0.00001$], fever disappearance rate [$RR = 1.25$, 95% CI: (1.04, 1.50), $p = 0.02$], fatigue disappearance rate [$RR = 1.28$, 95% CI: (1.00, 1.63), $p = 0.05$], and chest CT improvement rate [$RR = 1.24$, 95% CI: (1.14, 1.34), $p < 00001$]. Beneficial effects of the integrated medicine therapy were also seen in C-reactive protein (CRP) level [$WMD = -4.14$, 95% CI: (−6.38, −1.91), $p = 0.0003$] and white blood cell (WBC) count [WMD = 0.35, 95% CI: (0.11, 0.58), $p = 0.004$]. Subgroup analyses showed that, when the treatment time is <2 weeks, the effect of integrated medicine treatment is more obvious in improving the overall effective rate, clinical symptoms (fever, fatigue, and cough), the CRP level, and WBC count compared with that of the CWM treatment. For patients with severe and non-severe COVID-19, integrated medicine is more effective in improving fever and cough symptoms and WBC count than using CWM alone.

Conclusion: The results of the current meta-analysis suggested that the integrated medicine can improve the clinical symptoms, chest CT and infection indicators of COVID-19 patients. Even if the treatment time is <2 weeks, the effect of

integrated medicine in improving symptoms is more obvious compared with the treatment of CWM. However, the results should be interpreted cautiously due to the heterogeneity among the studies.

Keywords: integrated Chinese and Western medicine, COVID-19, efficacy, systematic review, meta-analysis

INTRODUCTION

Since the occurrence of the novel coronavirus pneumonia (NCP) in December 2019, the situation has increasingly become severe, and the disease continues to spread, which has had a significant impact on health and lives of people (1). Coronavirus disease 2019 (COVID-19) is a highly contagious viral pneumonia caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (2). People are usually susceptible to SARS-CoV-2, and there are different clinical manifestations (3). Mild symptoms usually include fever, dry cough, diarrhea, and fatigue. Patients with severe symptoms will rapidly develop acute respiratory distress syndrome (ARDS), multiple organ failure (MODS), and even death (3, 4). Globally, SARS-CoV-2 has infected more than 100 million people and has claimed 3.68 million lives worldwide but continues to cause effect, according to a report from the World Health Organization (as of 5:18 p.m. CEST, June 3, 2021) (5). Many COVID-19 vaccines have been developed at an unprecedented rate to prevent the occurrence of COVID-19 (5). However, apart from conventional Western medicines (CWMs), such as antiviral, antibacterial, expectorants, and bronchodilators, there is no specific drug for SARS-CoV-2, and COVID-19-targeting inhibitors are still under development (6). Given the complexity of the COVID-19, we should make more efforts to understand the pathophysiology of this new disease and look for alternative therapies that are novel, safe, and effective.

It is worth noting that the third edition of the COVID-19 diagnosis and treatment plan edited by the National Health Commission of China proposed the application of Chinese medicine (7). Traditional Chinese medicine (TCM) learns from the anti-epidemic experience accumulated in traditional medicine and may prevent the occurrence and development of diseases. Studies have shown that TCM has the characteristics of multicomponents acting on multitargets at multipathways and with broad-spectrum antiviral, anti-inflammatory activity, immunomodulatory, and organ-protective effects in the treatment of COVID-19 (8, 9). The integrated TCM and CWM (hereafter referred to as "integrated medicine") therapy as a key component of the COVID-19 treatment regimen effectively prevented the spread of the COVID-19 epidemic in China (7).

Previously published meta-analysis found that Chinese herbal medicines or integrated medicine therapy had better effects in the treatment of COVID-19, but conclusions were limited by the relatively high heterogeneity and low accuracy of the data included (10–14). With the increase in the publications on the latest COVID-19 research, in order to test the efficacy of integrated medicine to the greatest extent, we conducted a systematic review and meta-analysis to objectively evaluate the effectiveness of integrated medicine in the treatment of COVID-19.

MATERIALS AND METHODS

Systematic Search

We conducted a comprehensive search of seven different electronic databases, namely, PubMed, Excerpta Medica Database (Embase), Web of Science, SinoMed, China National Knowledge Infrastructure (CNKI), Chongqing VIP (CQVIP), and Wanfang Databases from inception to April 12, 2021, for randomized controlled trials (RCTs) investigating the role of integrated medicine in patients with COVID-19. We developed the search strategy with the assistance of an expert medical librarian, and the search terms were as follows: COVID-19, coronavirus disease 2019, SARS-CoV-2, novel coronavirus pneumonia, NCP, novel coronavirus, Chinese herbal medicine, traditional Chinese medicine, classical Chinese herbal formulas, Chinese herb, and medicine. We used the Medical Subject Headings database for the identification of synonyms and combined them with keywords as a search strategy (**Supplementary Appendix**). We also checked the references in the list of eligible publications for other related articles. No limits were set on language, publication year, or type of publication.

Study Inclusion Criteria

The inclusion criteria were constructed in accordance with the principle of PICOS. (1) Participants: patients with COVID-19 were included in the study, and the gender, age, and nationality of the patient were not restricted. (2) Type of interventions: the treatment group was treated with integrated TCM and CWM. The dosage forms of TCM included decoction, tablet, pill, powder, granule, capsule, cream, oral liquid, plaster, and injection. The CWM treatment in both the treatment group and the control group had to be the same in terms of usage and dosage. (3) Type of controls: the control group was treated with CWM, including antiviral, antibacterial, antitussive, expectorant, and antiasthmatic drugs and symptomatic supportive treatment. (4) Outcomes: the primary outcome measure was: overall effective rate; the secondary outcome measures were fever disappearance rate, fatigue disappearance rate, cough disappearance rate, chest CT improvement rate, C-reactive protein (CRP) (mg/L), erythrocyte sedimentation rate (ESR), procalcitonin (PCT) (ng/L), white blood cell (WBC) count (10^9/L), and lymphocyte (LY) count (10^9/L). (5) Study design: RCTs were eligible.

Overall effective rate = (clinical recovery cases + significantly effective cases + effective cases)/total cases × 100%. According to "Evaluation standard of curative effects of traditional Chinese medicine on COVID-19" (15), the curative effect is divided into: ① Clinical recovery: clinical symptoms and signs of TCM disappeared or basically disappeared, and the score decreased

by ≥90%; ② significantly effective: TCM clinical symptoms, signs improved significantly, 70% ≤ score decreased <90%; ③ effective: TCM clinical symptoms and signs were improved, 30% ≤ score decreased <70%; ④ invalid : TCM clinical symptoms, signs were not significantly improved, or even worse, scores decreased <30%. Score changes refer to "Evaluation standard of curative effects of traditional Chinese medicine on COVID-19" (15), including symptoms such as fever, cough, and fatigue and are scored according to the severity.

Study Elimination Criteria

The elimination criteria were as follows: (1) duplicate studies; (2) studies in which the experimental group was subjected to other TCM therapies, such as acupuncture, moxibustion, cupping, massage, qigong, and taiji therapy, in addition to the CWM; (3) studies in which the control group was treated with a form of TCM or integrated medicine; (4) studies in which data could not be extracted.

Data Extraction and Management

After removing duplicates, two reviewers (BY and YB) independently screened the titles and abstracts of each study in accordance with the inclusion and exclusion criteria. The full texts were subsequently obtained and evaluated by two reviewers (BY and YB) separately. Any discrepancies were resolved by consensus through discussion with the corresponding author (GF). Two reviewers (BY and YB) extracted the data from the included studies independently and double-checked the data using prepared data extraction forms, including authors, publication date, journal, title, sample size, study design, mean age, diagnostic criteria, subtypes of COVID-19, detailed information on methodology, intervention details, duration of treatment, and outcome measures.

All included pieces of literature were managed by Endnote (Version X8). When relevant details were insufficiently reported in studies, the authors were contacted by e-mail or phone if necessary.

Quality Assessment of Included Studies

In accordance with the Cochrane Collaboration's update tool for assessing the risk of bias (16), two reviewers (BY and YB) assessed the quality of the studies independently, and disagreements were resolved by discussion or consultation with the corresponding author (GF). The evaluation of the methodological quality of each item included random sequence generation, allocation concealment, blinding of outcome assessment, blinding of participants and personnel, incomplete outcome data, selective outcome reporting, and other forms of bias.

Statistical Analysis

Based on the Cochrane Handbook for Systematic Reviews of Interventions, the SDs of the change from baseline to post-therapy were calculated using the following formula ($R = 0.5$):

$$SD(C) = \sqrt{SD\,(B) \wedge 2 + \ SD\,(F) \wedge 2 - \ (2{*}R\,{*}SD(B) * SD(F))}$$

where SD (B), SD (F), and SD (C) represent the SDs of the baseline, final, and change, respectively; from continuous data, we took a weighted mean difference (WMD) with 95% confidence interval (CI), while dichotomous data were expressed as relative risk (RR) with 95% CI. Statistical heterogeneity was tested by the χ^2-based Cochran Q statistic and I^2 statistic. If I^2 was ≤50% and $p > 0.10$, we used a fixed-effects model to pool the estimations across the studies, where, I^2 score >50% or $p \leq 0.10$ indicates important heterogeneity. A random-effects statistical model was used when data showed significant heterogeneity.

As long as there is significant heterogeneity, we search for potential sources of heterogeneity. For example, if the results of a study are completely out of the range of the other studies, then we will look for possible reasons to explain the difference and conduct a sensitivity analysis to investigate the causes of heterogeneity in methodological quality. Subgroup analysis was planned to assess the impact on heterogeneity from different clinical trials where possible, including studies with treatment duration (<2 weeks and ≥2 weeks), subtypes of COVID-19 (severe type of COVID-19 and non-severe types of COVID-19), and risk bias for sequence generation (low risk for sequence generation and unclear risk for sequence generation).

Moreover, potential publication bias was assessed by Begg's tests. Results were considered as statistically significant for $p < 0.05$. All statistical analyses were performed using Stata (Version 14.2, Stata Corporation, College Station, TX, United States) and RevMan (Version 5.3.0, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014).

RESULTS

Search Results and Study Characteristics

As shown in **Figure 1**, the search of the electronic databases and reference lists yielded a total of 10,122 potentially relevant citations, of which 6,924 were duplicates and 3,042 were excluded after screening the titles and abstracts. We assessed 153 full-text articles and included 19 RCTs in the review (17–35). Finally, a total of 19 studies met the inclusion criteria and were included for qualitative synthesis and systematic review. Baseline characteristics of the included studies are depicted in **Table 1**.

The included studies were conducted in China between 2020 and 2021. Among them, 15 studies were published in Chinese literature and 4 in English literature. In these studies, 1,853 participants were included; the sample sizes ranged from 6 to 147, and the follow-up duration ranged from 5 to 21 days. The COVID-19 subtypes of the participants included in this study mainly include four types, such as mild, ordinary, severe, and critical, not including rehabilitation patients. The treatment groups in the included studies were treated with integrated medicine, while the control groups were treated with CWM.

Risk of Bias

According to the prespecified criteria, in the 19 included studies, the participants were randomly assigned to the integrated medicine group or CWM group; only four studies (22, 25, 30, 32) did not describe the method of randomization and were categorized as unclear risk. Except for two studies (33, 34), none of the studies provided information about allocation concealment and were categorized as an unclear risk in selection

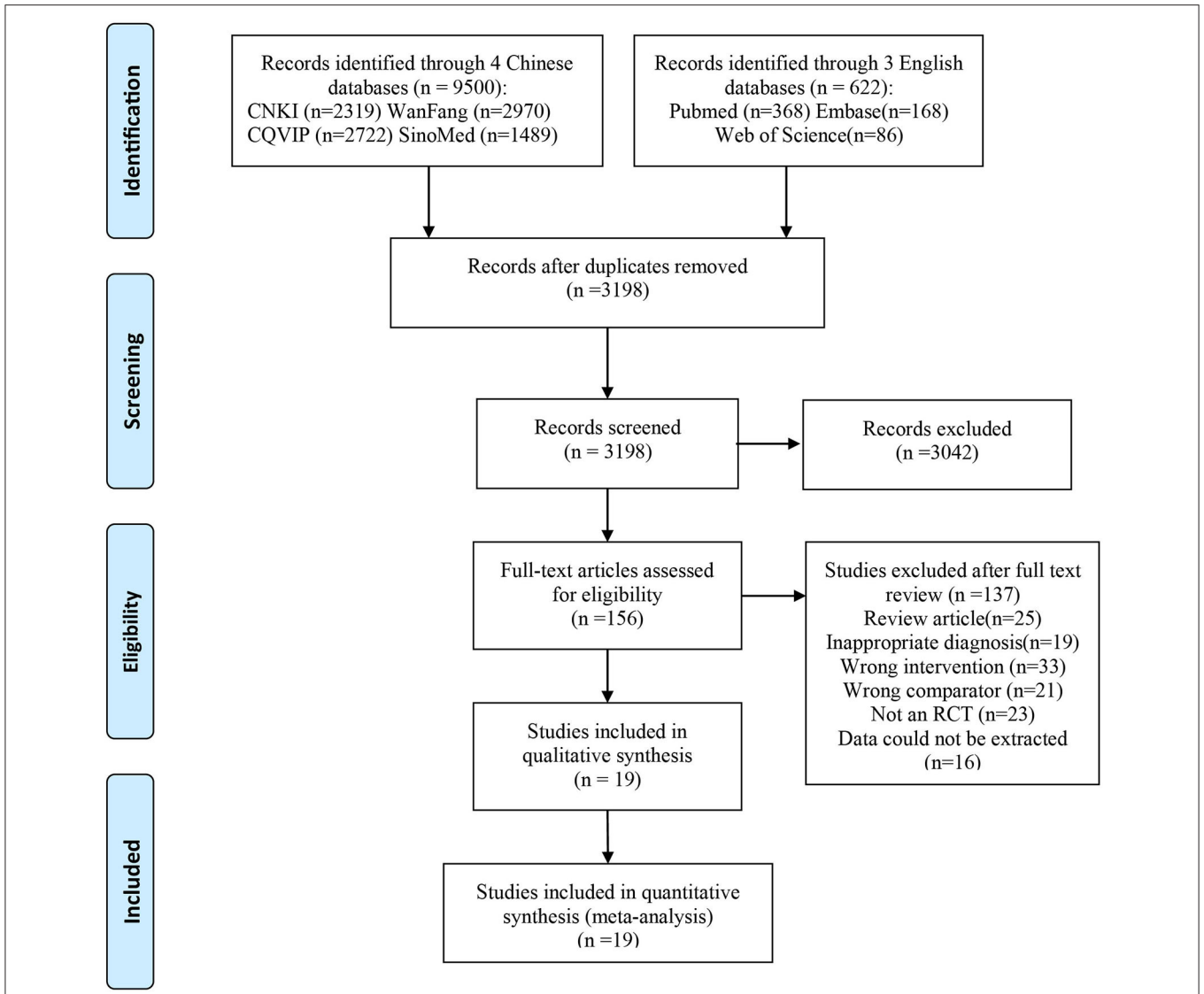


FIGURE 1 | Flow diagram of study selection.

bias. The most common weaknesses in the study methods were that none of the studies described blinding of outcome assessment, so they were evaluated as an unclear risk in detection bias. Furthermore, drugs were administered in different ways in the treatment and control groups in all the studies, and blinding in participants and personnel was easily broken. Therefore, all the studies were categorized as high risk in performance bias. Fifteen studies had incomplete outcome data and no follow-up, so they were classified as studies with unclear risk in attrition bias, while the remaining studies (17, 33–35) were classified as low risk because they had reported exclusions and the number of cases. Only one study (23) was classified as high risk in reporting bias, since the study did not report all the outcome indicators described in the methodological section; five studies (17, 24, 33–35) were classified as low risk because they have been clinically registered in the Chinese Clinical Trial Registry (ChiCTR) or USA National Institutes of Health Register (ClinicalTrials.gov) and had a registration number; and the remaining 13 studies were categorized as unclear risk in reporting bias since it is unclear whether an RCT is registered. The risk of other bias was considered high in the seven studies (17, 18, 20, 25–27, 35) because the drug dose of the control group is unknown, while other studies had complete data and no other bias. The summaries of the risk of bias assessment are illustrated in **Figure 2**.

Outcome of Integrated Medicine for COVID-19

Primary Outcome Measure

Overall Effective Rate

Six studies evaluated the effects of integrated medicine on the overall effective rate (17, 21, 22, 25, 26, 29). There were 301 patients in the integrated medicine group and 301 in the CWM

TABLE 1 | Baseline characteristics of the included studies.

Study author	Study design	Registration number	Types of COVID-19	Sample size		Intervention	Comparison	Duration (days)	Outcomes
				(I)	(C)				
1. Ai et al. (18)	Single-site RCT	-	Ordinary	33	34	Pneumonia No.1 prescription, pneumonia recovery formula (100 ml, two times/day) + CWM	CWM including A, B, C, D	12	②③④
2. Ding et al. (19)	Single-site RCT	-	Mild, ordinary, severe, critical	51	49	Qingfei Touxie Fuzheng recipe (150 ml, two times/day) + CWM	CWM including A, B, C, D	10	②④⑤⑥⑦
3. Duan et al. (20)	Single-site RCT	-	Mild	82	41	Jinhua Qinggan granules (2 bag, three times/day) + CWM	CWM including A, B	5	②③④
4. Fu et al.① (22)	Single-site RCT	-	Mild, ordinary	32	33	Toujie Quwen granule (one dose, two times/day) + CWM	CWM including A, B, C, D	10	①⑤⑥⑧⑨⑩
5. Fu et al.② (21)	Single-site RCT	-	Ordinary	37	36	Toujie Quwen granule (one dose, two times/day) + CWM	CWM including A, C, D	15	①⑨
6. Li and Zhang (25)	Single-site RCT	-	Severe	6	6	Qingfei Paidu decoction (one dose, two times/day) + CWM	CWM including A, B, C, D	6	①⑨
7. Qiu et al. (27)	Single-site RCT	-	Ordinary	25	25	Maxing Xuanfei Jiedu decoction (150 ml, three times/day) + CWM	CWM including A	10	⑤
8. Yu et al. (31)	Single-site RCT	-	Mild, ordinary	147	148	Lianhua Qingwen granule (30 mg, three times/day) + CWM	CWM including A, B, C	7	⑤⑥⑧⑨⑩
9. Zhang et al. (32)	Single-site RCT	-	Ordinary	80	40	Honeysuckle oral liquid (50 ml, three times/day) + CWM	CWM including A, C, D	10	②③④
10. Hu et al. (17)	Multiple-site RCT	ChiCTR 2000029434	Ordinary	142	142	Lianhua Qingwen granule (4 capsules, three times/day) + CWM	CWM including A, B, D, E	14	①⑤
11. He et al. (23)	Single-site RCT	–	Mild	36	35	Buzhong Yiqi decoction (one dose, two times/day) + CWM	CWM including A	10	⑦
12. Jin et al. (24)	Multiple-site RCT	ChiCTR 2000029558	Ordinary	20	18	Compound Yin Chai granule + Qingqiao detoxification granule (15 g, four times/day) + CWM	CWM including A, B, C, D	21	②③④⑤⑥⑧⑨
13. Liu et al. (26)	Single-site RCT	-	Mild	44	44	Lianhua Qingwen capsule (1.4 g, three times/day) + pneumonia 2 concerted prescription (one dose, two times/day) + CWM	CWM including A	21	①
14. Sun et al. (28)	Single-site RCT	-	Mild, ordinary	32	25	Lianhua Qingke granule (1 bag, two times/day) + CWM	CWM including A, C, D	14	②③④⑤
15. Wang et al.① (29)	Single-site RCT	-	Ordinary	40	40	Shengmai powder + Shenling Baizhu powder (200 ml, two times/day) + CWM	CWM including A, C, D	-	①⑤⑥⑦⑨⑩
16. Wang et al.② (30)	Single-site RCT	-	Ordinary	70	70	Qingfei Paidu decoction (100 ml, 2 times/day) + CWM	CWM including A, B, C, D	10	⑥⑨
17. Wang et al. (33)	Single-site RCT	NCT 04251871	-	24	23	Keguan-1 (19.4g, two times/day) + CWM	CWM including A, D	14	⑤③
18. Wu et al. (35)	Single-site RCT	ChiCTR 2000034795	Mild, ordinary, severe	22	20	Xuanfei Baidu decoction (200 ml, two times/day) + CWM	CWM	7	②③④
19. Xiao et al. (34)	Single-site RCT	ChiCTR 2000029601	-	58	63	Lianhua Qingwen granule (1 bag, three times/day) + CWM	CWM including A, B	14	③④

T, treatment group; C, control group; RCT, randomized controlled trial; CWM, conventional Western medicine; A, antiviral medications; B, antimicrobial medication; C, symptomatic therapies (expectorant, antitussive drugs); D, supportive therapy (gamma globulin, methylprednisolone); E, immunosuppressant; ① Overall effective rate; ② Fever disappearance rate; ③ Fatigue disappearance rate; ④ Cough disappearance rate; ⑤ Chest CT improvement rate; ⑥ C-reactive protein (CRP) (mg/L); ⑦ Erythrocyte sedimentation rate (ESR); ⑧ Procalcitonin (PCT) (ng/L); ⑨ White blood cell count (WBC) (10^9/L); ⑩ Lymphocyte count (LY) (10^9/L).

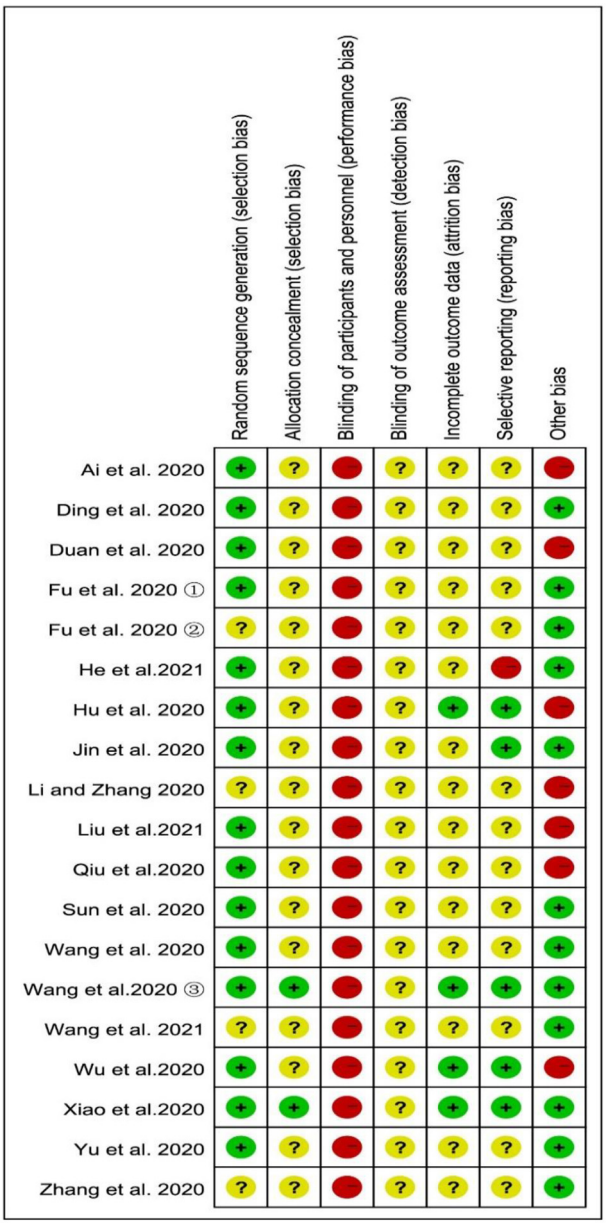


FIGURE 2 | Risk of bias summary.

group. Integrated medicine exhibited a significant improvement on the overall effective rate [$RR = 1.17$, 95% CI: (1.10, 1.26), $p < 0.00001$] (**Figure 3**).

Secondary Outcome Measures

Fever, Fatigue, and Cough Disappearance Rate

Seven studies (18–20, 24, 28, 32, 35) involving 521 patients reported the fever disappearance rate after treatment. Seven studies (18, 20, 24, 28, 32, 34, 35) involving 429 patients reported the fatigue disappearance rate after treatment. Eight studies (18–20, 24, 28, 32, 34, 35) including 606 participants reported the cough disappearance rate after treatment. Compared with patients treated with CWM, patients treated with integrated medicine have a higher fever disappearance rate [$RR = 1.25$, 95% CI: (1.04, 1.50), $p = 0.02$] (**Figure 4A**) and fatigue disappearance rate [$RR = 1.43$, 95% CI (1.17, 1.74), $p = 0.0004$] (**Figure 4B**). Besides, as for the cough disappearance rate, there were no significant differences between integrated medicine and CWM [$RR = 1.28$, 95% CI: (1.00, 1.63), $p = 0.05$] (**Figure 4C**). The pooled analysis showed no statistical heterogeneity among the included studies of fatigue disappearance rate ($p = 0.23$, $I^2 = 26\%$). However, significant heterogeneity was observed in cough disappearance rate ($p = 0.006$, $I^2 = 64\%$) and fever disappearance rate ($p = 0.06$, $I^2 = 51\%$).

Chest CT Improvement Rate

Nine studies (17, 19, 22, 24, 27, 28, 31, 33, 34) reported the comparison of chest CT improvement rate between integrated medicine treatment and CWM treatment, and no heterogeneity was observed ($p = 0.70$, $I^2 = 0\%$). There were 512 patients in the integrated medicine group and 504 in the CWM group. Meta-analysis suggested that chest CT improvement rate is significantly improved by integrated medicine treatment [$RR = 1.24$, 95% CI: (1.14, 1.34), $p < 00001$] (**Figure 5**).

CRP

Seven studies (19, 21, 22, 24, 29–31) evaluated the therapeutic effects of integrated medicine on the CRP level. There were 397 patients in integrated medicine group and 394 in the CWM group. The meta-analysis demonstrated that the integrated medicine was superior to the CWM in improving the CRP level [$WMD = -4.14$, 95% CI: $(-6.38, -1.91)$, $p = 0.0003$] (**Figure 6**). However, significant heterogeneity was observed among the included studies for CRP ($p < 0.0001$, $I^2 = 81\%$).

WBC

Seven studies (21, 22, 24, 25, 29–31) involving 703 patients were included to evaluate the efficacy of the integrated medicine on WBC count. Meta-analysis suggested that WBC count was significantly improved by integrated medicine treatment [$WMD = 0.35$, 95% CI: (0.11, 0.58), $p = 0.004$] (**Figure 7**), but the heterogeneity was high among the included studies for WBC ($p = 0.0003$, $I^2 = 76\%$).

ESR, PCT, and LY

Three studies (19, 23, 29) reported on the improvement of the ESR level, where two studies (19, 23) showed favorable effects of integrated medicine treatment for the ESR ($p < 0.05$), and the other study (29) reported no significance. Four studies (22, 24, 29, 31) evaluated the therapeutic effects of integrated medicine on the PCT level after the intervention, where two studies (22, 31) showed a positive effect toward integrated medicine treatment for the PCT ($p < 0.05$), and the other two studies (24, 29) reported no significance. Four studies (21, 22, 29, 31) reported the effect of integrated medicine on LY and showed favorable effects of integrated medicine treatment for LYs ($p < 0.05$).

Sensitivity Analysis

Significant heterogeneity was observed among the included studies for the fever disappearance rate ($I^2 = 51\%$), cough

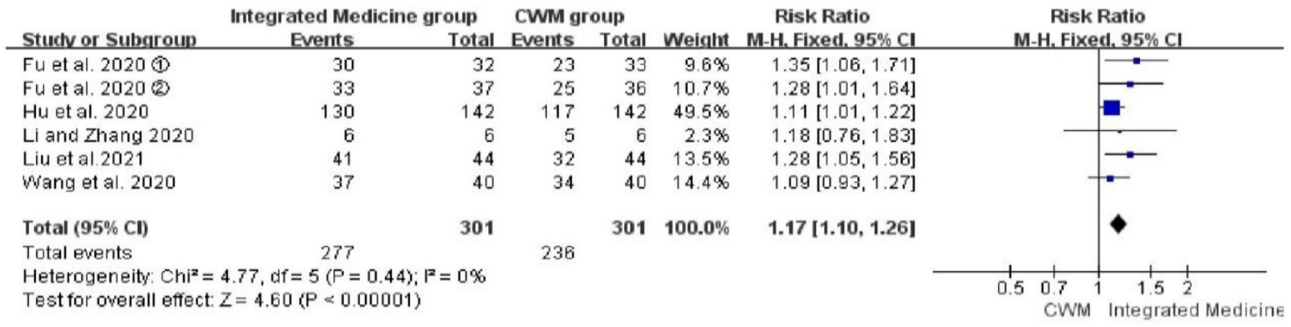


FIGURE 3 | Forest plot of the overall effective rate.

disappearance rate (I^2 = 64%), CRP (I^2 = 81%), and WBC (I^2 = 76%). Therefore, we conducted a sensitivity analysis to investigate the source of the heterogeneity. The study conducted by Xiao et al. did not mention the different subtypes of COVID-19 cases, and the heterogeneity may be caused by different degrees of severity of the disease in the participant. After excluding that study, the heterogeneity decreased significantly (I^2 = 0%), and the results showed that the cough disappearance rate was significantly improved by integrated medicine treatment [RR = 1.41, 95% CI: (1.18, 1.68), p = 0.0001] (**Supplementary Figure 1**). Sensitivity analysis shows that excluding any study for each result will not change the fever disappearance rate, CRP, and WBC results, indicating that the conclusion is reliable.

Subgroup Analysis

Subgroup Analysis Based on Treatment Duration

Subgroup analysis showed that the integrated medicine treatment significantly improved the overall effective rate, CRP, and WBC compared with CWM treatment, regardless of whether the treatment time exceeds 2 weeks ($p < 0.05$). The test for subgroup effects revealed that treatment duration-related subgroup differences were statistically significant in the CRP (p = 0.002, I^2 = 90.1%) but not statistically significant in the overall effective rate (p = 0.31, I^2 = 2.4%) and WBC (p = 0.19, I^2 = 41.4%) (**Supplementary Figure 2**).

Subgroup analysis also showed that the integrated medicine treatment significantly improved the rate of fever, fatigue, and cough disappearance on studies of treatment duration < 2 weeks (fever disappearance rate: five trials, RR 1.28, 95% CI 1.11 to 1.47; fatigue disappearance rate: four trials, RR 1.72, 95% CI 1.25 to 2.35; cough disappearance rate: five trials, RR 1.44, 95% CI 1.15 to 1.81). However, the test for subgroup effects revealed that treatment duration-related subgroup differences were not statistically significant (overall effective rate: $p = 0.31$, $I^2 = 2.4\%$; fever disappearance rate: $p = 0.48$, $I^2 = 0\%$; fatigue disappearance rate: $p = 0.06$, $I^2 = 70.8\%$; cough disappearance rate: $p = 0.05$, $I^2 = 73.4\%$) (**Supplementary Figure 3**).

Subgroup Analysis Based on Subtypes of COVID-19

In terms of the fatigue disappearance rate and overall effective rate, there was only one study on the type of severe and there was no difference between the groups. Subgroup analysis showed that the integrated medicine treatment significantly improved the overall effective rate, fatigue disappearance rate, and CRP on non-severe type of COVID-19 (overall effective rate: RR 1.17, 95% CI 1.10 to 1.26; fatigue disappearance rate: RR 1.56, 95% CI 1.18 to 2.07; CRP: WMD −3.53, 95% CI −4.31 to −2.76) (**Supplementary Figure 4**).

Subgroup analysis also showed that the integrated medicine treatment significantly increased the fever disappearance rate, cough disappearance rate, and WBC regardless of whether the type of COVID-19 is severe or non-severe as compared with the CWM treatment ($p < 0.05$). The test for subgroup effects revealed that COVID-19 type-related subgroup differences were statistically significant in the WBC ($p = 0.003$, $I^2 = 88.8\%$) but not statistically significant in the overall effective rate ($p = 0.97$, $I^2 = 0\%$), fever disappearance rate ($p = 0.45$, $I^2 = 0\%$), fatigue disappearance rate ($p = 0.65$, $I^2 = 0\%$), cough disappearance rate ($p = 0.89$, $I^2 = 0\%$), and CRP ($p = 0.30$, $I^2 = 8.7\%$) (**Supplementary Figure 5**).

Subgroup Analysis Based on Risk Bias for Sequence Generation

Subgroup analysis showed that the integrated medicine treatment significantly improved the overall effective rate, fever disappearance rate, fatigue disappearance rate, cough disappearance rate, CRP level, and WBC count on studies of low risk for sequence generation compared with CWM treatment ($p < 0.05$). However, the test for subgroup effects revealed that risk bias-related subgroup differences were statistically significant in the CRP ($p = 0.04$, $I^2 = 75.2\%$) but not statistically significant in overall effective rate ($p = 0.45$, $I^2 = 0\%$), fever disappearance rate ($p = 0.12$, $I^2 = 58.5\%$), fatigue disappearance rate ($p = 0.50$, $I^2 = 0\%$), cough disappearance rate ($p = 0.18$, $I^2 = 44.3\%$), and WBC ($p = 0.17$, $I^2 = 46.5\%$) (**Supplementary Figure 6**).

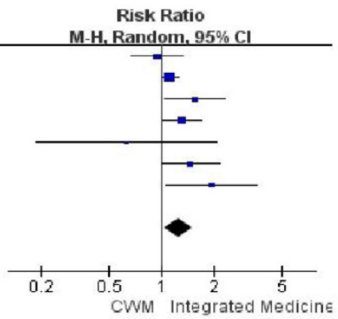
Evaluation of Publication Bias

Assessment of publication bias using Begg's test showed that there was no potential publication bias among the included trials (fever

A

Study or Subgroup	Integrated Medicine group Events	Total	CWM group Events	Total	Weight	Risk Ratio M-H, Random, 95% CI
Ai et al. 2020	21	33	23	34	15.2%	0.94 [0.66, 1.33]
Ding et al. 2020	50	51	43	49	29.8%	1.12 [1.00, 1.25]
Duan et al. 2020	53	82	17	41	13.0%	1.56 [1.05, 2.32]
Jin et al. 2020	18	18	15	20	19.7%	1.32 [1.01, 1.72]
Sun et al. 2020	4	32	5	25	2.2%	0.63 [0.19, 2.09]
Wu et al.2020	18	20	11	18	13.0%	1.47 [0.99, 2.19]
Zhang et al. 2020	13	30	15	68	7.2%	1.96 [1.07, 3.60]
Total (95% CI)		**266**		**255**	**100.0%**	**1.25 [1.04, 1.50]**
Total events	177		129			

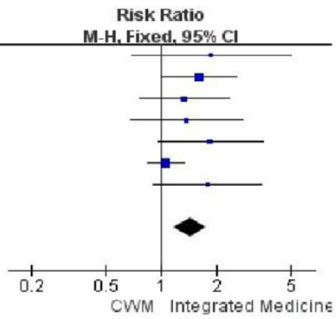
Heterogeneity: Tau² = 0.03; Chi² = 12.18, df = 6 (P = 0.06); I² = 51%
Test for overall effect: Z = 2.40 (P = 0.02)



B

Study or Subgroup	Integrated Medicine group Events	Total	CWM group Events	Total	Weight	Risk Ratio M-H, Fixed, 95% CI
Ai et al. 2020	9	33	5	34	6.1%	1.85 [0.69, 4.95]
Duan et al. 2020	45	82	14	41	23.0%	1.61 [1.01, 2.57]
Jin et al. 2020	12	18	10	20	11.7%	1.33 [0.77, 2.30]
Sun et al. 2020	14	32	8	25	11.1%	1.37 [0.68, 2.74]
Wu et al.2020	15	19	6	14	8.5%	1.84 [0.96, 3.52]
Xiao et al.2020	27	32	24	30	30.5%	1.05 [0.84, 1.33]
Zhang et al. 2020	9	21	14	58	9.2%	1.78 [0.91, 3.48]
Total (95% CI)		**237**		**222**	**100.0%**	**1.43 [1.17, 1.74]**
Total events	131		81			

Heterogeneity: Chi² = 8.15, df = 6 (P = 0.23); I² = 26%
Test for overall effect: Z = 3.53 (P = 0.0004)



C

Study or Subgroup	Integrated Medicine group Events	Total	CWM group Events	Total	Weight	Risk Ratio M-H, Random, 95% CI
Ai et al. 2020	15	33	9	34	8.4%	1.72 [0.88, 3.37]
Ding et al. 2020	38	51	28	49	17.0%	1.30 [0.97, 1.74]
Duan et al. 2020	41	82	12	41	11.2%	1.71 [1.01, 2.88]
Jin et al. 2020	14	18	13	20	13.9%	1.20 [0.80, 1.79]
Sun et al. 2020	29	32	16	25	16.4%	1.42 [1.03, 1.94]
Wu et al.2020	13	17	7	18	9.0%	1.97 [1.04, 3.72]
Xiao et al.2020	25	32	31	34	19.2%	0.86 [0.69, 1.06]
Zhang et al. 2020	8	80	6	40	4.9%	0.67 [0.25, 1.79]
Total (95% CI)		**345**		**261**	**100.0%**	**1.28 [1.00, 1.63]**
Total events	183		122			

Heterogeneity: Tau² = 0.07; Chi² = 19.70, df = 7 (P = 0.006); I² = 64%
Test for overall effect: Z = 1.93 (P = 0.05)

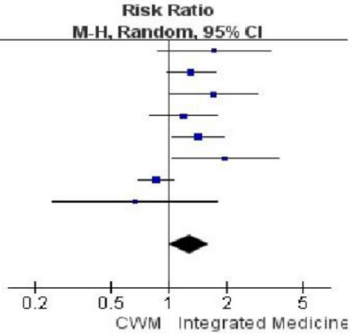


FIGURE 4 | Forest plot of fever disappearance rate **(A)**, fatigue disappearance rate **(B)** and cough disappearance rate **(C)**.

Study or Subgroup	Integrated Medicine group Events	Total	CWM group Events	Total	Weight	Risk Ratio M-H, Fixed, 95% CI
Ding et al. 2020	32	51	21	49	7.0%	1.46 [0.99, 2.15]
Fu et al. 2020 ①	16	32	13	33	4.2%	1.27 [0.73, 2.19]
Hu et al. 2020	119	142	91	142	29.9%	1.31 [1.13, 1.51]
Jin et al. 2020	17	18	16	20	5.0%	1.18 [0.92, 1.51]
Qiu et al.2020	5	25	2	25	0.7%	2.50 [0.53, 11.70]
Sun et al. 2020	31	32	18	25	6.6%	1.35 [1.05, 1.73]
Wang et al. 2020	37	40	32	40	10.5%	1.16 [0.97, 1.38]
Xiao et al.2020	21	24	16	23	5.4%	1.26 [0.92, 1.71]
Yu et al. 2020	102	148	93	147	30.7%	1.09 [0.92, 1.28]
Total (95% CI)		**512**		**504**	**100.0%**	**1.24 [1.14, 1.34]**
Total events	380		302			

Heterogeneity: Chi² = 5.54, df = 8 (P = 0.70); I² = 0%
Test for overall effect: Z = 5.01 (P < 0.00001)

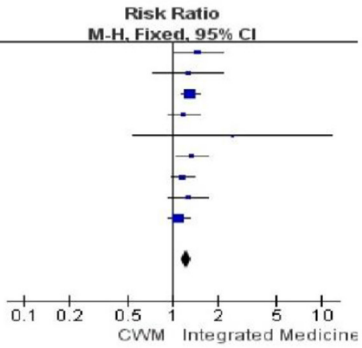


FIGURE 5 | Forest plot of chest CT improvement rate.

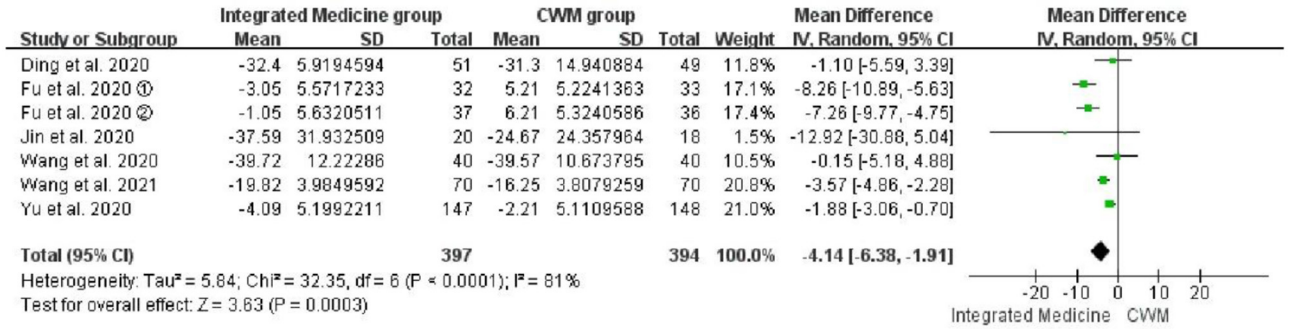


FIGURE 6 | Forest plot of CRP (mg/L).

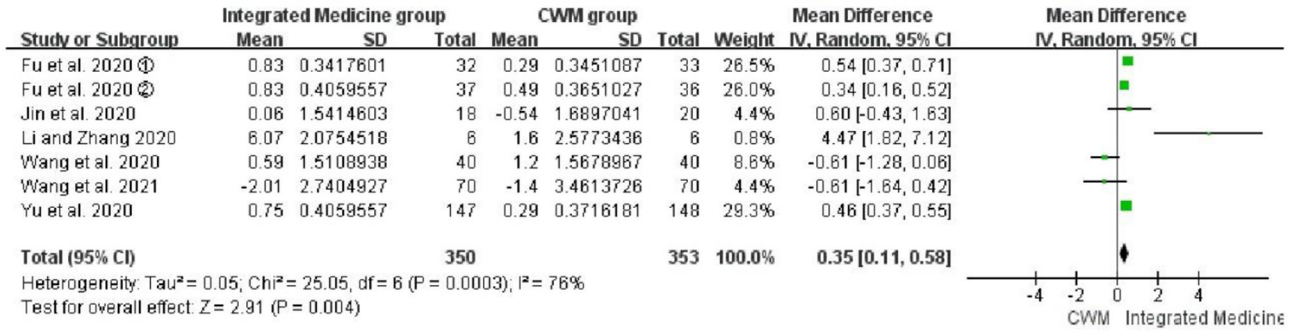


FIGURE 7 | Forest plot of white blood cell (10^9/L) count.

disappearance rate: $z = 1.50$, $P = 0.133$; cough disappearance rate: $z = 0.62$, $p = 0.536$; CRP: $z = 0.30$, $p = 0.764$; WBC: $z = 0.60$, $p = 0.548$).

DISCUSSION

Summary of Evidence

Traditional Chinese medicine has a long history and plays an important role in the current medical treatments in China. COVID-19 is a severe viral infection and lacks specific drugs. TCM is involved in the treatment of patients with different degrees of severity in the COVID-19 diagnosis and treatment program of China. Therefore, this study systematically reviewed, summarized, and disseminated the best evidence through strict inclusion and exclusion criteria to provide better evidence for COVID-19 treatment decisions.

In Chinese medicine, the dosage, composition, and course of treatment can be adjusted according to the condition of the patient. After a comprehensive search of seven databases, 19 RCTs included in this meta-analysis used 16 different herbs or proprietary Chinese medicines, which means that, in terms of treatment, Chinese medicine can make more choices for the best treatment compared with Western medicine. Our results showed that clinical symptoms such as fever and fatigue, as well as overall effective rate, chest CT, CRP, and WBC, were more improved in the integrated medicine group than in the CWM group. In addition, our imprecise results also showed that integrated medicine did not improve the cough disappearance rate compared with Western medicine. When we excluded studies that led to increased heterogeneity by sensitivity analysis, we found that the integrated medicine group improved the cough symptoms better than the CWM group. For COVID-19, more than 2 weeks of treatment course is suggested (36). However, our research also shows that, when the treatment time is <2 weeks, the effect of integrated medicine treatment is more obvious in improving overall effective rate, clinical symptoms, CRP level, and WBC count compared with the treatment of CWM. For patients with severe and non-severe COVID-19, integrated medicine is more effective in improving fever and cough symptoms, and WBC count than CWM.

According to the TCM viewpoint, some experts believe that, although, the cold and dampness are blocked in the early stage of COVID-19, the cold and dampness often turn into heat, and it is easy to manifest as damp heat (37, 38). The damp-heat virus invades the lungs from the nose and mouth, causing lung dysfunction and blockage of body fluids. Therefore, patients with COVID-19 usually have a dry cough with little sputum and difficulty breathing. The symptoms of dry cough and lack of sputum are inconsistent with lung pathological changes. During the dissection process, it was found that there was a large amount

of mucus secretions and pulmonary interstitial edema in the airways of the patient, but the exudate was very viscous, and these secretions were difficult to discharge (39, 40). Because the terminal airways are blocked by secretions, the patients have severe breathing difficulties. Even if sputum suction, oxygen therapy, and ventilator adjuvant treatment are given, it is not conducive to the removal of deep "phlegm"; instead, it makes the sputum thick or forms sputum scabs, and a large amount of retention in the lungs aggravates lung ventilation dysfunction and even leads to respiratory failure. The changes in chest CT in this study reflect that TCM has a significant effect in improving sputum drainage in the treatment of COVID-19.

Abnormal inflammation indicators are the most common indicators of viral infection. SARS-CoV-2 can also cause immune cascade of the body, resulting in systemic inflammatory response syndrome (SIRS), diffuse intravascular coagulation (DIC), and MODS (41). It has been reported that the stormy release of a large number of inflammatory cytokines correlated with mortality (42). Studies have shown that, in the early stage of COVID-19, CRP levels positively correlate with the lung disease and can reflect the severity of the disease (43). In addition, the baseline levels of CRP can be used as independent predictors of mortality in COVID-19 patients (44). TCM has multiple targets; it not only has antiviral effects, but it also has therapeutic effects in the occurrence, progression, and outcome stages of the cytokine storm. The change in CRP levels in this study reflects the efficacy of TCM in the treatment of COVID-19 to improve inflammation.

In summary, the currently available evidence suggests that integrated medicine treatment can be an effective treatment for COVID-19, when the treatment time is 5–21 days.

Limitations of the Current Review

Although, we followed the Cochrane method for meta-analysis, conducting comprehensive literature retrieval, repeatedly and independently screening literature, and abstracting data, our meta-analysis still has some limitations.

First, the general information of the patient is not provided in detail, such as the baseline age, underlying disease, subtype of COVID-19 participants, disease course, treatment duration, and the type and dosage of Western medicine used in the control group. Moreover, different laboratory measuring instruments and different normal value ranges may be responsible for the high heterogeneity in laboratory measurement outcomes (CRP, WBC, ESR, PCT, and LY). In addition, the use of different herbs in different interventions may be responsible for the observed high heterogeneity of the pooled effect size estimates. Furthermore, the method of random sequence generation is unclear, and most of the studies lack details of allocation concealment and blinding, leading to possible selection bias and implementation bias in the included studies. The above factors will cause clinical heterogeneity and methodological heterogeneity before performing meta-analyses. At the same time, due to the relatively small number of studies involving ESR (three RCTs), PCT (four RCTs), and LY (four RCTs) in the included studies, the insufficient population sample size may lead to statistical heterogeneity, so we have only described the results of each included RCT.

However, there are many obstacles for the control group drugs to be consistent with the preparations of TCM and to eliminate the unique smell of TCM, which might lead to an unblinding of the study. Therefore, it is difficult to implement double-blind Chinese medicine in clinical trials. In data processing, none of the studies reported cases of withdrawal and loss, and due to the lack of long-term follow-up data, there may be insufficient reports of adverse reactions. Finally, we are unable to assess the effects of integrated medicine on other clinically meaningful endpoints, such as the time when 2019-nCoV RT-PCR is negative, and composite events (the total number of patients diagnosed as type critical and all-cause death).

Research Implications

Considering the limitations of the current trials, the correct methods of allocation concealment and blinding should be recommended when designing future clinical trials in accordance with the Consolidated Standards of Reporting Trials (CONSORT) (45) and TCM guidelines (Standards for Reporting Interventions in Controlled Trials of TCM) (46). In order to optimize the effectiveness of TCM treatment of COVID-19, the design, quality, and reporting of RCTs should be improved, especially the allocation concealment. Although, blinding may be difficult for patients treated by TCM, blinding should be feasible for medical workers, outcome evaluators, and analysts. In addition, it is necessary to actively explore the preparation of placebos, which may be a way to solve the problem of double-blindness of TCM. Future studies may need to refer to the core outcome sets that have been developed, such as a core outcome set for clinical trials on coronavirus disease 2019 (COS-COVID-2019) (36), as an outcome measure for different subtypes of COVID-19, to avoid wasting research resources. Considering the inaccuracies of the included studies, future RCTs should include larger sample size, longer treatment time, and longer follow-up periods to confirm the efficacy of integrated medicine and to formulate the optimal regimens.

Clinical Practice Implications

The results of the current meta-analysis suggested that the integrated medicine can improve the symptoms of patients with COVID-19. Even if the treatment time is <2 weeks, compared with only CWM treatment, the effect of integrated medicine in improving symptoms is more obvious. In addition, integrated medicine treatment also can effectively improve the chest CT and infection indicators (CRP and WBC) of patients with COVID-19, which may be related to the promotion of sputum drainage in the lungs and anti-inflammatory by Chinese medicine. However, due to the low quality of the evidence and the small sample size, the results of the meta-analysis of ESR, PCT, and LY need further research and verification. This study provides an initial set of evidence for potentially recommending integrated medicine as a treatment plan for COVID-19. The treatment based on syndrome differentiation is one of the characteristics and advantages of TCM treatment. Therefore, each facility utilizing TCM can choose herbal medicines according to the type of syndrome of COVID-19 when using TCM for treatment or research.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

BY and Y-MB ran the search strategy. BY, Y-MB, X-ZW, and A-XL collected the data. BY and Y-MB re-checked the data. BY performed the analysis and LS re-checked the analysis. BY and Y-MB assessed the quality of studies and LS re-checked the quality. BY wrote the manuscript. Y-MB, J-ZH, JZ, and JY edited the manuscript. G-JF and LS designed and administrated the study. All the authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.622707/full#supplementary-material

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Monitoring and Analysis of COVID-19 Pandemic: The Need for an Empirical Approach

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The current worldwide pandemic produced by coronavirus disease 2019 (COVID-19) has changed the paradigm of mathematical epidemiology due to the high number of unknowns of this new disease. Thus, the empirical approach has emerged as a robust tool to analyze the actual situation carried by the countries and also allows us to predict the incoming scenarios. In this paper, we propose three empirical indexes to estimate the state of the pandemic. These indexes quantify both the propagation and the number of estimated cases, allowing us to accurately determine the real risk of a country. We have calculated these indexes' evolution for several European countries. Risk diagrams are introduced as a tool to visualize the evolution of a country and evaluate its current risk as a function of the number of contagious individuals and the empiric reproduction number. Risk diagrams at the regional level are useful to observe heterogeneity on COVID-19 penetration and spreading in some countries, which is essential during deconfinement processes. During the pandemic, there have been significant differences seen in countries reporting case criterion and detection capacity. Therefore, we have introduced estimations about the real number of infectious cases that allows us to have a broader view and to better estimate the risk. These diagrams and indexes have been successfully used for the monitoring of European countries and regions during the COVID-19 pandemic.

Keywords: COVID-19, risk indexes, risk diagram, epidemic monitoring, COVID-19 outbreak

INTRODUCTION

The current pandemic produced by coronavirus disease 2019 (COVID-19) is strongly impacting the world. With more than 150 million confirmed cases and more than 3 million reported deaths, the pandemic has been a worldwide tragedy, with consequences impacting far beyond these numbers. In addition to the health disaster in all the countries in the world, the control measures

had important consequences, not only the expected socioeconomic derivatives but also emotional (1, 2), educational (3, 4), or cultural (5, 6) consequences, to cite but a few. Therefore, this emergency situation has required constant monitoring at multiple levels—from the city neighborhood tracking of local outbreaks to a global continental perspective for socioeconomical decisions coordinated at the interstate level. Different political actors need different pieces of information to take decisions regarding mobility, schools, or the redirection of health resources, among others.

Unfortunately, the spreading dynamics of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is largely unknown and certainly not sufficiently characterized to develop mechanistic models that properly predict its propagation in the medium term. For example, there is uncertainty in the literature regarding the influence of temperature and humidity in its transmission, with reports indicating both a very small (7) and a relatively large (8) effect. The apparent clustering behavior (9) of the transmission adds an important layer of uncertainty regarding under which conditions the virus propagates optimally. This renders complicated mechanistic models of propagation useless in the sense of providing useful quantitative information. Relevant indicators for policy makers must come from empirical epidemiological models of the cumulative cases and fatalities of the pandemic.

One of these indicators is the effective reproduction number, R_t, that is normally assessed by means of an SIR model (i.e., a compartment model based on Susceptible-Infectious-Recovered flows) (10) or likelihood-based estimation procedures based on the generation time interval method (11). These kinds of models require previous parametrization. In this sense, we propose a more transparent and empiric way to characterize the spreading of the epidemic that we call ρ_t. This index measures the ratio between new cases at an interval of 5 days. It is thus a parametrization-free parameter that we will show is closely related to R_t. When combined with the evaluation of active cases, it provides an empirical quantification of the epidemiological risk in a given region.

The manuscript is structured as follows. First, the *Methods* describe the empirical indexes used on daily tracking of epidemics (12). Then, the *Results* section shows that they are good short-term predictors, allowing a proper evaluation of the state of the epidemic.

METHODS

The reported data from government sources about the pandemic are large and normally not unified in their criteria. They must be properly assessed and curated to obtain useful and truthful information, especially about the trend of its spreading. Our main aim is to analyze whether the situation is improving or getting worse, so that it can be used by policy makers when deciding different socioeconomic measures. To address this issue, we have developed or adapted three indexes to compare different situations and evaluate the resurgence risk: an empiric estimation of the reproduction number, an index of the contagious pool and a risk index of the effective potential growth. Moreover, and looking for an effective and proper communication of the epidemic situation in a certain country, we have built a discrete scale that assesses the level of incident cases. These indexes are adapted to COVID-19 but can also be used in any pandemic or epidemic.

Empiric Indexes

Empiric Reproduction Number

Classically, epidemiology uses the effective reproduction number (R_t) (9) to measure the velocity at which the epidemic is propagated during an outbreak. It is a measure of the mean number of new infections caused by an infectious individual. Let R_0 be the value of R_t before the epidemic starts, that is, at $t = t_0$. To compute these parameters, SIR and SEIR (susceptible-exposed-infectious-removed) models (13) are traditionally used. However, these models are difficult to address COVID-19 pandemic due to the high number of unknowns about inherent parameters (14). In addition, classical SEIR models are driven by susceptible population availability, while the evolution of this pandemic is mainly governed by the control measures like confinement or social conscience regarding the hygiene rather than by susceptible population.

Other methods to calculate R_t are also available based on the estimation of the generation time between two correlated infections and the probability of infection along the disease of an individual (11). The lack of complete knowledge of such factors at the beginning of the epidemic suggested to assume a naiver description.

We propose an empiric definition of the propagation rate (ρ_t), which is defined as the number of newly infected in the last 3 days divided by the number of newly infected during 3 days τ days ago:

$$\rho_t\left(t-1,\tau\right)=\frac{nc\left(t-2\right)+nc\left(t-1\right)+nc\left(t\right)}{nc\left(t-2-\tau\right)+nc\left(t-1-\tau\right)+nc\left(t-\tau\right)}$$

where $nc(t)$ is the number of new cases at time t, and τ is the incubation period, which in COVID-19 case is estimated to be around 5 days (15, 16). Furthermore, 5 days also correspond to the average generation time (time between generations) (17), giving rise to a simple first-order approximation to the effective reproduction number R_t. Then, similarly to the use of R_t, if $\rho_t > 1$, the epidemic is growing because the number of new cases today is bigger than the number of new cases 5 days ago. Otherwise, the incidence of new cases is lower and the epidemic is reducing. When $\rho_t = 1$, the epidemic is not growing nor reducing and the new number of cases is maintained.

Propagation rate is very sensitive to noise effects. Thus, at initial and final stages, when the number of new cases is small, the behavior of ρ_t does not represent the reality. Besides, the temporal evolution of ρ_t is subject to human reporting data effects, such as the weekend effect (12). To address these issues, we define ρ_T as the moving average of ρ_t for T days:

$$\rho_T\left(t,\tau\right)=\frac{1}{T}\sum_{i=-\frac{T-1}{2}}^{\frac{T-1}{2}}\rho_t\left(t+i,\tau\right).$$

In the following, we will set $T = 7$ days in order to avoid the weekend effect (18). Note that this definition is only valid for odd values of T. Otherwise, one would find non-integer values of t, which is the time variable in days and must be an integer.

The 14-Day Attack Rate: A Measure of Contagious People

Parameters ρ_t and ρ_7 are useful to identify if an epidemic is growing; however, it is not the same to obtain a ρ_t bigger than 1 with a large amount of potentially contagious individuals or, on the contrary, if the fraction of potentially contagious individuals is small. The number of contagious individuals is a difficult quantity to estimate since contagious people are not necessarily detected. An index commonly used to follow active cases in COVID-19 is the 14-day attack rate (i.e., new cases of last 14 days per 10^5 inhabitants, A_{14}) (19, 20), which is defined as follows:

$$A_{14}(t) = \frac{N(t) - N(t-14)}{population} \cdot 10^5,$$

where N is the number of cumulative reported cases in a country, and *population* is the population of the country or region under consideration.

Nevertheless, the reported cases criterion is very different through the countries due to many facts: type of test reported, reporting frequency, update of reported data temporal series, number of available tests, percentage of diagnosed cases, biased subpopulations that are over/underdiagnosed, etc. (21). Thus, the number of reported cases is not as representative as one would expect. As for the reported deaths, there is also variability among countries but at lower levels (22). Then, it is possible to calculate the diagnosis rate (DR) from these data, which allows us for the estimation of the real number of cases (N_{est}) (22). This estimation agrees with different seroprevalence testing done afterward (23). We can define the estimated 14-day attack rate ($A_{14,EST}$) as:

$$A_{14,EST}(t) = \frac{N_{EST}(t) - N_{EST}(t-14)}{Population} \cdot 10^5.$$

Assuming a constant diagnosis rate, this equation can be simplified:

$$A_{14,EST}(t) = \frac{A_{14}(t)}{DR(t)}.$$

By symmetry, we will name the 14-day attack rate evaluated with reported data as $A_{14,REP}$.

Effective Potential Growth

The effective potential growth (EPG) is a risk index that evaluates the potential epidemic growth at short term. It is defined as the product between the mean propagation rate of the last 7 days (ρ_7), which reflects the velocity at which the epidemic is spreading, and the 14-day attack rate ($A_{14,REP}$), which accounts for the contagious population that could propagate at that rate:

$$EPG_{REP}(t) = \rho_7(t) \cdot A_{14,REP}(t).$$

In fact, this product provides, under constant conditions, an order of magnitude of the expected number of new cases that will be diagnosed (i.e., that will be reported) for the next 14 days per 10^5 inhabitants. However, EPG_{REP} is a magnitude that changes over time, so it can be used for evaluating the risk associated with this potential growth at any moment during the epidemic. This index was used to decide which Catalan Sanitary Regions were deconfined, among other criteria.

If we want to evaluate the risk based on the estimated pool of contagious population, $A_{14,EST}$, we obtain the expression:

$$EPG_{EST}(t) = \frac{EPG_{REP}(t)}{DR(t)}.$$

The Biocom-Cov Scale

Popular language often uses sea-related vocabulary to describe the dynamics of COVID-19 in a region or country: first wave to refer to the first peak, secondary waves to describe subsequent outbreaks, or tsunami to refer to a totally uncontrolled outbreak, among others. The Douglas Scale is a discrete way of classifying the situation of the sea that considers, among others, the height of the waves. We propose a discrete way of classifying the situation with regard to daily new cases in what we name Biocom-Cov scale.

Looking at orders of magnitude, 200 active cases per 100,000 inhabitants pose an impossible challenge, while 20 active cases can be dealt with by public health officials if they are properly found and the structure of test and trace is in place. Assuming that active cases are well-represented by A_{14}, corresponding average daily new cases would be $200/14 = 14.3$ daily new cases. Then, the 14 daily new cases are placed as the threshold for the highest level. Here, 100 active cases per 100,000 inhabitants are a highly problematic situation from the control perspective. This situates another important threshold at 7–8 daily new cases per 100,000 inhabitants. Similarly, five daily new cases per 100,000 should count as rather high situation, and two daily cases (around 30 active cases) should be the limit of moderate. With these ideas in mind, we build the scale shown in **Table 1**, which gives a complete and accurate picture of the situation.

TABLE 1 | Biocom-Cov scale to assess the epidemic degree of a region.

Pandemic degree	Daily new incident cases per 10^5 inhabitants
0	0
1	0–0.1
2	0.1–0.5
3	0.5–1.25
4	1.25–2
5	2–3
6	3–5
7	5–8
8	8–14
9	>14

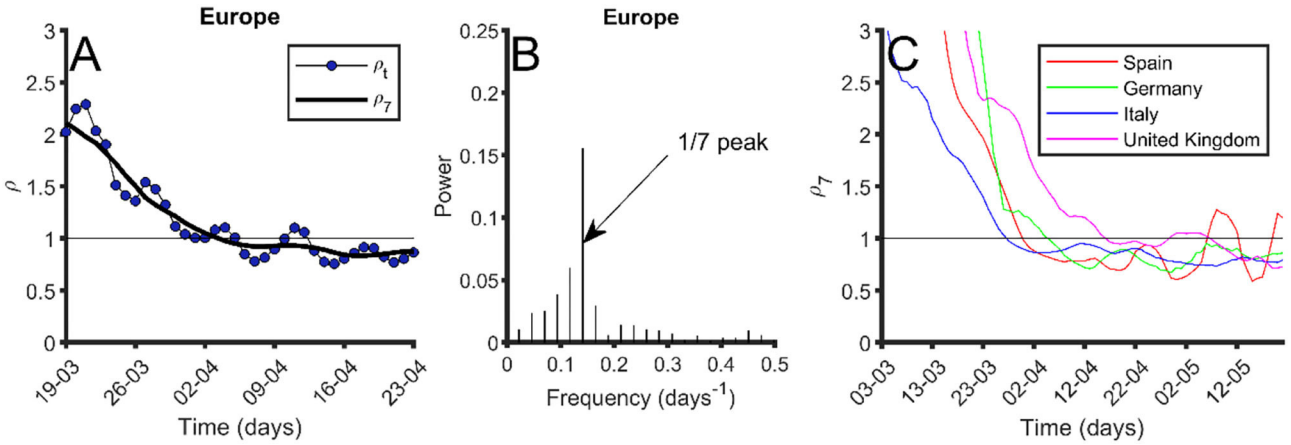

FIGURE 1 | Index $\rho_t$evolution and periodicity. **(A)** In blue dots, evolution of ρ_t in Europe; black thick line, 7-day moving average of ρ_t (ρ_7). **(B)** Fourier transform of ρ_t. The peak at frequency 1/7 days^{-1} is pointed out. **(C)** Seven-day moving average of ρ_t (ρ_7) for different European countries: Spain (red), Germany (green), Italy (blue), and United Kingdom (purple). Data were obtained from the European Center for Disease Prevention and Control (24) on May 25, 2020.

RESULTS

ρ_t, an Indicator of Contagious Velocity

The evolution of the empiric reproduction number, ρ_t, is crucial to evaluate the dynamics of the pandemic. The number of new cases increases until ρ_t gets smaller than 1. A stable ρ_t below 1 is needed to reduce the new number of cases. The blue dots in **Figure 1A** show the weekend effect in ρ_t at the European level caused by a weekend delay in data recording. Therefore, oscillations with a 7-day periodicity are observed. **Figure 1B** shows the Fourier transform of ρ_t, where the 7-day oscillation period is clearly identified. Therefore, the 7-day moving average in ρ_t is necessary to study the epidemic dynamics, given by ρ_7. **Figure 1A** shows the comparison between daily ρ_t and the averaged ρ_7. The comparison of ρ_7 through various countries allows for the exploration of differences in COVID-19's dynamics among countries. **Figure 1C** shows the evolution of ρ_7 in Germany, Italy, Spain, and United Kingdom. Italy was the first European country where the pandemic started, while the fastest pandemic control (i.e., decrease in ρ_7) was in Germany. Spain followed a control dynamics similar to the one in Italy, even slightly faster. Italy, Germany, and Spain have managed to stabilize ρ_7 below 1. Nevertheless, ρ_7 is still sensitive to fluctuations in data, which is the reason why it increases above 1 in two short periods for Spain. This country experienced some problems with data reporting the last weeks, and a couple of spikes in new data masks the real decreasing global trend. United Kingdom shows a slower control of the epidemic, with a ρ_7 that remained around 1 for several weeks, and that finally dropped below 1.

Reported and Estimated Risk Indexes

Risk indexes ρ_7, A_{14}, and EPG (on their reported and estimated versions) have been used in the daily tracking of the epidemic in European and several non-European countries. Index ρ_7 provides a quantification of the velocity at which the epidemic is being spread; the higher, the worse. Index A_{14} provides a way to quantify active cases, i.e., it is an indicator of the contagious people that can spread the virus at the velocity ρ_7. Finally, EPG evaluates the risk that results from both indexes. A high ρ_7 with a very low A_{14} does not represent high risk, and this is reflected by low values of EPG. We consider two types of EPG, EPG_{REP} and EPG_{EST} evaluated, respectively, with the data reported by countries and with the estimated real incidence.

Figure 2 shows the values of several variables and indexes (including ρ_7, $A_{14,REP}$, $A_{14,EST}$, EPG_{REP}, and EPG_{EST}) for different countries on May 22, 2020. At that date, countries at the highest risk according to EPG_{REP} were Perú, Brazil, and USA. If we look at the risk given by EPG_{EST}, the most worrying situation was that of Brazil followed by Sweden, UK, USA, and Perú. A comparison between the estimated and reported EPG is useful in determining which countries are underreporting.

Figure 2 also incorporates the Biocom-Cov degree of each country. As shown, this scale immediately facilitates a good visualization of the country situation beyond the need for other precise numerical indicators. In order to bypass the weekend effect, we assign the Biocom-Cov degree looking at the average of daily new cases in the last week.

Time evolutions of $A_{14,EST}$, EPG_{EST} and EPG_{EST} are shown in **Figure 3** for the five European countries with the highest number of total reported cases (UK, Spain, Italy, France, and Germany). **Figure 3A** shows that Italy was the first country in Europe to reach the peak of contagious cases. Spain is the country that arrived at the highest number of contagious people per 10^5 inhabitants, nearly doubling that of Italy (217 vs. 124), which was the country with the second highest incidence. UK curve shows that this country has transited a plateau rather than a peak, thus illustrating the delay in controlling the epidemic.

Figure 3B shows the evolution of EPG_{REP} in these countries. Spain was the one that reached the highest risk on March 28, 2020, when it had an EPG_{REP} of 214. This index also provides similar risk levels achieved by the other four countries, which at

	Reported data								Indexes			
Country	Cumulative cases	Attack rate /10^5 inh.	Cumulative deaths	Mortality /10^5 inh.	Active cases (last 14 days)	14-day attack rate /10^5 inh.	Estimated active cases (last 14 days)	Estimated 14-day attack rate /10^5 inh.	ρ_7	EPG_{REP}	EPG_{EST}	Biocom-Cov degree
United States of America	1,577,287	476.5	94,702	28.6	320,315	96.8	2,104,747	635.9	1.01	98	642	7
Brazil	310,087	145.9	20,047	9.4	174,981	82.3	1,444,359	679.5	1.45	119	985	7
United Kingdom	250,908	369.6	36,042	53.1	44,193	65.1	661,072	973.8	0.76	49	739	6
Spain	233,037	495.5	27,940	59.4	9,819	20.9	117,591	250.1	0.66	14	164	3
Italy	228,006	377.1	32,486	53.7	12,148	20.1	175,873	290.9	0.77	16	225	3
Germany	177,212	211.5	8,174	9.8	9,912	11.8	48,617	58.0	0.84	10	48	3
Turkey	153,548	182.1	4,249	5.0	19,827	23.5	56,700	67.2	0.88	21	59	4
France	144,163	220.9	28,215	43.2	6,384	9.8	134,187	205.6	0.91	9	187	3
Iran	129,341	154.0	7,249	8.6	26,206	31.2	157,794	187.9	1.21	38	228	5
Peru	108,769	329.9	3,148	9.5	50,243	152.4	172,374	522.8	1.28	196	671	8
China	84,079	5.8	4,638	0.3	103	0.0	571	0.0	0.93	0	0	0
Canada	81,313	215.4	6,152	16.3	16,391	43.4	149,029	394.9	0.96	42	377	5
Mexico	59,567	46.2	6,510	5.0	29,951	23.2	433,997	336.6	1.25	29	419	5
Netherlands	44,700	260.9	5,775	33.7	2,926	17.1	38,828	226.6	0.78	13	176	3
Ecuador	35,306	200.1	2,939	16.7	5,008	28.4	53,893	305.5	1.41	40	432	6
Sweden	32,172	318.6	3,871	38.3	7,549	74.7	100,545	995.6	0.94	71	939	7
Switzerland	30,611	353.7	1,637	18.9	568	6.6	3,058	35.3	0.74	5	26	2
Portugal	29,912	293.3	1,277	12.5	3,197	31.4	14,401	141.2	1.13	35	160	5
Ireland	24,391	494.0	1,583	32.1	2,006	40.6	13,370	270.8	0.62	25	167	4
Poland	20,143	53.2	972	2.6	5,096	13.5	28,757	76.0	0.97	13	74	3
Romania	17,585	91.4	1,151	6.0	3,086	16.0	21,712	112.9	0.84	13	95	3
Japan	16,513	13.1	796	0.6	966	0.8	4,917	3.9	0.59	0	2	1
Austria	16,332	181.3	633	7.0	659	7.3	2,617	29.1	0.99	7	29	3
Denmark	11,182	193.1	561	9.7	1,099	19.0	5,625	97.1	0.82	16	80	3
Czech Republic	8,754	81.7	306	2.9	723	6.8	2,752	25.7	1.35	9	35	3
Norway	8,268	152.5	234	4.3	273	5.0	792	14.6	0.69	3	10	2
Finland	6,493	117.2	306	5.5	820	14.8	4,227	76.3	0.95	14	72	3
Hungary	3,678	38.1	476	4.9	500	5.2	6,996	72.4	1.07	6	78	2
Greece	2,853	27.4	168	1.6	175	1.7	1,063	10.2	0.99	2	10	2

Scale											
Worst	Worst	Worst	Worst	Worst	Worst	Worst	Worst	Worst	2.0	100	1000
Best	Best	Best	Best	Best	Best	Best	Best	Best	0.0	0	0

FIGURE 2 | Table with last indexes and reported cases value as of May 22, 2020. Left to right columns are: country, cumulative reported cases, number of total cases per 10^5 inhabitants (attack rate), cumulative number of reported deaths, number deaths per 10^5 inhabitants, reported number of new cases last 14 days (active cases), reported active cases per 10^5 inhabitants (14-day attack rate), estimated number of new cases for the last 14 days (active cases), estimated active cases per 10^5 inhabitants (14-day attack rate), 7-day moving average empiric reproduction number (ρ_7), effective potential growth of reported cases (EPG_{REP}), estimated effective potential growth (EPG_{EST}), and Biocom-Cov degree. Each column has its own color scale as seen at the bottom of the figure. Data were obtained from the European Center for Disease Prevention and Control (24) and World Health Organization (25) on May 23, 2020.

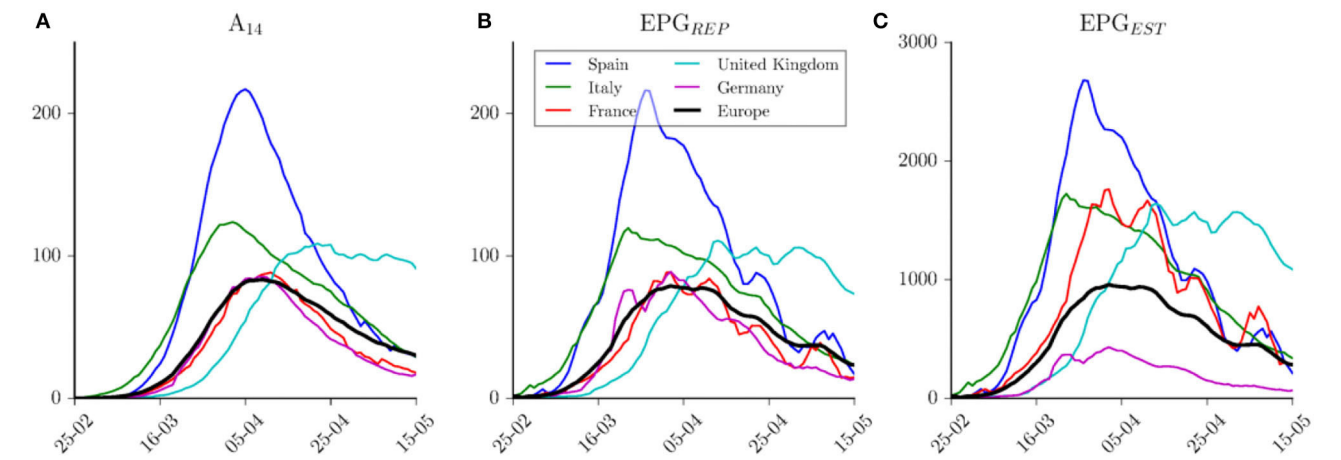

FIGURE 3 | Indices evolution among time. **(A)** Evolution of reported 14-day attack rate per country. **(B)** Effective Potential Growth evolution per country computed with reported cases. **(C)** Effective Potential Growth evolution per country computed with estimated cases. Blue lines for Spain, green for Italy, red for France, cyan for United Kingdom, purple for Germany, and black for Europe. Data were obtained from the European Center for Disease Prevention and Control (24) on May 16, 2020.

the same time would be similar to the one reached by Europe as a whole. Moreover, if we compare the same countries using the estimated EPG_{EST} (**Figure 3C**), we observe that Germany has been in a better situation than the one reflected by reported data when compared with other countries. According to this index, Italy, UK, and France have had a similar level of estimated risk but at different moments, slightly higher (i.e., worse) than the one shown by European average. Risk reduction in Spain is faster than the one observed in Italy; in fact, they are in a similar level now. Germany and France show a similar EPG_{REP} but totally different estimated EPG_{EST}. The number of reported deaths in this pair of countries is quite different, with a six-fold increase

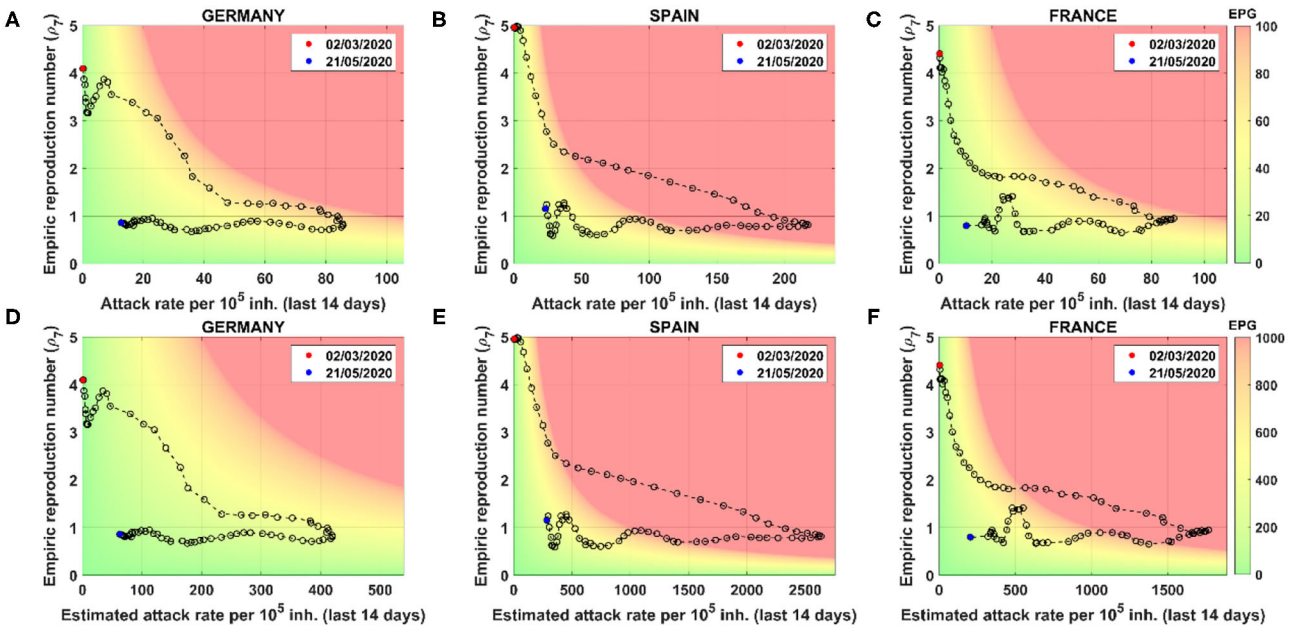

FIGURE 4 | Risk diagrams for different countries using reported and estimated cases. Each point represents a different day. Starting and final days are marked by date. Color code depends on Effective Potential Growth (EPG). Two color codes are used: one for reported (top right bar) and another for estimated (bottom bar). Using reported cases: **(A)** Germany, **(B)** Spain, and **(C)** France risk diagrams. Using estimated cases: **(D)** Germany, **(E)** Spain, and **(F)** France risk diagrams. Note that x-scales are different, but plots can be compared through the color background.

between them. This leads to the estimation of a higher diagnosis rate in Germany than the one in France (25 vs. 7%) (22). From this analysis, it is important to note that the time scale in the reduction of the number of active cases is larger than the time scale observed during the growing phase.

Risk Diagrams: A Tool to Evaluate Risk

The risk diagram is a tool to visualize the evolution of a region or country in terms of ρ_7 (y-axis), A_{14} (x-axis), and EPG (background color) with either reported or estimated data. **Figure 4** shows the risk diagrams for France, Spain, and Germany. In the upper part, risk diagrams using reported data (**Figures 4A–C**), in the bottom part, risk diagrams using estimated data (**Figures 4D–F**). We consider a risk situation (red) for $EPG_{REP} > 100$ and $EPG_{EST} > 1{,}000$.

The color code is related to the ability of a country or region to do contact tracing, setting at 1,000 estimated real cases the red as the threshold where it is impossible to carry it out. The maximum number of daily PCR tests per 10^5 people performed sustainably has been of the order of 50–100 (26) in affected countries. At this level of testing, it would take between 10 and 20 days to process all active cases, which is precisely the time it takes for infected people to get seriously sick or die. Unless the infrastructure is scaled up dramatically in the future, 1,000 active cases are impossible to test and trace nowadays.

The general dynamics along the risk diagram is quite similar for all countries. At the beginning of the pandemic, the attack rate is low while the propagation velocity is high ($\rho_7 > 1$). When restrictions and physical distancing measures are applied, the velocity of propagation drops down, but, since it is still above 1, the attack continues to increase. The inflection point is achieved when ρ_7 crosses the threshold of 1. At that moment, the number of new infected cases starts to decrease; meanwhile, ρ_7 remains below 1. Then, the curve moves toward the green zone.

Analyzing case-by-case, we see that Spain was the country that was in a worse situation since they went further in the risk zone, where there are more than 214 cases per 10^5 inhabitants expected for the next 14 days. Looking at the estimated diagrams, we see that Germany was the country with a better estimated real situation, since they did not reach the danger zone.

The analysis of a full country by using only this risk diagram could lead to a misleading visualization of the real situation. In these figures, we are studying the situation of these countries by considering them as a whole. However, the situation in the different regions of each country could be very different, and a deeper analysis must be done. The fragmentation of the country into several regions allows us to better understand the situation as well as how the propagation has occurred. This information is crucial for policymakers to properly develop novel strategies during confinement and deconfinement. For instance, the regional variability for the cases of Spain is shown in **Figure 5**. COVID-19 risk diagrams are updated daily at Català et al. (12).

Risk Diagrams as a Tool to Detect Uncontrolled Outbreaks

Most European countries have overcome an initial peak and then entered a long tail that is expected to finish when herd immunity is achieved or an efficient vaccine is available. Meanwhile, the

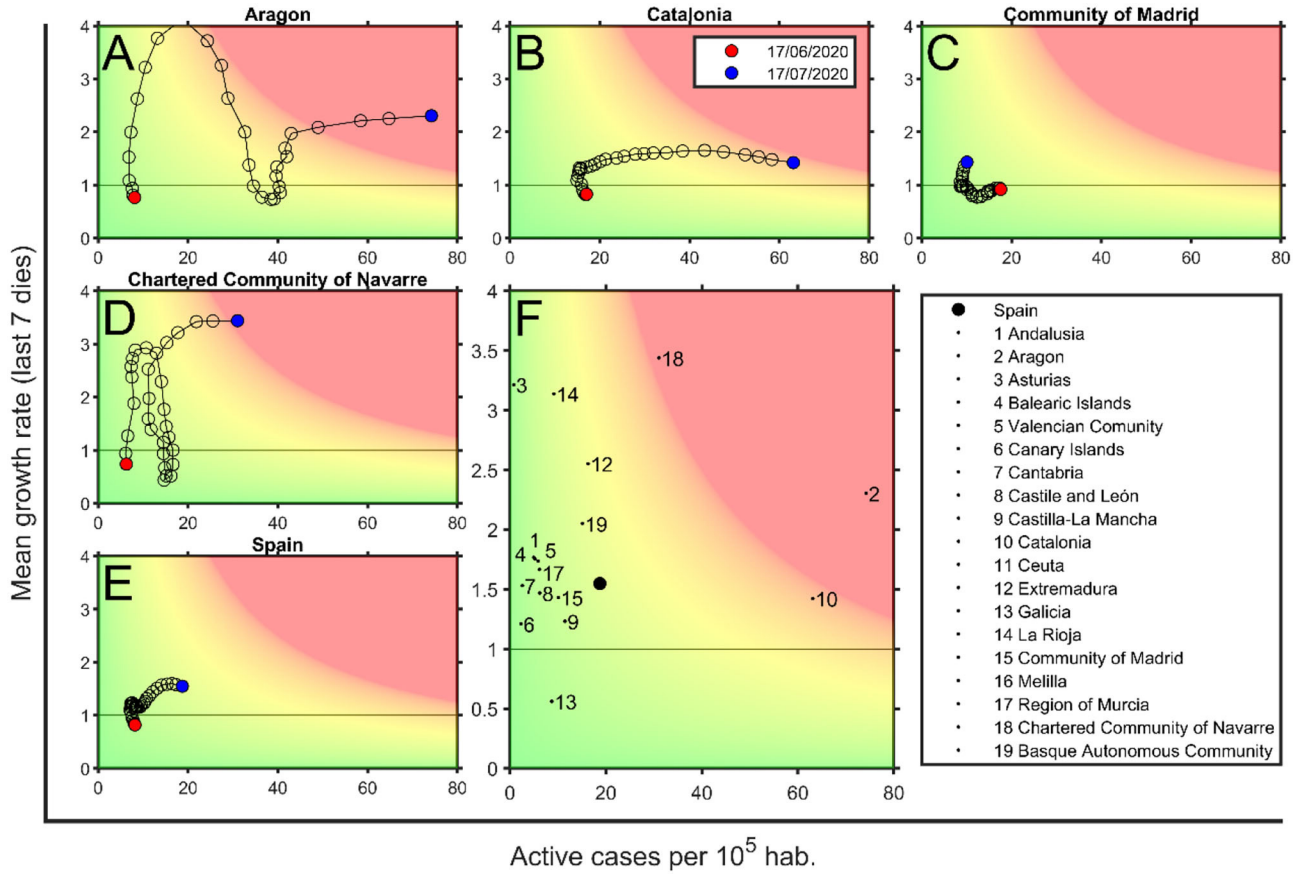

FIGURE 5 | Risk diagrams for Spain and some of its regions on July 17, 2020. **(A)** Aragon, **(B)** Catalonia, **(C)** Community of Madrid, **(D)** Community of Navarra, and **(E)** Spain risk diagrams. Each circle corresponds to a day, last day is marked with a black filled circle. **(F)** Spain region situation, where each dot is the position for each Spanish region on July 17, 2020 (see legend), and filled black circle is the situation of Spain. Background color depends on the Effective Potential Growth (EPG) risk. Red marks EPG = 100 and green is for EPG = 0; there is a linear degradation between both. Zones with an EPG higher than 100 are also marked in red. Data are obtained from Datadista (27) and Instituto de Salud Carlos III (28) on July 18, 2020.

main concern of regions and countries is the early detection of outbreaks and their evaluation from the risk perspective, so that physical distancing measures or mobility restrictions can be imposed, if necessary. The main threshold between control and uncontrol is the presence of community contagions, i.e., the loss of contagious chain traceability.

In risk diagrams, the loss of traceability is represented by a red zone, where control by test and trace is not possible. **Figure 6** shows the usefulness of risk diagrams to distinguish local controlled outbreaks from uncontrolled ones. In this figure, we show controlled and uncontrolled outbreaks that start with an initial increase in ρ_7 up to 2. First, we can observe two outbreaks in the control zone as a certain increase in ρ_7 that is not followed by an increase in active cases (Tarragona province). Therefore, this is observed as a simple up–down trajectory of the plot in the low risk zone. Contrarily, an outbreak that is not well-delimited and immediately controlled drags the curve to the right (Lleida). While this dynamics occurs in the yellow zones, control with soft measures is possible (in this case, a slowing down of the de-escalation process). When a new increase in new cases leads the curve toward the red zone, it strongly suggests the presence of community transmission and the need for restrictions in mobility or social interactions.

DISCUSSION

We have shown that ρ_7 and A_{14} are good indicators for assessing epidemiological risk in regions or countries. They can be replaced by alternative ways of measuring spreading rate and contagious potential, but an indicator for each one must be considered if the risk is to be evaluated properly. In particular, the proposed way to assess ρ_7 is very sensitive to changes in the transmission dynamics, which can be especially useful to detect those changes. A slight change in this methodology, applying the 7-day moving average to the cases (nc) instead of the ρ_7, provides a more stable evaluation of the transmission rate, which is less affected by artifacts such as changes in the diagnosis protocols or underreporting of holidays.

We have proposed the EPG index as a simple way to account for both factors. During the growth phase (pre-peak) of the epidemic, EPG is used to track the dynamics of the epidemic, and when it increases above a threshold,

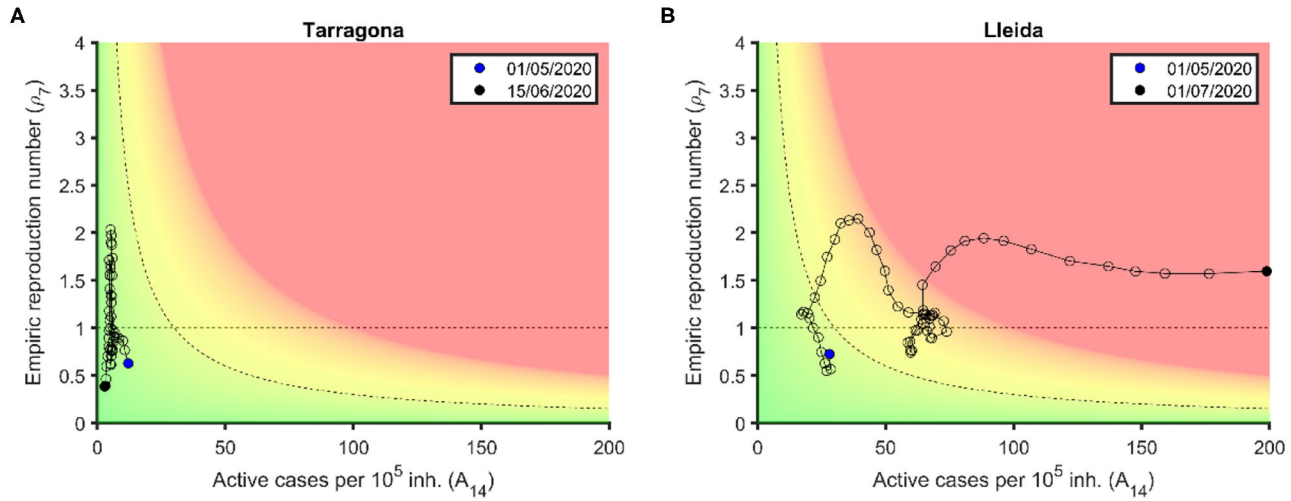

FIGURE 6 | Controlled and uncontrolled outbreaks in the risk. **(A)** Tarragona province: Two consecutive increases in ρ_7 occur in the green zone and can be controlled with simply test and trace. **(B)** Lleida province: An initial increase in ρ_7 occurs in the green-yellow zone and can be controlled through a slowing down of de-escalation process, while a second increase in ρ_7 enters the red zone and becomes an uncontrolled outbreak. Data are obtained from Datadista (27) and Instituto de Salud Carlos III (28) on October 23, 2020.

EPG can indicate the need for new control measures. Nevertheless, we consider the main focus of this index to be the management of the deconfinement process. It is essential that de-escalation phases take into account the relative epidemiological risk of the region or country in the context of the health system robustness and operability, together with the capability to incorporate contact tracing strategies that avoid new outbreaks. In addition, an increase in EPG also can be used as an alarm symptom when looking for secondary outbreaks.

Risk diagrams are a good way to visualize the situation and dynamics of countries in this sense. Its color scale is based on EPG values and its relation with the ability to trace given by the testing infrastructure typical in European countries. This scale, however, can be particularly adapted to each country, considering the level of incidence that the country can assume with the local ability to test and trace. Finally, those countries with a diagnostic level below 10% should try to incorporate estimations on the management of the epidemic (for instance, using EPG_{EST} instead of EPG_{REP}).

Most important limitations of any empirical approach to the COVID-19 pandemic are related to the diagnosis effort and bias (22). An insufficient diagnostic effort may affect not only an underreporting of cases but also an underreporting of deaths. In addition, holiday periods that modify the basic structure of 5 working days plus 2 holidays per week can generate artifacts on the observed data. In any case, and in conclusion, the use of empirical indexes like EPG and the risk diagrams can help with the monitoring of the COVID-19 epidemic and to address relevant questions, for example, the classification analysis of the evolution of the cases or the appearance of new outbreaks. In particular, any change in historical trends due to the appearance of a more transmissible variant or to the increase in the vaccination coverage among a certain population can be easily detected using such an empiric approach, since it lacks a mechanistic hypothesis to be revised.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CP, P-JC, and DL conceived the study. MC, MM, DC, PP, and TU wrote the codes and prepared tables and figures. MC, MM, SA, EA-L, DL, P-JC, and CP analyzed the results, discussed the implications, and proposed reformulations of the indexes. MC, MM, PP, SA, EA-L, and CP prepared the draft version. All authors read and approved the final version.

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REVIEW
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Combating the COVID-19 Pandemic: Experiences of the First Wave From Nepal

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Unprecedented and unforeseen highly infectious Coronavirus Disease 2019 (COVID-19) has become a significant public health concern for most of the countries worldwide, including Nepal, and it is spreading rapidly. Undoubtedly, every nation has taken maximum initiative measures to break the transmission chain of the virus. This review presents a retrospective analysis of the COVID-19 pandemic in Nepal, analyzing the actions taken by the Government of Nepal (GoN) to inform future decisions. Data used in this article were extracted from relevant reports and websites of the Ministry of Health and Population (MoHP) of Nepal and the WHO. As of January 22, 2021, the highest numbers of cases were reported in the megacity of the hilly region, Kathmandu district (population = 1,744,240), and Bagmati province. The cured and death rates of the disease among the tested population are ∼98.00 and ∼0.74%, respectively. Higher numbers of infected cases were observed in the age group 21–30, with an overall male to female death ratio of 2.33. With suggestions and recommendations from high-level coordination committees and experts, GoN has enacted several measures: promoting universal personal protection, physical distancing, localized lockdowns, travel restrictions, isolation, and selective quarantine. In addition, GoN formulated and distributed several guidelines/protocols for managing COVID-19 patients and vaccination programs. Despite robust preventive efforts by GoN, pandemic scenario in Nepal is, yet, to be controlled completely. This review could be helpful for the current and future effective outbreak preparedness, responses, and management of the pandemic situations and prepare necessary strategies, especially in countries with similar socio-cultural and economic status.

Keywords: COVID-19, pandemic, preparedness, response, spatial distribution, public health, Nepal

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INTRODUCTION

The unanticipated outbreak of the novel coronavirus was first reported in Wuhan, China, in December 2019; it transmits from human to human *via* droplets and aerosol (1). The WHO declared Coronavirus Disease 2019 (COVID-19) as a Public Health Emergency of International Concern (PHEIC) on January 30, 2020, and a pandemic on March 11, 2020 (2). As a result, countries

worldwide adopted various mitigative measures (3, 4) and eradication strategies (5), aiming to reduce potentially enormous damage and reach zero cases, respectively. However, significant gaps in advance preparedness and the implementation of response plans resulted in the rapid spread of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) globally with 219 nations reporting it as of January 22, 2021[1] (6).

The Federal Democratic Republic of Nepal is a landlocked country in South Asia bordered by India in the south, east, and west, and China in the north. Its population, gross domestic product (GDP), and human development index (HDI) are 29.24 million[2], 30.64 billion[3], and 0.579[4], respectively. The constitution of Nepal (2015) consists of a three-tier (federal, province, and local) governmental system. Each tier has the constitutional power to enact laws and mobilize its resources. In Nepal, the first case of COVID-19 was reported on January 23, 2020, in a 32-year-old Nepalese man who returned from Wuhan, China. Two months after the first case, the second case was diagnosed through domestic testing on March 23 in a returnee from France (7). Subsequently, the Government of Nepal (GoN) imposed early interventions approved by the WHO, including a travel ban and the Indo-Nepal and China-Nepal borders closure[5] (8) to delay the possible onset of the detrimental effects of the outbreak across the country.

This review presents a 1-year (up to January 22, 2021) scenario of COVID-19 in Nepal, reviews the strategies employed by the GoN to control COVID-19, and provides suggestions for the prevention and control of current and future pandemics. Federal, provincial, and district-level daily cases of COVID-19 [confirmed by real-time PCR (qRT-PCR), cured, and death] in Nepal from January 23, 2020, to January 22, 2021, were obtained from the Ministry of Health and Population (MoHP), GoN[6]. Searches using the website of MoHP of Nepal, PubMed, the WHO, the worldometer official website, and Google were conducted to gather the information on the number of deaths, cured, and confirmed cases of COVID-19 and reports describing the approach taken by the government to contain COVID-19 in Nepal. The search terms included "COVID-19 in Nepal" and "Prevention and management of COVID-19 in Nepal." Data used in this article were extracted from relevant documents and websites. The figures were constructed by using Origin 2016 and GIS 10.4.1. We did not consult any databases that are privately owned or inaccessible to the public.

TABLE 1 | Prevalence and case fatality ratio (CFR) of COVID-19 of top leading countries, neighbor countries of Nepal, and SAARC as of Jan 28, 2021.

Countries	Prevalence ratio on tested population (%)	Case fatality ratio (%)
TOP LEADING COUNTRIES		
USA	8.596	2.687
Brazil	31.681	2.721
Russia	3.767	2.170
NEIGHBOR COUNTRIES		
India*	0.099	1.460
China	0.055	5.297
SAARC		
Nepal	13.129	0.755
Pakistan	6.910	2.275
Bangladesh	14.769	1.661
Bhutan	0.188	0.128
Sri Lanka	3.710	0.542
Maldives	3.867	0.099
Afghanistan	22.281	4.799

**Belong to SAARC and top leading countries.*

EPIDEMIC STATUS OF COVID-19 IN NEPAL

The MoHP of Nepal confirmed the first and second cases of COVID-19, respectively, in January and March, in an interval of 2 months[1] (9). As of January 22, 2021, 268,948 COVID-19 positive cases were reported, with 263,546 recovered, and 1,986 death cases[6]. This data showed nearly 0.74% death and about 98% recovery rate in Nepal. The case fatality rate (CFR) was 0.5% up to March 30 in Nepal (9). The CFR in the USA, Brazil, and Russia is similar (∼2%), whereas in the South Asian Association of Regional Cooperation (SAARC) countries, the CFR varied from ∼0.09 to ∼4.7 % (**Table 1**). In total, 2,035,301 qRT-PCR tests were performed in Nepal, indicating about 13.47% current prevalence of COVID-19 among the qRT-PCR tested population as compared with 2.5% as of March 31, 2020[2]. As of reviewing, the prevalence of COVID-19 among the qRT-PCR tested population is higher than the neighboring countries, China (∼0.055%) and India (∼0.099%) (**Table 1**). In addition, up to the third quarter of 2020, <1% of the confirmed COVID-19 cases were symptomatic across all age groups, while the proportion of symptomatic cases progressively increased beyond 55 years of age from 1.3 to 9%[7,8]. Unlike Nepal, higher

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[6]Ministry of Health and Population. *COVID-19 Update*. (2020). Available online at: https://covid19.mohp.gov.np/ (accessed January 15, 2021).

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[8]World Health Organization. *WHO Nepal Situation Updates-22 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/22-who-nepal-sitrep-covid-19.pdf?sfvrsn=df7c946a_2 (accessed January 15, 2021).

symptomatic cases were reported from other parts of the world during the same period (10). Understandably, the scenario of the proportion of symptomatic to asymptomatic cases remains to vary between countries and care facilities. Few possible reasons for low symptomatic cases reported in the Nepalese population may be poor health-seeking behavior and utilization of tertiary health care services (11) for mild symptomatic cases, home isolation without a diagnosis, and a high rate of self-medication practices (12).

Among the provinces, Bagmati province (n = 144,278) has the highest number of confirmed cases in Nepal, followed by province no. 1 (n = 30,422) and Lumbini (n = 30,308) (**Figure 1A**). As depicted in **Table 2**, the confirmed cases of COVID-19 are distributed throughout the country in all the administrative districts. The total number of confirmed cases is highest in the Kathmandu district (n = 103,523) followed by Lalitpur (n = 16,106), Morang (n = 13,236), and Rupandehi (n = 9,708) districts and lowest in Manang (n = 20), Mugu (n = 37), Mustang (n = 43), and Humla (n = 44) districts (**Table 2**).

Among 268,948 confirmed cases, 174,193 were males, and 94,755 were females, with a male-to-female sex ratio of 1.85. The largest number of infected cases was reported in the age group 21–30 years (26.92%, n = 72,396), followed by the age group of 31–40 years (26.26%, n = 70,648) (**Figure 1B**); however, the number of death cases was higher in the age group 61–70 (23%, n = 458) (**Figure 1C**). A higher death trend in old age is also observed in Europe, America, and Asian countries (13, 14). Overall, male death was ∼2.33 times the death rate of females. Reports have indicated that men are at greater risk of around two time of acquiring severe outcomes of COVID-19, including hospitalizations, intensive care unit (ICU) admissions, and deaths (15). The enhanced susceptibility of males for COVID-19 associated adverse events may be correlated with the hormonal and immunological differences between males and females (15, 16). Among a total of 1,986 fatal cases (Male: n = 1,391; female: n = 595), over half (n = 1,166) were observed in senior adults (≥60 years). One early study among the Nepalese children suggested that male children were more commonly infected than female children (17).

Among 1,986 fatal cases (mean age: 66.15 years), 623 (31.37%), 721 (36.30%), and 642 (32.32%) were with no report of comorbidities, with single comorbidities, and with multiple comorbidities, respectively. In cases with single comorbidities, the highest incidence was reported in respiratory disease (n = 184) followed by hypertension (n = 117), renal disease (n = 107), diabetes (n = 77), liver disease (n = 44), and cardiovascular disease (n = 36) (**Figure 2**). Similar results are reported from other parts of the world (18). The detailed epidemiological trend analysis of COVID-19 in Nepal is shown in **Figure 3**.

Geographically, Nepal is divided into three distinct ecological zones, mountain, hilly, and low-plain land from north to south. Politically, Nepal is divided into 7 provinces, 77 districts, and 753 local bodies. There were multiple peaks of active cases of COVID-19 in Nepal: active cases rapidly increased from early May to early July 2020, then increased slowly up to late July and increased at a higher rate again up to the end of December, and then decreased sharply (**Figure 3A**). The spatial distribution of COVID-19 confirmed cases, recovery, and deaths were compared (**Figures 3B–D**). Approximately, 64.84% of the total confirmed cases were reported from the hill regions, with single megacity Kathmandu contributing nearly half, 33.31% of lowland-plain areas, and 1.85% of Himalayan regions. The reported cases in the megacities are relatively higher than in the other regions. The higher number of cases in megacities may be correlated with dense populations in these areas (8). In the earlier months, the testing facilities and contact tracing were limited only to few districts, including the capital, Kathmandu, which gradually became available in other parts of the country. However, the testing frequency and testing facilities are still not homogeneous due to the lack of required technical resources and professional workforces (19)[9].

THE RESPONSE OF NEPAL GOVERNMENT TO COVID-19

Nepal has adopted many readiness and response-related initiatives at the federal, provincial, and local government levels to fight against COVID-19. Initially, the government had set health desks and allocated spaces for quarantine purposes at the international airport and at the borders, crossing points of entry (PoE) with India and China[10], to withstand the influx of many possible infected individuals from India and other countries. The open border and the politico-religious relationship with India and migrant workers returning from the Middle East, and other countries were a source of rapid transmission to Nepal[10],[11]. The Nepal-China official border crossing points have remained closed since January 21, 2020. On March 24, 2020, the GoN imposed a complete "lockdown" of the country up to July 21, 2020. As part of the lockdown, businesses were closed, the restriction was imposed on movement within the country, workplaces were closed, travel was banned, and air transportation was halted[11],[12]. In addition, for COVID-19 preparedness and response, the GoN developed a quarantine procedure and issued an international travel advisory notice. Closing the border was critical as Nepal and India share open borders across which citizens travel freely for business and work.

[9]World Health Organization. (2020). *WHO Nepal Situation Updates-16 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/16--who-nepal--sitrep-covid-19-07082020-final.pdf?sfvrsn=53c5360f_2 (accessed January 15, 2021).

[10]Bhattarai, KD. *South Asian Voices: COVID-19 and Nepal's Migration Crisis*. Available online at: https://southasianvoices.org/covid-19-and-nepals-migration-crisis/ (accessed January 15, 2021).

[11]*GRADA WORLD Nepal: Government announces nationwide lockdown from March 24–31/update*. Available online at: https://www.garda.com/crisis24/news-alerts/326601/nepal-government-announces-nationwide-lockdown-from-march-24-31-update-4 (accessed January 15, 2021).

[12]Gautam D. *NDRC. Nepal's Readiness and Response to COVID-19*. (2020). Available online at: https://www.preventionweb.net/files/71274_71274nepalsreadinessandresponsetopa.pdf (accessed January 15, 2021).

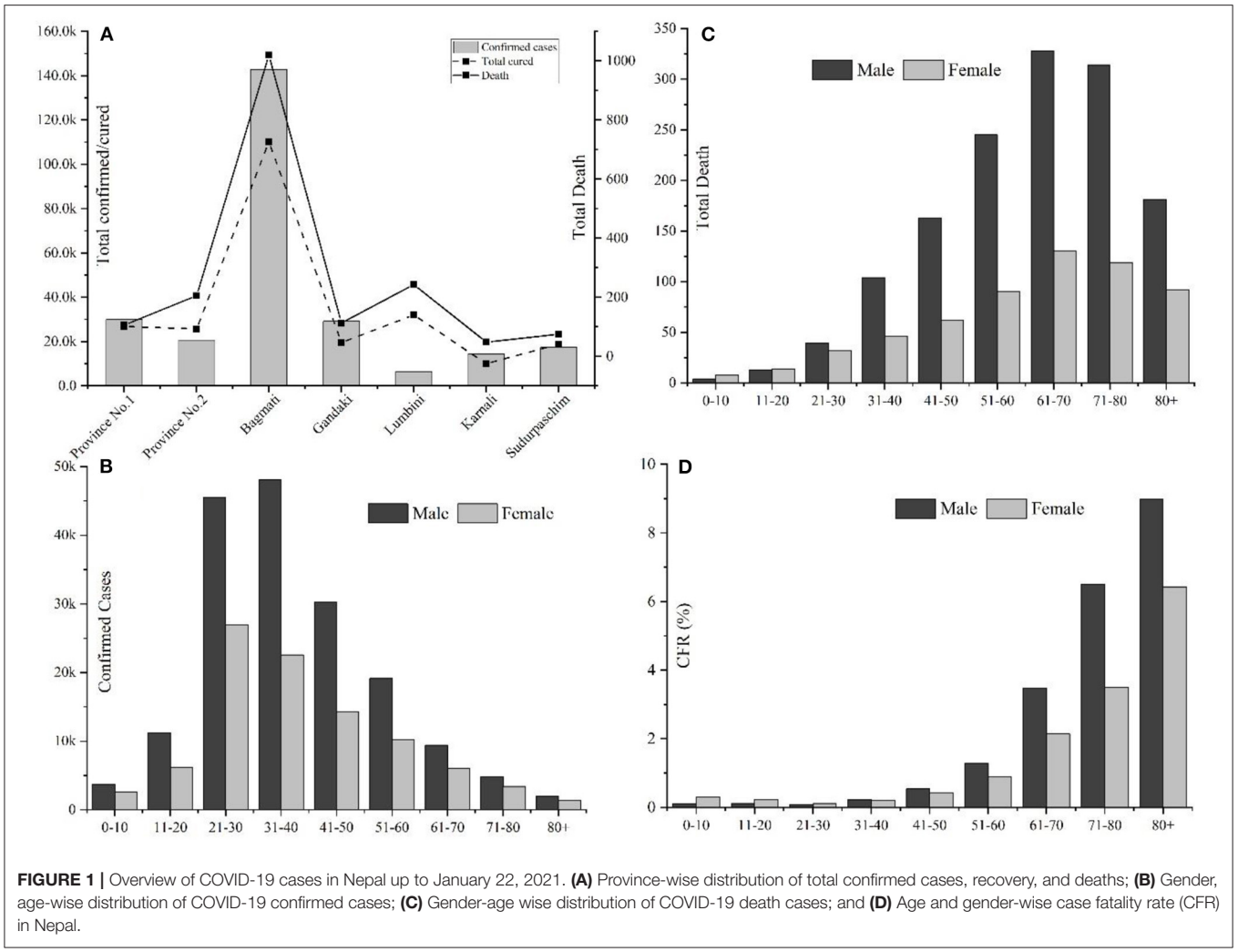

FIGURE 1 | Overview of COVID-19 cases in Nepal up to January 22, 2021. **(A)** Province-wise distribution of total confirmed cases, recovery, and deaths; **(B)** Gender, age-wise distribution of COVID-19 confirmed cases; **(C)** Gender-age wise distribution of COVID-19 death cases; and **(D)** Age and gender-wise case fatality rate (CFR) in Nepal.

The GoN underestimated both the short and long-term impacts of border closure[11]. Around 2.8 million Nepali migrant workers work in India. Though the GoN discussed holding these workers in India with its Indian counterpart[13], this plan did not materialize. Nepal has 1,690 km-long open borders with India, which could not keep migrant workers long despite the restrictions implemented by both governments[12]. As a consequence, the majority of COVID-19 cases were in the districts along the Indo-Nepal border. The decision of the government to lockdown the country from March 10, 2020, without sufficient preparation pushed daily wage laborers in urban areas to lose their jobs, and, hence, they were trapped without food or money. Ultimately, after a couple of days of lockdown, both migrant workers and daily wage laborers started walking the long way home due to the economic crisis.

As per the cabinet decision on March 25, 2020, Nepal established a COVID-19 response fund, developed a relief package[13], and distributed relief to families in need through a "one door policy"[13] designed to reduce the COVID-19 impact; however, there were several gaps: the selection of families was unfair, GoN delayed the procurement of relief, relief packages did not include cash, and relief materials were inadequate and substandard [14,15]. The government has not adequately taken into account the impact of COVID-19 on the socio-economic sector. For instance, people participated in meetings, rallies, political demonstrations, and protests, where the virus could quickly spread among a large group of people. The government has, yet, to develop a stimulus package for social and economic recovery

[13]*Building Back Better (BBB) from COVID-19: World Vision Policy Brief on Building Back Better from COVID-19*. (2020). Available online at: https://www.wvi.org/sites/default/files/202005/World%20Vision%20Policy%20Brief%20on%20Building%20Back%20Better_25%20May%202020.pdf (accessed January 15, 2021).

[14]World Health Organization. *WHO Nepal Situation Updates-1 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/who-nepal--sitrep-covid-19-20apr2020.pdf?sfvrsn=c788bf96_2 (accessed January 15, 2021).

[15]GoN MoHP. *Health Sector Emergency Response Plan COVID-19 Pandemic 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/health-sector-emergency-response-plan-covid-19-endorsed-may-2020.pdf?sfvrsn=ef831f44_2 (accessed January 15, 2021).

TABLE 2 | District wise distribution of confirmed cases, recoveries, and deaths due to COVID-19 and total population in Nepal.

S.N.	District	Confirmed#	Cured#	Death#	Total Population*	S.N.	District	Confirmed#	Cured#	Death#	Total Population*
1	Kathmandu	103,523	100,584	738	174,4240	40	Lamjung	1,142	1,127	2	167724
2	Lalitpur	16,106	15,806	69	468,132	41	Bajhang	1,115	1,079	12	195159
3	Morang	13,236	13,166	58	965,370	42	Pyuthan	1,103	1,076	15	228102
4	Rupandehi	9,708	9,496	93	880,196	43	Arghakhanchi	1,049	1,023	4	197632
5	Bhaktapur	9,245	9,139	60	304,651	44	Sindhupalchok	1,038	1,010	16	287798
6	Sunsari	9,145	9,111	26	763,487	45	Dolakha	802	772	10	186557
7	Chitawan	8,065	7,953	68	579,984	46	Dadeldhura	793	776	2	142094
8	Kaski	7,668	7,544	20	492,098	47	Baitadi	735	706	3	250898
9	Kailali	6,111	6,036	34	775,709	48	Bajura	733	724	5	134912
10	Banke	5,123	4,960	45	491,313	49	Udayapur	682	669	2	317532
11	Jhapa	5,033	4,993	23	812,650	50	Salyan	665	656	5	242444
12	Makawanpur	4,348	4,290	42	420,477	51	Sindhuli	660	631	4	296192
13	Dang	4,189	4,119	6	552,583	52	Ramechhap	590	567	19	202646
14	Kabhrepalanchok	3,642	3,584	33	381,937	53	Parbat	546	489	28	146590
15	Parsa	3,513	3,449	50	601,017	54	Ilam	495	482	5	290254
16	Dhanusha	3,131	3,097	29	754,777	55	Jumla	475	454	2	108921
17	Nawalpur	3,100	3,068	20	310,864	56	Darchula	426	409	15	133274
18	Sarlahi	2,861	2,841	18	769,729	57	Sankhuwasabha	399	392	3	158742
19	Rautahat	2,819	2,785	30	686,722	58	Rolpa	341	319	4	224506
20	Surkhet	2,514	2,492	13	350,804	59	Myagdi	302	278	2	113641
21	Kapilbastu	2,314	2,262	14	571,936	60	Kalikot	295	292	3	136948
22	Siraha	2,294	2,270	20	637,328	61	Okhaldhunga	279	272	3	147984
23	Palpa	2,246	2,214	2	261,180	62	Dhankuta	278	269	5	163412
24	Saptari	2,187	2,141	13	639,284	63	Rukum West	267	255	12	154,272
25	Bara	2,182	2,153	21	687,708	64	Rasuwa	238	227	1	43300
26	Mahottari	2,073	2,041	29	627,580	65	Khotang	224	220	0	206312
27	Bardiya	2,004	1,927	50	426,576	66	Solukhumbu	207	204	2	105886
28	Kanchanpur	1,948	1,929	4	451,248	67	Panchthar	192	179	8	191817
29	Tanahu	1,868	1,835	13	323,288	68	Bhojpur	166	163	3	182459
30	Dhading	1,762	1,742	8	336,067	69	Terhathum	164	155	1	101577
31	Achham	1,761	1,754	4	257,477	70	Taplejung	161	160	0	127461
32	Dailekh	1,666	1,655	6	261,770	71	Jajarkot	123	119	1	171304
33	Parasi	1,642	1,596	40	332,644	72	Rukum East	114	105	3	53184
34	Gorkha	1,550	1,490	30	271,061	73	Dolpa	60	55	2	36700
35	Nuwakot	1,530	1,456	17	277,471	74	Humla	44	37	4	50858
36	Doti	1,448	1,444	1	211,746	75	Mustang	43	42	0	13452
37	Baglung	1,275	1,241	7	268,613	76	Mugu	37	35	2	55286
38	Gulmi	1,244	1,208	12	280,160	77	Manang	20	15	1	6538
39	Syangja	1,201	1,184	10	289,148	**Total**		**268948**	**263546**	**1986**	26494504

*Source: *National Population Census, 2011; #Ministry of Health and Population. COVID-19 Update. Available from: https://covid19.mohp.gov.np/ (Accessed on January 23, 2021).*

at the micro and macro levels. As the government has allocated $788 million for the health sector for the fiscal year (July–June 2020), a budget of 32% larger than the previous fiscal year, it should address the COVID-19 impact on the socio-economic front[16]. There is an opportunity to integrate all fragmented social protection schemes to strengthen socio-economic conditions and to emphasize more tremendous efforts, capacities, and resources to cope with the likely impacts of the COVID-19 pandemic[16].

In addition, a minimal standard of quarantine as per the "Quarantine Operation and Management Protocol" (2076 B.S.) and "Standards for Home Quarantine" were imposed for all provinces[16,17]. The Sukraraj Infectious and Tropical Disease

[16]Gautam, D. *The COVID-19 Crisis in Nepal: Coping Crackdown Challenges. National Disaster Risk Reduction Centre, Kathmandu, Nepal. Issue 3, 2020.* Available online at: https://www.alnap.org/help-library/the-covid-19-crisis-in-nepal-coping-crackdown-challenges (accessed January 30, 2021).

[17]Gautam, D. *Fear of COVID-19 Overshadowing Climate-Induced Disaster Risk Management.* Available online at: https://www.spotlightnepal.com/2020/05/08/fear-covid-19-overshadowing-climate-induced-disaster-risk-management/ (accessed January 30, 2021).

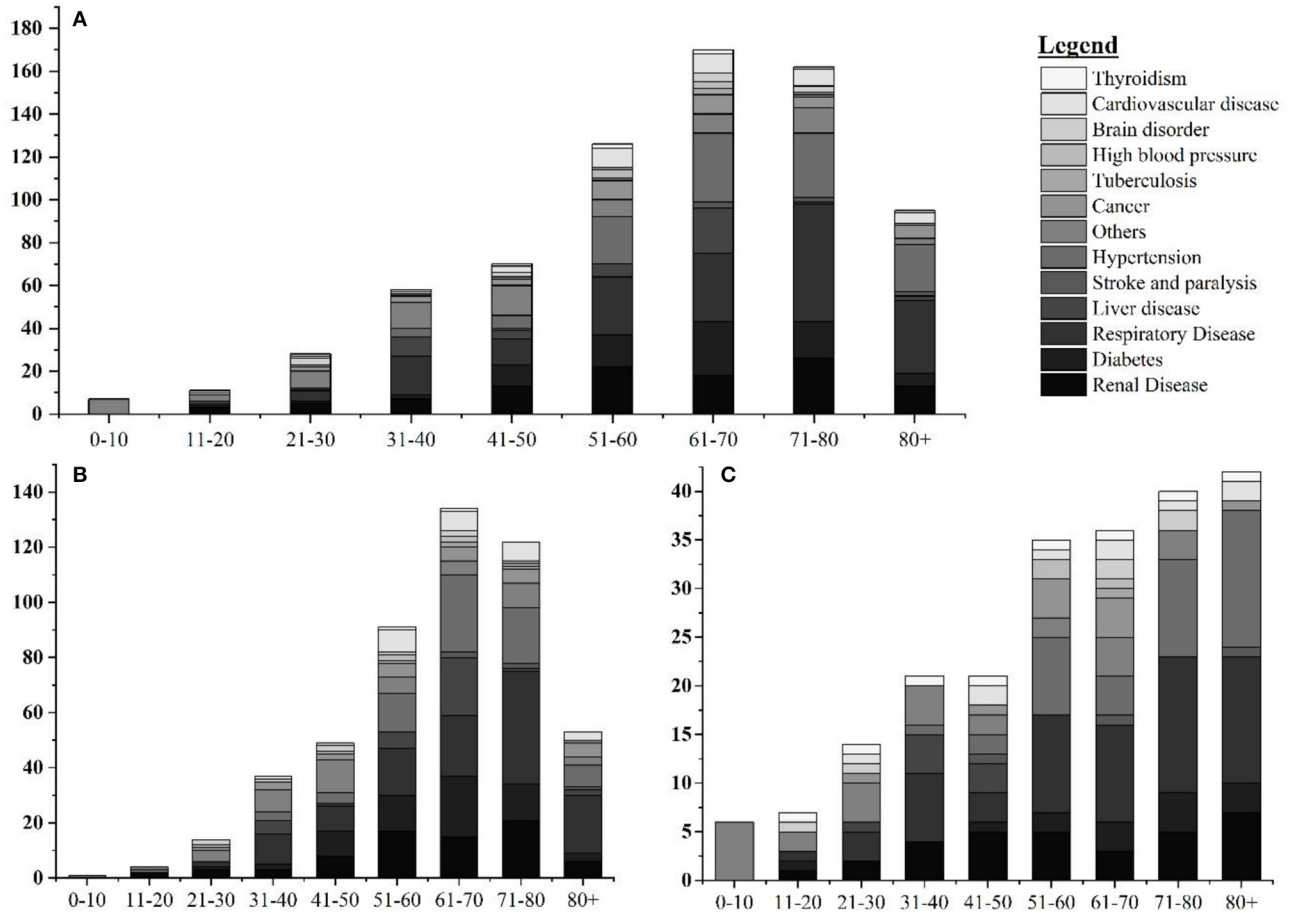

FIGURE 2 | Age and gender-wise distribution fatal cases with single comorbidities. **(A)** Age-wise distribution of leading single comorbidities among COVID-19 deaths; **(B)** age-wise distribution of leading single comorbidities among COVID-19 deaths in Nepal in male; and **(C)** age-wise distribution of leading single comorbidities among COVID-19 deaths in Nepal in female.

Hospital (SITDH) in Teku, Kathmandu, was designated by GoN as the primary hospital for COVID-19 cases along with Patan Hospital, the Armed Police Forces Hospital, in the Kathmandu Valley, followed by twenty-four hubs, and four satellite hospitals across the country[18]. Similarly, MoHP updated the National Public Health Laboratory (NPHL) capacity for confirmatory laboratory diagnosis of the COVID-19 from January 27, 2020, followed by the regional laboratory. The interim guideline for the establishing and operating of molecular laboratories for COVID-19 testing in Nepal was imposed to make uniformity in the test results[14]. Furthermore, the NPHL organized the training of trainers for laboratory staff in collaboration with the Medical Laboratory Association of Nepal[19]. Ministry of Health and Population established two hotline numbers (1115 and 1133) to address public concerns, and prepared and disseminated regular press briefings, and improved its websites to channel appropriate information to the public. Besides, MoHP also conveyed decisions, notices, and situation updates periodically through its websites. Further, the Health Emergency Operation Centre (HEOC) of MoHP launched a "Viber communication group" to circulate updates on COVID-19[11,13]. Early testing and timely contact tracing are crucial restrictive policies to control the spreading of the SARS-CoV-2 virus (20, 21); however, in the earlier days of the pandemic, Nepal could not perform enough diagnostic tests and timely contact tracing; it resulted in a crucial time lag in identifying and isolating COVID-19 patients and caused delays in the ability of government to respond to the pandemic adequately. To alert and improve the testing and tracing response of the government, youth-led protests were carried out in different parts of the country[20]. Health Sector Emergency Response Plan was implemented in

[18]Pradhan TR. *The Kathmandu Post. Nepal Goes Under Lockdown for a Week Starting 6am Tuesday*. Available online at: https://kathmandupost.com/national/2020/03/23/nepal-goes-under-lockdown-for-a-week-starting-6am-tuesday (accessed January 30, 2021).

[19]World Health Organization. *WHO Nepal Situation Updates-3 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/who-nepal--situpdate-3-covid-19-06052020.pdf?sfvrsn=714d14c4_2 (accessed January 30, 2021).

[20]Jha IC. *The Rising Nepal. MoHP Sets Forth Standards for Home Quarantine*. Available online at: https://risingnepaldaily.com/featured/mohp-sets-forth-standards-for-home-quarantine (accessed January 30, 2021).

May 2020, focusing on the COVID-19 pandemic. This plan intends to prepare and strengthen the health system response capable of minimizing the adverse impact of the COVID-19 pandemic. Government of Nepal devised a comprehensive plan on March 27, 2020, for quarantining people who arrived in Nepal from COVID-19 affected countries. The GoN had initially airlifted 175 Nepalese from six cities across Hubei Province of China on February 15, 2020, followed by Middle East countries, Australia, and so on[13].

Ministry of Health and Population engaged in developing, endorsing, improving, and disseminating contextualized technical guidelines, standard operating procedures (SOPs), tools, and training in all other critical aspects of the response to COVID-19, for instance, surveillance, case investigation, laboratory testing, contact tracing, case detection, isolation and management, infection prevention and control, empowering health and community volunteers, media communication and community engagement, rational use of personal protective equipment (PPE), requirements of drugs and equipment for case management and public health interventions, and continuity of essentials services[13] (15). The major contextualized technical guidelines, SOPs, tools, and training materials developed by GoN to respond to COVID-19[21,22,23,24,25,26,27,28,29,30] were listed in **Table 3**.

Ministry of Health and Population and supporting organizations, such as United Nations Development Program (UNDP), UNICEF, and World Vision managed crucial supplies of PPE, facemasks, gloves, and sanitizers to ensure the protection of frontline workers and supporting staffs[13,30,31,32]. The frontline media of the nation increased online awareness programs *via* the involvement of celebrities, doctors, and experts of microbiology and infectious diseases on physical distancing and the importance and use of masks and sanitizers to prevent the COVID-19 contagion. In addition, camping programs were launched by the involvement of youth volunteers of the community in central Nepal[33].

Government of Nepal received funds from the World Bank ($29 million), the United States of America ($1.8 million), and Germany ($1.22 million) to keep people protected from COVID-19 through health systems preparedness, emergency response, and research. In addition, support from UNICEF and countries, including China, India, and the USA, in the form of emergency medical supplies and equipment were received within January 2020 to March 2020. Private companies, corporate houses, business organizations, and individuals have also contributed to the prevention, control, and treatment fund of coronavirus ($13.8 million), established by GoN to cope with COVID-19. The Prime Minister Relief Fund is also expected to be utilized. The GoN allowed international NGOs to divert 20% of their program budget to COVID-19 preparedness and response; for instance, the Social Welfare Council has allocated $226 million[31,33,34,35,36,37].

The GoN has formed a committee to coordinate the preparedness and response efforts, including the MoHP, Ministry of Home Affairs, Ministry of Foreign Affairs, Ministry of Finance,

[21]The Kathmandu Post. *Youth-Led Protests Against the Government's Handling of Covid-19 Spread to Major Cities*. (2020). Available online at: https://kathmandupost.com/national/2020/06/12/youth-led-protests-against-the-government-s-handling-of-covid-19-spread-to-major-cities (accessed January 30, 2021).

[22]World Health Organization. *WHO Nepal Situation Updates-2 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/who-nepal--sitrep-covid-19-29apr2020.pdf?sfvrsn=dac001bf_2 (accessed January 30, 2021).

[23]World Health Organization. *WHO Nepal Situation Updates-4 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/who-nepal--situpdate-4-13052020.pdf?sfvrsn=630b68ea_6 (accessed January 30, 2021).

[24]World Health Organization. *WHO Nepal Situation Updates-18 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/18-who-nepal-sitrep-covid-19-23082020.pdf?sfvrsn=6fb20500_2 (accessed February 5, 2021).

[25]World Health Organization. *WHO Nepal Situation Updates-5 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/5-who-nepal--situpdate-covid-19-20052020-final.pdf?sfvrsn=7552c8ba_4 (accessed February 5, 2021).

[26]World Health Organization. *WHO Nepal Situation Updates-7 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/7-who-nepal--situpdate-covid-19-03062020-final.pdf?sfvrsn=87f582d6_2 (accessed February 5, 2021).

[27]World Health Organization. *WHO Nepal Situation Updates-8 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/8-who-nepal--situpdate-covid-19.pdf?sfvrsn=ce5ecb07_2 (accessed February 5, 2021).

[28]World Health Organization. *WHO Nepal Situation Updates-10 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/10-who-nepal--situpdate-covid-19-24062020.pdf?sfvrsn=c7f99a61_8 (accessed February 5, 2021).

[29]World Health Organization. *WHO Nepal Situation Updates-13 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/13--who-nepal--situpdate-covid-19-17072020-v4.pdf?sfvrsn=fc0f19cc_2 (accessed February 5, 2021).

[30]World Health Organization. *WHO Nepal Situation Updates-17 on COVID-19, 2020*. (2020). Available online at: https://www.who.int/docs/default-source/nepal-documents/novel-coronavirus/who-nepal-sitrep/17-who-nepal-sitrep-covid-19-15082020.pdf?sfvrsn=68a53b32_2 (accessed February 10, 2021).

[31]UN. *Nepal Information Platform, COVID-19 Nepal: Preparedness and Response Plan*. Available online at: http://un.org.np/reports/covid-19-nepal-preparedness-and-response-plan (accessed February 10, 2021).

[32]*UNICEF for Every Child, Supporting COVID-19 Readiness and Response in the West of Nepal*. Available online at: https://www.unicef.org/nepal/stories/supporting-covid-19-readiness-and-response-west-nepal (accessed February 10, 2021).

[33]UNDP. *Enhancing Public Awareness on COVID-19 Through Communications*. Available online at: https://www.np.undp.org/content/nepal/en/home/presscenter/articles/2020/Enhancing-public-awareness-of-COVID-19-through-communications.html (accessed February 10, 2021).

[34]The World Bank. *The Government of Nepal and the World Bank sign $29 Million Financing Agreement for Nepal's COVID-19 (Coronavirus) Response*. Available online at: https://www.worldbank.org/en/news/press-release/2020/04/03/world-bank-fast-tracks-29-million-for-nepal-covid-19-coronavirus-response (accessed February 10, 2021).

[35]Khatri PP. *The Rising Nepal. Govt Receives Over Rs 1.59 Bln In Anti-COVID-19 Fund*. Available online at: https://risingnepaldaily.com/main-news/govt-receives-over-rs-159-bln-in-anti-covid-19-fund (accessed February 10, 2021).

[36]Dahal A. *Govt Does U-Turn to Let NGOs Hand Out Medical Supplies, Food, Cash directly*. Available online at: https://myrepublica.nagariknetwork.com/news/govt-does-u-turn-to-let-ingos-hand-out-medical-supplies-food-cash-directly/ (accessed February 10, 2021).

[37]Rijal A. *The Rising Nepal. China Gives Anti-Corona Medical Aid*. Available online at: https://risingnepaldaily.com/main-news/china-gives-anti-corona-medical-aid (accessed February 10, 2021).

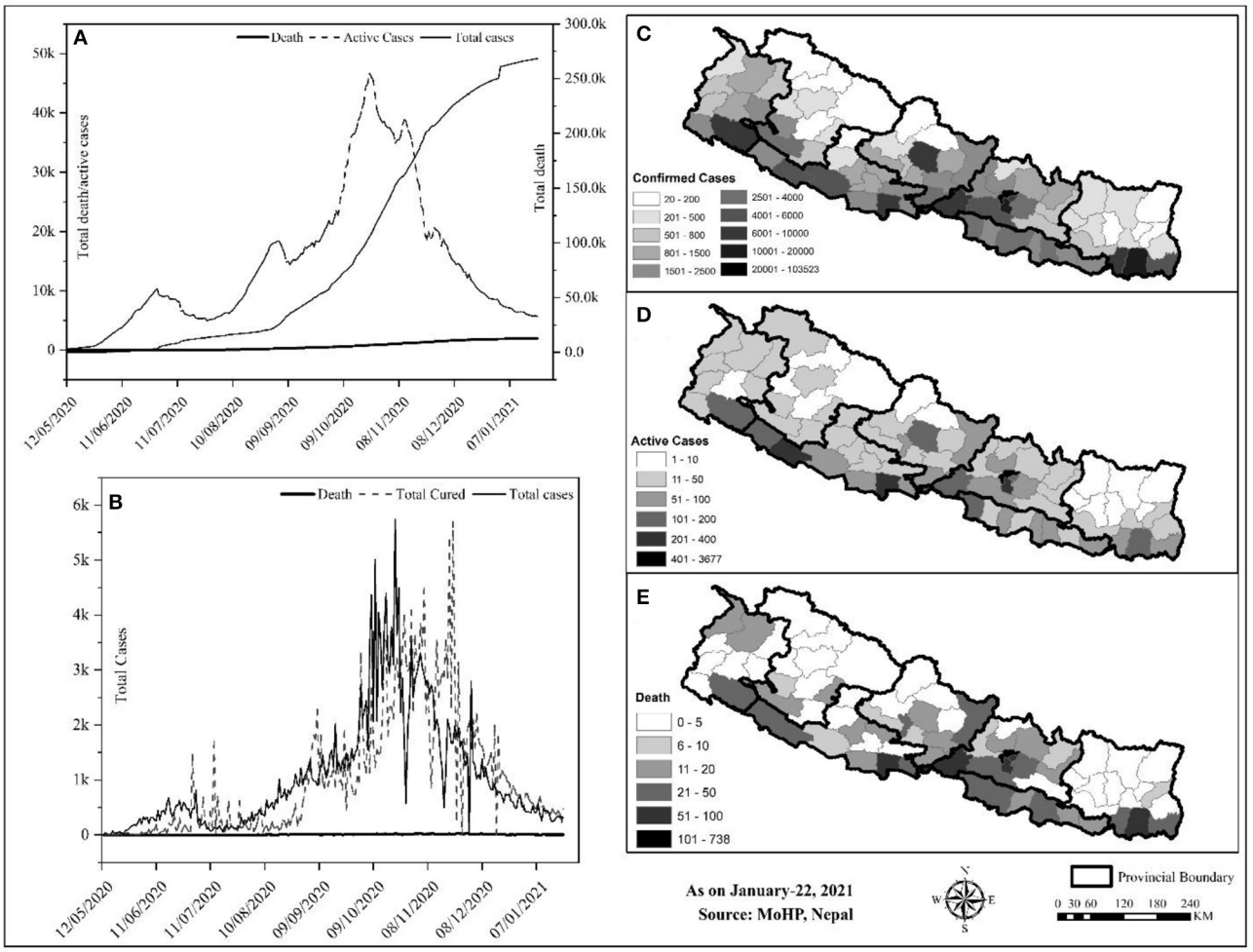

FIGURE 3 | Trend and spatial distribution of COVID-19 cases in Nepal. **(A)** Cumulative trend analysis of COVID-19 cases, **(B)** daily case wise trend analysis of COVID-19, **(C–E)** spatial distribution of infected, recovered, and death cases.

Ministry of Culture, Tourism and Civil Aviation, Ministry of Urban Development, Nepal Army, Nepal Police, and Armed Police Force. The Humanitarian Country Team (HCT) includes the Red Cross Movement and civil society organizations (national and international NGOs). Under the joint leadership of the office of Resident Coordinator and the WHO, the HCT has initiated contingency planning and preparedness interventions, including the dissemination of communications materials to raise community-level awareness across the country[21]. The clusters led by the GoN and co-led by the International Astronomical Search Collaboration (IASC) cluster leads and partners are working on finalizing contingency plans, which will be consolidated into an overall joint approach with the Government and its international partners. The UN activated the provincial focal point agency system to support coordination between the international community and the GoN at the provincial level[21].

However, despite these robust efforts implemented by GoN, few lapses existed. Examples are the following: issues of inconsistent implementation of immigration policies usually at Indo-Nepal borders[38,39,40], shortage and misuse of crucial protective suits and other supplies in hospitals, the ease and the end of lockdown, lack of poor infrastructure facilities, and continuous spread of COVID-19 across the country (19). The GoN decided to lift the lockdown effective from July 22, 2020, completely; however, the socio-administrative and health measures with the potential for high-intensity transmission (colleges, seminars, training, workshops, cinema halls, party palaces, dance bars, swimming pools, religious places, etc.)

[38]Nepali Sansar. *Nepal Receives 23 Tons 'COVID-19 Medical Equip' As Gifts from India*. (2020). Available online at: https://www.nepalisansar.com/coronavirus/nepal-receives-23-tons-covid-19-medical-equip-as-gifts-from-india/ (accessed February 10, 2021).

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TABLE 3 | Major contextualized technical guidelines, standard operating protocols, tools, and training materials developed by the Government of Nepal (GoN) to respond to COVID-19.

S.No.	Date	Major contextualized technical guidelines, SOPs, tools, and training materials
1	April, 2020	Guidelines for the management of front-line healthcare service providers and other workers involved in the management of COVID-19 cases
2	April, 2020	Interim guidance for the operation of nutrition rehabilitation in the context of COVID-19
3	April, 2020	Interim pocketbook of clinical management of COVID-19 in the healthcare setting and Infection Prevention and Control pocket booklet; interim guideline for the establishment and operationalization of molecular laboratory for COVID-19 testing in Nepal; a guideline on safety measures to be taken at the point of entry
4	May, 2020	Standard operating procedure of cleaning and decontamination of the ambulance used in COVID-19. The Department of Ayurveda and alternative medicine has recently published national guidelines on preventive measures and management protocol for COVID-19 in Nepal
5	May, 2020	Guidelines for the management of dead bodies of people who died from COVID-19, COVID-19 cases isolation management, and COVID-19 emergency medical teams (EMDT) mobilization
6	June, 2020	MoHP has issued a guideline on minimum standards for donor agencies/partner organizations for COVID-19 logistics support to the MoHP-2020
7	July, 2020	Guidance on testing of high-risk groups and random testing of people in communities at Kathmandu Valley including other high-risk COVID-19 affected districts to detect community transmission
8	August, 2020	Standards for the service delivery of senior citizens in the context of COVID-19

remained closed until the following directive as of September 1, 2020. Long route bus services and domestic and international passenger flights were halted until August 1, 2020[41]. A high-level committee at the MoHP has requested all satellite hospitals (public, private, and others) to allocate 20% of their beds for COVID-19 cases. The respective hub hospitals coordinate with the HEOC and satellite hospitals to manage COVID-19 cases[42]. After lifting lockdown for 3 weeks, the federal government has given authority to local administrations to decide on restrictions and lockdown measures as COVID-19 cases continue to rise. In addition, the authority to impose necessary restrictions if COVID-19 active cases surpass the threshold of 200 was given to the Chief District Officer (CDO)[43]. Since March 2020, all the central hospitals, provincial hospitals, medical colleges, academic institutions, and hub-hospitals were designated to provide treatment care for COVID-19 cases. At this stage of operation, the major challenges for the COVID-19 response were managing quarantine facilities, lack of enough human resources, having limited laboratories for testing, and availability of limited stock of medical supplies, including PPEs[14]. To the best of our knowledge, this pandemic is the most extensive public health emergency the GoN faced in its recent history.

There is no doubt that GoN has taken major initiatives to fight the COVID-19 pandemic. The MoHP, together with associated national and international organizations are closely monitoring and evaluating the signs of outbreaks, challenges, and enforcing the plan and strategies to mitigate the possible impact; however, many challenges and difficulties, such as management of testing, hospital beds, and ventilators, quarantine centers, frontline staffs, movement of people during the lockdown, are yet to be solved[18,30,3844,45,46,47]. Therefore, in the opinion of the authors, we recommend some steps to be implemented as soon as possible to mitigate and lessen the impacts of COVID-19 (**Table 4**).

To strengthen its coordination mechanism, the government formed a team to monitor conditions and measures applied to control the outbreak; a COVID-19 coordination committee[11] to coordinate the overall response, and a COVID-19 crisis management center[14] to coordinate daily operations; however, these teams and committees did not function efficiently because roles and authorities were not delegated to ministries and government. A new institution was created, instead of using the National Disaster Risk Reduction and Management Authority (NDRRMA)[48], which enhanced additional confusion. The

[41] Raturi K. *How Is Nepal Tackling COVID Crisis & Reverse Migration of Workers?* Available online at: https://www.thequint.com/voices/opinion/india-nepal-border-coronavirus-pandemic-migrant-workers-exodus-reverse-migration-unemployment (accessed February 10, 2021).

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[48] *National Disaster Risk Reduction and Management Authority, Ministry of Home Affairs, Government of Nepal*. Available online at: https://covid19.ndrrma.gov.np/ (accessed February 10, 2021).

TABLE 4 | Major steps taken by GoN and way forward in the response to COVID-19 outbreak.

S. N.	Date	Key steps taken by the GoN	Way ahead for Nepal
1	January, 2020	Early warning and reporting system (EWARS)—daily and weekly bulletin: Nepal	Increase the tracing and testing
2	March, 2020	Formed a high-level coordination committee led by the Deputy Prime Minister	Impact analysis and current and post-pandemic recovery plans and strategies
3	March, 2020	Non-pharmacological interventions such as lockdown, social distancing, quarantine, travel restrictions, media awareness	Capacity building training for healthcare personal and front-line workers
4	March, 2020	Postponed less essential scheduled campaigns program, for instance, Visit Nepal 2020	Training and monitoring for vaccination
5	March, 2020	Discussion with experts to outline new strategies, frame action plans and implement interventions	
6	April, 2020	Collaboration, networking, and coordination with ministries, global health sectors, NGOs, and INGOs. Instructed INGOs to divert up to 20% of their program budget to tackle COVID-19	Preparation of a robust database and information system
7	April, 2020	Guidelines for SOP/Tools/Protocol for management of COVID-19	
8	April, 2020	Establishment of grain banks for needy families	
9	May, 2020	Acceptance of donation fund and set up an emergency COVID-19 fund at the federal level, province level, and local level	Strengthening of laboratory and hospital facilities, and motivation for frontline staffs
10	May, 2020	Significantly strengthened ICU, ventilators, laboratory facilities, expansion of laboratory and testing	Systematization of quarantine centers and isolation beds
11	August, 2020	Procurement and supplies of PPE and Coronavirus Insurance Program	Volunteer mobilization and increase awareness and knowledge for citizens

MoHP is responsible for overall policy formulation, planning, organization, and coordination of the health sector at federal, provincial, district, and community levels during the COVID-19 pandemic situation. Allegedly, there is an opportunity to strengthen coordination among the tiers of governments by following protocols and guidance for effective preparedness and response. For example, some quarantine centers were so poorly run that, in turn, could potentially develop into breeding grounds for the COVID-19 transmission[15].

Finally, this study only focuses on analyzing COVID-19 data extracted from the MoHP database for 1 year. Furthermore, we did not quantify the effectiveness of the strategies of GoN and the role of non-governmental organizations and authorities to combat COVID-19 in Nepal.

CONCLUSION

This study provides an insight into the impacts of the COVID-19 pandemic from the Nepalese context for the period of first-wave from January 2020 to January 2021. Despite the several initiatives taken by the GoN, the current scenario of COVID-19 in Nepal is yet to be controlled in terms of infections and mortality. A total of 268,948 confirmed cases and 1,986 deaths were reported in one year period. The maximum number of cases were reported from Bagmati province ($n = 144{,}278$), all of the 77 districts were affected. The cases showing highly COVID-specific symptoms were low (<1%) in comparison with the reports across the globe (10), which may be because the average age of the Nepalese population is younger than many of the highly affected European countries. The other reasons may be differences in demographic characteristics, sampling bias, healthcare coverage, testing availability, and inconsistencies relating to the reporting of the data included in the current study. Both the number of infections and deaths are higher in males than in females. Despite the age, testing and positivity, hospital capacity and hospital admission criterion, demographics, and HDI index, the overall case fatality was reported to be less than in some other developed countries (**Table 1**). Consistent with reports from other countries (22, 23), the death rate is higher in the old age group (**Figure 1**). Spatial distribution displayed the cases, which are majorly distributed in megacities compared with the other regions of the country.

Based on this assessment, in addition to the WHO COVID-19 infection prevention and control guidance[49], some recommendations, such as massive contact tracing, improving bed capacity in health care settings and rapid test, proper management of isolation and quarantine facilities, and advocacy for vaccines, may be helpful for planning strategies and address the gaps to combat against the COVID-19. Notably, the recommendations provided could benefit the governmental bodies and concerned authorities to take the appropriate decisions and comprehensively assess the further spread of the virus and effective public health measures in the different provinces and districts in Nepal. In this review, we have summarized the ongoing experiences in reducing the spread of COVID-19 in Nepal. The Nepalese response is characterized by nationwide lockdown, social distancing, rapid response, a

[49]World Health Organization. *Infection Prevention and Control Guidance - (COVID-19)*. (2021). Available online at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/advice-for-public (accessed February 10, 2021).

multi-sectoral approach in testing and tracing, and supported by a public health response. Overall, the broader applicability of these experiences is subject to combat the COVID-19 impacts in different socio-political environments within and across the country in the days to come.

AUTHOR CONTRIBUTIONS

BB: Conceptualization, writing, and original draft preparation. KB, BB, and AG: data curation. BB, RP, TB, SD, NP, and DG: writing, review, and editing. All authors contributed to the article and approved the submitted version.

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Acute Kidney Injury and Early Predictive Factors in COVID-19 Patients

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Objectives: Our objective was to explore the incidence and early predictive factors of acute kidney injury in coronavirus disease 2019 (COVID-19) patients.

Method: We established a retrospective cohort of 408 patients who were admitted to Shenzhen Third People's Hospital in Shenzhen, China, between January 1 and March 31, 2020. Clinical outcomes and renal function were monitored until April 12, 2020, with a median follow-up duration of 21 days [interquartile range (IQR) = 14–33].

Results: When first admitted to hospital (baseline), 19.36% (79/408) presented renal dysfunction [estimated glomerular filtration rate (eGFR) <90 ml/min/1.73 m^2]. During follow-up, 3.9% (16/408) developed acute kidney injury (AKI). Age ≥60 years [hazard ratio (HR) = 4.78, 95% CI = 1.10–20.69], PaO_2/FiO_2 ratio <300 (HR = 3.48, 95% CI = 1.04–11.62), and higher creatinine (HR = 1.04, 95% CI = 1.01–1.07) at baseline independently predicted the risk of AKI. Respectively, 25.0% (102/408), 3.9% (16/408), 0.5% (2/408), 1.0% (4/408), and 0.2% (1/408) experienced G2, G3a, G3b, G4, and G5 as their most severe category during hospitalization, while 69.4% (283/408) had normal eGFRs throughout the follow-up period. When finally discharged from hospital, there were 12.5% (51/408) of patients with abnormal eGFRs.

Conclusions: COVID-19 patients can be at risk of AKI and continuous eGFR decline during hospitalization, which can be early predicted by baseline factors. Some individuals still had renal dysfunction when finally discharged from hospital.

Keywords: coronavirus, COVID-19, SARS-CoV-2, creatinine, eGFR, acute kidney injury

BACKGROUND

Coronavirus disease 2019 (COVID-19) is an emerging respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). According to the updated information of the "Coronavirus disease 2019 (COVID-19) Situation Report" by WHO, a total of 169,604,858 confirmed cases and 3,530,837 deaths were reported globally as of May 30, 2021 (1). Unfortunately, targeted drugs have not been available to date, and the number of infections is still growing worldwide. For the foreseeable future, COVID-19 could constantly pose a great threat to human health.

Most of the published articles on COVID-19 highlighted the lungs as the main organ involved in the disease (2–4), and a series of studies have also reported data regarding injury in the liver (5–7), the cardiovascular system (8, 9), and the gastrointestinal tract (10). Unfortunately, a few studies have also reported an increased incidence of renal injury following diagnosis of COVID-19, e.g., acute kidney injury (AKI) in COVID-19 patients varying from 0.1 to 29% (11–15). Meanwhile, a large cohort study from China revealed that 44% of COVID-19 patients developed proteinuria or hematuria, 15.5% had an increase of blood creatinine, and 14.1% presented an increase of blood urea nitrogen; these kidney dysfunction-related events were identified as independent associated factors for mortality (14). Another study on renal histopathological analysis of 26 autopsies of patients with COVID-19 reported that immunostaining with a SARS-CoV nucleoprotein antibody was positive in the tubule epithelium (16), which provided direct evidence of the invasion of SARS-CoV-2 into the kidney tissue. In addition, systemic hypoxia, abnormal coagulation, and possible drug or hyperventilation-relevant rhabdomyolysis could also contribute to kidney injury in COVID-19 patients (17, 18).

In summary, the existing studies mainly reported various incidences of AKI or described abnormalities in kidney laboratory tests using cross-sectional data; however, the longitudinal changes of renal function and the early predictive factors of renal dysfunction in COVID-19 patients have not been well characterized and evaluated yet. Therefore, it is quite urgent to add longitudinal data regarding renal function in COVID-19 patients. In this study, we aimed to investigate the dynamics of renal function and the risk of AKI in COVID-19 patients during hospitalization and explore the predictive factors in the early stage.

METHODS

Study Design and Data Collection

All confirmed COVID-19 patients admitted to Shenzhen Third People's Hospital between January 1 and March 31, 2020, were enrolled. Shenzhen Third People's Hospital, located in Guangdong, China, is the designated hospital with the largest number of COVID-19 cases outside Hubei in China. All the subjects with COVID-19 enrolled in this study were diagnosed according to the WHO interim guidance. The clinical outcomes of COVID-19 and renal function were monitored up to April 12, 2020. During the follow-up period, patients may include one or more hospital admission(s), e.g., there were 43 patients who were re-admitted to the hospital due to recurrence of positive SARS-CoV-2. The inclusion criteria were: (1) subjects diagnosed with COVID-19; (2) subjects with the records well-documented; and (3) subjects with longitudinal follow-up, i.e., renal function testing [i.e., estimated glomerular filtration rate (eGFR) and creatinine] with at least across 2 days during follow-up. Subjects with missing data at baseline for eGFR, creatinine, or severity of COVID-19 were excluded. Finally, 408 COVID-19 patients who met the above eligibility criteria were selected for analysis in this study.

The study protocol was consistent with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of Shenzhen Third People's Hospital (2020-183). All subjects provided signed informed consent.

Confirmation of COVID-19

The presence of SARS-CoV-2 was detected using the real-time reverse transcription polymerase chain reaction (RT-PCR) method. Two pairs of primers targeting the open reading frame 1ab (ORF1ab) and the nucleocapsid protein (N) were amplified and examined. Each sample was run in triplicate with a positive and negative control set, as suggested. These diagnostic criteria were based on the recommendations of the Chinese Center for Disease Control and Prevention (CDC). Samples identified as positive for SARS-CoV-2 by the local laboratory, further confirmed by the Key Laboratory of Shenzhen CDC, China.

Clinical Evaluation, Follow Up, and Outcomes

Baseline was defined as the first hospital admission due to COVID-19. At baseline and during follow-up, all subjects included in this study underwent routine examination, monitoring of renal function, and SARS-CoV-2 nucleic acid testing with a median interval of 3 days. The median follow-up period of patients was 21 days (IQR = 14–33).

Serum creatinine was used for assessing AKI-related events and eGFR was used for monitoring whether a decline occurs in renal function during follow-up. The eGFRs were classified into six categories according to the Kidney Disease Improving Global Outcomes (KDIGO) guideline (19): G1: $\geq$90 ml/min/1.73 m^2 (normal); G2: 60–89 ml/min/1.73 m^2 (mildly decreased kidney function); G3a: 45–59 ml/min/1.73 m^2 (mild-moderate loss of kidney function); G3b: 30–44 ml/min/1.73 m^2 (moderate–severe loss of kidney function); G4: 15–29 ml/min/1.73 m^2 (severe loss of kidney function); and G5: <15 ml/min/1.73 m^2 (kidney failure). Abnormal eGFR is defined as <90 ml/min/1.73 m^2, i.e., for which the eGFR is at a category of G2, G3a, G3b, G4, or G5. Urine tests were used to assess the proportion of proteinuria or hematuria for patients at baseline.

The main outcome was the incidence of AKI, which was defined as an elevation in serum creatinine of $\geq$0.3 mg/dl (26.5 μmol/L) or $\geq$1.5 times compared to a previous time point. The time to event was defined as the period from the date of the first hospital admission to the date of occurrence of the defined outcome. Patients were censored at death, discharge, or the last follow-up visit.

Statistical Analysis

All analyses were carried out using R software version 3.6.1. Firstly, we described the baseline characteristics for all subjects and for subgroups stratified by baseline renal function (eGFR <90 vs. $\geq$90 ml/min/1.73 m^2).

Secondly, we calculated the total proportions of AKI occurrence during follow-up and then summarized these incidences stratified by baseline characteristics (age, sex, and eGFR), using χ^2 test or Fisher's exact test to compare the proportions between subgroups. Furthermore, we performed

Cox regression analysis to examine the early predictive factors of an AKI event. Covariates were included in the multivariable model if they had a p-value < 0.1 in the univariable Cox regression analysis, or if they were considered to be key factors from the clinical perspective.

Thirdly, we analyzed the dynamics of eGFR during follow-up. Specifically, we investigated the most severe renal dysfunction of each individual during follow-up and summarized the proportion of patients who still had abnormal eGFRs when finally discharged from the hospital.

All significance tests performed were two-sided. Values of $p < 0.05$ were deemed statistically significant, and 95% confidence intervals (CIs) were calculated for point estimates.

RESULTS

Baseline Characteristics

The baseline characteristics of the subjects are summarized in **Table 1**. At baseline, 19.36% (79/408) presented eGFR <90 ml/min/1.73 m^2 at baseline. In the subgroup of patients who had renal dysfunction at baseline, the eGFR categories G2, G3a, G3b, and G4 accounted for 86.1% (68/79), 11.4% (9/79), 1.3% (1/79), and 1.3% (1/79), respectively, but no patient had renal failure (G5) at baseline. Compared to those who had normal renal function at baseline, patients with renal dysfunction at baseline had higher median values of age (62 vs. 41 years), BMI (24.4 vs. 22.8 kg/m^2), chest computed tomography (CT) score (14 vs. 9.5), C-reactive protein (CRP) (17.07 vs. 7.04 mg/L), urea (5.09 vs. 3.71 mmol/L), creatinine (89 vs. 59 μmol/L), and erythrocyte sedimentation rate (ESR) (42 vs. 25 mm/h), while they had lower median values of the PaO_2/FiO_2 ratio (P/F ratio) (368.57 vs. 432.38), platelet count (152 vs. 192 $\times$ 10^9/L), and lymphocyte (1.13 vs. 1.36 $\times$ 10^9/L) (all $p < 0.05$). Meanwhile, the renal dysfunction subgroup had a higher proportion of males (68.4% vs. 43.2%), a higher percentage of severe COVID-19 patients at baseline (13.9% vs. 3.0%), and a higher proportion of individuals who had more than one comorbidity (50.6% vs. 34%), compared to the group with normal renal function at baseline (all $p < 0.05$). Before hospital admission due to COVID-19, 65/408 (15.9%) patients took a long-term course of medicines for comorbidities (**Supplementary Table 1**). None of patients had chronic kidney disease (CKD) before hospital admission according to medical history records. Additionally, 389 out of 408 patients had urine dipstick at baseline. Urine protein was negative in 84.32% (328/389), while positive with +2 to +3 in 2.05% (8/389). Hematuria was negative in 89.46% (348/389), but positive with +2 to +3 in 4.39% (17/389) (**Supplementary Table 2**).

AKI Occurrence During Follow-Up

Overall, 3.9% (16/408) developed AKI during follow-up (**Table 2**). With a subgroup analysis by baseline characteristics, we observed that patients aged ≥60 years, with at least one comorbidity, or with severe COVID-19 at baseline had a higher incidence of AKI events compared to the control groups, i.e., 10.6% in the group aged ≥60 years vs. 1.6% in the group aged <60 years ($p < 0.001$), 7.6% in patients who had at least one comorbidity vs. 2.4% in those without comorbidity ($p = 0.031$), and 14.3% in the baseline severe COVID-19 group vs. 3.4% in the baseline non-severe COVID-19 group ($p = 0.043$). Most of the drugs used before COVID-19 were antihypertensives and hypoglycemics, which are not nephrotoxic and less likely to cause AKI. Although three patients took steroids, none of them experienced AKI during hospitalization. We then summarized the occurrences of AKI stratified by medicine use during hospitalization (**Supplementary Table 3**). Patients who took steroids during hospitalization had a higher incidence of AKI events compared to those who did not take steroids (9.1 vs. 2.0%, $p = 0.003$). Similarly, the incidence of AKI was 8.6% in those taking antibiotics vs. 1.5% in those without using antibiotics ($p < 0.001$) (**Supplementary Table 3**).

Risk Factors to Early Predict AKI

The multivariable Cox regression analysis showed that age ≥60 years [hazard ratio (HR) = 4.78, 95% CI = 1.10–20.69], a P/F ratio <300 (HR = 3.48, 95% CI = 1.04–11.62), and a higher creatinine (HR = 1.04, 95% CI = 1.01–1.07) at baseline independently predict the risk of AKI during follow-up (**Figure 1**).

Dynamics of eGFR During Follow-up

Most Severe Renal Dysfunction of Each Individual During Follow-Up

We observed that 25.0% (102/408), 3.9% (16/408), 0.5% (2/408), 1.0% (4/408), and 0.2% (1/408) respectively experienced G2, G3a, G3b, G4, and G5 as their most severe category during follow-up, although 69.4% (283/408) of patients had their eGFRs maintained at normal levels (i.e., eGFR category maintained at G1) throughout the follow-up period (**Figure 2A**). For those patients ($n = 125$) who had abnormal eGFRs during follow-up, 63.2% (79/125) had renal dysfunction at baseline. We observed that five, three, two, three, and one patient(s) in each most severe category (G2, G3a, G3b, G4, and G5) experienced AKI during hospitalization, respectively (**Supplementary Table 4**). In the subgroup with normal renal function at baseline, 12.8, 0.9, and 0.3% of patients respectively had G2, G3a, and G3b as the most severe eGFR category during follow-up (**Figure 2B**). In contrast, for the subgroup with renal dysfunction at baseline, 75.9, 16.4, 1.3, 5.1, and 1.3% respectively had G2, G3a, G3b, G4, and G5 as their most severe eGFR category during follow-up (**Figure 2B**).

Proportion of Patients With Abnormal eGFR When Finally Discharged From Hospital

At the end of follow-up, 87.50% (357/408) of patients had normal renal function (stayed at G1), while 11.76% (48/408), 0.49% (2/408), and 0.25% (1/408) were at G2, G3a, and G3b categories, respectively, and no patient had eGFR <30 when discharged from the hospital. For those patients ($n = 51$) who had abnormal eGFRs at the end of follow-up, the median [Q1, Q3] values of eGFR at baseline and at the end of follow-up, as well as the lowest eGFR during hospitalization, were 72.4 [65.4, 85.0], 79.0 [71.6, 83.5], and 67.4 [58.4, 74.9] ml/min/1.73 m^2, respectively; 80.4% (41/51) of patients had renal dysfunction at baseline. In other words, 48.1% (38/79) of

TABLE 1 | Baseline characteristics of the selected COVID-19 patients for this study.

	Overall	Normal renal function at baseline	Renal dysfunction at baseline	*p*-value
Number of patients	408	329	79	
Age, median (IQR) (years)	47 (34–60)	41 (32–56)	62 (55–69)	<0.001
Age ≥60 years	104 (25.5)	57 (17.3)	47 (59.5)	<0.001
Male	196 (48.0)	142 (43.2)	54 (68.4)	<0.001
BMI, median (IQR) (kg/m^2)	23.0 (21.2–25.6)	22.8 (20.8–25.2)	24.4 (22.0–26.6)	0.002
Case severity				<0.001
Mild	43 (10.5)	39 (11.9)	4 (5.1)	
Moderate	344 (84.3)	280 (85.1)	64 (81.0)	
Severe	19 (4.7)	10 (3.0)	9 (11.4)	
Critical	2 (0.5)	0 (0.0)	2 (2.5)	
Time from illness onset to admission, median (IQR) (days)	3 (1–6)	3 (1–6)	3 (2–6)	0.574
Number of comorbidities				<0.001
0	289 (70.8)	250 (76.0)	39 (49.4)	
1	88 (21.6)	67 (20.4)	21 (26.6)	
2	23 (5.6)	8 (2.4)	15 (19.0)	
3	8 (2.0)	4 (1.2)	4 (5.1)	
Comorbidity types				
Diabetes	22 (5.4)	12 (3.6)	10 (12.7)	0.004
Hypertension	58 (14.2)	35 (10.6)	23 (29.1)	<0.001
Cardiovascular disease	35 (8.6)	15 (4.6)	20 (25.3)	<0.001
Cancer	5 (1.2)	3 (0.9)	2 (2.5)	0.545
Chronic liver disease	38 (9.3)	30 (9.1)	8 (10.1)	0.951
P/F ratio, median (IQR)	420.48 (356.67–476.02)	432.38 (372.38–485.71)	368.57 (317.26–406.90)	<0.001
CT score, median (IQR)	10.00 (4.00–16.00)	9.50 (2.75–15.00)	14.00 (10.00–22.000	<0.001
ALT, median (IQR) (U/L)	21.00 (15.00–31.00)	20.00 (14.00–29.50)	25.30 (19.00–34.70)	0.001
AST, median (IQR) (U/L)	26.10 (21.00–35.52)	25.00 (20.00–33.90)	31.00 (24.75–42.10)	<0.001
TBIL, median (IQR) (μmol/L)	10.90 (8.30–16.15)	10.70 (8.20–15.40)	12.60 (8.70–21.55)	0.015
GGT, median (IQR) (U/L)	23.30 (16.00–36.25)	22.00 (15.00–35.00)	30.00 (20.90–40.00)	0.003
Fibrinogen, median (IQR) (g/L)	3.84 (3.08–4.60)	3.67 (3.03–4.52)	4.27 (3.75–5.04)	<0.001
Platelet count, median (IQR) (×10^9/L)	186.00 (148.75–230.25)	192.00 (154.00–236.00)	152.00 (131.50–190.00)	<0.001
Lymphocyte count, median (IQR) (×10^9/L)	1.31 (0.99–1.80)	1.36 (1.04–1.91)	1.13 (0.88–1.54)	0.001
C-reactive protein, median (IQR) (mg/L)	8.75 (3.43–24.77)	7.04 (2.64–22.23)	17.07 (8.66–37.37)	<0.001
Fibrosis-4, median (IQR)	1.38 (0.80–2.37)	1.27 (0.73–1.85)	2.67 (1.66–4.00)	<0.001
AST-to-platelet ratio index, median (IQR)	0.32 (0.23–0.48)	0.30 (0.22–0.43)	0.46 (0.33–0.63)	<0.001
eGFR, median (IQR) (ml/min/1.73 m^2)	105.18 (93.44–115.82)	109.38 (99.81–118.84)	77.88 (67.88–84.97)	<0.001
eGFR categories (ml/min/1.73 m^2)				<0.001
G1: ≥90	329 (80.6)	329 (100.0)	0 (0.0)	
G2: 60–89	68 (16.7)	0 (0.0)	68 (86.1)	
G3a: 45–59	9 (2.2)	0 (0.0)	9 (11.4)	
G3b: 30–44	1 (0.2)	0 (0.0)	1 (1.3)	
G4: 15–29	1 (0.2)	0 (0.0)	1 (1.3)	
Urea, median (IQR) (mmol/L)	3.92 (3.21–4.81)	3.71 (3.10–4.42)	5.09 (4.25–5.90)	<0.001
Creatinine, median (IQR) (μmol/L)	63.00 (53.00–77.00)	59.00 (50.00–72.00)	89.00 (74.00–99.50)	<0.001
Urea/creatinine, median (IQR)	60.73 (49.23–74.97)	61.29 (49.57–78.94)	58.39 (47.67–67.31)	0.065
ESR, median (IQR) (mm/h)	28 (14–48)	25 (14–45)	42 (25–63)	<0.001
Fever	274 (67.2)	215 (65.3)	59 (74.7)	0.146

BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; GGT, γ-glutamyl transpeptidase; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; P/F ratio, PaO_2/FiO_2 ratio; IQR, interquartile range.

patients with abnormal eGFR at baseline had recovered before discharged from the hospital; among of them, 4 of 38 (10.5%) patients experienced AKI during hospitalization. Moreover, at the end of follow-up, seven had a deteriorating eGFR category compared to their baseline eGFR category; their median [Q1, Q3] values of eGFR at baseline and at the end of follow-up,

TABLE 2 | Total proportions of AKI occurrence during follow-up stratified by baseline characteristics.

Stratifying variables at baseline	Subgroups		Patients without AKI events	Patients with an occurrence of AKI event	Proportion	*p*-value
Overall			392	16	16/408 (3.9%)	
Age at baseline	Age <60 years	$n = 304$	299	5	5/304 (1.6%)	0.0002
	Age ≥60 years	$n = 104$	93	11	11/104 (10.6%)	
Baseline eGFR	Baseline eGFR ≥90	$n = 329$	319	10	10/329 (3.0%)	0.121
	Baseline eGFR <90	$n = 79$	73	6	6/79 (7.6%)	
No. of comorbidities[a]	None	$n = 289$	282	7	7/289 (2.4%)	0.031
	At least one	$n = 119$	110	9	9/119 (7.6%)	
Severity of COVID-19 at baseline	Non-severe	$n = 387$	374	13	13/387 (3.4%)	0.043
	Severe	$n = 21$	18	3	3/21 (14.3%)	

eGFR, estimated glomerular filtration rate.

[a]*Types of comorbidities: hypertension, cardiovascular disease, diabetes, liver diseases, and cancer.*

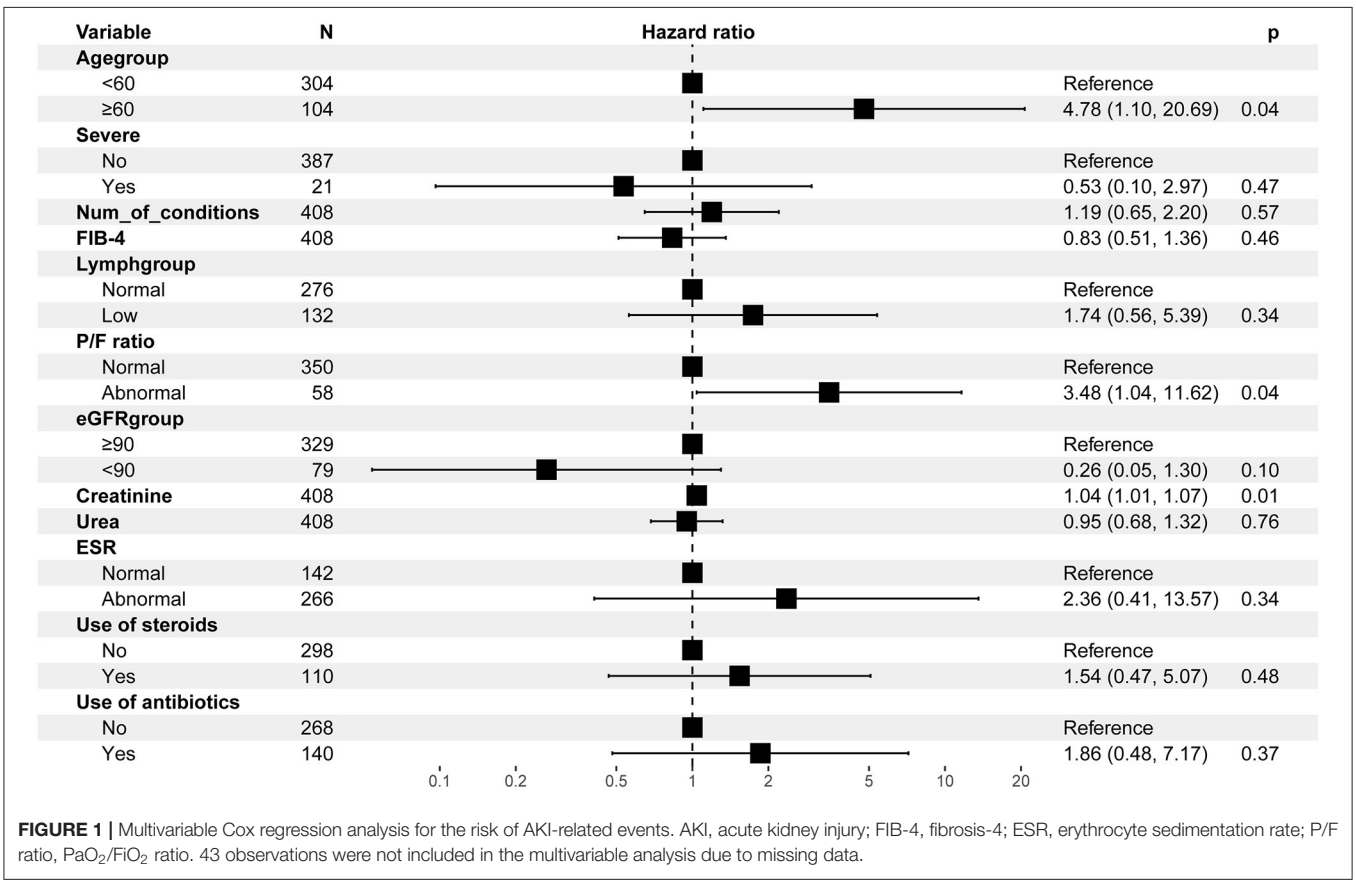

FIGURE 1 | Multivariable Cox regression analysis for the risk of AKI-related events. AKI, acute kidney injury; FIB-4, fibrosis-4; ESR, erythrocyte sedimentation rate; P/F ratio, PaO_2/FiO_2 ratio. 43 observations were not included in the multivariable analysis due to missing data.

as well as the lowest eGFR during hospitalization, were 92.2 [91.7, 96.3], 84.3 [79.8, 86.4], and 74.6 [63.3, 82.0] ml/min/1.73 m^2, respectively.

Renal Function of Patients With Readmission

For the 43 patients who were re-admitted to hospital due to the recurrence of positive SARS-CoV-2, nine (20.9%), eight (18.6%), six (13.9%), and three (7.0%) patients had abnormal renal function at the first hospital admission, at the first discharge, at re-admission, and at the second discharge, respectively (**Supplementary Table 5**).

DISCUSSION

Based on a retrospective cohort study of 408 patients, this study reveals that COVID-19 patients can be at risk of AKI; moreover, a large proportion of patients still had abnormal eGFRs when

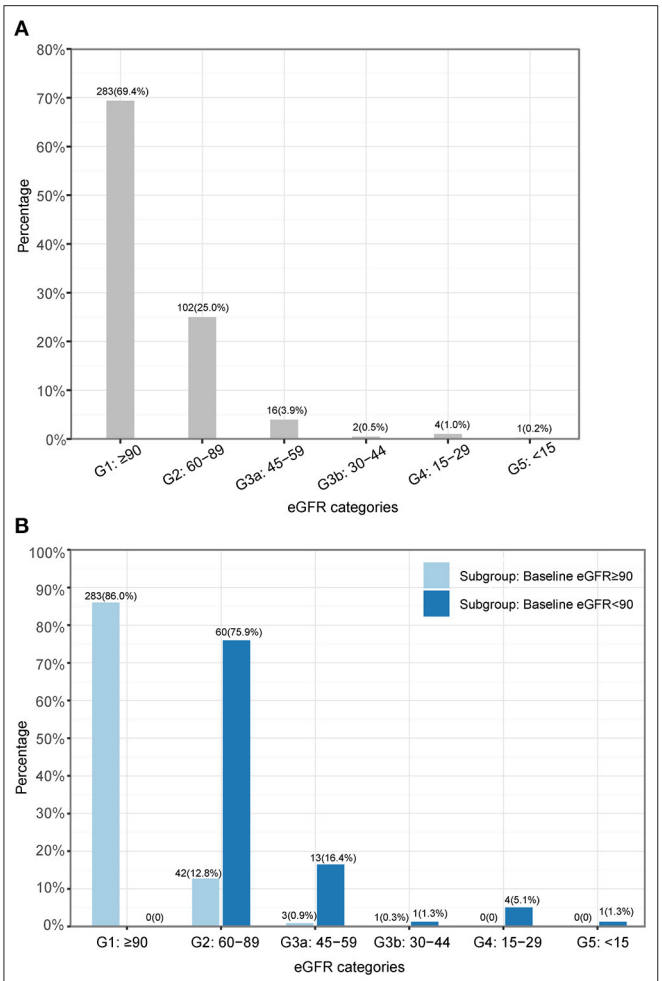

FIGURE 2 | The most severe renal dysfunction of each individual during follow-up. (**A**) Overall. (**B**) Subgroups. *eGFR*, estimated glomerular filtration rate.

finally discharged from the hospital. Furthermore, this study found that age ≥60 years, a low P/F ratio (<300), and a higher creatinine at baseline independently predict the risk of AKI. This study added more evidence concerning the longitudinal changes of renal function in COVID-19 patients and provided important information to support the management of COVID-19.

Since SARS-CoV-2 is considered to predominantly enter alveolar epithelial cells with angiotensin-converting enzyme 2 (ACE2) as its receptor, the lungs become the most severely damaged organ (11, 13). Unfortunately, a few studies have provided evidence of SARS-CoV-2 invading the kidney tissue (16, 20). In our study, we observed that the occurrence rate of AKI was 3.9%, which was similar to the pooled incidence rate of AKI (3%) in COVID-19 patients from a meta-analysis (21). Furthermore, we found that the incidence rate of AKI was significantly higher in subgroups with age ≥60 years, or who had at least one comorbidity, or with severe COVID-19 at baseline, although the overall incidence of AKI was relatively low in the whole cohort. This implied that physicians should pay more attention to these special patients, and it is quite essential to frequently monitor the renal function of these patients. It had been confirmed in previous studies that even a small rise in creatinine, a key parameter of defining AKI, is independently associated with an increased mortality in non-COVID-19 inpatients (22); recently, a study also found that AKI was associated with a higher risk of in-hospital mortality in patients with COVID-19 (23). In this study, we could not investigate the association of AKI with mortality as few (a total of three) patients died in our study. However, it is important to investigate the predictive factors in order to identify the risk of AKI as early as possible in clinical practice. This study found that age ≥60 years, P/F ratio <300, and a higher creatinine at baseline independently predict the risk of AKI. In addition to the direct effect of SAS-CoV-2 on the kidney, a lung–kidney crosstalk might be another important mechanism that causes AKI. A recent study has reported that 68% of 357 patients with acute respiratory distress syndrome (ARDS) developed AKI (24). Cytokine overproduction in lung–kidney bidirectional damage and the injury of tubular cells secondary to renal medullary hypoxia caused by ARDS could be the potential reasons of the high risk of AKI in patients with ARDS (25). The P/F ratio is commonly used to determine the onset or the severity of acute lung injury (ALI) and ARDS. Therefore, a lung–kidney crosstalk could explain the independent predictive value of a low P/F ratio (<300) on the incidence of AKI.

Typically, persistence of abnormal eGFR for >3 months is one criterion used to determine CKD (19). In this study, we could not determine whether patients had CKD as the longest follow-up duration of our study cohort was <3 months. However, a decline of the eGFR is the precondition of potential CKD (22). Thus, we investigated the dynamics of eGFR to reflect the influence of COVID-19 on the renal function. We found that 19.36% of patients with COVID-19 had abnormal eGFRs at baseline. Although the overall dynamic of eGFR presented a trend of restoration, we observed that 13.97% of patients had an eGFR decline by one or more categories during follow-up. Moreover, at the end of follow-up, 12.5% of patients still had abnormal eGFRs; among these patients, seven had a deteriorating eGFR category compared to their baseline eGFR category. This implied that not all the renal dysfunction caused by COVID-19 was transient. It is essential to monitor the renal function of all COVID-19 patients during the hospitalization and to provide continuous attention on the risk of CKD for those patients who still had abnormal eGFRs even when they have been discharged from the hospital.

The evidence described in the KDIGO guideline suggests that a moderate decline of eGFR (<25%) is also associated with an increased risk of all-cause mortality and end-stage renal disease (ESRD) (26, 27). Thus, it is crucial to investigate the most severe category of eGFR of patients during follow-up both for assessing kidney impairments caused by COVID-19 and for predicting potential long-term severe outcomes. When examining the most severe renal dysfunction during follow-up for each individual, we found that only 69.4% had maintained a normal eGFR through follow-up; however, a total of 30.6% of patients experienced an abnormality in their eGFRs. Furthermore, it was observed that 1.2% experienced more severe than the G2 category as their most severe renal dysfunction in the subgroup with normal baseline

eGFR. In contrast, this was significantly higher (i.e., increasing to 24.1% from the baseline 13.9%) in the subgroup with abnormal baseline eGFR. Our results suggested that patients with baseline renal dysfunction had a higher risk of progression to more severe kidney impairments (10.1% vs. 1.2%). Therefore, the evaluation of renal function in patients with COVID-19 at presentation is essential for identifying an already occurring kidney impairment early. More importantly, intensive monitoring of renal function is crucial for COVID-19 patients who had kidney impairments when admitted.

This study is not without limitations. Firstly, information on preexisting chronic conditions were collected based on patients' self-reports, and the renal dysfunction might have existed before the SARS-CoV-2 infection, but unknown to patients, which might influence our results. Secondly, the majority of patients received routine urine test at hospital admission, but not after then, so we could not differentiate the glomerular and tubular injuries caused by COVID-19. Thirdly, due to the follow-up duration of <3 months, we were unable to assess whether patients developed chronic kidney dysfunction due to COVID-19. Long-term follow-up studies are necessary to assess the occurrence rate of CKD and its outcomes.

In conclusion, COVID-19 patients can be at risk of AKI and continuous eGFR decline. Age ≥60 years, P/F ratio <300, and a higher creatinine at baseline independently predict the risk of AKI. The results from this study imply that it is necessary to evaluate and monitor the renal function of COVID-19 patients, especially for those who had renal dysfunction at baseline. Furthermore, our results suggest that it is necessary to continue to monitor renal function for those who still had abnormal eGFRs even when finally discharged from the hospital.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Shenzhen Third people's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JL and TW conceived the study and contributed equally. JC and F-SW supervised the study. JC organized the ethics approval and had full access to all of the data in the study. TW designed the data analysis pipeline and wrote the codes. JL run the codes on the data. JL, TW, and QH interpreted the data. JL, QC, DH, and LS collected clinical data. JL and TW wrote the manuscript. All the authors critically read, edited, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.604242/full#supplementary-material

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PERSPECTIVE
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Estimated Dissemination Ratio—A Practical Alternative to the Reproduction Number for Infectious Diseases

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Policymakers require consistent and accessible tools to monitor the progress of an epidemic and the impact of control measures in real time. One such measure is the Estimated Dissemination Ratio (EDR), a straightforward, easily replicable, and robust measure of the trajectory of an outbreak that has been used for many years in the control of infectious disease in livestock. It is simple to calculate and explain. Its calculation and use are discussed below together with examples from the current COVID-19 outbreak in the UK. These applications illustrate that EDR can demonstrate changes in transmission rate before they may be clear from the epidemic curve. Thus, EDR can provide an early warning that an epidemic is resuming growth, allowing earlier intervention. A conceptual comparison between EDR and the commonly used reproduction number is also provided.

Keywords: epidemics, survaillance, mathematical models, COVID-19, reproduction number R, estimated dissemination ratio

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KEY POINTS

Estimated Dissemination Ratio (EDR) is a simply calculated, replicable, easily explained and robust measure of the trajectory of an outbreak. Examples from the current COVID-19 outbreak in the UK illustrate these merits.

INTRODUCTION

As the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic emerged, policymakers, planners, and frontline workers scrambled to understand the spread and likely impact of this new virus and viral pneumonia it can cause, namely the Coronavirus Disease 2019 (COVID-19). During an epidemic, these public health teams need rapid and reliable information on the progress of the epidemic.

Epidemics have a trajectory and for the planning of responses it is important to understand today what the situation is likely to be tomorrow or next week and in particular, the number of new cases likely to arise. Several different quantitative measures have been used to address these questions. One of the most commonly used is the reproduction number, R, which has a long history

(1–3). R can be described in plain words as "the average number of next-generation cases caused by each current case." This simple definition refers to a generic R which encompasses the basic reproduction number, R_0, when a primary case is introduced to a susceptible population (1, 2, 4) or a reproduction number R_t determined as a function of time during an epidemic (3, 5). In general, R is a measure of the rate of transmission of infection and plays a key role in the management of epidemics. Despite being widely used, however, R is itself a complex measure that is difficult to estimate in real time and can easily be misinterpreted by practitioners (6).

An alternative to R_t, as an indicator of the rate of transmission of infection during an epidemic, is the Estimated Dissemination Ratio (EDR). At its simplest, EDR is a direct measure of the relative change in the number of cases over time. EDR is a measure that has been used for many years in animal health to monitor progress and control of epidemics, for example with foot and mouth disease (7–10). It has become an established tool for decision support and policy formulation (11–13). As the name implies, EDR can also be interpreted as an estimate of dissemination, or transmission, of infection, since the change in the number of cases over time depends directly on the rate of transmission. EDR gives an estimate of the slope of the epidemic curve and indicates whether an epidemic is accelerating, plateauing—through being brought under control, or declining. It can be an important tool in planning.

Here we discuss the use and value of EDR and provide a conceptual comparison with R_t as a parameter in epidemic management.

CALCULATION OF EDR

EDR is a simple ratio of cases counted in a set period, divided by the number of cases counted in the preceding period of the same duration. EDR is intentionally simple to calculate using case counts that are available during an epidemic. It can be easily calculated in a transparent, consistent and readily comparable manner. The EDR at day t can be calculated by using two consecutive periods of n days as follows:

$$EDR = \frac{\text{cases in the days } [\mathrm{t}-\mathrm{n}+1, \mathrm{t}]}{\text{cases in the days } [\mathrm{t}-2\mathrm{n}+1, \mathrm{t}-\mathrm{n}]} \qquad (1)$$

For example, for a 7-day period ($n = 7$), EDR is simply given by the cases reported this seven days (between day $t - 6$ and day t) divided by the cases reported in the previous seven days (between day $t - 13$ and day $t - 7$).

A 7-day period will be used for illustration in this paper. This choice can be convenient in many applications since it helps to smooth out any anomalous "weekend effects" as seen for example during the current COVID-19 epidemic (14). However, periods other than 7 days could be used and, as discussed below, could be more convenient depending on the specific application.

Since EDR refers to the ratio of cases in two intervals of time, the specific point in time to which EDR is attributed is to some extent arbitrary. Here, we attribute the EDR to the day on which it is calculated, i.e., to the last day in the period used for the numerator in Equation 1. For instance, this is the same convention used to attribute EDR in the freely available "epiR" software package (15). With this choice, a 7 days EDR can be regarded as an indicator of the progression of the epidemic in the last week relative to the week before.

INTERPRETATION AND USE OF EDR

The EDR can be interpreted as an approximate indicator of the infection transmission rate. Indeed, assuming that the periods used to calculate EDR approximate the generation interval of the infection, the cases counted in the numerator of EDR (Equation 1) can be considered to be largely generated by contagious transmission from the cases counted in the denominator of EDR. To use EDR as an indicator of infection transmission rate it is important to calculate EDR for time intervals close to the generation time of the disease at hand.

When using EDR to draw inferences about transmission rate, it is important to note that an EDR calculated on current case count data and attributed to the last day in the numerator of Equation 1 will reflect transmission events occurring in the "denominator period" of the EDR–i.e., EDR is a retrospective indicator of transmission rate occurring one generation interval previously.

A graph of EDR over time should be interpreted along with the epidemic curve (case counts or case rates indicating the overall progression and size of the epidemic). Whether there are 2,000 cases in a period following 1,000 cases in a preceding period *or* 20 cases following 10 cases, the EDR equals 2 in both situations. However, disease control decisions might well differ given the different scales. Both the size of the outbreak and the rate at which it is changing (as indicated by EDR) are important. This same consideration applies equally when R_t is used for epidemic management.

An EDR of 1 at a given time indicates that the number of new cases was stable in the preceding periods used to calculate the EDR. An EDR above (or below) 1 indicates that the daily new case numbers increased (or declined) in recent days.

In addition to whether EDR is above or below 1 (cases increasing or decreasing), the absolute value of EDR provides further indication of the speed of increase or decrease of the epidemic curve. When using a 7-day period, an EDR of 2 means that cases are doubling every week, while an EDR of 1.4 means that cases may double in just over 2 weeks. Conversely an EDR of 0.5 means that cases are halving every week; while an EDR of 0.7 means that cases would halve in just under 2 weeks. When EDR is close to 1 (e.g., 0.9–1.1) case numbers are not changing rapidly, but while the situation may not be rapidly deteriorating, neither is it rapidly improving. When EDR is close to 1 it is especially important to also consider the absolute number and spatial distribution of cases. Otherwise, one might miss situations in which the number of cases is maintained at a level from which relaxation of control would result in high case numbers within a relatively short time. The ideal goals in managing an epidemic could be: First reduce the transmission of infection in such a way that the number of new cases fall rapidly (i.e., EDR well-below

1) and, second, maintain this decline until the number of cases is low enough to ensure that individual outbreak clusters can be effectively contained.

Further to looking at the value of EDR at a given time, it is more useful to analyse the trends of EDR. A sustained increase of EDR, when EDR is already >1, indicates an increased transmission rate that will inevitably lead to an acceleration of the increase of cases. More interestingly, an increase of EDR, when EDR is <1, can be observed alongside a decreasing epidemic curve: this indicates that the rate of decline is reducing. This EDR increase would allow us to identify a resurgence of infection which may be difficult to recognize from the epidemic curve. Conversely, an EDR decrease may be observed for an increasing epidemic curve whose rate of increase is reducing, for example, because of interventions implemented to suppress the infection.

PRACTICAL EXAMPLES OF THE APPLICATION OF EDR IN THE COVID-19 OUTBREAK IN THE UK

Figure 1 shows the epidemic curve and EDR graph for the UK during the COVID-19 epidemic from 10-Mar-20 to 21-Apr-20. EDR was estimated using periods of 7 days (Equation 1). This period is close to the generation interval of COVID-19 (16) and we expect the obtained EDR to be a suitable indicator of the infection transmission rate.

The epidemic curve shows a rapid daily increase in new cases over the period from about 21 March to 1 April which suggests little, if any, control was being achieved. However, the EDR graph shows a sustained and steady downward trend from 18-Mar-20. This suggests a gradual decrease in the transmission of infection over this period. Because EDR is a retrospective indicator of transmission rate, the suggestion is that the transmission rate began falling from around 11 March.

A lockdown was ordered in the UK on 24 March 2020 to suppress the spread of SARS-CoV-2. The declining trend in EDR before this date suggests that transmission was already being slowed before the lockdown, most likely by voluntary home working and reduction in social contact among the population in response to concern over the situation and advice from various sources.

The value of EDR here is that whilst the daily case numbers are increasing quickly, the decreasing EDR shows that there is progress toward control of the epidemic. In this situation, EDR gives an early indication that control measures are working.

Later in the epidemic, EDR can be used to detect rises in infection rates before they become clear in the case data. This has been more difficult to clearly illustrate because of the changing testing criteria and testing capacity available in the UK which has complicated the picture. **Figure 2** show the period from 23-Jun-20 to 30-Aug-20 when testing capacity was stable at between 200,000 and 225,000 tests per day and testing criteria were also stable (17).

EDR started rising consistently from 1 July and was above 1 from 13 July, staying so consistently. The initial increase of EDR suggests a slowing down of the decrease of the epidemic curve before 8 July which gives early signs of a resurge that would indicate a need for action. Despite the signs, no action was taken during this period to prevent a resurge of the virus (18, 19) and cases doubled from around 550 per day on average in early July to around 1,100 by 23 August. As an aside, the EDR fell below 1 on 21 August. It is unclear why this happened as no new interventions were put into place—perhaps a change in testing or reporting, but it can be observed that there was also a slight contemporaneous fall in the new case 7-day moving average.

It would be preferable to use a more dramatic example from a later period of the epidemic but many other conditions have changed since September in the UK including a rapid rise in reported testing capacity and an intensification of testing groups such as school-age children and university students.

Note that in the two examples above, the graphs show EDR and an average daily case number. The latter could equally be replaced with a case rate (for example case per week per 100,000 population) if required.

Especially when using EDR to compare between areas or between time periods, the methods by which cases are defined, searched for, counted and registered must be clearly described. There must be a consistent method of case counting, across the whole period for which comparison of EDR and/or monitoring change is required (*this applies equally to calculations of the reproduction number R*). In practice, this means that changes in either case definition or case searching (surveillance) must be taken into account. Case counts must come from the same population for all times being compared: if surveillance starts to cover a wider population, more cases might be found that are not epidemiologically linked.

CONCEPTUAL COMPARISON WITH R_t

R_t and EDR are similar in several respects. First, both measures indicate the progress of an epidemic and can be used to quantify the infection transmission rate during epidemics. Both EDR and R_t take values smaller than one for declining epidemics and values larger than one for accelerating epidemics. Apart from the value 1, the two quantities will typically not take identical values for accelerating or declining epidemics.

Despite EDR being qualitatively similar to R_t, the definition and calculation methods of these quantities are significantly different. In principle, an exact estimate of R_t requires knowing who infected whom during an epidemic. In some cases, it may be possible to construct an epidemic tree to calculate R_t by simply counting the number of individuals infected by each case (5). For many epidemics, however, it is not known who infected whom and one has to rely on less precise observations such as the epidemic curve. In these situations, estimates of R_t can be obtained from epidemic curves by fitting mechanistic epidemiological models based on disease-specific assumptions (20–24). Fitting mathematical models to data is often a technically involved task. In addition, models fitted to an epidemic are not easily generalizable to other epidemics. Wallinga and Teunis proposed a more generic method to estimate R_t which only requires case incidence data and the

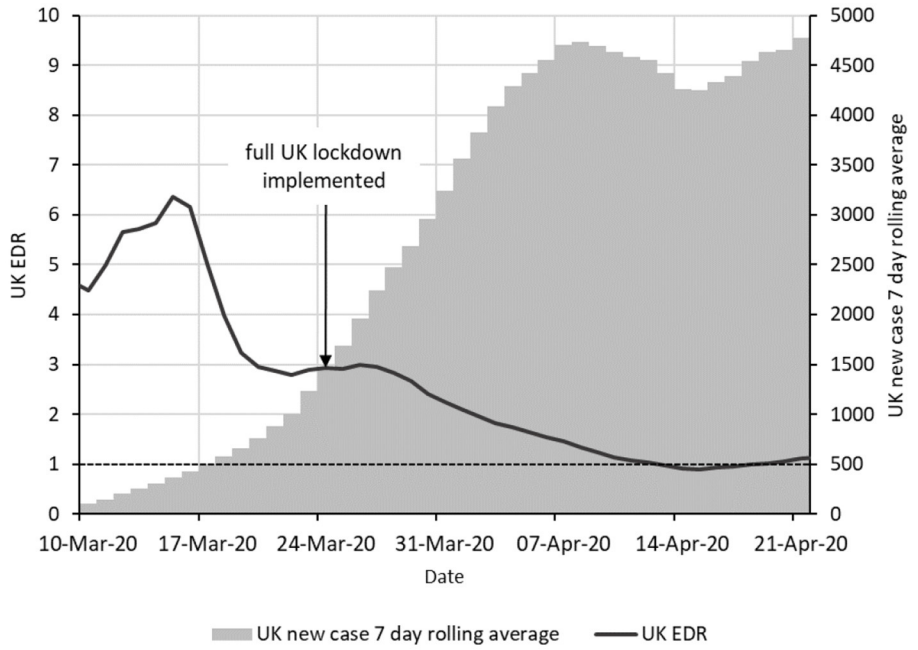

FIGURE 1 | Epidemic curve and EDR for the COVID-19 epidemic in the UK from 1 March to 20 April 2020. The epidemic curve is indicated as vertical bars giving a 7-day moving average of new cases. The solid line shows the EDR estimated using periods of 7 days. The horizontal dashed line shows the boundary with EDR = 1.

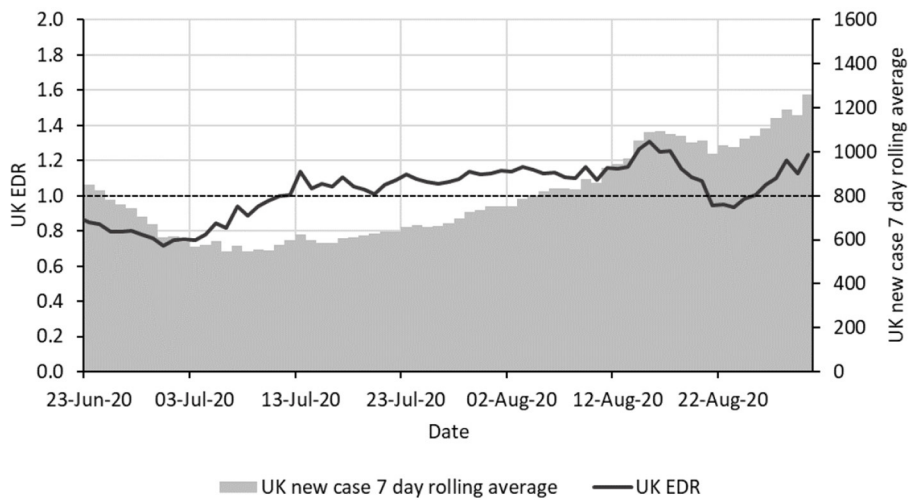

FIGURE 2 | Epidemic curve and EDR for the COVID-19 epidemic in the UK from 23 June to 30 August 2020 with the same format as in **Figure 1**.

generation interval distribution (3). This method and some extensions (25, 26) are widely used to estimate R_t. The main drawback of these methods is that they require estimates for the generation interval distribution [or the serial interval as a proxy (3, 25, 26)] which is likely to be missing for emerging diseases. In addition, these methods usually involve advanced mathematical concepts that are not necessarily handled by every public health practitioner. As a consequence, these methods are often perceived as a "black box" of assumptions that are not under the control of practitioners.

In contrast to R_t, EDR only relies on epidemic curves and can be easily estimated without knowledge of advanced mathematical concepts. The period used to calculate EDR can be interpreted as a parameter of the model but its specific value is not absolutely crucial to observe informative trends in EDR. It is interesting, however, that in the particular case in which the period used to

calculate EDR approximates the generation time, estimates of R_t and EDR are expected to be similar to each other.

DISCUSSION

EDR is intuitive, simple to calculate, easy to explain and relies only on the time series for the number of cases (or other epidemiological observables). As a pragmatic measure of the change in the number of cases over time, EDR can help understand the trajectory of an epidemic in real time. Being unit free, EDR (as with R) should always be used in combination with the epidemic curve, allowing both the scale and trajectory of an epidemic to be taken into consideration. The EDR can be a useful indicator of transmission rate as shown here for the COVID-19 outbreak in the UK and previously demonstrated in animal epidemics (7–12). At the very least, EDR is useful as a direct, transparent, measure of the direction (up, down, stable) and the rate of change of the epidemic, but a more in-depth analysis also allows more nuanced interpretation.

EDR is a conservative measure. This is particularly advantageous when EDR is falling. As a retrospective measure, it does not indicate a change until clearly present. Another advantage of displaying EDR along with the daily case totals is that while daily totals can vary considerably from day to day, EDR uses aggregate cases over consecutive multi-day periods, which smooths out the inevitable day-to-day variation.

Being unit free, and given a consistent case definition within a country or territory, EDR can also be used to compare the degree of control between areas. This can be a valuable tool for learning from the experiences of the impact of control measures in different areas.

EDR aims at quantifying the progression of epidemics in a way similar to the reproduction number R_t. Despite some similarities, EDR is significantly easier to estimate than R_t both in terms of the information required and the mathematical expertise involved. Indeed, a strength of EDR is that it can be readily estimated from epidemic curves. A caveat is that EDR can only capture information of epidemics at the population level. In contrast, R_t could in principle resolve features of transmission at the level of individuals in cases in which epidemic trees could be reconstructed (5).

CONCLUSION

EDR is a transparent measure of the progress of an epidemic with clear potential as a tool to support planning and monitoring of the public health response and impact. It can be used from local to national scales and is readily communicated to the public. The combination of epidemic curve plus EDR is a simple measure of the direction and rate of change in case numbers. This combination can be used as a simple measure to inform epidemic control as well as to explain the progress of outbreaks or to assess the impact of control measures. These multiple uses of EDR together with its clarity should encourage the engagement of frontline workers and the public.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

FP-R, NT, and NH retrieved the data and analyzed it. All authors planned the project, designed the research, wrote the manuscript, reviewed the manuscript, and approved the final version of it.

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Excess Mortality in Italy During the COVID-19 Pandemic: Assessing the Differences Between the First and the Second Wave, Year 2020

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COVID-19 dramatically influenced mortality worldwide, in Italy as well, the first European country to experience the Sars-Cov2 epidemic. Many countries reported a two-wave pattern of COVID-19 deaths; however, studies comparing the two waves are limited. The objective of the study was to compare all-cause excess mortality between the two waves that occurred during the year 2020 using nationwide data. All-cause excess mortalities were estimated using negative binomial models with time modeled by quadratic splines. The models were also applied to estimate all-cause excess deaths "not directly attributable to COVD-19", i.e., without a previous COVID-19 diagnosis. During the first wave (25th February−31st May), we estimated 52,437 excess deaths (95% CI: 49,213–55,863) and 50,979 (95% CI: 50,333–51,425) during the second phase (10th October−31st December), corresponding to percentage 34.8% (95% CI: 33.8%–35.8%) in the second wave and 31.0% (95%CI: 27.2%–35.4%) in the first. During both waves, all-cause excess deaths percentages were higher in northern regions (59.1% during the first and 42.2% in the second wave), with a significant increase in the rest of Italy (from 6.7% to 27.1%) during the second wave. Males and those aged 80 or over were the most hit groups with an increase in both during the second wave. Excess deaths not directly attributable to COVID-19 decreased during the second phase with respect to the first phase, from 10.8% (95% CI: 9.5%–12.4%) to 7.7% (95% CI: 7.5%–7.9%), respectively. The percentage increase in excess deaths from all causes suggests in Italy a different impact of the SARS-CoV-2 virus during the second wave in 2020. The decrease in excess deaths not directly attributable to COVID-19 may indicate an improvement in the preparedness of the Italian health care services during this second wave, in the detection of COVID-19 diagnoses and/or clinical practice toward the other severe diseases.

Keywords: COVID-19, surveillance, mortality from all causes, excess mortality, Italy

INTRODUCTION

Italy has been the first European country to experience the spread of the Sars-Cov2 virus at the end of February 2020, with the first related death occurring on February 21. A first epidemic wave was observed between the end of February and May 2020, with a peak observed in March and April (1, 2). The first wave was characterized by a great geographical heterogeneity: the northern regions were the most affected, also in terms of mortality, while in the center and the South, the epidemic had a lower impact. On the contrary, during the second wave (October–December), the virus spread was more homogeneous throughout the country (1, 2).

Following a 2-month lockdown period, the number of new cases and deaths was largely reduced during the summer period (from June to September 2020), while a second epidemic wave occurred in the country from the second half of October (3). The two epidemic waves were characterized by a substantial number of COVID-19 related deaths (4). The estimation of the excess deaths from all causes is considered the most reliable method to comprehensively evaluate the impact of COVID-19 on mortality (5). The assessment of total deaths can help to better estimate the overall impact of COVID-19, by overcoming possible issues related to underreporting of COVID-19 deaths and by assessing "indirect mortality," i.e., caused by health systems not being able to cope with other acute or chronic conditions (6). The purpose of this work was to highlight the differences in excess deaths between the two waves that occurred in 2020.

We, then, estimated the excess mortality during the first wave by comparing it with the second in terms of geographical distribution, sex and age groups. Therefore, we also estimated the excess mortality "not directly attributable to COVID-19" to assess the possible impact of deaths without a COVID-19 diagnosis during the two waves in Italy.

METHODS, OUTCOMES, DATA SOURCES, AND THE CHOICE OF THE STUDY PERIODS

The primary outcome variable of the study was the daily excess mortality, defined as "the difference between the observed numbers of deaths in specific time periods and the number of expected deaths in the same time periods" (7). We estimated daily expected deaths as the average of the previous 5 years (i.e., 2015–2019) and observed daily deaths were those that occurred in the year 2020 during the same periods.

A secondary outcome was the daily excess mortality "not directly attributable to COVID-19", defined as the difference between all-cause excess deaths, as just mentioned, minus the total number of deaths after a COVID-19 diagnosis. The estimates for excess mortality were based on data provided by the Italian National Institute of Statistics (ISTAT) which, on an annual basis, gathers all-cause mortality data by day and by geographical unit by the "provinces," which are administrative local units of Italy (8). Data on deaths with a COVID-19 diagnosis came from national COVID-19 surveillance (9). The surveillance system contains data on all laboratory-confirmed (by real-time-PCR, RT-PCR) cases of COVID-19, as already published by Riccardo et al. (10). Deaths were considered as COVID-19 related when occurring in persons who tested positive for Sars-Cov2 *via* RT-PCR and reported to the national COVID-19 surveillance (9).

To estimate the overall excess mortality during 2020, we took into consideration the period from 1st January to 31st December. Then, we divided the study period according to the waves observed in the excess mortality pattern. We defined wave or phase, as periods characterized by "a rising number of excess deaths >0 with a defined peak, followed by a decline in deaths, in which excess deaths had decreased", as already reported for the COVID-19 epidemic (11). Therefore, we defined "transitional phase" as the time period elapsed between waves. After a preliminary graphical analysis of data [**Supplementary Figure 1** by locally estimated scatterplot smoothing (LOESS) method], we divided the study period, on the basis the previous definitions, into the following phases: (i) the first wave, from 25th February 2020 to 31st May 2020; (ii) the transitional phase, from 1st June 2020 to 9th October 2020 and (iii) the second wave, from 10th October 2020 to 31st December 2020 (last date mortality data update). In this regard, we also performed a sensitivity analysis using data from national COVID-19 surveillance (9), confirming similar waves in the pattern of deaths after a COVID-19 diagnosis (**Supplementary Figure 2**).

STATISTICAL ANALYSIS

We used statistical models to estimate both excess mortality by counts and percentage with 95% CI. In detail, we applied negative binomial models to account for over dispersion observed in the death counts distribution (i.e., to obtain a model with a chi-squared/degree of freedom more closer to 1). The daily count of deaths was the outcome variable of the employed models, whose covariates were the following: time, a categorical variable coded as 1 for the year 2020 and as 0 for the previous pre-pandemic years (2015–2019) and the interaction of the two. The time period from 1st January to 31st December was modeled by quadratic spline functions to account for seasonality. To estimate the excess mortality, we summed the exponentiated linear prediction obtained from the described model (and its 95% CIs), as reported elsewhere (12). The estimates of excess deaths

TABLE 1 | All-cause excess deaths estimates, Italy - year 2020.

	Deaths in 2020	Expected deaths[a]	Excess deaths	95% CI
Italy	746 146	645 620	100 526	(97 575–103 560)
Northern regions[b]	376 181	301 886	74 295	(72 697–75 925)
Central regions[c]	141 550	131 647	9 903	(9 650–10 158)
Southern regions[d]	228 415	212 087	16 328	(15 948–16 712)

[a]*The average of deaths occurred in 2015–2019;* [b]*Northern regions: Piedmont, Valle d'Aosta, Liguria, Lombardy, Trentino-Alto Adige, Veneto, Friuli-Venezia Giulia, and Emilia-Romagna;* [c]*Central regions: Tuscany, Umbria, Marche, Latium;* [d]*Southern regions: Abruzzo, Molise, Campania, Apulia, Basilicata, Calabria, Sicily, and Sardinia.*

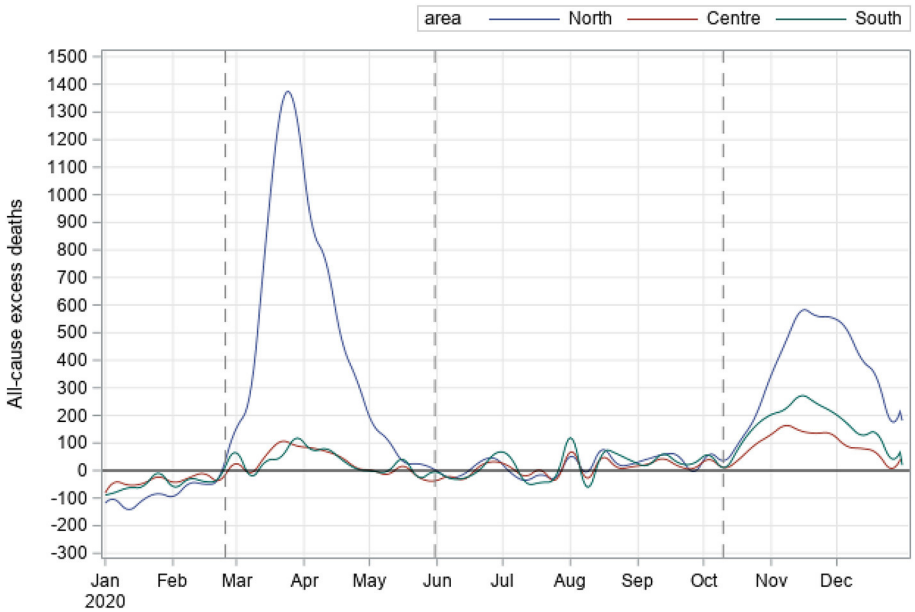

FIGURE 1 | All-cause excess deaths during the COVID-19 pandemic by geographical macro-area, Italy - year 2020.

with its 95% CI were obtained considering the time period of 1st January−31st December, and according to the waves observed, as well as the transitional phase, as we defined. Furthermore, we applied separate models for three macro-geographical areas (northern regions, central and southern regions), as well as for sex and age groups (0–49, 50–79 and 80+ years). The same negative binomial models were also applied for the secondary outcome, as defined previously, to give an estimate of excess mortality "not directly attributable to COVID-19" during the two epidemic waves.

Finally, given the arbitrariness of the reference period considered (i.e., 2015–2019), we made a sensitivity analysis by repeating the negative binomial models for overall all-cause excess deaths choosing as a reference each of the 5 years separately. All statistical analyses were performed using SAS 9.4.

RESULTS

During the year 2020 (from 1st January to 31st December), overall all-cause excess deaths in Italy were 100,526 (95% CI: 97,575–103,560) (**Table 1**); the corresponding percentage of excess mortality was 15.6% (95%:14.6%–16.6%). With regards to the macro-geographical area (**Table 1**), the majority of all-cause excess deaths were observed in northern regions of Italy: 74,295 (95% CI: 72,697–75,925) with a corresponding percentage increase of 24.6%; followed by south: 16,328 (95% CI: 15 948–16 712), with a percentage increase of 7.5% and Central Italy: 9,903 (95% CI: 9 650–10 158), with a percentage increase of 7.7%.

The results of the sensitivity analyses were reported in **Supplementary Figure 3** (a, b, c, d, and e) and **Supplementary Table 1**: the estimates of overall all-cause excess deaths were not significantly different from that reported in **Table 1** [i.e., 100,526 (95% CI: 97 575–103 560)] when chosen as reference last 2 years, i.e., 2018 or 2019. Instead, when considering as a reference the preceding years 2015, 2016, or 2017, the overall all-cause excess deaths were lower when chosen, as a reference, 2015 or 2017, whilst it was higher when we considered 2016.

Estimates of All-Cause Excess Deaths According to the Defined Phases

As described in the Methods section, we divided the overall study period (the year 2020) into three phases, mainly individuated by the waves, as clearly seen in **Supplementary Figure 1**. Northern Italy was the most hit macro-area in terms of excess deaths during both waves (**Figure 1**). We estimated counts and percentage terms for northern regions: 46,342 (95% CI: 43,922–48,891), which corresponded to 59.1% (95% CI: 52.6%–66.4%) during the first wave; whilst during the second wave, we estimated 30,023 excess deaths (95% CI: 29,675–30,371), which corresponded to 42.2% (95% CI: 41.7%–45.1%). It is important to note that excess deaths estimate increased in both, central and southern, regions in terms of counts and percentage in Central Italy, from 2,879 (95%: 2,735–3,028), equivalent to 8.3% (95% CI: 7.4%—9.4%), during the first wave to 7,691 (95%: 7,548–7,833), corresponding to 25.7% (95% CI: 24.2%–27.3%), in the second wave; in southern Italy, from 3,216 (95% CI: 3,046–3 394), corresponding to 5.7% (95% CI: 5.1–6.4%), to 13,265 (95% CI: 13,072–13,458), equivalent to 28% (95% CI: 26.7%–29.4%), during the second wave. **Table 2** shows excess all-cause deaths also estimated during the 3 phases, overall and according to sex and age groups. The overall estimate of excess during the first wave was: 52,437

TABLE 2 | All-cause excess deaths estimates according to the the two waves and the transitional phase.

	Deaths in 2020	Expected deaths[a]	Excess deaths	95% CI	Excess deaths %	95% CI
First wave						
Italy	221 447	169 010	52 437	(49 213–55 863)	31.0%	(27.2%–35.4%)
Males	108 307	80 924	27 383	(25 831–29 023)	33.8%	(29.8%–38.3%)
Females	113 140	88 086	25 054	(23 645–26 541)	28.4%	(25.1%–32.2%)
Age groups						
0–49	4 735	5 018	−283	(−306; −261)	−5.6%	(−6.9%; −4.6%)
50–79	71 631	56 840	14 792	(13 998–15 623)	26.1%	(23.0%–29.4%)
80+	145 081	107 152	37 928	(35 813–40 173)	35.4%	(31.5%–39.8%)
Transitional phase						
Italy	170 026	166 643	3 383	(3 333–3 433)	2.0%	(1.96%–2.1%)
Males	81 267	79 868	1 399	(1 378–1 420)	1.7%	(1.6%–1.8%)
Females	88 759	86 775	1 984	(1 956–2 012)	2.3%	(2.2%–2.4%)
Age groups						
0–49	4 764	5 571	−807	(−851; −763)	−14.5%	(−16.9%; −7.9%)
50–79	55 722	56 834	−1 112	(−1 130; −1 092)	−2.0%	(−2.1%; −1.9%)
80+	109 540	104 238	5 302	(5 220–5 384)	5.1%	(4.9%–5.3%)
Second wave						
Italy	197 502	146 523	50 979	(50 533–51 425)	34.8%	(33.8–35.8%)
Males	98 204	70 770	27 434	(27 109–27 758)	38.8%	(37.3–40.3%)
Females	99 298	75 753	23 545	(23 270–23 819)	31.1%	(29.9–32.3%)
Age groups						
0–49	4 125	4 235	−110	(−118; −103)	−2.6%	(−3.1%; −2.0%)
50–79	63 983	49 070	14 913	(14 698–15 127)	30.4%	(29.0–31.8%)
80+	129 394	93 218	36 176	(35 801–36 550)	38.8%	(37.5–40.1%)

[a]*Average of deaths occurred in 2015–2019.*

(95% CI: 49,213–55,863), whilst in absolute terms, we estimated a slight decrease during the second wave: 50,979 (95% CI: 50,333–51,425). Of note, when considering the excess deaths on percentage terms, we estimated an increase in excess deaths during the second wave with respect to the first, although not statistically significant: 34.8% (95% CI: 33.8%–35.8%) vs. 31% (95% CI: 27.2%–35.4%) (**Table 2**). Concerning sex (**Figure 2A**), we estimated a higher number of excess deaths in males, both on absolute and percentage terms (**Table 2**): 27,383 (95% CI: 25,831–29,023), excess deaths for males during the first wave, with a percentage increase of 33.8%, whilst during the second wave, we estimated 27,434 (95% CI: 27,099–27,748) excess deaths, corresponding to a percentage increase of 38.8%. Excess deaths of 25,054 (95% CI: 23,645–26,541) was estimated for females during the first wave (28% increase), whereas excess deaths of 23,545 (95% CI: 23,256–23,806) (31.1% increase) in the second wave.

During both the waves, the age groups with the largest numbers of excess deaths were 80 or over, as shown in part b of **Figure 2**. Specifically (**Table 2**), we estimated all-cause excess deaths of 37,929 (95% CI: 35,813–40,173) during the first wave, with a corresponding percentage increase of 35.4% and 36,176 (35,811–36,542) during the second wave, with a percentage increase of 38.8% (**Table 2**). Excess deaths during the first wave were 14,792 (95% CI: 13,970–15,663) in the age group 50–79, corresponding to a percentage increase of 26.1%, whilst during the second wave, corresponding to a percentage increase of 30.4%. We estimated all-cause excess deaths of −283 (95% CI: −306; −261) in age group <50 during the first wave, whilst it increased slightly [−110 (95% CI: −117; −103)] during the second wave, in terms of the percentage, this corresponded to a decrease of −5.6% during the first wave and −2.6% during the second wave. The estimates of excess deaths during the transitional phase were 3,383 (95%: 3,333–3,433), corresponding to a percentage increase of 2%. Of note, during this phase, the worst-hit age group in terms of the number of excess deaths was again 80 or over, with 5,302 deaths (95%: 5,220–5,384), corresponding to an increment of 5.1%.

Estimates of All-Cause Excess Deaths Not Directly Attributable to COVID-19 According to the Two Defined Waves

Figure 3 and **Table 3** show the estimates of excess deaths that were not directly attributable to COVID-19, i.e., without a previous COVID-19 diagnosis. During the first wave, we estimated excess deaths that occurred without a previous COVID-19 diagnosis of 18,307 (95% CI: 17,197–19,487), corresponding to 10.8% (95% CI: 9.5%–12.4%). The estimate of excess deaths without a previous COVID-19 diagnosis decreased significantly during the second wave, being 11,318 (95% CI:

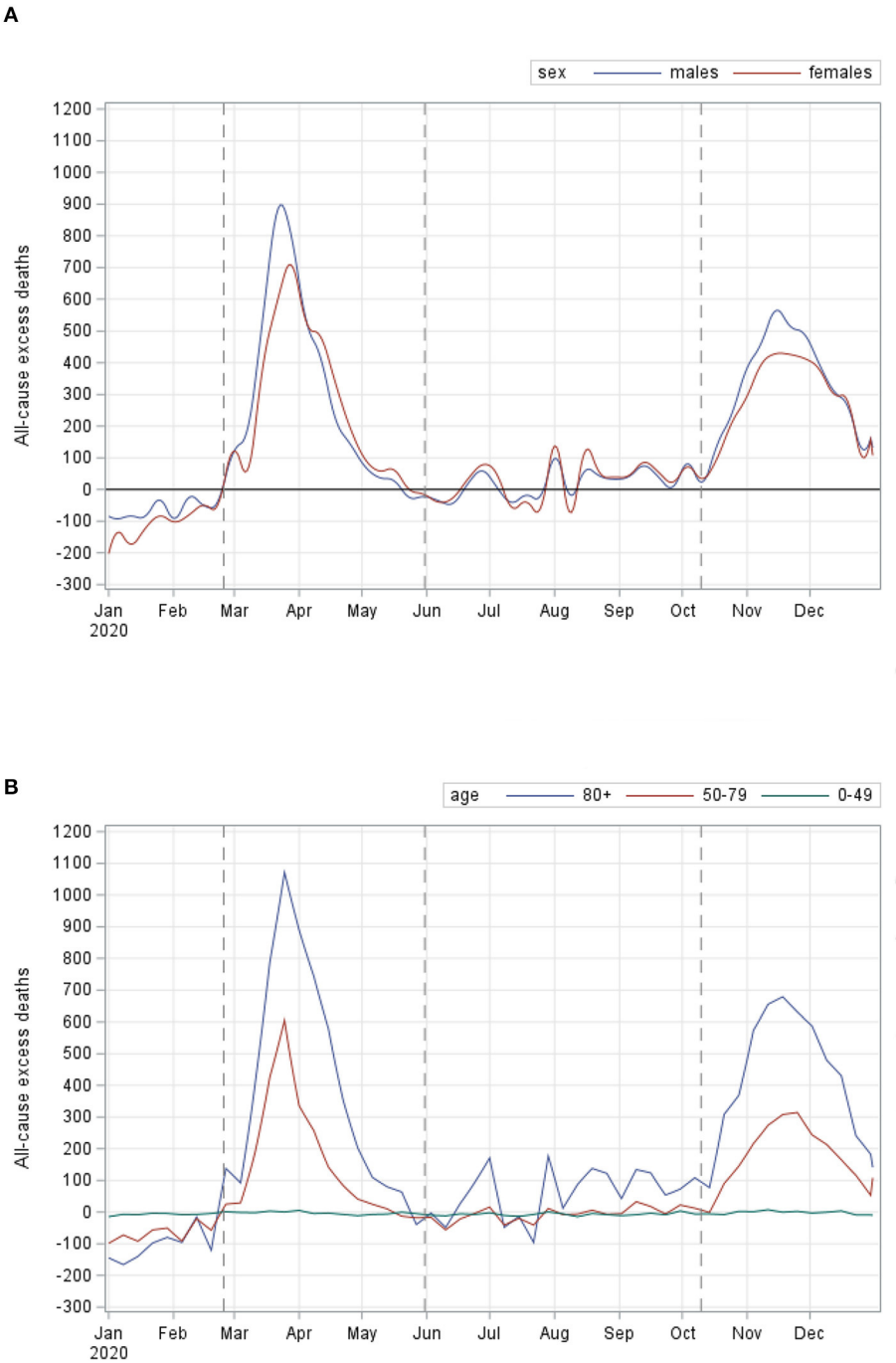

FIGURE 2 | All-cause excess deaths during the COVID-19 pandemic by sex **(A)** and by age groups **(B)**, Italy - year 2020.

11,221–11,414), corresponding to 7.7% (95% CI: 7.5–7.9%). In Northern Italy (results were not showed in **Table 3**), we estimated percentage of excess death without a previous COVID-19 diagnosis of 22% (95% CI: 19.6%–24.8%) during the first wave, whilst the second wave, the estimate was 8.8% (95% CI: 8.5%–9.2%). On the contrary, in the rest of Italy, excess deaths without a previous COVID-19 increased from 1.1 (95% CI: 1.0–1.2%) to 6.7% (95%CI: 6.4–7.1%). A significant decrease in excess deaths not directly attributable to COVID-19 was observed in both sexes during the second wave (**Table 3**), as also in all age groups except for those aged <50, for whom we observed a slight increase [during the first wave: −652 (95% CI: −702; −604), during the second wave: −532, (95% CI: −563; −501)].

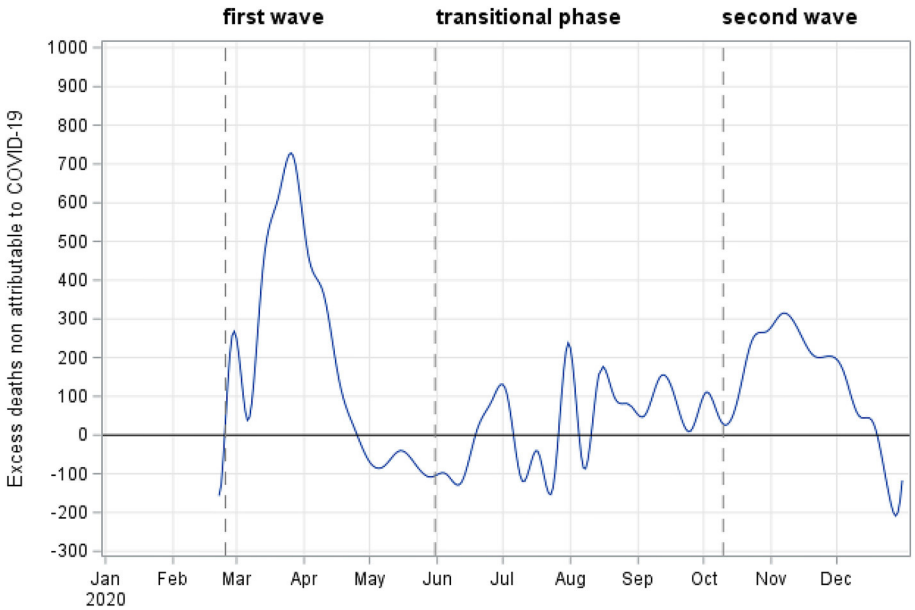

FIGURE 3 | Excess deaths not directly attributable to COVID-19 during the pandemic, Italy - year 2020.

TABLE 3 | Estimates of excess deaths "not directly attributable to COVID-19".

	Deaths *non* COVID-19[a]	Excess deaths *non* COVID-19[a]	95% CI	Excess deaths *non* COVID-19%[a]	95% CI
First wave					
Italy	187 322	18 307	(17 197–19 487)	10.8%	(9.5–12.4%)
Males	88 520	7 593	(7 166–8 047)	9.3%	(8.3–10.6%)
Females	98 802	10 714	(10 108–11 356)	12.2%	(10.7–13.8%)
Age groups					
0–49	4 366	−652	(−702; −604)	−12.3%	(−15.8%; −10.6%)
50–79	57 986	1 145	(1 091–1 201)	2.0%	(1.8–2.3%)
80+	124 970	17 815	(16 863–18 821)	16.6%	(14.8–18.7%)
Second wave					
Italy	157 841	11 318	(11 221–11 414)	7.7%	(7.5–7.9%)
Males	76 108	5 338	(5 273–5 403)	7.5%	(7.2–7.8%)
Females	81 733	5 980	(5 909–6 051)	7.9%	(7.6–8.2%)
Age groups					
0–49	3 703	−532	(−563; −501)	−12.6%	(−14.8%; −10.6%)
50–79	50 080	1 010	(997–1 023)	2.1%	(2.0–2.2%)
80+	104 058	10 840	(10 724–10 957)	11.6%	(11.2–12.0%)

[a]*Deaths "not directly attributable to COVID-19", i.e., deaths defined as "without a previous COVID-19 diagnosis".*

DISCUSSION

This study provides estimates of the excess mortality, i.e., the difference between the observed number of deaths during a given time period and the expected number of deaths in the same period, occurred in Italy in 2020. The COVID-19 pandemic scenario in Italy during this period can be summarized in three phases, similarly to other European countries (5): a first wave, from late February to the end of May, characterized by a sharp increase of cases and deaths and by a high territorial concentration, in Italy mostly in the north; a transitional phase, from June to mid-September, with a low diffusion of the virus (13); and a second wave, starting from the end of September 2020, when the cases increased rapidly again until the first half of November (9) and then decreased again. We estimated an increase in all-cause excess deaths during the second wave from 31% (95% CI: 27.2%–35.4%) to 34.8% (95% CI: 33.8%–35.8%), although not statistically significant. This increase may have

depended on factors including different pattern of the Sars-Cov2 diffusion observed during the second wave, both in terms of quantitative and the geographical distribution (9). In particular, a significant decrease in excess mortality in the northern regions (from 60% during the first phase to 42% in the second phase) was coupled with a significant increase in the rest of Italy. This result may be due to greater preparedness of the health care services in the north of Italy during the second wave, although the overall contribution to excess mortality during the whole year 2020 remained significantly higher in the north than in the rest of Italy.

The curve tracking all-cause excess deaths increased much faster during the first wave than in the second wave, even though the percentage of all-cause excess deaths was higher during the second wave. This result may be explained by the different mitigation measures adopted in Italy during the two phases. During the first phase, Italy was the first European country (and second only to China in the world) to adopt a hard national lockdown in March and April, whereas a different containment strategy was adopted in the autumn of 2020, based on regional parameters that resulted in a three-color classification of regions (yellow, orange, and red). Each color corresponded to a different risk scenario, from the lowest to the highest and characterized by different prevention measures against the diffusion of COVID-19 (refer to ministerial decree published in the Official Gazette, General Series No. 275, 4th November 2020, ordinary supplement No. 41).

Focusing on gender differences, as widely reported in the literature (14) and despite a similar incidence of COVID-19 cases reported for men and women (9), men presented a higher mortality risk than women (15). This difference appeared to be more marked during the second wave, even though not significantly.

Not surprisingly, the highest contribution to excess mortality during the whole period covered by this study was observed among people aged 80 years and older, with a further increase during the second wave (from 35% in the first to 39% in the second wave). It is important to note that this age group is particularly relevant in Italy, where it accounts for 7.4% of the population, compared with an EU average of 5.9%.

Another core finding of this study is that the excess mortality observed among people aged less than 50, although of negative sign, increased slightly during the second wave. The most likely explanation is that the COVID-19 related deaths recorded in this part of the population concern, in most cases, people who were already suffering from serious diseases, that is, a very fragile component of the population (16).

During the second wave, we also estimate a decrease in the excess deaths that are not directly attributable to COVID-19 (i.e., without a previous COVID-19 diagnosis), which we approximated by using a proxy. This finding can be interpreted as an improvement recorded by the national health system in the diagnosis and treatment of other severe diseases that were previously delayed by the presence of COVID-19, which was overburdening the health care services during the first wave. As a result, there was a decline in the indirect effects of the COVID-19 pandemic on other diseases during the second wave when compared with the first one.

Another explanation for the decrease in excess deaths not directly attributable to COVID-19 could be the improvement in the detection of COVID-19 related cases *via* diagnoses completed before death. To support this aim, the National Institute of Statistics has recently reported data on specific causes of deaths (see https://www.istat.it/it/archivio/256854), which showed that COVID-19 was the first cause of death in Italy during the period March–April 2020: overall, 60% of deaths were attributable to COVID-19, 10% to pneumonia, most likely related to COVID-19 and 30% to other causes. While findings are yet to be published for the second wave, the decline of excess deaths not directly attributable to COVID-19 estimated in this study also seems to confirm the higher proportion of deaths attributable to COVID-19 in this second phase.

We are aware that a limitation in our study may be given by the choice of the reference period (2015–2019) to estimate the excess deaths, which is rather arbitrary. Different reference periods may produce different results. We hypothesize, however, that the period chosen may provide a good benchmark as it includes both years characterized by a high mortality rate, such as 2015, and years as 2016 or 2018 characterized in Italy by lower mortality. This resulted in a lower or higher excess estimate over the years as confirmed by the supplementary analysis. In addition, as the number of deaths in a given year depends on both the multiple factors that affect survival and the age structure of the population (13), it is appropriate to choose a reference period that is as close in time as possible to the observed periods.

The lack of data on specific causes of death was a second limitation of the study. Thus, to estimate the impact of the other deaths "not directly attributable to COVID-19" we used a proxy, defined as the difference between all-cause excess deaths minus the total number of deaths after a COVID-19 diagnosis.

Furthermore, we did not consider ambient temperature that is also an important factor that can cause excess mortality (17) and the two waves occurred in two seasons with different temperature patterns. Although we applied the quadratic spline function of time to account for seasonality, there might still exist residual confounding caused by temperature.

Overall, while it is true that only a specific analysis of the actual causes of each death would allow for a fully-fledged understanding of the differences between age groups, we hypothesize that all-cause excess mortality is an effective epidemiological tool that includes both the direct and indirect effects of the COVID-19 disease.

An important strength of this study is that it was conducted by using high-quality national data provided by the ISTAT, which are exhaustive with regards to total mortality and the best available with regard to the COVID-19 cases and related deaths, as detected by the national surveillance system of the National Institute of Health (ISS). This reliable dataset has enabled to identify some findings that may contribute to a better

understanding of the COVID-19 pandemic in Italy. Finally, this study includes suggestions for future research avenues that may further help in the identification of more effective public health measures.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not provided by the participants' legal guardians/next of kin because COVID-19 surveillance data are collected in the National Institute of Health in Italy (ISS) and in February 27th, 2020, the Italian Presidency of the Council of Ministers, being compliant with the European General Data Protection Regulation (UE GDPR 2016/679) authorized the processing of personal data related to COVID-19 by the ISS and other public institutions (such as ISTAT too) for reason of public interest in the area of public health. Further, data are already anonymised.

CONSENT TO PARTICIPATE

COVID-19 surveillance data are collected in the ISS in Italy and on 27th February 2020, the Italian Presidency of the Council of Ministers, being compliant with the European General Data Protection Regulation (UE GDPR 2016/679) authorized the processing of personal data related to COVID-19 by the ISS and other public institutions, such as ISTAT, for the reason of public interest in the area of public health.

CONSENT FOR PUBLICATION

All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

AUTHOR CONTRIBUTIONS

MD performed the statistical analyses and drafted the manuscript. GM contributed to the design of the study and revised the advanced draft of the manuscript. SB elaborated surveillance data and revised critically the manuscript. VM elaborated mortality data. SP coordinated and supervised national mortality data. MB and GC elaborated national mortality data and revised critically the manuscript. XA, FR, MF, and MV elaborated surveillance data and revised critically the manuscript. MS, AM-U, and MDM elaborated surveillance data. GO revised critically the manuscript. PP is the head of the Italian coronavirus disease surveillance system and revised the manuscript. AB coordinated and supervised the surveillance data collection and contributed to the conception and design of the study by revising critically the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.669209/full#supplementary-material

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COVID-19 Vaccination: Concerns About Its Accessibility, Affordability, and Acceptability

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By the mid of June 2021, after an almost 1.5-year-long COVID-19 pandemic that has significantly affected the world in multiple ways, various vaccines against COVID-19 have arrived and started worldwide. Yet, economic, (geo)political, and socio-cultural factors may influence its uptake at individual and country levels. Several issues will (and already have been reported in media) revolve around this vaccination regarding its accessibility, affordability, and acceptability at an individual level and a country level. Given that in this commentary, we provoke a discussion: Who—a country as well as the individuals—would have access to it, and who would economically afford it, and who would accept it? Centering these intriguing questions, we revisit the body of literature that explicates vaccine hesitancy, refusal, and resistance, and we also draw on the current literature and media reports about vaccination against COVID-19. We suggest that these backdrops need essential attention so that everyone can afford, accept, and have access to it. Otherwise, the current risk in the face of a year-old pandemic will continue.

Keywords: COVID-19, vaccination, immunization, disparities, vaccine hesitancy, refusals, low-income countries, high-income countries

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INTRODUCTION

Since the coronavirus disease (COVID-19) has overwhelmed the entire world, the longing and production of a vaccine against it have also significantly increased over time (1). By December 2020, ∼200 candidate vaccines for COVID-19 went under preclinical and clinical evaluation (2). After several successful trials, various vaccines, e.g., Pfizer–BioNTech, Moderna, and Oxford–AstraZeneca, have arrived and been started in some countries, such as the United Kingdom and Russia. The European Union (EU) has also commenced the vaccination first vaccinating older people and healthcare providers.

This phenomenal discovery would undoubtedly end the almost 1.5 year-old pandemic. Nonetheless, concerns can be raised about its affordability, accessibility, and acceptability at individual and country levels. Unsurprisingly, economic, (geo)political, and socio-cultural factors may influence the vaccine's uptake: Which country can afford the vaccine and which individuals can have access to it and then will accept/refuse the vaccine?

First, low-income countries, such as Papua New Guinea (PNG) and Pakistan, at the first point cannot afford the COVID-19 vaccine. Second, the vaccine may face critical challenges that may affect its uptake. The underlying reasons include various (a) forms of mistrust—i.e., between

citizens and government, between laypeople and global stakeholders, and between governments and global stakeholders; (b) forms of institutionalized inequalities and inequities appropriated at local, national, and global levels; and types of rumors and conspiracy theories about vaccination (1, 3). The routine vaccination uptake in Pakistan is still way behind as per the World Health Organization (WHO) recommended 95% (3). Consequently, viruses like measles and polio are still prevalent in the country and cause severe outbreaks. These complex processes may affect the vaccine's affordability, accessibility, and acceptability, especially in the face of the "infodemic" surrounding COVID-19 (4, 5). For instance, many people in Pakistan have considered the pandemic "Western" "plot," "political game," or "fake" (6–8), and consequently, they have refused polio vaccination (9). Therefore, this commentary first provides an overview of the vaccination, the situational analysis of the COVID-19 vaccination, and factors that may affect its uptake, its such as various forms of disparities.

VACCINATION: A BRIEF OVERVIEW

Vaccination emerged as the most effective public health intervention to prevent communicable diseases, save lives, and reduce disease burden. Following the "Pasteur's Germ Theory," Edward Jenner produced the smallpox vaccine in 1798 (10). Globally, many clinical and public health professionals have been studying the nature of immunological memory for the last 100 years (11). From the public health perspective, vaccination is considered a substantial measure to immunize people for reducing vaccine-preventable diseases (VPDs) (12). The success of vaccination programs highly relies on herd immunity at a population level (13), as an increase in herd immunity may result in a lower intensity of infection in the population and thus a lower risk of infection among unvaccinated persons (14).

Despite the remarkable success of vaccination programs, vaccines are neither 100% efficacious nor 100% effective (15). One common argument frequently found in anti-vaccination literature is that people still get the disease after a vaccine (16). That means the lack of vaccine efficacy or lack of adequate protection are generally used for vaccination failure (17), while a failure indicates that the vaccine has not been administered appropriately for any reason (15). Such incomplete coverage, vaccine–vaccine interactions, and manufacturing-related issues are also crucial factors involved in vaccine failure.

In addition to vaccine failure, "vaccine hesitancy" and vaccine reluctancy are growing in public due to a lack of confidence in the vaccine and those who administer it (3). "Vaccine hesitancy" can be defined as a set of beliefs, attitudes, and behaviors that many people hold to decline, delay, or doubt a vaccine (18). Vaccination came under public suspicion when a large population refused pertussis vaccination in the 1980s, and afterward a decrease in measles, mumps, and rubella (MMR) vaccines (19). Rumors and conspiracy theories have since long been associated with vaccinations across the world, which ultimately affect the vaccination uptake (1, 3, 20–22). These narratives are social phenomena that are meaningful within a context (1, 3). Since this hesitancy affects routine vaccine coverage, ultimately resulting in vaccine-preventable disease outbreaks and epidemics, the success of the current COVID-19 vaccine seems unachievable without dealing with these perceptions and practices.

COVID-19 VACCINATION: SITUATIONAL ANALYSIS

The 2020 COVID-19 pandemic compelled the global scientific community to find the solution in terms of therapeutics and vaccines to control it. Many vaccine candidates joined the endeavor to produce an effective vaccine (2). Several trials were conducted. And finally, a few candidates were successful in producing and supplying vaccines as vaccination started in several countries, notably in the EU at the end of December 2020.

Concerning the acceptance and vaccination strategies to run COVID-19 immunization programs, numerous surveys, and studies have been conducted. For instance, one study in China was carried out in March 2020 for evaluating the risk perception, impacts of COVID-19, and attitudes, acceptance, and preferences of COVID-19 vaccines. The results showed that around 91.3% adult population reported that they would accept COVID-19 vaccination (23). In contrast, a national cross-sectional survey in the UK found that around 64% of participants are likely to accept the stated vaccine (24). Similarly, a global survey of 19 countries concluded that 71.5% of participants showed a positive response regarding the COVID-19 vaccine (25).

Nonetheless, many people are reluctant to receive the COVID-19 vaccine as they are concerned about side effects. The media reported a recent survey in Germany that only 33% of the population showed a willingness to receive the vaccine; however, they were slightly more hesitant about the side effects (26). While about 19% of people said, they do not want to receive this vaccine at all (26).

Similarly in Pakistan, there are a considerable number of people who are hesitant to get the shot of the COVID-19 vaccine. While revising this paper in May 2021, extremely critical rumors have started, such as those who receive the COVID-19 vaccine will die after 2 years. And, for instance, during our data collection for the project on COVID-19 led by Inayat Ali, one respondent shared, "Today got the second dose of COVID-19. During my visit to the vaccination center, I observed either people are well-aware or full of fear. Everyone was asking which vaccine you are injecting Sinopharm or Astra Zeneca. This double mind standard is set by the media, I guess."

In this regard, the scientific literature is scant in Pakistan. Yet, two studies have focused on healthcare workers to observe their perceptions of the COVID-19 vaccine. One study focusing on healthcare workers found that out of 5,237 responses, 70.25% accepted COVID-19 vaccination, 24.51% were reluctant thus wanted to delay until more data was available, and 0.05% rejected being vaccinated. Vaccine acceptance was more in young (76%) and female gender (63.3%) who were engaged in a tertiary care hospital (51.2%) and provision of direct patient care (61.3%) (27). The reason women refused to receive the vaccine was due to doubts about the effectiveness of the vaccine (31.48%), while the

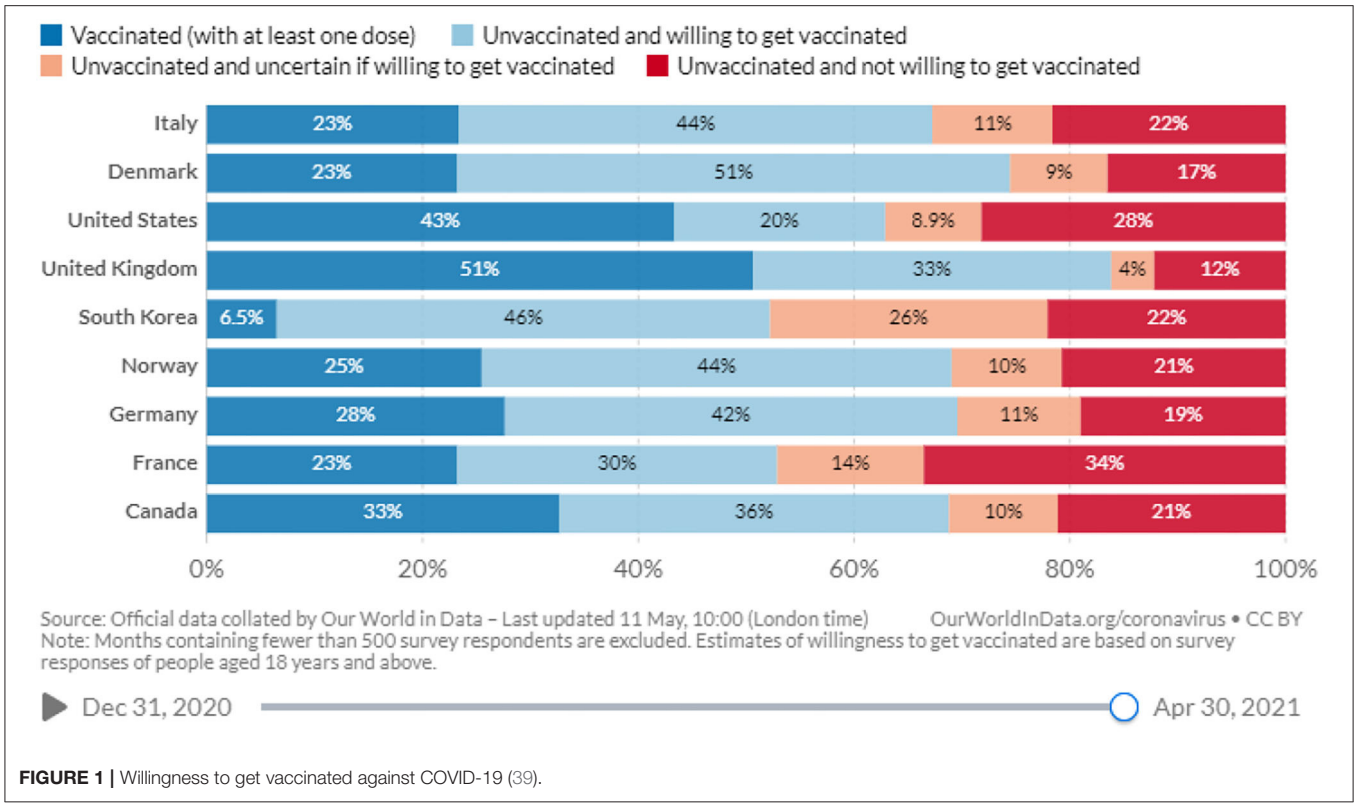

FIGURE 1 | Willingness to get vaccinated against COVID-19 (39).

reason men refused to receive the vaccine was because of previous exposure to COVID-19 (42.19%) and the side effect profile of the vaccine (33.17%). The study obtained responses from 555 doctors 89% of them worked in healthcare facilities where they could encounter COVID-19 patients, and 32% tested positive previously; 81% of them shown acceptance as they wanted to be vaccinated while 19% were not convinced to be vaccinated (27). It was interesting to know that 46% of them were anxious to be offered Astra-Zeneca or Pfizer (28).

Determinants of Vaccine Hesitancy: Focusing on COVID-19 Vaccination

Several rumors and conspiracy theories have already surfaced about COVID-19 and affected its people's perceptions and preventive behaviors (4, 6, 8). Analogously, old narratives have also emerged concerning the COVID-19 vaccine, such as, about its potentially harmful side effects, illnesses, and even death. An extensive antivaccine content has frequently been shared on social media, which directly or indirectly would shape vaccination opinions and may cause vaccine hesitancy (29).

Individual freedom is a critical factor (30), as it sparks controversy among parents who feel deprived of their freedom to make decisions about their children's health (31). This is a complex decision that involves emotional, socio-cultural, spiritual, and political factors as much as cognitive factors (32). During the current pandemic, about 34% of the British population opposed the government's decision to make vaccination legally compulsory for all people (33).

Trust deficiency is another crucial issue in contemporary society, especially regarding science and knowledge. Many studies of vaccination decision-making and perceptions are closely linked to trust and mistrust in health professionals, government, or public health institutions (3, 6, 34, 35). One study in Italy highlighted that people's trust in science and vaccination decreased between the first and the second phase of the pandemic (36). Moreover, many people refuse to vaccinate in the US due to vaccine-specific concerns, such as the need for more information, anti-vaccine attitudes or beliefs, and a lack of trust (37). Similarly, numerous people in America and Canada preferred natural COVID-19 immunity. They showed mistrust in vaccine benefits and concerns about unforeseen future effects, and commercial profiteering from pharmaceutical companies [(38); see **Figure 1**].

Adequate knowledge and awareness appear to be another important factor. One recent review reported that lack of knowledge and understanding of the benefits of vaccination, inconsistent recommendations by providers, and uncertainties about cost benefits might be some critical causes of low adult vaccination coverage (40). Similarly, another study found that education about herd immunity and local vaccination coverage could be a useful tool for increasing willingness to vaccinate, generating benefits both to individuals and communities (41).

Likewise, one study from Italy proposed that educational initiatives and other interventions are equally important steps to develop the appropriate awareness in people about COVID-19 vaccination (42).

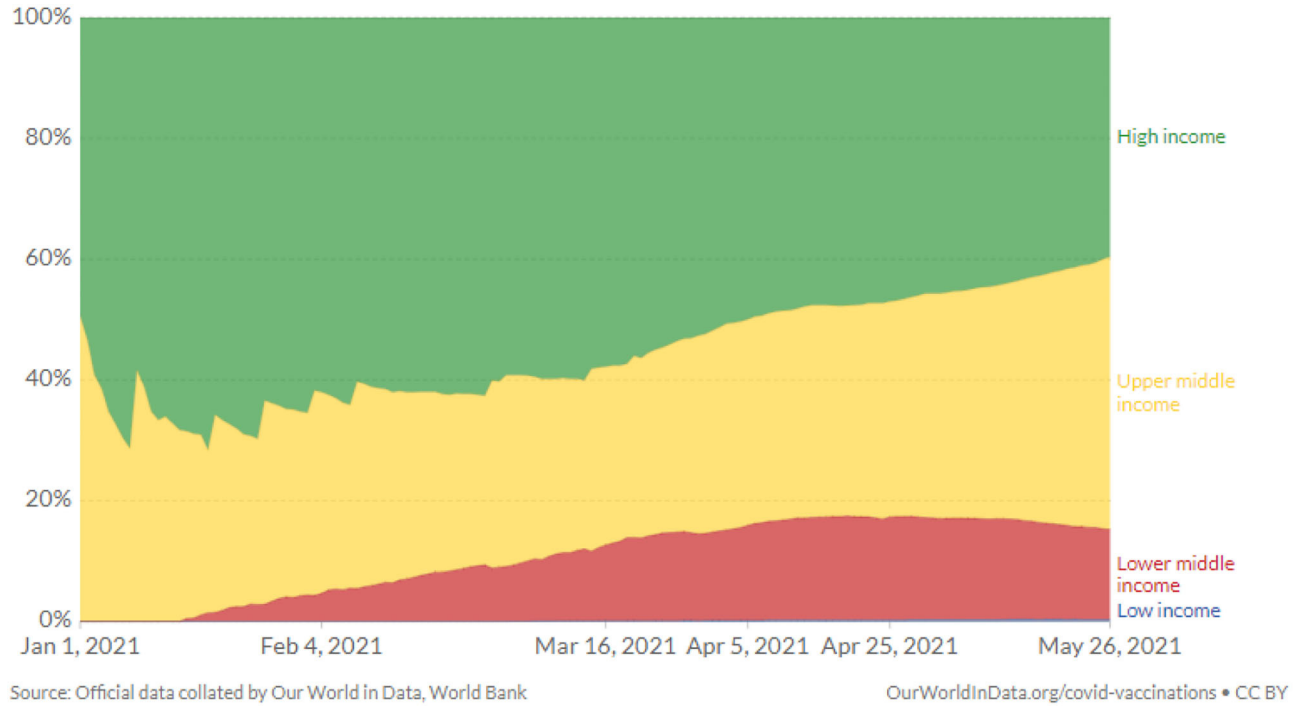

FIGURE 2 | COVID-19 vaccine doses administered by country income group (39).

COVID-19 Vaccine Affordability: Structured Disparities at Play

By sharing the antibody testing data, one study suggested that about 90% of people are susceptible to COVID-19 and around 60–70% (5.6 billion) of the global population have to be immunized to achieve herd immunity (43). Given that, we reiterate here that will COVID-19 vaccine be available to all people worldwide anxious to accept and want this vaccine? Most probably, it would not be possible due to the existing economic and political conditions of the world.

Recent reports specified that the EU stockpiled around 2 billion vaccine doses, and some high-income countries have already secured large numbers of doses of different candidate vaccines without knowing which one may prove helpful (44). However, low-income countries are far away from the state of purchasing and implementing COVID-19 vaccination programs (see **Figure 2**). Along with the challenges in resources and manufacturing, there are issues associated with distribution and acceptance. For instance, the vaccine is available in high-income countries as they can afford it, but people refuse to receive it. Here they have a choice to decide either to opt for it or leave it. In contrast, until low-income countries are given vaccines by high-income countries as "donations," they cannot afford it due to limited economic resources and high population pressure. A small village of Ice Land may have access to an effective vaccine, but the Akha village of Nepal indeed would face significant challenges to have it. At least, this village would not have easy access.

Therefore, the implementation of COVID-19 immunization programs will likely be more affected in low-resource settings. Similarly, it is essential to pursue comprehensive vaccination strategies in parallel to minimize reluctance, hesitancy, and refusal at global, regional, and country levels.

Given these backdrops, it is necessary to reimagine "the culture of public health" (45) since diverse local, national, and global contexts where various economic, (geo)political, and socio-cultural factors are entangled (1). Dealing with these contexts is essential so that every country and individual may quickly and indiscriminately afford, accept, and have access to the COVID-19 vaccine to end over a year-old pandemic. Vaccination proves useful when over 95% of the population receives the vaccine that helps to create what can be called "communal immunity." Otherwise, even in the presence of an effective COVID-19 vaccine, everyone would be at a similar risk prevalent during these days of the pandemic. The risk multiplies due to globalization since travel is more comfortable than in the past—how a microorganism swiftly travels, we have seen it in the case of COVID-19.

CONCLUSION

Based on the Pasture's work, many vaccines have been developed over time. Vaccination done right is a great preventive measure against various communicable diseases, including COVID-19. By revising this paper (in the mid of June 2021), the vaccine against COVID-19, such as Pfizer–BioNTech, Moderna, and AstraZeneca, Sinopharm has arrived and started across the

world. However, by writing this paper (December 2020), this vaccination only started in several high-income countries. Like other vaccines, there are genuine concerns about the COVID-19 vaccine who can afford it, who will have access to it, and who will accept it. We provoke that the current vaccination would significantly be determined by the pre-existing socio-cultural, economic, and (geo-)political disparities. Economically rich countries and individuals will have easy access to it, but low-income countries and populations will face significant issues to buy and receive it. We, therefore, suggest that it is important to take these different contexts into account for indiscriminately accessibility, affordability, and acceptability. Otherwise, the same risk that has been visible during the pandemic would continue even in the presence of an effective COVID-19 vaccine.

Moreover, based on the current narratives surrounding the COVID-19 vaccination, we invite ethnographically and sociologically rich accounts to study, analyze, and report the results of factors that not only discourage vaccination uptake but also pay significant attention to those factors that encourage vaccination uptake. Also, there are a plethora of narratives about different vaccines of COVID-19 that which one is more effective. Such detailed studies hold great importance, as these will play a pivotal role to address concerns that shape particular perceptions and practices of vaccination.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: all data are cited.

AUTHOR CONTRIBUTIONS

IA: conceptualization, writing the first draft, analysis, revision, and validation. SA: conceptualization, revision, and validation. SI: conceptualization, writing the first draft, and validation. All authors contributed to the article and approved the submitted version.

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Community-Based Measures to Against the COVID-19: An Experience From Vietnam With Consecutive 3 Months of no New Infection in the Community During the First Wave of Pandemic

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Vietnam has faced a high risk of transmission of COVID-19 during the pandemic. Despite the specific challenges that come with a low-resource country, the Vietnamese government has provided a sustainable response, demonstrating both great capacity and rapid decision-making to manage the entirety of the COVID-19 outbreak with lessons learned from the SARS and H1N1 epidemics in 2003 and 2009, respectively. A rapid response, specific epidemiological F0–F5 tracing system, and public education are some of the key measures that have helped Vietnam to control the outbreak. As of July 15, 2020, Vietnam has reported 373 accumulated confirmed cases and no deaths within the last 90 consecutive days of no new infections in the community. Vietnam can now consider declaring an end to the COVID-19 crisis on their part.

Keywords: outbreak, control measures, epidemiological F0–F5 tracing system, Vietnam, COVID-19

INTRODUCTION

The outbreak of COVID-19 spread rapidly during the period of the traditional Lunar New Year Festival in Vietnam, China, and Taiwan; the occasion saw a stream of millions of people who were expected to visit their home countries (1). Given the high risk of virus transmission since the first case report of COVID-19 in China on December 1, 2019 (2), and the first case outside China in Thailand on January 13, 2020 (3), the Vietnamese government took numerous preventive strategies. Indeed, airports and hospitals established additional stations to deploy body temperature scanning for passengers and patients who had entered Vietnam in the last 21 days. Early detection and high priority surveillance of infected cases as well as strict monitoring of airports, seaports, and national borders were seriously applied. On January 30, 2020, when the World Health Organization declared COVID-19 a Public Health Emergency of International Concern with 7.736 confirmed cases in China and 82 confirmed cases in 18 other countries (4), Vietnam had stopped issuing visas for Chinese citizens and foreign travelers who visited China in the past 14 days. All Vietnamese citizens had been urged to complete their health status declarations via Bluezone, a bluetooth-based mobile application that helps to notify its users if they come into contact with a COVID-19 patient (5). All passengers who entered Vietnam from affected areas must report all information, including their

TABLE 1 | F0–F5 evaluation system for epidemiological tracing contacting people who are related to a confirmed case of COVID-19 in Vietnam.

Level	Definition and the attitutde of tracking
F0	Positive with COVID-19 (isolation and treat in the hospital)
F1	Suspected of COVID-19 or having close contact with F0 (should wear mask, maintain a distance to other people for at least 2 m, should go to the hospital for isolation, inform to the Government and F2 about their situation
F2	Having close contact with F1 (should wear mask, maintain a distance to other people for at least 2 m, should stay at home for isolation and inform to the Government and F3 about their situation)
F3	Having close contact with F2 (should wear mask, should stay at home for isolation, and inform to the Government and F4 about their situation)
F4, F5	Having close contact with F3, F4 (should wear mask, should stay at home for isolation, and inform to the Government)

contact details, COVID-19 symptoms, and current and previous locations at entry ports before being sent to temporary hospitals for 14-day self-isolation management. The field hospitals were built from scratch and were also launched in the suburbs.

HOW THE VIETNAMESE GOVERNMENT MANAGED THE FIRST WAVE OF THE PANDEMIC

The Vietnamese Centers for Disease Control and Prevention, which had been developed and improved since the outbreak of SARS in 2003 and H1N1 in 2009, cooperating with the Ministry of Health, had promptly advised the government to achieve effective and rapid control of various items. These included (1) social distancing and barriers, restriction of movement, avoiding public transport, closure of schools and public areas, prohibition of gatherings of more than 15 people in air-tight areas, obligatory use of face masks and hand hygiene practice, and enhanced environmental ventilation. In addition, advice also looked at (2) preventative measures, such as quarantining suspicious cases, isolating confirmed cases, performing epidemiological tracing, and contacting people who are related to a confirmed case of COVID-19 by using the F0 to F5 evaluation system (**Table 1**). Since February 14, Vietnamese citizens coming home from abroad and foreign citizens arriving in Vietnam must be quarantined for 14 days and tested for COVID-19. Further issues included (3) advice on socio-economic management was also given: adjustment of relevant health insurance policies, tax, the workforce, and food and daily necessities; (4) mobilization of armed forces to control the air, sea, and land borders; and (5) social media education to fight fake news and the spread of misinformation.

On March 19, the Vietnamese government temporarily achieved a triumph for the first stage of this pandemic with 85 confirmed cases and no cases of death since the first Vietnamese case of COVID-19 was acquired from China on January 23, 2020 (6, 7). Vietnam also had a period of 2 weeks that was free of new cases before having a series of confirmed and suspected cases from March 6 onwards after a stream of Vietnamese people returning from overseas (8). Subsequently, Vietnam has entered a new period characterized by susceptibility to COVID-19 infection due to the high number of clusters in the community and the loss of source tracing. On March 20, the Government decided to impose a lockdown on Bach Mai hospital, which is the biggest national healthcare center in the North of Vietnam and which sees a high volume of outpatients, since the two female nurses were tested positively to COVID-19 without a detectable source. The Prime Minister was quick to implement a lockdown level of 3 and 4 that saw the strict application of contact investigation strategy using the F0–F5 evaluation system. All of Bach Mai Hospital, including its 7,664 staff members, was quarantined following positive testing results for SARS-CoV-2 in 27 employees working in catering (9). Contact tracing in the community was carefully and quickly processed in an additional 52,239 individuals. Among them, 27,893 F1 and F2 persons were subsequently detected and placed under quarantine using ∼30,000 RT-PCR tests. The national lockdown requested that citizens stay at home and just go out for daily necessities, such as food shops, pharmacies, urgent medical appointments, and work. School and universities were closed. Despite the school closures, education systems moved to online activities as an alternative. Online teaching and learning platforms were encouraged and widely used. Most businesses began shutting down except those that were essential. Suspension of public transportation followed, and travel between cities was extremely limited. A total of 3 week later, the Bach Mai Hospital crisis was consequently contained without further spread. As of July 15, 2020, our report revealed 373 confirmed cases of COVID-19, with 352 patients (94.4%) having recovered from COVID-19, and no cases of death (10). Vietnam had 90 consecutive days of being free from new acquired COVID-19 cases in the communities (**Figure 1**). At present, the Vietnamese government remains vigilant of the infectious virus to cope with its next wave. The AstraZeneca vaccine had been approved to use across the country. The same tactics coupled with a serious attitude are maintained.

KEY WEAPONS DURING A TIME OF CRISIS: RAPID RESPONSE, A SPECIFIC EPIDEMIOLOGICAL F0–F5 TRACING SYSTEM, AND PUBLIC EDUCATION

The Vietnamese Government quickly ramped up its responses and prepared for combat following the first case that originated in China (7). A national declaration of an epidemic was officially announced on February 1, 2020, while there were still only six confirmed cases. The prompt reaction at the begining of February remains a key point of success, as demonstrated in other countries. In Romania, rapid implementation of containment measures, including the declaration of a state of emergency and swift application of 14-day self-isolation,

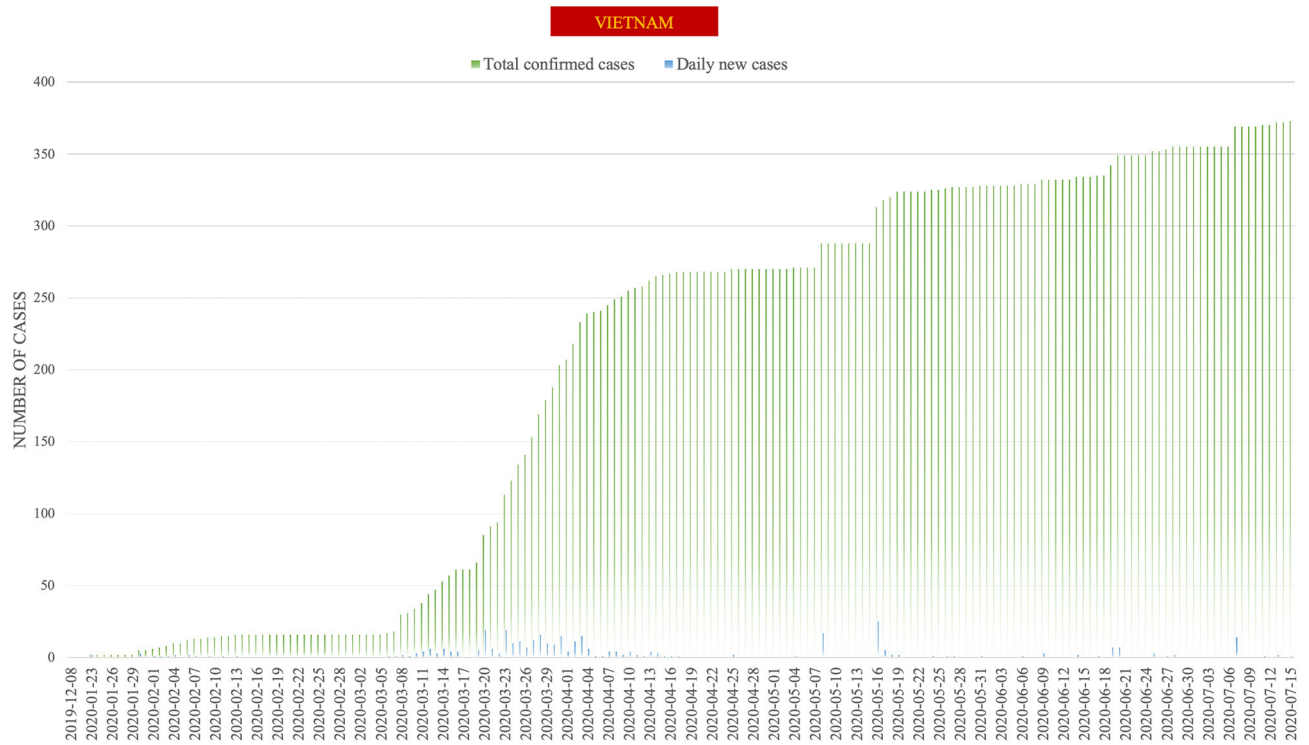

FIGURE 1 | Epidemic curve of new cases of COVID-19 in Vietnam. The total confirmed cases are presented in green and the daily new cases in blue. The using data was retrieved from the daily reports released by the World Health Organization.

coupled with the establishment of a nationwide information campaign dramatically lessened the surge of infectious cases (11). In the same manner, it took China 10 days to construct 45 makeshift hospitals in response to the burden of new confirmed cases. Early reactions from the government in association with sufficient emergency responses significantly contributed to the reopening of Wuhan, the initial epicenter of COVID-19, just after 76 days of strict restrictions (12). Furthermore, aggressive prevention with epidemiological tracing using the F0–F5 evaluation system represent an unique but effective measure to mitigate the crisis. Regarding financial assisstance, given the limited average budget of households for healthcare services as well as the insufficient coverage of the insurance, the Vietnamese government had anticipated an overwhelming burden of medical expenditures on a large scale during the outbreak. Since Vietnamese healthcare consumers are unable to afford healthcare payments equal to or higher than US$90 (VND 2 million), especially in terms of uninsured, non-married, and unemployed individuals (13), the government offered budget support packages of US$2.6 billion in which a sum of US$80 (VND 1.8 million) was distributed monthly between April and June (14). Another important implication is the consistent coordination across sectoral ministries/the government departments in dealing with an event. Right after the notice of suspected patients who returned to Vietnam by airlines, the Ministry of Transport rapidly alerted the relevant agencies about the passengers' information and the corresponding flight numbers so that quarantine could be implemented quickly and efficiently. Passengers were also given instructions from airlines regarding the current regulations on disease prevention and control in Vietnam. In parallel, the Ministry of National Defense took responsibility for the establishment of the quarantine areas and transporting all relevant people from or passing through regions hit by the epidemic and into other areas. Recognizing the risks of over-reaching the airports' capacities, the Ministry of Public Security had quickly promulgated necessary regulations to shorten the duration of immigration procedures. Side by side, the Ministry of Health and Foreign Affairs closely updated the epidemic reports from around the world and hourly reported to the Prime Minister any adequate and important findings.

On the other hand, the Government, with regular announcements on daily news of the crisis and up-to-date developments in terms of COVID-19 treatments, provided official and exact information to the public. The introduction of the website http://ncov.moh.gov.vn and the application of the NCOVI and Vietnam Health apps on mobile phones (15), which provided full information regarding preventive measures, new cluster detection, testing data, and live consultation for any inquiries related to the outbreak, sufficiently responded to the demand of citizens about the reliable sources of information. Furthermore, the dissemination of necessary information was universally enhanced by the official press and social media. The publication of ∼15,000 articles from 14 online newspapers from January 9 to April 4, coupled with the hourly COVID-19

update in the two main social media channels, Facebook (more than 57% of the population), and the local app Zalo (with 100 million users), remarkably improved the awareness of all citizens about the infectious disease (16). Besides, a Vietnamese scientist had also engaged in the global research community by publishing and sharing data related to COVID-19. High-quality publications attracted attention from numerous readers and also significantly contributed to the global database and knowledge (7, 17, 18). As a consequence, all Vietnamese people felt safe, proud, and confident in the Government. The whole country is courageous and united in the efforts to combat COVID-19. Therefore, maintaining a high level of consciousness and responsibility for citizens is crucial and effective during this critical period, as previously reported in Jordan and South Korea (19, 20).

IS IT THE RIGHT WAY, VIETNAM?

Lessons learned from responding to the Ebola outbreaks in Africa (2014–2016 and 2018–2020) emphasize the crucial role of community engagement as well as rapid response in fighting against the crisis (21). Early detection of a new infection, comprehensive contact tracing, and strict quarantine of confirmed cases are some of the backbone policies of success in African countries. Vietnam, with the unique tracing system F0–F5 classification, has also proven its effectiveness in rapidly locating the new infected cluster and therefore maximally limit the spread of the virus. Furthermore, Vietnamese citizens seem in favor of adopting lockdown strategies and be in line with the full response of the authorities. Regarding non-pharmaceutical intervention, the implementation of compulsory quarantine, school closure, and bans of public gatherings in New York City during the 1918–1919 influenza pandemic reduced the total mortality and induced greater delays in reaching peak mortality (22). These physical and lockdown restrictions, such as the closure of borders and cities, non-essential businesses, and educational facilities, social distancing and barriers, and the shift to work from home also helped to flatten the new infection curve in Australia (23). Similarly, we also witnessed the rapid application of social distancing and barriers as well as epidemiologically preventive measures in Vietnam since the first day of the outbreak. The current situation of a consecutive 3 months without a new community infection might be the best answer for the aforementioned question.

CONCLUSION

At the beginning of the pandemic, the strong public support for the response measures and a strong culture of surveillance were key points in the struggle for victory. Using strict and cross-sectoral control measures combined with social and economic support measures, the Vietnamese government gained the support of all Vietnamese citizens during the first wave of the pandemic. Various factors were involved: the evacuation flight to rescue 30 Vietnamese citizens in Wuhan, China; the free treatment and laboratory investigation of all confirmed and suspected COVID-19 cases; the warm attitude of hospital staff, high quality of surveillance, and appropriate daily regimen for quarantined people at temporary medical camps; and the stabilization of food and daily necessities. These all contributed to building the citizens' belief in the government. Following the epidemics of SARS in 2003 and H1N1 in 2009, coupled with strong support from the United States Center for Disease Control and Prevention (US CDC) and the WHO, the Vietnamese government have provided a sustainable response and executed a rapid response to manage the entirety of the COVID-19 outbreak. A serious control policies balancing with humanity in a well-organized and well-trained teams had responded adequately to the inquiries of Vietnam during this time. Vietnam can now consider declaring an end to the COVID-19 crisis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

NN: conceptualization, software, writing—original draft preparation, visualization, and investigation. B-TD: writing—original draft preparation, visualization, and investigation. NH: supervision, writing—reviewing, and editing. All authors contributed to the article and approved the submitted version.

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Coronavirus Disease-2019 Survival in Mexico: A Cohort Study on the Interaction of the Associated Factors

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The pandemic caused by the new coronavirus Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) is currently affecting more than 200 countries. The most lethal clinical presentation is respiratory insufficiency, requiring attention in intensive care units (ICU). The most susceptible people are over 60 years old with comorbidities. The health systems organization may represent a transcendental role in survival.

Objective: To analyze the correlation of sociodemographic factors, comorbidities and health system organization variables with survival in cases infected by SARS-CoV-2 during the first 7 months of the pandemic in Mexico.

Methods: The cohort study was performed in a health system public basis from March 1st to September 30th, 2020. The included subjects were positive for the SARS-CoV-2 test, and the target variable was mortality in 60 days. The risk variables studied were: age, sex, geographic distribution, comorbidities, health system, hospitalization, and access to ICU. Bivariate statistics (X^2-test), calculation of fatality rates, survival analyses and adjustment of confusing variables with Cox proportional-hazards were performed.

Results: A total of 753,090 subjects were analyzed, of which the 52% were men. There were 78,492 deaths (10.3% of general fatality and 43% inpatient). The variables associated with a higher risk of hospital mortality were age (from 60 years onwards), care in public sectors, geographic areas with higher numbers of infection and endotracheal intubation without management in the ICU.

Conclusions: The variables associated with a lower survival in cases affected by SARS-CoV-2 were age, comorbidities, and respiratory insufficiency (with endotracheal intubation without care in the ICU). Additionally, an interaction was observed between the geographic location and health sector where they were treated.

Keywords: Mexico, SARS-CoV-2, survival analysis, cohort study, comorbidity, COVID-19 outbreak

INTRODUCTION

The Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) outbreak has been declared as a pandemic by the World Health Organization. The first case of this disease in Mexico was reported on April 28th, 2020. Until April 1, 2021, 131,435,555 cases of Coronavirus Disease-2019 (COVID-19) were registered worldwide, with a global fatality rate of 2.17% (1). At that time, 2,443,755 cases and 204,147 deaths were confirmed in Mexico, with a fatality rate of 9% (2). The disease is highly contagious, and although fatality remains low, there is a constant increase in the number of new cases in Mexico with a higher fatality rate than that observed globally (1).

Several changes in some epidemiological indicators have been observed worldwide: according to the initial findings in China, it was indicated that older adults presented more severe symptoms. In Europe, the population over 60 years old was the most affected, which coincide with being the community with the highest life expectancy in the world (3–6). In Latin-America, COVID-19 is also frequently observed in the population under 60 years of age, whereas in Brazil, 47% of cases occur between 20 and 59 years of age, and this was associated with the presence of comorbidities such as obesity, diabetes and hypertension, which are frequent at early ages (6–9). There is no specific treatment so far; a decrease in SARS-CoV-2 fatality cases was achieved with an appropriate care, including prompt hospitalization, mechanical ventilation, and attention in an intensive care unit (10, 11).

The hospital reconversion in Mexico and other countries has enabled an increase in resources (more hospitals, ventilators, and intensive care units (ICU) for patients with acute and severe COVID-19. According to daily reports in Mexico, there was an increase in the number of ICU beds or "beds with ventilators" from 2,446 to 11,346 over a 10-month period (12). However, the changes in fatality rates were not substantial (12). A hypothesis derived from early assessments showed that comorbidities in the Mexican population have a negative impact on survival, especially in cases of diabetes, arterial hypertension, and obesity (13, 14). In addition, there are other related conditions to death, such as the hospital's number and the health services quality that are variously distributed in the country. These variables must be weighted to identify the riskiest conditions. The experience in the results presented in Mexico may be helpful for other countries with similar social, economic and health system conditions, for a better chance in their public health strategies.

The aim of this study was to analyze the survival of confirmed cases with SARS-CoV-2 in the first 7 months of the national pandemic, assessing the impact of different factors as age, sex, comorbidities, healthcare system organization, medical unit geographic location, modality of care received, and access to ICU.

Abbreviations: CDMX, City of Mexico; COVID-19, Coronavirus Disease-2019; EDOMEX, State of Mexico; ICU, intensive care unit; IMSS, Instituto Mexicano de Seguridad Social; IMV, invasive mechanical ventilation; ISSSTE, Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado; PEMEX, Petroleos de México; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus-2; SEDENA, Secretaría de la Defensa Nacional; SEMAR, Secretaría de la Marina; SS, Secretaría de Salud.

MATERIALS AND METHODS

The data used was from the open database of the Viral Respiratory Disease Epidemiological Surveillance Systems published daily by the Ministry of Health of Mexico (2). Among the variables available in the databases, the type of institution of the National Health System that provided care, federative entity where the medical unit was located, type of care (ambulatory, hospitalized), date of admission, gender, age, place of residence, date of symptoms onset, intubated or inpatient in ICU, presence of comorbidities, smoking history, pregnancy, and date of death if occurred were included. The confirmation of SARS-CoV-2 infection was performed by real-time polymerase chain reaction technique in certified laboratories by the National Institute of Epidemiological Reference.

A cohort of patients with positive SARS-CoV-2 test result was integrated from February 28th to September 30th, 2020. The day 0 of every patient was considered as the disease confirmation date and tracking was done till day 60 or the date death. The exposition variables were age (categorized in groups: <2, 2.1–5, 6–10, 11–20, 21–30, 31–40, 41–50, 51–60, 61–70, 71–80, and >80 years), sex, federative entity (32 States of Mexico). The following comorbidities were registered: obesity, diabetes, arterial hypertension, asthma or chronic obstructive pulmonary disease (COPD), immunosuppression and other risk factors such as smoking and pregnancy. Main health institutions of the country were analyzed: private institutions, Secretaría de Salud (SS), Instituto Mexicano de Seguridad Social (IMSS), Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado (ISSSTE), Petroleos de México (PEMEX), Secretaría de la Marina (SEMAR), Secretaría de la Defensa Nacional (SEDENA), State Hospitals, and the group of "other public" that assist <2% of the population. The outcome variables also included were: hospitalization, pneumonia, invasive mechanical ventilation (IMV), and admission to ICU. The main outcome variable was survival till 60 days.

Statistical Analysis

Descriptive statistical analyses of the variables of interest were performed by calculating the relative frequencies, and independent hypothesis tests (X^2) were used to assess correlations between qualitative variables. The softened risk density in patients hospitalized vs. those in ambulatory care was analyzed using Nelson–Aalen estimation (**Supplementary Material**). The analyses of survival were performed using Kaplan–Meier method with the log rank test. Finally, different models of Cox regression were constructed to evaluate the effect of the different clinical, demographic, and socio-economic factors on the period of death. The criteria for introducing the model were p-value < 0.05 or biological plausibility. Finally, three different models were built: the total population, hospitalized patients, and the ICU. Internal validation of each model was performed by calculating each patient's probability in the cohort with the formula, $\lambda(t) = \lambda_0(t)\exp(\beta^T X)$, and estimating the value of the area under the curve (AUC) with the death variable (15). The entire analysis was performed using the Stata 16.1 version.

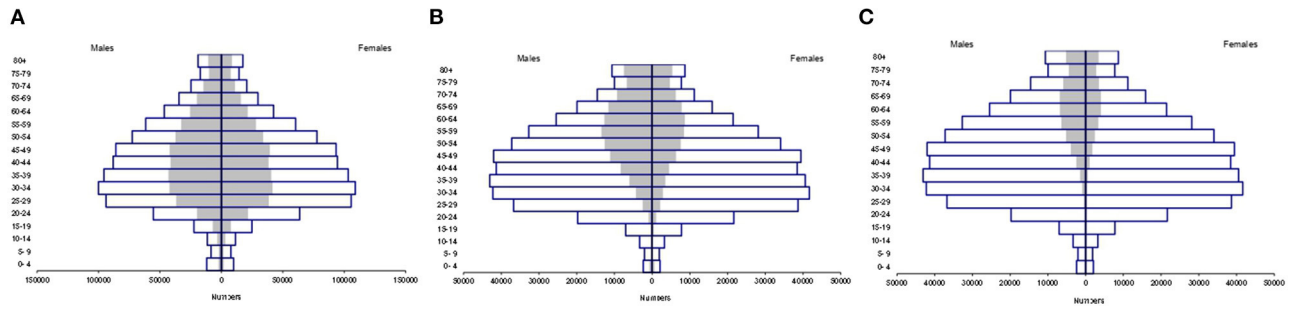

FIGURE 1 | Distribution according to age group and sex, in Mexico. **(A)** Distribution of suspected cases (white bar) and positive cases (gray bar) according to age group and sex, in Mexico. **(B)** Distribution of positive cases (white bar) and hospitalized cases (gray bar) according to age group and sex, in Mexico. **(C)** Distribution of patients who died from SARS Cov-2 by age group and sex in Mexico. Positive cases (white bar) and deaths (gray bar).

RESULTS

A total of 1,735,597 observations were included in the original basis, of which were eliminated for analysis 893,324 (51.5%) who corresponded to patients with negative SARS-CoV-2 test, and 89,186 (5.1%) individuals whose results were not available.

Analysis was made with 753,090 patients with positive SARS-CoV-2 test, 52% were males, and >55% of the population was grouped between 20 and 59 years old (**Figure 1A**). Male sex was predominant in all age groups requiring hospitalization. Both men (70.2%) and women (61.5%) aged >80 years required a higher proportion of hospitalizations. The lowest hospitalization proportion was observed in younger people (15–24 years old group), **Figure 1B**.

A total of 78,534 deaths were registered. The lowest fatality rate was observed in people <18 years of age (0.86%), followed in ascending order by patients of age groups 19–40 (7.03%), 40–60 (35.9%), and >60 (56.1%), the male population being the most predominant (**Figure 1C**).

Patients <18 years of age had a lower percentage of comorbidities, and 14% of infected children had one comorbidity. The comorbidities observed in the descending order of frequency were obesity (3.7%), asthma (3.7%), immunosuppression (2.9%), diabetes mellitus (0.7%), arterial hypertension (0.7%), and chronic renal failure (0.7%). The greater comorbidities frequencies were observed in children of 11–18 years old; obesity (6%), asthma (4%), and immunosuppression (1.5%) were highlighted. It must be noted that 4.7% of infected people <18 years of age were obese and represented 0.8% of the total obese population infected with SARS-CoV-2 (**Table 1**).

The principal comorbidities observed among people aged ≥19 years included arterial hypertension (20%) and obesity (19%) followed by diabetes mellitus (16%), asthma (2.6%), chronic renal failure (1.9%), and immunosuppression (1%) in the descending order of frequency. The largest number of patients with some comorbidity is grouped between 19 to 60 years of age (**Table 1**).

A significant statistical difference was observed between the proportions of comorbidities in the group of non-surviving and surviving patients (**Table 2**). Higher fatality rates could be found in the age group of >60 years. Further, the high frequency of comorbidities in the age group of 40–60 years is of concern.

According to geographical distribution (**Table 3**), a large number of positive cases and deaths were concentrated in Mexico City (CDMX) and Mexico State (EDOMEX), which are states with the largest population in our country. The national case fatality rate was 10.4%, whereas that in CDMX and EDOMEX was 7.98 and 16.2%, respectively.

Some entities with lower population density showed greater case fatality rates, such as the States of Morelos, Baja California, Sinaloa, Chiapas, and Hidalgo. In other states, population density was not associated with case fatality rate.

23.8% of positive SARS-Cov-2 patients required hospitalization of which 17.5% received endotracheal intubation and 8.6% were admitted in an ICU. The proportion of patients who required endotracheal intubation and who could be attended to in an ICU was variable in different states; however, the case fatality rate was higher in those states with a higher rate of intubated patients with lower admissions in ICU than others.

In study period 23.8% (179,288) of the patients with SARS-CoV-2 infection were hospitalized (**Table 4**); among these, 76.8% had pneumonia as the main diagnosis, 20.1% required intubation and 8.6% was inpatient in an ICU. Nationally, 89.3% of the cases were attended in three institutions: IMSS (48.1%), SS (33.6%) and ISSSTE (7.6%). The institutional case fatality rate (CFR) was 43.7%. Among the patients admitted to IMSS, 30% had pneumonia, 10.9% who were intubated required IMV, and 2% were attended to in an ICU. Among patients admitted at SS, 30.8% had pneumonia, 6.2% required IMV, and 4.8% were treated in an ICU. Private hospitals attended to 2.1% of hospitalized patients with COVID-19. Among these, 22% required IMV and 27% were admitted in an ICU. The CFRs for IMSS, SS, and private hospitals were 50.7, 39.2, and 19.4%, respectively.

Other's health system hospitals where ICU attention was greater were SEDENA (29.9%), private hospitals (27.2%), and SEMAR (24.1%). These units had lower CFR, 14.9, 4.3, and 7.1%, respectively.

In non-hospitalized patients, the survival at 60 days was 95.4%; in contrast survival in hospitalized patients was 75%, with an survival average of 11 days ($p < 0.05$) (**Figure 2A**).

TABLE 1 | Comparison of presentation of comorbidities according to age in patients with SARS Cov-2.

Years	Diabetes			COPD			Asthma			Immunosupression			Hypertension			Cardiovascular Disease			Obesity			Chronic Renal Disease			Tabaquism		
	n	% Total	% Group	n	% Total	% Group	n	% Total	% Group	n	% Total	% Group	n	% Total	% Group	n	% Total	% Group	n	% Total	% Group	n	% Total	% Group	n	% Total	% Group
<2	75	0.10%	0.90%	18	0.10%	0.20%	109	0.40%	1.20%	270	2.00%	3.10%	101	0.10%	1.20%	298	1.40%	3.40%	147	0.10%	1.70%	43	0.20%	0.50%			
2-5	11	0.00%	0.20%	4	0.00%	0.10%	187	0.60%	3.90%	227	1.60%	4.70%	17	0.00%	0.40%	56	0.30%	1.20%	45	0.00%	0.90%	18	0.10%	0.40%			
6-10	39	0.00%	0.40%	11	0.10%	0.10%	376	1.20%	4.30%	303	2.20%	3.40%	35	0.00%	0.40%	68	0.30%	0.80%	228	0.10%	2.60%	54	0.30%	0.60%			
11-20	366	0.30%	0.90%	45	0.30%	0.10%	1643	5.30%	4.00%	711	5.10%	1.80%	360	0.20%	0.90%	283	1.30%	0.70%	2428	1.40%	6.00%	316	1.60%	0.80%	1579	1.90%	3.90%
21-30	2915	2.10%	1.50%	337	2.20%	0.20%	6515	21.10%	3.40%	1345	9.70%	0.70%	4957	2.80%	2.60%	1088	5.10%	0.60%	22644	13.10%	11.70%	1544	7.90%	0.80%	18169	21.80%	9.40%
31-40	10889	7.90%	4.40%	677	4.40%	0.30%	7637	24.80%	3.10%	2029	14.70%	0.80%	15475	8.70%	6.30%	1898	8.80%	0.80%	41038	23.80%	16.70%	2273	11.60%	0.90%	22359	26.80%	9.10%
41-50	27922	20.30%	12.50%	1511	9.90%	0.70%	6758	21.90%	3.00%	2550	18.40%	1.10%	35098	19.70%	15.80%	3012	14.00%	1.40%	44269	25.60%	19.90%	3008	15.40%	1.40%	16707	20.00%	7.50%
51-60	39374	28.60%	24.00%	2651	17.30%	1.60%	4104	13.30%	2.50%	2587	18.70%	1.60%	47397	26.60%	28.90%	4089	19.00%	2.50%	33903	19.60%	20.60%	4324	22.10%	2.60%	11643	14.00%	7.10%
61-71	32597	23.60%	35.20%	3740	24.50%	4.00%	2107	6.80%	2.30%	2105	15.20%	2.30%	39651	22.20%	42.90%	4486	20.90%	4.90%	18075	10.50%	19.60%	4335	22.10%	4.70%	6980	8.40%	7.60%
71-80	17397	12.60%	37.10%	3657	23.90%	7.80%	990	3.20%	2.10%	1181	8.50%	2.50%	24353	13.60%	52.00%	3917	18.20%	8.40%	7761	4.50%	16.60%	2530	12.90%	5.40%	4012	4.80%	8.60%
>80	6114	4.40%	29.30%	2636	17.20%	12.60%	411	1.30%	2.00%	518	3.70%	2.50%	10969	6.10%	52.60%	2318	10.80%	11.10%	2283	1.30%	10.90%	1127	5.80%	5.40%	1764	2.10%	8.60%

COPD, Chronic Obstructive Pulmonary Disease.

In addition, in hospitalized patients, there was a direct association between age and survival ($p < 0.05$) (**Figure 2B**). Patient survival was higher (average: 33 days) among those hospitalized in private than in public institutions. Lower survival (average: 10 days) was observed in patients admitted to IMSS (**Figure 2C**).

On comparing the probability of survival based on the number of comorbidities, patients with three or more comorbidities were found to have a lower probability of survival (**Figure 2D**).

The adjustment of the effect of the variables with mortality was performed using three Cox proportional models (**Table 5**). The individual probability of outcome presentation by each subject according to each model was calculated. In the first prognostic model, the mortality of all subjects was explained, including all variables with a p-value < 0.05 (sex, age group, health service, comorbidities, the need for hospitalization, and pneumonia), resulting in 10 associated variables, two related to the health system (private practice with protective effect), five comorbidities and their sum, pneumonia development, and need for hospitalization. The summed probability of the model showed an area under the curve (AUC) of 0.86.

The second model variables (sex, age >60 years, type of health service, comorbidities, ICU, and IMV) were adjusted in hospitalized patients and demonstrated the association of 12 variables, which differed from model 1 and additionally included the following: age >60 years, male, asthma, smoking, and pregnancy. In terms of independent care in the ICU and AMV, the variables demonstrated an association with the risk of death; however, the combination of both offered a protective effect. The AUC value was determined as 0.78.

The last model was carried out in ICU patients, demonstrating a primary association between the type of health services and comorbidities. The AUC value was observed to be 0.65.

DISCUSSION

We have provided an analysis of the first 7 months from the start of the COVID-19 pandemic in Mexico. CFR has been considered higher for Mexico compared with many other countries. However, during the first 3 months of the pandemic, sampling of the people suspected to have SARS-Cov-2 virus was limited; "sentinel monitoring" was used (only one in every 10 suspected ambulatory cases and all hospitalized cases were sampled). Therefore, CFR denominator was underestimated (diagnosis confirmation bias). The number of samples gradually increased and included all clinically suspected patients. Only 14 tests per 1,000 people were carried out in Mexico, which was in contrast to that observed in a study carried out in Chile, where 130 PCR tests were carried out per 1,000 people, obtaining a fatality rate of 4.16% (16).

Considering the COVID-19 registered deaths around the world, Mexico is placed as the 3th country of the world with major number of deaths, behind the United States and Brazil

TABLE 2 | Differences between survivors and non-survivors with SARS CoV-2.

Variables	Survival		No survival		*p*-value
	n	%	*n*	%	
Sex					
Male	340,076	50.40%	50,340	64.10%	<0.0001
Female	334,244	49.60%	28,152	35.90%	
Group Age					
<2 years	2,383	0.40%	121	0.20%	<0.0001
2.1–5.9	2,557	0.40%	33	0.00%	
6–9.9	4,255	0.60%	39	0.00%	
10–18.9	15,628	2.30%	102	0.10%	
19– <40	284,456	42.20%	4,337	5.50%	
40– <50	151,988	22.50%	9,252	11.80%	
50– <60	114,663	17.00%	17,446	22.20%	
60– <70	60,976	9.00%	21,766	27.70%	
70–79.9	26,723	4.00%	16,741	21.30%	
80+	10,691	1.60%	8,655	11.00%	
Type					
Ambulatory	564,547	83.70%	8,977	11.40%	<0.0001
Hospitalization	109,773	16.30%	69,515	88.60%	
Comorbidities					
Diabetes	85,614	12.70%	30,096	38.30%	<0.0001
COPD	7,085	1.10%	3,813	4.90%	<0.0001
Asthma	17,889	2.70%	1,578	2.00%	<0.0001
Immunocompression	6,159	0.90%	1,935	2.50%	<0.0001
Hypertension	109,956	16.30%	35,221	44.90%	<0.0001
Cardiovascular disease	10,572	1.60%	4,175	5.30%	<0.0001
Obesity	116,071	17.20%	19,182	24.40%	<0.0001
Renal chronic disease	8,635	1.30%	5,529	7.00%	<0.0001
Smoking	48,622	7.20%	6,264	8.00%	<0.0001
Outcomes					
Pneumonia	79,584	11.80%	58,078	74.00%	<0.0001
Invasive mechanical ventilation	5,898	0.90%	25,539	32.50%	<0.0001
Intensive care unit	7,514	1.10%	7,890	10.10%	<0.0001

The value of p was calculated by X^2 test.

(1). Age has been considered one of the most outstanding death risk factors in most countries of the world. The percentage of deaths in the first wave in people over 60 years of age in Italy and China were 96.5 and 81%, respectively. A total of 98% of the deceased were older than 50 years in England, 97.5% were older than 45 years in USA, and 56.2% were over 60 years of age in Mexico (5, 17, 18). It is of interest that 19.5% of the deceased individuals in Mexico were between 21 and 50 years of age, which corresponds to a young and economically active population. In Mexico, a mandatory lockdown was not imposed, and Mexican population with low and middle income, has no savings capacity; hence, people had the necessity to work, this factor could influence on the higher disease incidence and higher mortality rates observed in young people with risk factors for developing a serious disease (13, 14).

In our country infected patients aged < 20 years represented 3.1% of the total infected population; the CFR was 0.12%, which was similar to that observed in Spain, Italy, Germany, China, and South Korea (19, 20). The number of infected children in Mexico has been strikingly higher than that in countries. Little is known about COVID-19 and comorbidities in children (21). In the infected Mexican population aged <18 years, the percentage of comorbidities was low (14%); obesity (4.7%) and diabetes mellitus (0.8%) were not factors with a high incidence, which was in contrast to that observed in the adult population. Comorbidities were most frequent in children aged 11–18 years. Most of the Mexican children did not have any comorbidity. A Saudi Arabian cohort, which included population aged <1 and >5 years, was observed to have a high rate of hospitalization, with a low percentage of comorbidities (22).

TABLE 3 | Hospitalization, ICU care, endotracheal intubation, and fatality by State.

	Positive SARS CoV-2	Hospitalizated	Endotracheal Intubation	%	ICU	%	Death	% by State	% total	Fatality rate (x100)
Ags	7429	2008	392	19.52	72	3.59	663	33.02	0.37	8.92
B.C.N	19421	6512	1365	20.96	243	3.73	3552	54.55	1.98	18.29
B.C.S	10393	1197	287	23.98	171	14.29	478	39.93	0.27	4.60
Camp	6116	1854	256	13.81	243	13.11	829	44.71	0.46	13.55
Coah	26800	3910	591	15.12	138	3.53	1911	48.87	1.07	7.13
Col	5152	1486	354	23.82	196	13.19	579	38.96	0.32	11.24
Chis	6233	2249	577	25.66	246	10.94	1020	45.35	0.57	16.36
Chih	11449	3344	715	21.38	779	23.30	1397	41.78	0.78	12.20
Cd.Mx	153255	28134	6969	24.77	2576	9.16	12224	43.45	6.82	7.98
Dgo	9133	1322	258	19.52	193	14.60	639	48.34	0.36	7.00
Gto	41366	6313	793	12.56	499	7.90	2953	46.78	1.65	7.14
Gro	18962	3989	893	22.39	621	15.57	1899	47.61	1.06	10.01
Hgo	12517	4430	499	11.26	281	6.34	1998	45.10	1.11	15.96
Jal	27467	8095	1314	16.23	875	10.81	3332	41.16	1.86	12.13
Edo Mex	58490	25265	3916	15.50	990	3.92	9496	37.59	5.30	16.24
Mich	20677	3866	575	14.87	244	6.31	1653	42.76	0.92	7.99
Mor	5665	2543	329	12.94	105	4.13	1102	43.33	0.61	19.45
Nay	5948	1933	259	13.40	208	10.76	745	38.54	0.42	12.53
N.L	41172	8249	1069	12.96	902	10.93	3148	38.16	1.76	7.65
Oax	16999	3340	706	21.14	436	13.05	1421	42.54	0.79	8.36
Pue	32398	9237	1271	13.76	779	8.43	4115	44.55	2.30	12.70
Que	9546	2781	643	23.12	150	5.39	999	35.92	0.56	10.47
Q.Roo	11888	3423	920	26.88	391	11.42	1659	48.47	0.93	13.96
S.L.P	23407	3444	421	12.22	323	9.38	1712	49.71	0.95	7.31
Sin	18904	6473	1201	18.55	877	13.55	3218	49.71	1.79	17.02
Son	24874	5866	979	16.69	290	4.94	2914	49.68	1.63	11.72
Tab	32341	4675	515	11.02	417	8.92	2838	60.71	1.58	8.78
Tam	29084	4478	490	10.94	225	5.02	2281	50.94	1.27	7.84
Tlax	6584	1920	380	19.79	552	28.75	1029	53.59	0.57	15.63
Ver	33005	11239	1676	14.91	865	7.70	4333	38.55	2.42	13.13
Yuc	18630	4015	560	13.95	234	5.83	1633	40.67	0.91	8.77
Zac	7507	1698	264	15.55	283	16.67	722	42.52	0.40	9.62
Total	752812	179288	31437	17.53	15404	8.59	78492	43.78		10.43

Ags, Aguascalientes; B.C.N, Baja California Norte; B.C.S, Baja California Sur; Camp, Campeche; Coah, Coahuila; Col, Colima; Chis, Chiapas; Chih, Chihuahua; Cd.Mx, Ciudad de México; Dgo, Durango; Gto, Guanajuato; Hgo, Hidalgo; Jal, Jalisco; Edo Mex, Estado de México; Mich, Michoacán; Mor, Morelos; Nay, Nayarit; N.L, Nuevo León; Oax, Oaxaca; Pue, Puebla; Que, Querétaro; Q.Roo, Quintana Roo; S.L.P, San Luis Potosí; Sin, Sinaloa; Son, Sonora; Tab, Tabasco; Tam, Tamaulipas; Tlax, Tlaxcala; Ver, Veracruz; Yuc, Yucatán; Zac, Zacatecas.

TABLE 4 | Outcomes according to the Health System.

	Total SARS CoV-2	Hospitalizated			Pneumonia			Endotracheal Intubation			UCI			Death		
	n	n	% Total*	% Institution	Pneumonia	% Total**	% Institution	n	% Total**	% Institution	UCI	% Total**	% Institution	Death	% Total**	% Institution
S.S.A	420477	60251	33.6	14.3	55275	30.83	13.15	11148	6.22	18.50	8643	4.82	14.34	23670	13.20	39.29
I.M.S.S	237731	86265	48.1	36.3	53771	29.99	22.62	19431	10.84	22.52	1771	0.99	2.05	43809	24.43	50.78
I.S.S.S.T.E	32304	13581	7.6	42.0	11553	6.44	35.76	2239	1.25	16.49	1446	0.81	10.65	5251	2.93	38.66
PEMEX	7687	3686	2.1	48.0	3781	2.11	49.19	415	0.23	11.26	482	0.27	13.08	1204	0.67	32.66
SEDENA	5022	2799	1.6	55.7	1434	0.80	28.55	399	0.22	14.26	837	0.47	29.90	750	0.42	26.80
SEMAR	5051	1383	0.8	27.4	1163	0.65	23.03	370	0.21	26.75	334	0.19	24.15	354	0.20	25.60
Estatal	13999	3815	2.1	27.3	4375	2.44	31.25	726	0.40	19.03	380	0.21	9.96	1615	0.90	42.33
Private medicine	23071	5209	2.9	22.6	4746	2.65	20.57	1156	0.64	22.19	1419	0.79	27.24	1014	0.57	19.47
other	7470	2299	1.3	30.8	1564	0.87	20.94	203	0.11	8.83	92	0.05	4.00	825	0.46	35.89
total	752812	179288	23.81577		137662	76.78		36087	20.13		15404	8.59		78492	43.78	

*n/hospitalizated.

**n/hospitalizated by institution.

SS, Secretaría de Salud; IMSS, Instituto Mexicano del Seguro Social; ISSSTE, Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado; SEDENA, Secretaría de la Defensa Nacional; SEMAR, Secretaría de Marina.

There was a relationship in mortality between the economic States conditions and the health services distribution; for example, difference in the fatality rate was twice higher in the Edo Mex than in the Cd Mx despite that Edo Mex population is twice as high (16,992,418 vs. 9,209,944 people) (23), but the gross domestic product is 45% lower in Edo Mex, a condition reflected in the provision of health services; in Cd Mx the number of third level hospitals is 3.5 higher (56 vs. 16 hospital centers) (24). The impact of sociodemographic conditions on mortality in Brazil was evaluated by Braga-Ribeiro et al., who found an association between less education, more household crowding, lower income, and a higher population concentration in subnormal areas; mortality was found to be four times higher in a population with a lower degree of education compared with that having a higher degree of education, showing that socio-economic inequity impacts fatality during this pandemic (25).

Nation-wide, the proportion of confirmed cases that required hospitalization was 23.8%, 17.5% were intubated and 8.6% were inpatient in an ICU and 43.8% of hospitalized patients died with a CFR of 10.4%. A total of 50% of patients requiring endotracheal intubation received management outside an ICU; fatality was higher in such individuals than in than those who received attention in an ICU. There was variation according to every state, for example, in Baja California, 33% of the confirmed cases were hospitalized and 20.9% of these were intubated, but only 3.7% were admitted in an ICU. In this state, the CFR was 18.3%. In contrast Chihuahua, where 29% of the cases were hospitalized, 23.3% were admitted to an ICU, and 21.4% received attention outside an ICU, the CFR was 12.2%. In the analysis by Health Institutions, the differences between the two institutions that serve the largest proportion of the national population stand out; 10% of admitted patients required endotracheal intubation and 2% were treated in an ICU in the IMSS, which had a fatality rate of 19%, whereas 15% of admitted patients required endotracheal intubation and 14% were treated in an ICU at the SSA, which had a 6% fatality rate. Despite the fact that with health system policies there was an increase in the number of hospital beds and ventilators, the fatality rate in critically ill patients requiring endotracheal intubation was high, as observed in a previous study. This suggests that the quality of care was inadequate due to the lack of expertise of the medical and paramedical groups in ventilatory management and critical care medicine; however, expertise has improved as the pandemic progresses. Having only a sufficient number of beds with ventilators does not ensure optimal care or a better prognosis for patients with acute respiratory distress syndrome due to SARS-CoV-2. In this analysis, critical patients cared in ICU were not observed to be at higher risk of death vs. those who were intubated outside of an ICU, revealing infrastructure and specialized staff importance in care of such patients.

In this study due to the database characteristics, the period of disease progression and clinical conditions at the time of requesting medical care cannot be correlated, as well as the time interval between endotracheal intubation and displacement to ICU. In two studies in Mexico on patients receiving ICU care, the average time between the onset of symptoms and the inpatient

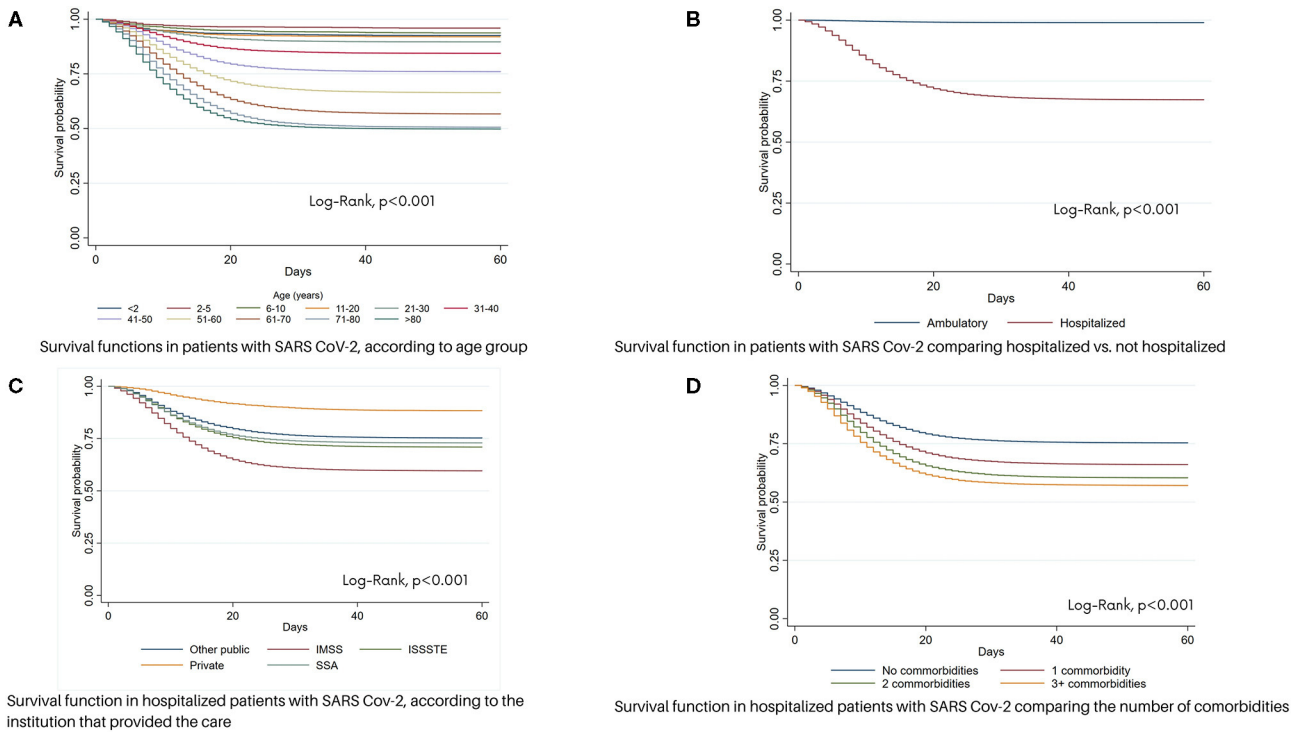

FIGURE 2 | Survival function in patients with SARS Cov-2 in México. **(A)** Survival functions in patients with SARS Cov-2, according to age group. **(B)** Survival functions in patients with SARS Cov-2 comparing hospitalized vs. non- hospitalized. **(C)** Survival function in hospitalized patients with SARS Cov-2, according to the institution that provided the care. **(D)** Survival function in hospitalized patients with SARS Cov-2 comparing the number of comorbidities.

hospitalization in ICU was 7 days (interquartile range 4.5–9), the mean of days from the presentation of symptoms to admission was 4.3 ± 3.4 days; from the admission to death was 5.9 ± 4.9 days; and from the presentation of symptoms to death was 10.1 ± 5.5 days (12, 13). There were 4.6% of non-hospitalized patients who died, which probably reflects health system problems during hospital service saturation and the low capacity of patients to recognize the severity of their condition and visit the hospital in a timely manner. The information collected however, was insufficient to delve into this topic.

The national database has been analyzed in other publications (13, 26). The three prognostic models presented in this work analyze the effect that risk variables have in different scenarios, particularly in patients in ICU; the analysis of Ñamendys Silva is consistent (12), who analyzed the decision made by the health authorities in Mexico during the second wave of infections, in December 2020, when the number of beds with a ventilator increased by 4.7 times (from 2,446 to 11,634) in hospital areas not equipped with intensive care, consequently, mortality was 12% higher, probably explained by human resources and the equipment had a lower quality of care. A recently published analysis in the pediatric population demonstrated the relationship among age, the clinical presentation with intubation, and the need for intubation as the variables associated with mortality (27).

Some limitations of this study include the following: (a) As each hospital center feeds the database and the results are issued as cases are added, reports on the outcomes are subject to the data being updated. Hence, it is possible that the population at cut-time may be underrated (follow-up bias); (b) Variables that assess the presence of comorbidities were obtained from questioning, and because the operational variables were not defined, a bias is highly probable (misclassification bias); (c) As hospitals followed a conversion strategy by using certain hospital areas as intensive care units, it is possible that the patients treated at this sites have been registered as intubated without ICU (registration bias); and (d) the construction of predictive models, using the PRISMA recommendations, shows that the variables analyzed could be insufficient to explain mortality fully. It is necessary to consider that, in the context of hospitalized patients, multiple variables that could be associated are involved (reporting bias).

CONCLUSION

In Mexico, mortality from SARS-CoV-2 was found to be associated with age and a history of comorbidities. The provision of services in the public sector is associated with mortality due to the relationship between IMV and access to intensive care areas.

TABLE 5 | Proportional Cox Regression for prognostic mortality in patients with SARS CoV-2.

(A) Prognostic variables associated in all population					
	β	**HR**	**95% CI**		***p*-value**
Social Security	1.221	3.391	3.304	3.481	0.0001
Private Hospital	−0.221	0.802	0.721	0.893	0.001
Diabetes	0.654	1.924	1.823	2.031	0.001
COPD	0.424	1.528	1.426	1.636	0.001
Hypertension	0.736	2.088	1.977	2.206	0.001
Immunossupresion	0.117	1.124	1.034	1.221	0.02
Chronic Renal Disease	0.26	1.297	1.216	1.383	0.002
Comorbidities (number)	0.29	1.336	1.276	1.4	0.0001
Hospitalization	2.83	17	16.25	17.78	0.0001
Pneumonia	1.124	3.07	2.96	3.17	0.0001
AUC = 0.86					
(B) Prognostic variables associated in patients hospitalizated					
Age>60 years	0.586	1.796	1.22	2.644	0.003
Male	0.151	1.163	1.15	1.176	0.0001
Social Security	0.156	2.304	2.176	2.44	0.02
Private Hospital	−1.382	0.251	0.204	0.309	0.0001
Health minstery	0.285	1.33	1.212	1.46	0.0001
Asthma	0.107	1.113	1.034	1.198	0.004
Tabaquism	0.157	1.17	1.097	1.248	0.0001
Pregnancy	1.351	3.86	3.075	4.846	0.0001
ICU	0.054	1.055	1.009	1.103	0.018
Advanced mechanical ventilation	0.724	2.063	2.038	2.089	0.0001
ICU and AMV	−0.142	0.868	0.829	0.908	0.0001
Comorbidities (number)	0.123	1.2	1.1	1.3	0.0001
AUC = 0.78					
(C) Prognostic variables associated in patients in ICU					
Male	0.075	1.078	1.042	1.114	0.001
Age 60 years	1.942	6.974	6.041	8.051	0.0001
Social Security	0.988	2.686	1.058	6.819	0.0001
Private	−1.451	0.234	0.117	0.469	0.0001
Diabetes	0.091	1.095	1.059	1.133	0.0001
Immunossupresion	0.108	1.114	1.023	1.214	0.0001
Obesity	0.054	1.055	1.018	1.093	0.0001
Chronic Renal Disease	0.171	1.186	1.108	1.269	0.0001
Comorbidities (number)	0.233	1.3	1.2	1.4	0.0001

AUC = 0.65; β, beta coefficient; CI, Confidence Interval; HR, Hazard Ratio.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent was not provided because Research subjects were obtained from the public database of the Secretariat of Health of Mexico. Access to the identity of the research subjects is not possible.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. Analysis: HM-G, AR-L, MK-K, and FS-S. Manuscript writing: HM-G, JFM-G, MK-K, RJ-J, JG-E, and FS-S.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.660114/full#supplementary-material

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CORRECTION
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Corrigendum: Coronavirus Disease-2019 Survival in Mexico: A Cohort Study on the Interaction of the Associated Factors

*Horacio Márquez-González[1], Jorge F. Méndez-Galván[2], Alfonso Reyes-López[3], Miguel Klünder-Klünder[4], Rodolfo Jiménez-Juárez[5], Juan Garduño-Espinosa[6] and Fortino Solórzano-Santos[7]**

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Keywords: Mexico, SARS-CoV-2, survival analysis, cohort study, comorbidity, COVID-19 outbreak

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A Corrigendum on

Coronavirus Disease-2019 Survival in Mexico: A Cohort Study on the Interaction of the Associated Factors

by Márquez-González, H., Méndez-Galván, J. F., Reyes-López, A., Klünder-Klünder, M., Jiménez-Juárez, R., Garduño-Espinosa, J., and Solórzano-Santos, F. (2021). Front. Public Health 9:660114. doi: 10.3389/fpubh.2021.660114

In the original article, there were some errors. "Comorbidities" was misspelled. All instances of the incorrect spelling should be corrected to read "comorbidities".

The acronym for chronic obstructive pulmonary disease was incorrectly spelled. This should be COPD.

In the original articles, there were some mistakes in **Table 2**.

The section "Taquism" should be named "Smoking". The row "Taquism" in the "Outcomes" section was deleted. "Immunossupression" should read "Immunocompression".

The corrected table appears below.

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

TABLE 2 | Differences between survivors and non-survivors with SARS CoV-2.

Variables	Survival		No survival		*p*-value
	n	%	*n*	%	
Sex					
Male	340,076	50.40%	50,340	64.10%	<0.0001
Female	334,244	49.60%	28,152	35.90%	
Group Age					
<2 years	2,383	0.40%	121	0.20%	<0.0001
2.1–5.9	2,557	0.40%	33	0.00%	
6–9.9	4,255	0.60%	39	0.00%	
10–18.9	15,628	2.30%	102	0.10%	
19– <40	284,456	42.20%	4,337	5.50%	
40– <50	151,988	22.50%	9,252	11.80%	
50– <60	114,663	17.00%	17,446	22.20%	
60– <70	60,976	9.00%	21,766	27.70%	
70–79.9	26,723	4.00%	16,741	21.30%	
80+	10,691	1.60%	8,655	11.00%	
Type					
Ambulatory	564,547	83.70%	8,977	11.40%	<0.0001
Hospitalization	109,773	16.30%	69,515	88.60%	
Comorbidities					
Diabetes	85,614	12.70%	30,096	38.30%	<0.0001
COPD	7,085	1.10%	3,813	4.90%	<0.0001
Asthma	17,889	2.70%	1,578	2.00%	<0.0001
Immunocompression	6,159	0.90%	1,935	2.50%	<0.0001
Hypertension	109,956	16.30%	35,221	44.90%	<0.0001
Cardiovascular disease	10,572	1.60%	4,175	5.30%	<0.0001
Obesity	116,071	17.20%	19,182	24.40%	<0.0001
Renal chronic disease	8,635	1.30%	5,529	7.00%	<0.0001
Smoking	48,622	7.20%	6,264	8.00%	<0.0001
Outcomes					
Pneumonia	79,584	11.80%	58,078	74.00%	<0.0001
Invasive mechanical ventilation	5,898	0.90%	25,539	32.50%	<0.0001
Intensive care unit	7,514	1.10%	7,890	10.10%	<0.0001

The value of p was calculated by X^2 test.

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Insights Into Excess Mortality During the First Months of the COVID-19 Pandemic From a Rural, Demographic Surveillance Site in Bangladesh

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Background: Coronavirus disease 2019 (COVID-19) has spread globally, and the government of each affected country is publishing the number of deaths every day. This official figure is an underestimate as it excludes anybody who did not die in a hospital, who did not test positive, who had a false result, or those who recovered on their own without a test.

Objective: This study aimed to measure the community level excess mortality using health and demographic surveillance in a rural area of Bangladesh.

Method: The study was conducted in Matlab, in a rural area of Bangladesh, with a Health and Demographic Surveillance System (HDSS) covering a population of 239,030 individuals living in 54,823 households in 142 villages. We examined the mortality in January-April from 2015 to 2020 and compared the mortality in 2020 with the historical trend of 2015–2019. Between 2015 and 2020, we followed 276,868 people until migration or death, whichever occurred first. We analyzed mortality using crude mortality rate ratio (MRR) and adjusted MRR (aMRR) from a Cox proportional hazard model. Mortality was analyzed according to age, sex, and period.

Results: During follow-up, 3,197 people died. The mortality rate per 1,000 person-years increased from 10 in 2019 to 12 in 2020. Excess mortality was observed among the elderly population (aged 65 years and above). The elderly mortality rate per 1,000 person-years increased from 80 in 2019 to 110 in 2020, and the aMRR was 1.40 (95% CI: 1.19–1.64). Although an increasing tendency in mortality was observed between 2015 and 2019, it was statistically insignificant.

Conclusions: The study reported a 28% increase in excess deaths among the elderly population during the first months of the pandemic. This all-cause mortality estimation at the community level will urge policymakers, public health professionals, and researchers to further investigate the causes of death and the underlying reasons for excess deaths in the older age-group.

Keywords: COVID-19, mortality, Bangladesh, elderly, sex

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INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1), first emerged in Wuhan city of Hubei province in China on December 31, 2020, when Chinese health officials informed the WHO about a cluster of 41 patients with mysterious pneumonia, supposedly connected to Huanan Seafood Wholesale Market (2). The detection time of the first cases varied from country to country; some counties detected early in the pandemic and some later. For instance, Bangladesh announced the first cases on March 8, 2020 (**Table 1**), and 2 days later on March 11, 2020, WHO declared COVID-19 as a pandemic (2).

The number of people migrating overseas from Bangladesh for employment annually is more than 400,000 (15). During the first months of 2020, more countries were placing lockdowns after the detection of the first cases of COVID-19 surfaced in each country. Bangladeshi migrant workers residing in these countries were compelled to return home to rural Bangladesh due to lack of income (16, 17). In addition, when Bangladesh announced its own lockdown on March 22, 2020 (18), after detecting the first three cases, an increasing number of people were leaving the capital city (19). Such movement of people opens the window for the spreading of the virus to more places in the country.

Official numbers of COVID-19 cases and deaths in Bangladesh are likely to be an underestimate of the real scenario because this mainly accounts for cases that have tested positive of coronavirus through laboratory confirmation (20) and deaths that are recorded in hospitals. What the numbers have missed could be cases of deaths and infections before testing started, and the first cases were confirmed when only six PCR laboratories were available throughout the country (21). After the tests were made available, false test results could have eliminated any real cases and unaccounted for people who did not step forward for tests fearing isolation and stigma (22) or those that recovered from taking treatment at home (23). In addition, it is also difficult to determine the cause of death in many instances to find out whether the person was COVID-19 positive if they died prior to testing (24).

Weak civil and vital registration statistics (CVRS) for the great majority of low- and middle-income countries (LMIC) (25) means that we have remarkably little insight into the magnitude of the excess mortality associated with the COVID-19 pandemic. This is in marked contrast to some high-income countries; for instance, during the first months of pandemic the United Kingdom published preliminary all-cause mortality data and COVID-19-related mortality data with a lag of only a few weeks unlike Bangladesh (26).

Although most countries are submitting daily data on the number of COVID-19 deaths to the WHO (27), without comparable all-cause mortality data, and all-cause mortality data for the equivalent time period over preceding years, it is difficult to estimate the excess mortality attributable to the pandemic. The excess mortality, of course, is not restricted to COVID-19 deaths alone; it also includes the non-COVID-19 deaths that arise from the loss of adequate care, as health systems become overstretched coping with COVID-19 cases.

Health and Demographic Surveillance System (HDSS) conducts surveillance of geographically prescribed populations for extended periods of time (28). They have, historically, provided estimates for many LMICs about the underlying birth, death, and fertility rates in the absence of an effective CVRS system (28). The Matlab HDSS is the longest running HDSS which has provided Bangladesh with some of its earliest data on rates of births and deaths (29). Utilizing these ongoing surveillance data, we are able to estimate the age and sex of mortality rates over the past 6 years and thereby to estimate the excess mortality in 2020 associated with the COVID-19 pandemic.

TABLE 1 | Timeline of detection of first cases of COVID-19.

Country	2019	2020		
	December	January	February	March
China (3, 4)	31			
United Kingdom (5)		6		
Thailand (3, 4)		13		
Japan (4)		15		
Republic of Korea (South Korea) (4)		20		
United States (3)		20		
France (6)		24		
Germany (6)		27		
India (7)		27		
United Arab Emirates (UAE) (8)		29		
Spain (9)		31		
Iran (3, 10)			19	
Italy (3)			21	
Belarus, Lithuania, Netherlands, New Zealand, and Nigeria (11)			27	
Saudi Arabia (12)				2
South Africa (13)				5
Bangladesh (14)				8

MATERIALS AND METHODS

Settings and Population

The Matlab HDSS covers a population of 239,030 individuals living in 53,823 households in 142 villages in a rural district of Bangladesh that is situated 60 km south of Dhaka (30). All households are visited every 3 months to enquire about marriages, pregnancies, births, migrations, and deaths. Annually in the Matlab HDSS area, 5,298 babies are born, 1,687 people died, 2,671 people moved out of Bangladesh, and 1,371 people returned from abroad (30).

A web-based software application has been designed and developed for Matlab HDSS. Thirty-one tablets (smartphones) are connected to the mobile internet through the network of mobile operators. Traditionally, community health research workers (CHRWs) visit households every 3 months and record

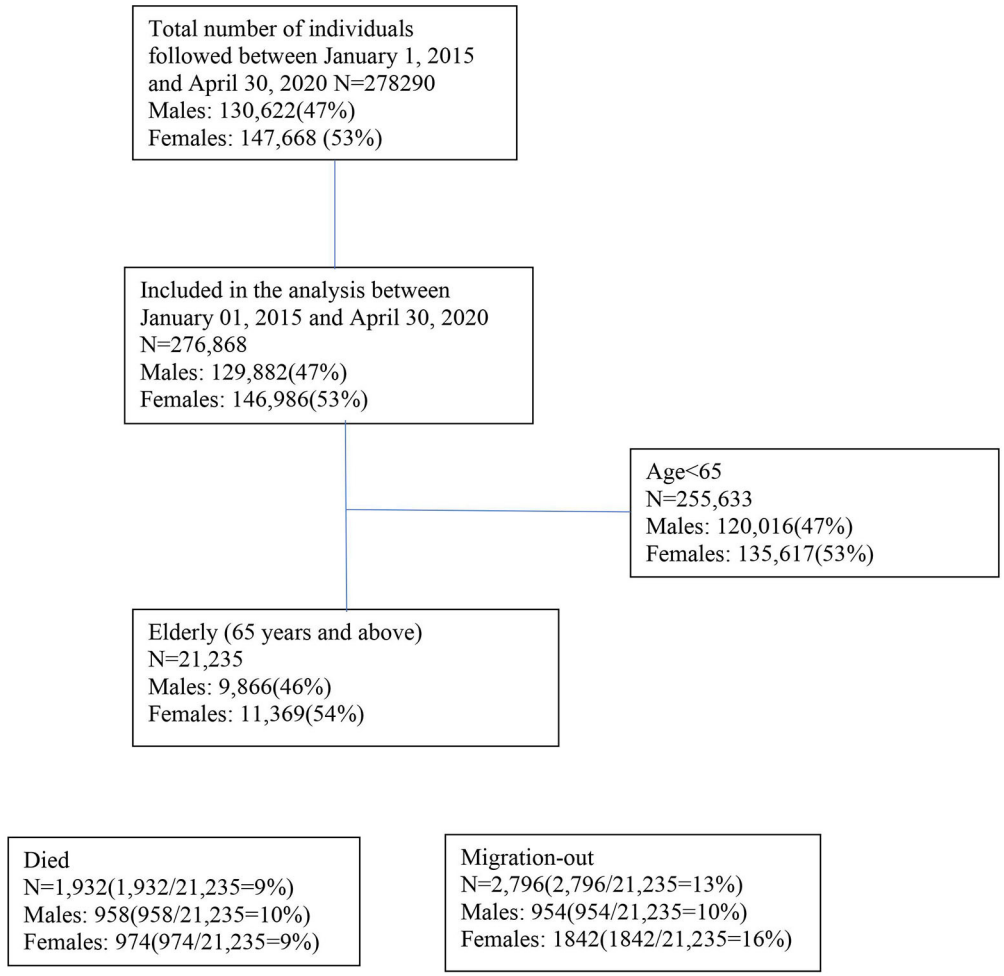

FIGURE 1 | Flowchart of the study population.

FIGURE 2 | Number of people returned from abroad between 2015 and 2020.

health and demographic events using these devices, and data are stored in the central database server. In the COVID-19 pandemic, to follow the precautionary guidelines in Bangladesh, CHRWs continued registering birth and death through mobile phones instead of household visits between March 25, 2020, and November 9, 2020. During data collection through mobile phones, CHRWs reached 85% of households in the first contact. Information of absent households (in the first contact) was updated in the next round.

Epidemiological and Statistical Methods

We analyzed mortality data in the study area between January 2020 and April 2020 to take into account the transmission of coronavirus, not only related to the movement of population from the capital city to rural areas after declaring the first lockdown on March 22, 2020, in Bangladesh but also the spread of the virus exacerbated by the migrants returning from abroad during the first months of the pandemic (January 2020-April 2020) (24, 31). The mortality rate is compared with the mortality rates for the period of January 1 and April 30 in the years from 2015 to 2019. Between 2015 and 2020, we followed 278,290 people until migration or death, whichever occurred first (**Figure 1**). We calculated the mortality rate between January and April as seasonality in mortality was observed in the study area (30) (**Figure 1**). Deaths due to COVID-19 vary by age (32) and sex (33). We presented age-specific and sex-specific mortality and population size as the denominator. We used Cox proportional hazard models with age as the underlying timescale to estimate the aMRR. We included sex (33), period (30), and village (30) as potential confounders in the adjusted models.

RESULTS

In-Migration

In the Matlab HDSS area between January 2020 and April 2020, 1,008 people returned from abroad, which is 2.39 times (95% CI: 2.29–2.55) higher than in 2019. Moreover, in 2020, in-migration was very high, which is 3.87 times higher (95% CI: 3.10-4.14) in January and February compared to March and April (**Figure 2**).

Mortality

During the follow-up period from January to April from 2015 to 2020, a total of 3,197 people died. In 2020, the number of people migrating back to Matlab from abroad was 406 in January, 375 in February, 207 in March, and 17 in April. From 2015 to 2019, the crude mortality rates (CMRs) per 1,000 person-years were 7.37 (95% CI: 6.75–8.05), 7.63 (95% CI: 7.01–8.31), 7.81 (95% CI: 7.18–8.50), 8.98 (95% CI: 8.29–9.73), and 9.70 (95% CI: 8.99–10.48), respectively. In 2020, the CMR was 11.61 (95% CI: 10.51–12.82) (**Table 2**). **Figure 3** shows the age by year mortality rates. Between 2015 and 2019, there is little evidence of substantial year-to-year variation in the mortality rate in any age-group. In 2020, the mortality rate in the 65+ age-group (older adults) is markedly higher than in the previous 5 years.

The mortality rate in older adults per 1,000 person-years increased from 80 in 2019 to 110 in 2020, and the aMRR was 1.39 (95% CI: 1.17–1.66) among older adults. The aMRR was

TABLE 2 | Mortality rate per 1,000 person-years according to age, sex, and calendar year.

Age group (in years)	Male						*P-value	Female						*P-value
	Mortality rate (deaths/pyrs)							Mortality rate (deaths/pyrs)						
	2015	2016	2017	2018	2019	2020		2015	2016	2017	2018	2019	2020	
0–14	3 (36/11,102)	3 (38/11179)	3 (28/10,988)	3 (29/10,787)	4 (44/10,472)	4 (19/5,406)	0.440	2 (22/10,999)	2 (20/10,992)	2 (20/10,992)	2 (24/10,808)	3 (27/10,527)	3 (14/5,393)	0.397
15–44	1 (16/11,618)	1 (16/11795)	1 (12/11,521)	2 (20/10,965)	1 (15/10,347)	1 (5/5,258)	0.997	1 (13/15,891)	1 (13/16,399)	1 (13/16,399)	1 (14/16,314)	2 (25/15,826)	1 (8/8,120)	0.148
45–64	12 (78/6,588)	9 (62/6652)	12 (79/6,605)	15 (98/6,454)	16 (102/6,367)	16 (51/3,285)	0.001	7 (51/7,207)	6 (45/7,154)	6 (45/7,154)	7 (49/6,960)	9 (59/6,713)	8 (28/3,500)	0.138
65+	67 (144/2,153)	68 (151/2205)	86 (183/2,133)	88 (185/2,102)	84 (169/2,015)	117 (126/1,081)	0.000	56 (142/2,539)	60 (154/2,560)	60 (154/2,560)	72 (182/2,524)	77 (186/2,405)	105 (136/1,289)	0.000
Total	9 (274/31,461)	8 (267/31830)	10 (302/31,247)	11 (332/30,308)	11 (330/29,201)	13 (201/15,030)	0.000	6 (228/36,635)	7 (261/37,349)	6 (232/37,105)	7 (269/36,606)	8 (297/35,471)	10 (186/18,303)	0.000

*Test for the trend in mortality from 2015 to 2020.

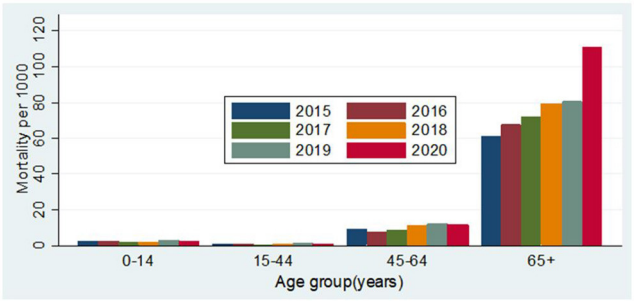

FIGURE 3 | Mortality per 1,000 person-years according to age and year.

1.38 (95% CI: 1.09–1.75) for men and 1.43 (95% CI: 1.16–1.77) for women. No significant difference was observed between men and women in increasing mortality from 2019 to 2020 (p = 0.910). It should be noted that the apparent increasing tendency in mortality observed in the 65+ age-group between 2015 and 2019 was not statistically significant (**Table 1**).

DISCUSSION

Main Findings

Compared to the five previous years, there is a clear, statistically significant difference in the mortality rate in the 65+ age-group during the COVID-19 period in Matlab. This result is strong indication of a pandemic effect. No such difference was observed in any of the younger age-groups. The age by mortality effect is consistent with the global data on excess mortality that shows the highest mortality risk in those who are aged 65 years or older (32, 34, 35).

Interpretation

The COVID-19 pandemic has had a significant negative impact on mortality rates in older people living in rural Bangladesh. It is not possible at this stage to determine the cause of death—although a verbal autopsy process, to be conducted in a few months, may give some insight into this. However, given the age effect in the excess mortality, the results strongly indicate either a direct or an indirect COVID-19 effect. The indirect effect may be attributable to a decreased likelihood of seeking life-saving care or a decreased capacity of the health system to manage non-COVID-19-related healthcare needs (36). For instance, the prevalence of hypertension is 53% among older age-groups (aged 65 years and above) in Bangladesh (37) and these hypertensive patients may not have been able to avail regular checkups or acquire medicines during the pandemic. Moreover, about 50% of deaths occur in the study area per year (30) due to conditions of chronic disease like heart disease and stroke. The pandemic situation may be responsible for deaths among such patients by preventing them from traveling the distance to a hospital that could provide them the immediate intensive care services that they required.

Strength and Weakness

We analyzed mortality from January to April, 2015–2020, which reduced any seasonality bias (30). Mortality rates were calculated using person-time techniques (38) that remain the basic epidemiological approach to estimating mortality, yet one of them is frequently missed in the calculation of mortality (39).

The data were only collected from one rural area, which may not reflect the situation in all rural Bangladesh. Indeed, Matlab appears to have better health outcomes than other rural areas of Bangladesh, and this may indicate that excess mortality rates would be worse elsewhere. The relatively short period of observation was to ensure the timely reporting of data.

We cited a number of news media-released reports as there was no detailed timeline information on these events during the first months of the pandemic.

CONCLUSIONS

Globally, the COVID-19 pandemic impacted the mortality of the overall population. COVID-19 pandemic attributed 30 deaths per 1,000 among the older age-group in the study area. We did not determine the deaths related to COVID-19 and non-COVID-19 causes. A further cause of death analysis will provide an estimate of excess deaths associated with COVID-19 and non-COVID-19 causes. It is important to examine whether the excess deaths as a result of access to healthcare and how the national COVID-19 policy operates on the decisions and actions of the people. Bangladesh needs to strengthen the CVRS system and national health statistics to monitor timely morbidity and mortality, especially in an epidemic or pandemic situation. Bangladesh should strive to strengthen its health systems by providing additional resources to make healthcare services more accessible to its residents irrespective of geographical locations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Review Committee of icddr,b. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SH and DR conceived and designed the study and are the guarantors of the study. SA prepared the data file. SH analyzed the data and wrote the first draft of the manuscript. SS contributed to the literature review. All authors contributed to the final version of the manuscript. All authors had full access

to all of the data (including statistical reports and tables) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis.

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Impact of COVID-19 Pandemic on the Clinical Activities in Obstetrics and Gynecology: A National Survey in China

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Objective: Few studies have quantified the influence of coronavirus disease 2019 (COVID-19) pandemic on medical providers. This is the first national study to investigate the impact of the pandemic on physicians practicing obstetrics and gynecology in China.

Methods: A two-stage, stratified, cluster sampling method was performed based on the city categories (category 1, fewer than 10,000 beds; category 2, 10,000–30,000; and category 3, more than 30,000) and public hospital levels (primary, secondary, and tertiary). Physicians practicing obstetrics and gynecology reported the relevant changes in their general clinical activities and changes in the management of specific diseases or conditions occurring during the periods that they were most strongly affected. These changes were compared by municipal and hospital characteristics.

Results: Questionnaires were collected from a representative sample of 11,806 physicians actively practicing obstetrics and gynecology in 779 hospitals from 157 cities of 31 provinces. Except emergency visits and online consultations, category 3 cities, tertiary hospitals and general hospitals had greater reductions in overall clinical activities than category 1 cities, primary hospitals and specialized hospitals (all adjusted $p < 0.05$), respectively. The differences also existed in the management of specific diseases and conditions, especially for less urgent conditions, including cervical cancer screening, instructions regarding contraception and miscarriage, and assisted reproduction (all $p < 0.05$).

Conclusions: During the COVID-19 pandemic, the clinical obstetrics and gynecology activities in China markedly decreased, with significant differences across municipal and hospital characteristics.

Trial Registration: This study was registered with ClinicalTrials.gov on July 27, 2020 (NCT04491201).

Keywords: COVID-19 pandemic, Mainland China, city category, hospital levels, obstetrics and gynecology, clinical practice

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has caused substantial damage to China since its outbreak and spread in the first half of 2020. As of August 24, 2020, the cumulative number of confirmed cases of COVID-19 reported in China was 84,967, among which 68,139 (80.2%) were from Hubei Province (1). Although there have been numerous studies performed pertaining to this pandemic, only a few studies have quantitatively assessed the impact of this pandemic on medical providers in China (2–4). In a survey of junior doctors in the United Kingdom, most units limited face-to-face antenatal clinics and suspended elective gynecology services (5). Other reports showed the impact of COVID-19 lockdowns on the treatment of gynecologic cancer patients (6, 7), admissions to gynecological emergency care (8, 9), emergency surgery (10), and maternal and newborn healthcare (11). However, these studies had limited sample sizes and voluntary response sampling methods, restricting the generalizability of their findings. As well as in other fields, the lack of sufficient health and legal protection for surgeons and patients may result in a special reduction in the volume of surgical interventions during COVID pandemic and the immediately following period, therefore, determining inability to ensure health care to all patients (12–14).

As reported by the WHO, people living with non-communicable diseases (NCDs) are more vulnerable to becoming severely ill with or dying from COVID-19. The more severe the transmission phase of the COVID-19 pandemic, the more NCD services are disrupted (15). Although most physicians in obstetrics and gynecology were not involved in the immediate response to the pandemic, they represent a major force at the crossroads of politics, social justice, and reproductive rights in the fight for the preservation of reproductive healthcare (16). A representative quantitative assessment of the changes in clinical activities of obstetrics and gynecology during the pandemic would not only provide vital and accurate information for developing coping strategies during this time (17, 18), but also offer suggestions for health care reform, leading to the development of more flexible, and effective health care systems (19).

As previous studies were confined to local regions or used convenience sampling methods hence providing limited information, we performed a national survey in China among registered physicians who practice obstetrics and gynecology in public hospitals. To our knowledge, this is the first nationally representative survey of physicians describing the impact of the COVID-19 pandemic on clinical activities. We particularly examined whether such an impact varied between different municipal and hospital characteristics. In this way we were to explore the practical effects of COVID pandemic on clinical practicing in the view of obstetricians and gynecologists.

METHODS

Sampling Design and Participants

This study used a stratified two-stage random cluster sampling design as to obtain a representative sample and minimize selection bias. Considering the vastness of the territory and large size of the population as well as the unbalanced distribution of healthcare resources across mainland China, all 31 provinces, municipalities and autonomous regions (the latter two have same administrative status as provinces) were included in the study. In the first stage, within each province, three strata of cities were generated according to the total number of hospital beds, namely, category 1 (fewer than 10,000), category 2 (10,000 to 30,000), and category 3 (more than 30,000). Two cities were randomly chosen from each stratum, if applicable. In the second stage, in each selected city, three strata of hospitals were generated according to the hospital levels, namely, primary, secondary, and tertiary. All physicians of obstetrics and gynecology in the chosen hospitals received a link to an electronic questionnaire (https://www.wjx.cn). The data were obtained from completed questionnaires, and were stored in the same online database. A more detailed sampling methods and results were described in **Supplementary Materials**.

The eligible participants were registered physicians working in the obstetrics and gynecology from public hospitals who agreed to participate in the survey. Participants were excluded if they were registered as assistant physician or midwife, or if they retired from routine medical practice. Participants presented their electronic consents when they submitted their questionnaire. The Institutional Review Board of Peking Union Medical College Hospital approved the study (No. S-K1291). This study was registered with ClinicalTrials.gov on July 27, 2020 (NCT04491201).

Data Collection

The questionnaire was developed based on the current clinical activities in China, and consisted of 31 items: 10 pertained to the participants' sociodemographics, one pertained to the period that was most strongly affected by the pandemic (January to June as multiple options), 7 pertained to general clinical activities (outpatient visits and appointments, emergency visits, surgical volumes, consultant requests, admission arrangements and online consultations), and 13 pertained to specific diseases or conditions (preconception counseling, prenatal examinations, prenatal diagnoses, instructions regarding contraception and miscarriage, assisted reproduction, outpatient surgeries and procedures, emergency obstetrical and gynecological surgeries, cervical cancer screening, treatment for benign neoplasms, malignancies and pelvic floor dysfunctions, and follow-up for malignancies). For each clinical activity, the responder was asked to select options to describe the changes during the pandemic from January to June 2020 as irrelevant to his/her specialty, complete shutdown, decreased by >50%, decreased by 25–50%, decreased by <25%, no change or increase. For the item "online consultations," based on the experience gained while constructing the questionnaire, the options consisted of irrelevant, decreased by >50%, decreased by <50%, no change, increased by <50%, and increased by >50%. For each item, the respondent was also asked to evaluate the changes after July 1, 2020, with the following options of the same, less than or more than the level in 2019. A team of 20 physicians from Peking Union Medical College Hospital had validated and modified

TABLE 1 | Demographics of the participants.

	Categories of cities*			Levels of hospital			Natures of hospital	
	Category 1	Category 2	Category 3	Primary	Secondary	Tertiary	General	Specialized
Age, mean (SD)	40.9 (9.2)	40.3 (9.0)	40.3 (8.9)	41.4 (9.1)	40.8 (9.0)	39.9 (9.0)	40.3 (9.0)	40.7 (9.0)
Female, %	92.6	91.7	89.6	93.8	93.2	89.2	91.0	92.1
Han Chinese, %	83.7	91.0	95.7	88.0	90.5	92.5	91.0	91.7
Married, %	86.6	85.6	84.5	87.1	86.5	84.3	85.3	86.1
Degrees, %								
Master or doctor of medicine	10.4	21.9	43.0	6.5	13.8	39.9	29.4	15.5
Bachelor of medicine	72.7	66.9	51.6	70.9	72.8	55.2	62.2	66.6
Others	17.0	11.2	5.4	22.6	13.4	4.9	8.4	17.8
Subspecialties, %								
Obstetrics	37.8	38.9	32.3	39.9	36.9	35.6	34.7	43.9
Gynecology	32.1	34.6	39.7	26.3	30.7	41.6	37.0	31.3
Others†	30.1	26.5	28.0	33.8	32.4	22.9	28.4	24.8
Working years, %								
No more than 10 years	41.8	46.0	46.7	38.8	42.3	49.3	46.7	40.7
11–20 years	25.9	26.7	27.3	24.0	28.4	26.6	26.5	27.4
More than 20 years	32.3	27.4	26.0	37.2	29.3	24.1	26.7	31.9
Professional title, %								
Chief doctor	29.1	32.6	31.0	28.1	30.1	33.4	32.5	27.8
Attending doctor	29.1	30.6	31.4	32.2	32.0	29.2	29.9	33.1
Resident doctor	21.7	21.7	20.8	20.2	20.4	22.4	22.1	19.1
Others‡	20.1	15.1	16.8	19.5	17.6	15.0	15.5	20.1

The percentages were calculated from a sample of 11,806 participants. SD, standard deviation.

**The categories of cities were based on the numbers of total hospital beds. In the cities of category 1, 2 and 3, the numbers of total beds were <10,000, 10,000–30,000, and more than 30,000.*

†*Including physicians on reproductive medicine, family planning and no subspecialty.*

‡*Including post-doctor and physicians refusing to report.*

the questionnaire, and these physicians were excluded in the formal survey.

Statistical Analysis

Unweighted demographic characteristics of all participants were stratified by the city categories, hospital levels, hospital natures (general vs. specialized hospitals for women health) and various provinces (Hubei Province vs. other provinces). Continuous variables are presented as the means with standard deviations, and categorical variables are presented as percentages. All the calculations were then weighted to represent obstetricians and gynecologists nationwide and analyzed with the "Survey data analysis" module in Stata (version 15.0, StataCorp, TX, USA). The weights incorporated sampling probabilities, non-response adjustments, and poststratification adjustments. The weighted percentages of changes in clinical activities and changes in the management of specific diseases or conditions were compared between various municipal and hospital characteristics mentioned above by χ^2-test. Multinomial logistic analysis was used to simultaneously examine the associations of city categories, hospital levels, and natures with changes in clinical activities. The results are presented as relative risk ratios (RRRs) and 95% confidence intervals (95% CIs). Unless otherwise stated, all analyses were performed with a two-sided significance level of 0.05 performed by Stata.

RESULTS

Sampling Design and Participating Results

Overall, 11,806 physicians from 779 hospitals in 157 cities of 31 provinces completed the questionnaires from August 1 to August 10, 2020, corresponding to 7.6% of the 155,787 registered, actively practicing obstetrics and gynecology physicians in China (20). The response rates of physicians and hospitals were 93.8 and 82.0%, respectively. More than one third (35.9%) physicians had the experiences of frontline working against COVID-19 infection. The 11,806 respondents consisted of 17.8, 51.2, and 20.9% of all physicians from category 1, 2, and 3 cities; consisted of 16.2, 31.1, and 52.7% of all from primary, secondary, and tertiary hospitals; and consisted of 78.3 and 21.7% of all from primary and specialized hospitals, respectively. Besides, 376 (3.2%) physicians were from Hubei Province. **Table 1** shows the demographic characteristics of the participants.

Impact of the COVID-19 Pandemic on Clinical Activities

With regard to the months during which their activities were the most strongly affected by the pandemic, 21.7, 87.1, 58.8, 21.7, 6.3, and 4.9% of the physicians chose January, February, March, April, May and June, respectively (**Figure 1**).

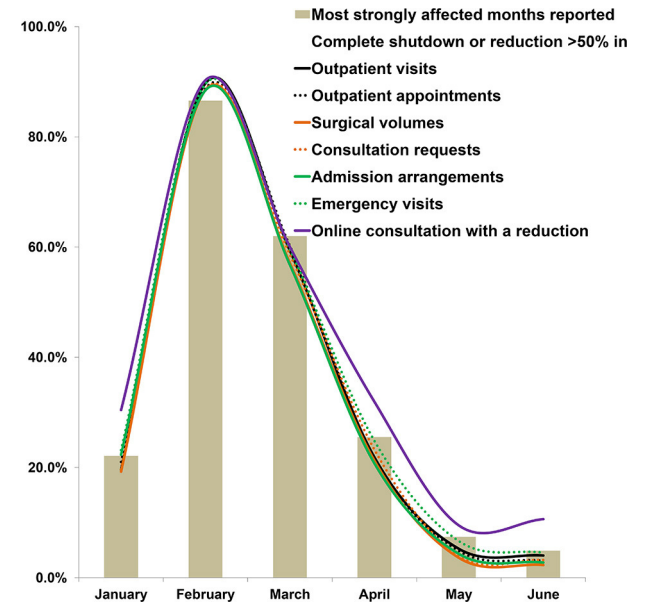

FIGURE 1 | The periods most affected during the COVID-19 pandemic and clinical activities with a >50% reduction or a complete shutdown as reported by physicians. The ratios have been weighted.

Changes of General Clinical Activities

As shown in **Table 2**, from January to June 2020, all clinical activities were reduced. Complete shutdown or a >50% reduction was reported to range from 45.1% (95% CI 43.2–47.0%) for outpatient visits to 20.8% (18.9–22.7%) for emergency visits. With regard to online consultations, 17.7% (95% CI 16.1–19.5%) and 51.6% (48.9–54.3%) of the physicians reported decreased and increased volumes, respectively. Except for emergency visits and online consultations, the proportions of activities with complete shutdowns or >50% reductions differed significantly according to various city categories, hospital levels and hospital natures (all $p < 0.05$, **Supplementary Tables 1–3**).

As shown in **Table 3**, a multivariable regression analysis revealed that, with the exception of emergency visits and online consultations, category 3 cities, tertiary hospitals, and general hospitals experienced more reductions across broad areas of clinical activities compared with category 1 cities, primary hospitals, and specialized hospitals (all adjusted $p < 0.05$), respectively. However, after July 1, 2020, these differences disappeared. With regard to emergency visits and online consultations, differences in reductions only existed in the comparison of various city categories and hospital natures (adjusted $p < 0.05$).

With the exception of outpatient visits and online consultations, physicians from Hubei Province and physicians from other provinces did not report any significant differences in complete shutdowns or >50% reductions (all $p > 0.05$, **Supplementary Table 4**). Significantly higher proportions of physicians reported reduction in online consultations ($p = 0.015$) and complete shutdowns or >50% reductions in outpatient visits ($p = 0.003$) from Hubei Province than physicians from other provinces.

Changes of Management of Specific Diseases or Conditions

As shown in **Table 4**, treatments for specific diseases or conditions decreased in parallel with the changes in general clinical activities. From the 11,806 respondents, the proportion of physicians reporting a complete shutdown or a >50% reduction ranged from 38.0% (35.4–40.6%) for assisted reproduction to 15.8% (95% CI 13.9–18.0%) for emergency obstetrical surgeries. However, unlike general clinical activities, disparities existed according to municipal and hospital characteristics (**Supplementary Tables 5–7**). The treatment and follow-up of malignancies did not significantly differ based on various municipal or hospital characteristics (all $p > 0.05$). Less urgent issues, including assisted reproduction, cervical cancer screening, instructions regarding contraception and miscarriage, and treatment for benign neoplasms or for pelvic floor dysfunctions, differed significantly across municipal or hospital levels and natures (all $p < 0.05$). Compared with other provinces, in Hubei Province, all clinical activities for specific conditions or diseases significantly decreased (all $p < 0.05$, **Supplementary Table 8**).

DISCUSSION

This was the first nationally representative survey of physicians describing the impact of the COVID-19 pandemic on clinical activities in China. In this national survey including Chinese obstetricians and gynecologists, all clinical activities except online consultations substantially decreased. Our findings provided a specific description and sceneria of the national reflection toward COVID pandemic in a medical speciality caring the health of the women and children. The data from our survey could offer a substantial basis for the discussion and reformation of health system coping with the global outbreak and persistence of severe pandemic. In our survey, cities with more hospital beds, hospitals with better resources, and general hospitals were more severely affected with regard to most clinical activities. There are several explanations for these differences. Larger, densely populated cities have a greater risk of infection; therefore, the general clinical activities were more severely impacted in these cities due to lockdown. Larger hospitals and general hospitals undertook the more pressing tasks of testing and caring for patients who had contracted COVID-19 than smaller hospitals and specialized hospitals for women health. In such conditions, medical staff and resources were significantly shifted to other priorities as an emergency measure spontaneously or according to the administrative regulations.

However, the need to shift resources and personnel to cope with an emerging crisis does not mean that the shift remains indefinitely sustainable (21). It is important to evaluate whether and how much this shift has exacerbated existing health inequities and to be proactive in creating policies that promote equity (22). A reform to create a more balanced, healthy medical service system may be warranted, and steps need to be taken after the pandemic to minimize the delay in routine care for

TABLE 2 | Changes in clinical activities during the pandemic.

	No. of participants (%)	During the imminent months of the pandemics (% [95% CI])			After July 1, 2020 (% [95% CI])		
		Complete shutdown or >50% reduction	Reduction by 25%-50%	Reduction <25% or no change	Less than 2019	Same as 2019	More than 2019
Outpatient visits	9,673 (81.9%)	45.1 (43.2-47.0)	32.6 (31.2–34.1)	22.3 (20.7–24.0)	49.0 (46.6–51.5)	42.6 (40.4–44.9)	8.3 (7.6–9.2)
Outpatient appointments	7,519 (63.7%)	27.4 (25.5–29.5)	26.2 (24.9–27.6)	46.4 (44.3–48.5)	44.5 (42.2–46.8)	47.5 (45.4–49.5)	8.0 (7.3–8.9)
Surgical volumes	9,398 (79.6%)	30.2 (28.2–32.2)	30.5 (28.9–32.2)	39.3 (37.3–41.2)	49.1 (46.7–51.6)	42.9 (40.7–45.3)	7.9 (7.1–8.8)
Consultation requests	7,827 (66.3%)	22.3 (20.6–24.2)	18.3 (17.0–19.6)	59.4 (57.4–61.4)	39.7 (37.0–42.3)	53.7 (51.0–56.5)	6.6 (5.8–7.5)
Admission arrangements	9,180 (77.8%)	27.4 (25.1–29.7)	29.1 (27.3–31.0)	43.5 (41.4–45.7)	48.2 (45.8–50.7)	43.8 (41.6–45.9)	8.0 (7.1–9.0)
Emergency visits	6,763 (57.3%)	20.8 (18.9–22.7)	25.4 (23.3–27.7)	53.8 (51.2–56.3)	42.2 (39.5–45.0)	49.4 (46.7–52.1)	8.4 (7.5–9.4)
Online consultations*	5,231 (44.3%)	17.7 (16.1–19.5)	30.7 (28.7–32.7)	51.6 (48.9–54.3)	29.9 (28.1–31.9)	47.9 (45.4–50.4)	22.2 (20.5–24.0)

95% CI, 95% confidence interval.

**The three percentage values during the imminent months of the pandemics denote decreasing, no change and increasing.*

women. In our study, cervical cancer screening and instructions regarding contraception and miscarriage had more significant reductions in cities with more hospital beds and in higher-level hospitals. These changes should be noted. With regard to cervical cancer screening, health professionals should focus on high-risk women and adhere to cost-effective policies, including self-sampling in the immediate postepidemic phase (23). The reduction in attention paid to contraception and miscarriage in large cities or high-level hospitals may reflect a substantial bias with regard to such topics (24), since the shutdown of or delays in contraception and safe abortion during COVID-19 will disproportionately impact the most vulnerable populations in low-income and middle-income regions and countries and lead to considerable increases in preventable mortality and lifelong disability (25).

Our survey provided insight into the management of specific diseases and conditions, including emergencies and less urgent medical issues. According to the survey, the changes in emergency visits, including changes in emergency gynecological or obstetrical surgeries, differed significantly between general and specialized hospitals. Although numerous reports on COVID-19 exist, only a few discussed the impact of COVID-19 pandemic on clinical activities. We used keywords of "clinical activity," "COVID-19," and "impact" had a search in clinical trials and reviews published in English in PubMed (https://pubmed.ncbi.nlm.nih.gov/), only 260 papers were available up to July, 2021. The COVID-19 lockdown substantially reduced admission to gynecological emergency departments, but triage allowed the separation of real emergencies from more deferrable emergencies (8), such as emergency surgeries (26). On the other hand, only less urgent or critical medical issues, including assisted reproduction, differed significantly according to the levels and characteristics of the cities and hospitals. While these services were temporarily disrupted, new strategies are needed to overcome these changes. It is essential for authorities and health care providers to identify patients who should be prioritized for the continuation of fertility care in a safe environment (27). Many guidelines or protocols are available to support prioritization in the field of obstetrics and gynecology (28, 29), and they should be considered on the basis of local resources and planning (30). In our study, we did not find significant differences in the treatment of gynecological malignancies according to city categories, hospital levels or hospital natures, which reflected the attention paid to these critical diseases across the country.

Our survey highlighted feasible innovative treatment strategies during the pandemic. According to the WHO report (15), telemedicine is currently one of the mitigation strategies most often used (27). As previously reported (31), and as expected during the design of the questionnaire, online consultations increased by 51.6% in our survey. The pandemic afforded ambulatory clinicians with the opportunity to expand care to vulnerable populations in ways that were previously underutilized, thus improving health equity (32) by adopting the necessary regulatory framework for the wide application of telemedicine (33). However, telemedicine has its own limitations with regard to examinations and procedures necessary for the diagnosis and treatment of gynecological and obstetrical diseases (34, 35). The quality and trustworthiness of social media are also questionable (36). Legal issues pertaining to telemedicine have yet to be resolved in China (37). Last, in our study, little evidences suggested telemedicine would provide a sufficient and satisfactory solution for the lack of direct clinical interviews during pandemic lockdown. A more exhaustive survey would prudently translate the changes of tendency in medical service into specific, quantified clinical activities, such as outpatient's visits, medication, and examination. However, in our study, in order to quantize the impact, we must include a lot more respondents as to decrease the greater bias caused by epidemiological and personal characteristics. In conclusion, as no study could forward direct evidences discovering and resolving the gaps between telemedicine and face-to-face interviews, we must keep discreet optimism toward the prosperity of telemedicine.

Our survey revealed critical differences in the changes in medical services among various regions of different situations with respect to the pandemic. The comparison between Hubei Province and other provinces in China suggested that general clinical activities did not significantly decrease in Hubei; however,

TABLE 3 | Relative risk ratios for the changes in clinical activities estimated from a multivariable regression model adjusted by municipal and hospital characteristics.

	During the imminent months of the pandemics				After July 1, 2020			
	Complete shutdown or >50% reduction		Reduction by 25%–50%		<2019		More than 2019	
	RRR* (95% CI)	*p*-values	RRR* (95% CI)	*p*-values	RRR† (95% CI)	*p*-values	RRR† (95% CI)	*p*-values
Outpatient visits								
Categories of cities								
Category 1	1.00		1.00		1.00		1.00	
Category 2	1.30 (1.00–1.69)	0.052	1.18 (0.95–1.47)	0.139	1.13 (0.90–1.42)	0.276	0.74 (0.60–0.92)	0.006
Category 3	1.39 (1.05–1.85)	0.024	1.30 (1.01–1.66)	0.038	1.29 (0.96–1.73)	0.093	0.57 (0.42–0.77)	<0.001
Levels of hospitals								
Primary	1.00		1.00		1.00		1.00	
Secondary	1.05 (0.81–1.37)	0.706	0.93 (0.74–1.16)	0.493	1.07 (0.84–1.37)	0.572	1.08 (0.80–1.47)	0.598
Tertiary	1.44 (1.04–1.99)	0.030	0.96 (0.75–1.23)	0.743	0.86 (0.62–1.20)	0.368	0.79 (0.57–1.09)	0.145
Natures of hospitals								
Specialized	1.00		1.00		1.00		1.00	
General	1.63 (1.16–2.28)	0.005	1.38 (1.11–1.71)	0.004	0.88 (0.69–1.13)	0.315	1.05 (0.78–1.40)	0.754
Outpatient appointments								
Categories of cities								
Category 1	1.00		1.00		1.00		1.00	
Category 2	1.34 (0.96–1.88)	0.084	1.04 (0.84–1.30)	0.716	1.09 (0.84–1.41)	0.508	0.70 (0.57–0.85)	0.001
Category 3	1.50 (1.04–2.16)	0.028	1.23 (0.98–1.54)	0.073	1.27 (0.93–1.74)	0.126	0.58 (0.45–0.74)	<0.001
Levels of hospitals								
Primary	1.00		1.00		1.00		1.00	
Secondary	1.09 (0.81–1.46)	0.565	0.95 (0.76–1.20)	0.686	1.02 (0.80–1.30)	0.864	1.44 (1.04–1.98)	0.029
Tertiary	1.63 (1.19–2.25)	0.003	1.01 (0.81–1.25)	0.957	0.75 (0.55–1.01)	0.061	0.99 (0.72–1.36)	0.953
Natures of hospitals								
Specialized	1.00		1.00		1.00		1.00	
General	1.56 (1.12–2.18)	0.01	1.43 (1.18–1.71)	<0.001	1.05 (0.82–1.34)	0.694	1.08 (0.85–1.37)	0.529
Surgical volumes								
Categories of cities								
Category 1	1.00		1.00		1.00		1.00	
Category 2	1.20 (0.83–1.72)	0.325	1.26 (0.92–1.72)	0.145	1.01 (0.80–1.28)	0.904	0.79 (0.63–0.99)	0.045
Category 3	1.55 (1.06–2.25)	0.023	1.38 (1.02–1.86)	0.037	1.21 (0.89–1.65)	0.22	0.66 (0.46–0.95)	0.025
Levels of hospitals								
Primary	1.00		1.00		1.00		1.00	
Secondary	1.06 (0.79–1.40)	0.706	1.02 (0.81–1.29)	0.861	1.03 (0.76–1.41)	0.828	1.46 (1.08–1.97)	0.013
Tertiary	1.40 (1.03–1.92)	0.034	0.94 (0.74–1.19)	0.583	0.78 (0.55–1.12)	0.174	1.01 (0.74–1.37)	0.972
Natures of hospitals								
Specialized	1.00		1.00		1.00		1.00	
General	2.18 (1.47–3.23)	<0.001	1.63 (1.33–2.00)	<0.001	0.94 (0.73–1.21)	0.612	0.85 (0.67–1.08)	0.19
Consultation requests								
Categories of cities Category 1	1.00		1.00		1.00		1.00	
Category 2	1.24 (0.90–1.69)	0.183	0.90 (0.68–1.20)	0.466	0.95 (0.73–1.23)	0.687	0.64 (0.51–0.80)	<0.001
Category 3	1.56 (1.12–2.17)	0.01	1.07 (0.80–1.44)	0.648	1.06 (0.77–1.48)	0.709	0.47 (0.31–0.71)	<0.001
Levels of hospitals								
Primary	1.00		1.00		1.00		1.00	
Secondary	1.30 (0.98–1.71)	0.068	1.29 (1.02–1.62)	0.032	0.99 (0.75–1.30)	0.93	1.22 (0.88–1.67)	0.227
Tertiary	1.91 (1.44–2.55)	<0.001	1.39 (1.11–1.73)	0.004	0.86 (0.59–1.24)	0.405	1.06 (0.75–1.48)	0.744
Natures of hospitals								
Specialized	1.00		1.00		1.00		1.00	
General	1.69 (1.21–2.35)	0.002	1.40 (1.15–1.71)	0.001	0.88 (0.65–1.19)	0.393	0.98 (0.72–1.32)	0.877

(Continued)

TABLE 3 | Continued

	During the imminent months of the pandemics				After July 1, 2020			
	Complete shutdown or >50% reduction		Reduction by 25%–50%		<2019		More than 2019	
	RRR* (95% CI)	*p*-values	RRR* (95% CI)	*p*-values	RRR† (95% CI)	*p*-values	RRR† (95% CI)	*p*-values
Admission arrangements								
Categories of cities								
Category 1	1.00		1.00		1.00		1.00	
Category 2	1.26 (0.91–1.76)	0.166	1.19 (0.89–1.58)	0.234	1.09 (0.87–1.36)	0.47	0.66 (0.51–0.85)	0.002
Category 3	1.64 (1.15–2.33)	0.006	1.24 (0.90–1.70)	0.186	1.20 (0.89–1.63)	0.232	0.51 (0.36–0.72)	<0.001
Levels of hospitals								
Primary	1.00		1.00		1.00		1.00	
Secondary	1.13 (0.81–1.56)	0.475	1.00 (0.77–1.30)	0.998	1.06 (0.77–1.44)	0.729	1.29 (0.95–1.74)	0.099
Tertiary	1.63 (1.14–2.34)	0.008	0.98 (0.75–1.29)	0.901	0.80 (0.57–1.14)	0.218	0.90 (0.64–1.26)	0.524
Natures of hospitals								
Specialized	1.00		1.00		1.00		1.00	
General	1.86 (1.22–2.82)	0.004	1.43 (1.16–1.76)	0.001	1.04 (0.80–1.34)	0.776	1.07 (0.80–1.44)	0.636
Emergency visits								
Categories of cities								
Category 1	1.00		1.00		1.00		1.00	
Category 2	1.12 (0.82–1.53)	0.461	1.03 (0.79–1.34)	0.824	1.02 (0.81–1.28)	0.893	0.85 (0.66–1.08)	0.179
Category 3	1.36 (0.95–1.96)	0.091	1.16 (0.85–1.60)	0.34	1.05 (0.74–1.47)	0.798	0.60 (0.44–0.83)	0.002
Levels of hospitals								
Primary	1.00		1.00		1.00		1.00	
Secondary	0.96 (0.69–1.33)	0.814	0.87 (0.68–1.10)	0.243	0.86 (0.66–1.11)	0.232	1.16 (0.81–1.68)	0.416
Tertiary	1.14 (0.87–1.49)	0.342	0.97 (0.77–1.21)	0.769	0.68 (0.51–0.91)	0.011	0.81 (0.56–1.16)	0.249
Natures of hospitals								
Specialized	1.00		1.00		1.00		1.00	
General	1.82 (1.36–2.43)	<0.001	1.39 (1.15–1.68)	0.001	1.06 (0.86–1.31)	0.569	1.10 (0.80–1.52)	0.551
Online consultations‡								
Categories of cities								
Category 1	1.00		1.00		1.00		1.00	
Category 2	0.89 (0.67–1.19)	0.425	0.68 (0.54–0.86)	0.001	0.92 (0.71–1.20)	0.552	0.91 (0.71–1.16)	0.445
Category 3	1.06 (0.74–1.50)	0.764	0.59 (0.44–0.79)	<0.001	1.07 (0.79–1.45)	0.68	1.20 (0.90–1.62)	0.215
Levels of hospitals								
Primary	1.00		1.00		1.00		1.00	
Secondary	0.87 (0.61–1.24)	0.445	0.95 (0.74–1.22)	0.693	0.78 (0.56–1.08)	0.128	1.37 (0.99–1.90)	0.058
Tertiary	0.81 (0.59–1.12)	0.202	0.83 (0.65–1.06)	0.134	0.67 (0.49–0.91)	0.012	0.94 (0.69–1.29)	0.702
Natures of hospitals								
Specialized	1.00		1.00		1.00		1.00	
General	1.38 (0.94–2.03)	0.098	1.10 (0.92–1.33)	0.298	1.08 (0.81–1.44)	0.593	1.06 (0.83–1.36)	0.637

The categories of cities were based on the total number of hospital beds. In categories 1, 2 and 3 cities, the total numbers of beds were fewer than 10,000; 10,000–30,000; and more than 30,000, respectively. 95% CI, 95% confidence interval;. RRR, relative risk ratio.

**With the response of "reduction <25% or no change" as reference.*

†With the response of "same as 2019" as reference.

‡The two percentage values during the imminent months of the pandemics denote no change and increasing, with the response of "reduction" as reference.

the management of all specific gynecological or obstetrical conditions declined significantly. These differences suggested the shift of medical sources to cope with COVID-19, including new assignments for obstetricians and gynecologists, since 80.2% of confirmed cases in China occurred in Hubei Province.

The large nationally representative sample and a comprehensive assessment of the impact on clinical activities were the strengths of our study. Specifically, our results revealed that COVID-19 pandemic had significantly different impact on the clinical activities across various municipal and hospital characteristics. However, there are several limitations in our study. We did not include private health services in the survey since they account for a very limited proportion of the total volume of the healthcare market in China. This study did not

TABLE 4 | Changes in the management of specific diseases or conditions.

	No. of participants (%)	Complete shutdown or >50% reduction, (% [95% CI])	Reduction by 25%−50%, (% [95% CI])	Reduction <25% or no change, (% [95% CI])
Preconception counseling	8,241 (69.8%)	34.3 (32.7–36.0)	31.7 (30.2–33.1)	34.0 (32.4–35.6)
Prenatal examinations	8,218 (69.6%)	26.9 (25.1–28.9)	32.2 (30.5–34.0)	40.8 (39.2–42.5)
Prenatal diagnosis	6,919 (58.6%)	27.6 (25.8–29.4)	28.0 (26.6–29.5)	44.4 (42.5–46.4)
Instructions for contraception and miscarriage	8,419 (71.3%)	29.0 (27.2–30.8)	23.4 (22.1–24.8)	47.6 (45.9–49.3)
Assistant reproduction	3,871 (32.8%)	38.0 (35.4–40.6)	21.5 (19.7–23.5)	40.5 (38.1–42.9)
Outpatient surgeries and procedures	7,196 (61.0%)	35.4 (33.6–37.4)	26.7 (25.1–28.3)	37.9 (36.0–39.8)
Emergent obstetrical surgeries	7,542 (63.9%)	15.8 (13.9–18.0)	20.7 (19.2–22.4)	63.4 (61.1–65.7)
Emergent gynecological surgeries	7,575 (64.2%)	21.0 (18.9–23.3)	20.3 (18.7–21.9)	58.8 (56.7–60.8)
Cervical cancer screening	7,434 (63.0%)	37.3 (35.4–39.2)	22.0 (20.6–23.4)	40.7 (39.0–42.5)
Treatment for benign neoplasm	7,278 (61.6%)	35.7 (33.7–37.7)	24.7 (23.0–26.5)	39.6 (37.9–41.4)
Treatment for malignancies	6,625 (56.1%)	25.8 (23.7–28.1)	20.1 (18.9–21.4)	54.0 (51.7–56.3)
Follow–up for malignancies	6,492 (55.0%)	26.5 (24.6–28.6)	19.6 (18.3–20.9)	53.9 (51.8–55.9)
Treatment for pelvic floor dysfunctions	6,551 (55.5%)	39.5 (37.9–41.1)	21.7 (20.3–23.1)	38.9 (37.0–40.7)

95% CI, 95% confidence interval.

explore the impact of the pandemic on the lives, professional careers, and mental health of obstetricians and gynecologists, although many reports have discussed the stress experienced by these clinicians (4, 38), which may be associated with changes of medical service during the pandemic (39). Physicians' reported qualitative assessment about changes in clinical activities lacks of uniform evaluation, which should be supported by more data from national statistical data. However, although personal feeling had its limitation of clear description, it indeed reflected an invaluable experience in caring their patients.

One of the most important limitations is that we did not consider the national and/or local policies and interventions in this study. The national policies would have played a great role of resumption of medical service and social economics. Just like the situations in other society activities, a temporal trend in the decrease or increase would be most strongly affected by the restrictions, measures of dealing with the pandemics by federal and local governments and organizations (40, 41). A successful control of COVID-19 pandemics depends on the unselfish devotion of the healthcare staffs, comprehensive society movement against pandemics, and national decisions and policies. Although widespread gaps in the quality of primary health care still exist in China (42), a series effective, rapid measure have been implemented to tackle the disease (43). The most serious outbreaks occurred in February and March, 2020, and accordingly, the most rigorous restrictions from personal, organizational and national requirements were performed (44–47). These restrictions, undoubtedly, would cause great changes in clinical activities. It is regret that we couldn't quantitatively take these changes in this analysis. However, since all the provinces and hospitals in China were under a series of relative consistent policies, changes among these administrations and different diseases have their significances in coping with COVID-19 pandemics. Authors' clinical experiences during the COVID-19 pandemics accorded with the trends discovered in this study. In the principle investigational hospital, one of the teaching tertiary hospitals, in February and March of 2020, only less half outpatient and inpatient workload was required for gynecologic services.

CONCLUSIONS

In this national, stratified, two-stage, random cluster sampling survey, clinical activities in obstetrics and gynecology were majorly reduced during the COVID-19 pandemic in China. Cities and hospitals with more resources or general hospitals were more severely affected, resulting in delays or other disparities in the medical care of vulnerable populations, such as women needing cancer screening or assisted reproduction. However, the magnitudes of the decline varied among other specific diseases or conditions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Peking Union Medical College Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

LZ, HZ, and JLv conceived of the original idea for the study, interpreted results, carried out the statistical analysis, edited the paper, and were overall guarantor. LL obtained ethical approval, contributed to the preparation of the data set, interpreted results, and contributed to drafts of the paper. YC, JF, TL, and JLa

contributed to the study design, data collection, interpretation of results, and commented on drafts of the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.633477/full#supplementary-material

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A Perspective on the Role of Point-of-Care "Immuno-Triaging" to Optimize COVID-19 Vaccination Distribution in a Time of Scarcity

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Vaccine bears hope to bring COVID-19 pandemic under control. With limited supply, vaccines must be utilized efficiently to provide protection to those who need it most. Currently, no practical framework has been proposed to ensure fair vaccine allocation at individual level, which is a recognized problem. We propose here an evidence-based decision-making framework for COVID-19 vaccine appropriation that prioritizes vaccine doses to individuals based on their immunological status, or immuno-triaging. To ensure successful implementation of the proposed framework, point-of-care (POC) immunodiagnostic testing is needed to quickly ramp up the testing capability. Considerations for deploying POC immunodiagnostic testing at such a large scale are discussed. We hope that the proposed immunological decision-making framework for evidence-based COVID-19 vaccine appropriation provides an objective approach to ensure fair and efficient utilization of the scarce vaccine resource at the individual level that also maximizes the collective societal benefit.

Keywords: COVID-19, vaccine, point-of-care, immunodiagnostics, vaccine distribution

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INTRODUCTION

Since its onset in January, 2020, COVID-19 has caused millions of deaths worldwide (1, 2). Several new SARS-Cov-2 variants have caused the death toll to increase rapidly in recent waves (3). In the current dire situation, COVID-19 vaccine finally brings a glimmer of light at the end of the tunnel. As of Jun 4th, 2021, a total of 102 COVID-19 vaccine candidates are under clinical evaluation (4), and 9 Emergency Use Listing (EUL) are issued by the World Health Organization (WHO) (5). Armed with these vaccines, the world will be ready to enter the second half of the battle against COVID-19 pandemic.

Without effective pharmacological interventions, molecular testing (nucleic acid amplification test) has been our best defense against COVID-19 to date (6). Besides molecular testing, a wide range of immunodiagnostic tests have been developed to detect the IgG and/or IgM against SARS-CoV-2, the viral pathogen that causes COVID-19. Emerging evidences suggest that T-cell immunity may play an equal, if not greater, role in protective immunity against SARS-CoV-2 (7, 8). As public health strategies shift toward vaccine and immunity, we believe POC immunodiagnostic should play a major role in vaccine appropriation in the second half of the battle against COVID-19.

Despite the unprecedented speed and scale of R&D effort in COVID-19 vaccine development, the supply of vaccines will be limited. Even though an ambitious goal of 1 billion doses by the end of 2020 is planned globally (9), this number is far from sufficient. It will take at least another year to produce enough doses for the world. In reality, some of the vaccine candidates in the production plan may not cross the finishing line, and some of the planned manufacturing capacity may be delayed due to disruption of supply chains. Even under optimal circumstances, the massive demand will put tremendous pressure on the global supply of biomedical products needed for vaccine production. Concerns have already been raised on potential shortage of horseshoe crab's blood (10), glass vial (11), and syringe (12) that are required for vaccine testing, storage and administration. In short, COVID-19 vaccine will be a scarce resource.

With limited supply for the first few months and likely years, a critical question is who gets the vaccine first. This is not an easy question to answer. To bring the COVID-19 pandemic under control in the shortest possible time, vaccines must be utilized efficiently to provide protection to those who need it most. Most frameworks proposed to guide equitable allocation of vaccines are primarily focused on targeting population groups (13, 14). For example, the WHO's fair allocation framework through COVAX is focused on mortality reduction and protection of health system by targeting groups including frontline healthcare works and age >65 with high risk factors (15). But only a limited number of practical frameworks, such as the allocation plans employed by individual US CDC jurisdictions (16), have been proposed to ensure more precise vaccine allocation at the individual level, which is a recognized problem (13). Moreover, because the development of multiple vaccine candidates is occurring in isolation and in parallel to compress the usual vaccine timeline from 10-15 years to 1–2 years, crucial information regarding the efficacy, longevity, safety, and deployment of the various vaccines will be variable, asynchronous, and evolve over time (17). Those who received early generation of vaccine with rapidly waning immune responses may require re-vaccination using an improved second-generation vaccine. There are patient-level differences in susceptibility to SARS-CoV-2 infection, variable immunity in asymptomatic individuals or those recovered from COVID-19, and emerging evidence of pre-existing immunity resulting from past exposure to other human coronaviruses (18).

Although there is no consensus on the correlation between seropositivity and protective immunity against SARS-CoV-2, immunodiagnostic testing is still the primary metric used to evaluate the efficacy of COVID-19 vaccines, and the decision on the necessity of booster vaccine is also based on the antibody titer (19, 20). As such, we propose here an immuno-triaging framework for both fair and precise COVID-19 vaccine appropriation in a time of scarcity that incorporates existing frameworks that target priority populations but also accounts for the immunological status of each individual (20). Due to the large testing scale and relatively simple assay format, POC immunodiagnostic testing, particularly in community settings and primary healthcare settings, can play a central role in establishing an equitable and evidence-based vaccine distribution at the individual level. The necessity and considerations for large-scale deployment of POC immunodiagnostic testing for immuno-triaging are discussed in this perspective. We hope that it could assist in making objective decisions based on scientific and medical evidence and lead to the most equitable and efficient utilization of a limited vaccine supply for the collective benefit of society.

IMMUNO-TRIAGING FOR EVIDENCE-BASED COVID-19 VACCINE APPROPRIATION

The decision-making flowchart of immuno-triaging developed by us is illustrated in **Figure 1**. When in ample supply, vaccines are administered without testing pre-existing immunity in mass vaccination campaigns. In the case of COVID-19 vaccine, which has an urgent and huge worldwide demand but limited supply in the foreseeable future. The collective benefit of immuno-triaging would outweigh the cost of immunodiagnostic testing when such testing is accessible. In this framework proposed by us, POC immunodiagnostic testing ensures equitable vaccine allocation, both on an individual level and societal basis. However, getting access to immunodiagnostic tests may be no less challenging than getting access to vaccines, which must be taken into consideration when implementing the framework. Here, Immunodiagnostic testing refers to serological tests or T-cell immune response tests.

There are three key steps in the main immuno-triaging decision path, and two side decision paths for high-risk groups of vaccine failure and immunity monitoring program post vaccination.

Main Decision Path

Step 1: Assess risk of contracting and spreading COVID-19 and identify high-risk groups to enroll in an active vaccination program

Prioritized access to vaccines should be given to groups with high risks of contracting and spreading the disease. The risks may be assessed by individual's baseline medical conditions and social risk factors using metrics such as social vulnerability index (SVI) (21). Low-risk groups in the initial assessment will be enrolled in future program when vaccines become more readily available. The assessment could be integrated into existing ethical frameworks (13–15) for the collective benefit of people by ensuring that certain groups are not disadvantaged due to morally irrelevant factors such as religion and race. Low-priority group will be re-admitted to the vaccination program when more vaccine doses are available.

Step 2: Assess existing immunity against COVID-19 in prioritized groups immuno-triaging of COVID-19 vaccines

Vaccines are appropriated according to immunodiagnostic evidence based on virus-specific antibodies, neutralizing antibodies, or in ideal scenario T-cell immune response if

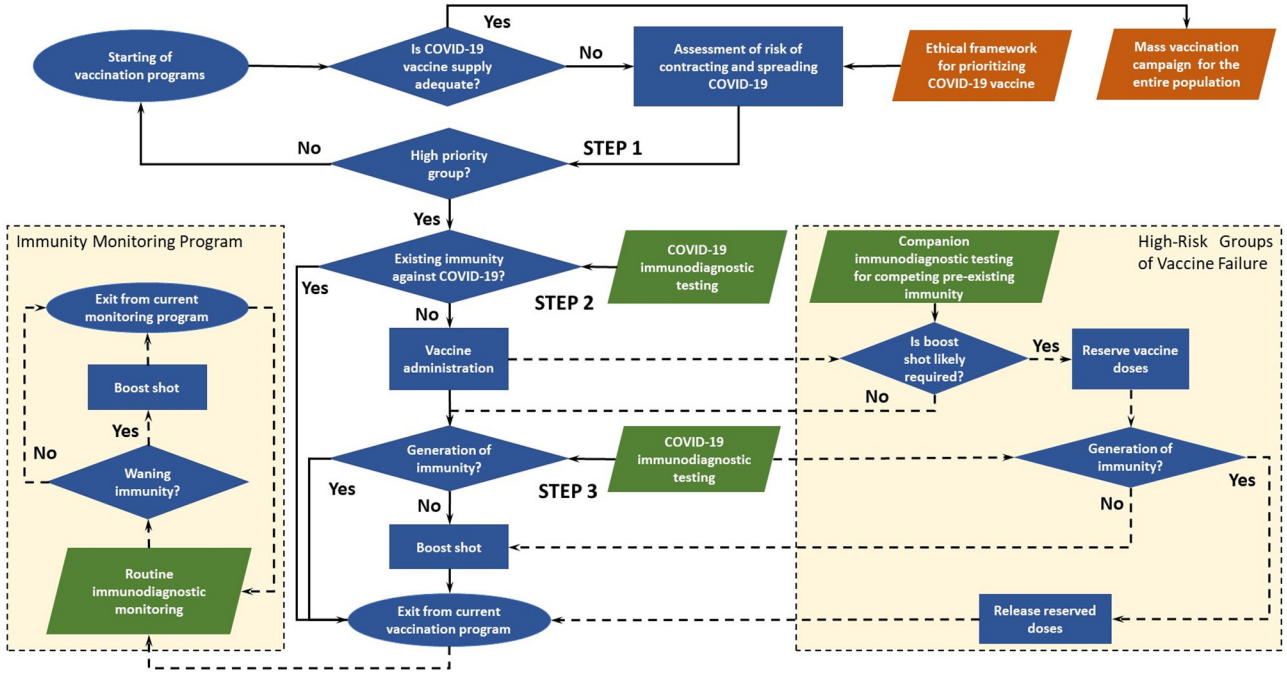

FIGURE 1 | Decision-making flowchart for immuno-triaging of COVID-19 vaccine.

condition allows. Qualitative testing based on ELISA or competitive ELISA could be used to determine the presence of IgM/IgG pr against SARS-CoV-2 (8, 18, 22–24). The cutoff level for protective immunity could be determined based on models like the one suggested by Khoury et al. (25). Vaccines are prioritized for individuals without existing immunity against COVID-19. Individuals with existing immunity due to virus exposure are relieved from current vaccination program. This group would be included in the immunity monitoring program, one of the side decision paths.

Step 3: Assess immune response and determine whether a booster vaccine is required

The efficacy of vaccines in clinical trials is evaluated by antibody titer or T-cell immune response, and the same strategy could be used to assess immune response in the framework. Although multiple doses are suggested for a number of vaccine candidates, it has been shown in clinical trials that the antibody titer in certain individuals reaches the threshold for seroconversion with only a single dose (19). These individuals could skip subsequent doses when vaccine supply is limited, and catch-up doses may be given if necessary when immunity wanes or vaccines become widely available. On the flip side, clinical trials for single-dose vaccine candidates reveal that booster vaccine may be required for individuals with pre-existing immunity against vaccine vectors (20). Therefore, to ensure efficient utilization of vaccines, the booster should be appropriated based on immunodiagnostic evidence.

High-Risk Groups of Vaccine Failure: Assess Pre-existing Immunity That Predicts the Likelihood of An Individual Requiring a Booster Vaccine

POC immunodiagnostic testing could be used to identify individuals who are predicted to require a booster vaccine based on immunodiagnostic evidence (20). In such a scenario, a dose is reserved to ensure the booster vaccine is available so that the first dose does not go waste. Immunodiagnostic testing should be conducted to check the immune response before the administration of the booster vaccine. If the individual successfully generates immunity without the booster vaccine, the reserved vaccine dose is released. Individuals who receive the booster vaccine could be included in the immunity monitoring program.

Immunity Monitoring Program: Tracking Immune Response and Checking for Waning Immunity

Routine immunodiagnostic monitoring tracks the dynamics of immune response post vaccination and identifies individuals who may no longer have protective levels of antibody or T-cell memory. Combined with cloud-based information platform, big-data tools (26), and other digital solutions (27, 28), routine measurement of these levels could guide future vaccination programs and public health responses. If immunodiagnostic testing results impart waning immunity, booster vaccine could be administered to these individuals.

After receiving the booster vaccine, Individuals may exit or remain in the routine monitoring program depending on available resources.

ESSENTIAL ROLE OF POC IMMUNODIAGNOSTIC TESTING IN IMPLEMENTING THE FRAMEWORK

POC immunodiagnostic testing is imperative for successful implementation of the framework. Currently, immunodiagnostic testing, particularly POC immunodiagnostic testing, is only recommended as a surveillance tool or a primary screening mechanism to supplement molecular testing (29). Molecular testing will continues serving as the primary diagnostic tool for patients acutely ill or exposed to COVID-19, but immunodiagnostic testing will be a key tool in immuno-triaging of COVID-19 vaccines.

Widespread deployment of POC immunodiagnostic testing would be prioritized because of the following considerations.

POC Immunodiagnostic Testing Will Enable Efficient COVID-19 Vaccine Utilization

To bring COVID-19 under control calls for efficient utilization of the scarce vaccine resource. Rapid test-to-decision workflow is of the outmost importance in the context of immuno-triaging. POC testing brings the testing capability to the site of patient care and offers sample-to-answer tests that require minimal user intervention. Besides reduced cost and demand for resources compared to centralized testing, one defining characteristic of POC testing is the rapid access to testing results for timely decisions on COVID-19 vaccine appropriation. Short wait time also eases patients' anxiety level, reduces the number of clinical visits (which is critical for vaccine adherence), and decreases the chance of infection while waiting for testing results.

Immunodiagnostic Testing Needs to Be Conducted at a Large Scale That Is Beyond the Capability of Existing Centralized Testing

As the focus of testing for COVID-19 shifts from identifying pathogen to determining immunity in the second half of the battle, immunodiagnostic testing will need to be conducted at a large scale over wide geographic regions. The huge test volume and scattered population distribution present a logistical nightmare for centralized testing schemes: sample delay, loss and mislabeling are inevitable when tests are conducted on such a large scale. If communication of testing results requires a long turnaround time, decisions on vaccine appropriation will be delayed. Decentralization will enable rapid ramp-up in immunodiagnostic testing capability and relieve the burden on central healthcare facilities. In regions with poor medical resource, POC immunodiagnostic testing may be the only viable option for implementing the proposed framework for fair and efficient vaccine utilization.

Large-Scale POC Immunodiagnostic Testing Is Feasible and Relatively Easy to Implement

The proposed immuno-triaging framework requires immunodiagnostic evidence at various stages over a period of several weeks or even months. Therefore, a high level of patient's compliance with the testing schedule is essential for efficient vaccine utilization. POC immunodiagnostic testing could enhance patient's adherence to the program due to its easy access, rapid turnaround time and timely clinical decision. In fact, COVID-19 testing based on POC immunoassays has already been deployed in large scale in Singapore (30). The decentralized arrangement makes active follow-up with patients a relatively easy task. Local recruits could establish effective partnership with local communities to promote the COVID-19 vaccination program for improved outcomes.

POC Immunodiagnostic Testing Enhances Patient and Community Engagement in COVID-19 Vaccination Program

The proposed immuno-triaging framework requires immunodiagnostic evidence at various stages over a period of several weeks or even months. Therefore, a high level of patient's compliance with the testing schedule is essential for efficient vaccine utilization. POC immunodiagnostic testing could enhance patient's adherence to the program due to its easy access, rapid turnaround time and timely clinical decision. The decentralized arrangement makes active follow-up with patients a relatively easy task. Local recruits could establish effective partnership with local communities to promote the COVID-19 vaccination program for improved outcomes.

CONCERNS WITH EXISTING COVID-19 POC IMMUNODIAGNOSTIC TESTS AND GAPS TO CLOSE

Test Accuracy

One major concern about widespread deployment of POC immunodiagnostic testing is the accuracy of test kits. At the moment, the majority of POC immunodiagnostic testing kits are based on lateral flow assay (LFA), and many of them show suboptimal performance in independent evaluations. Australian Therapeutic Goods Administration (TGA) has announced that all eight POC immunodiagnostic test kits evaluated in its Post Market Review so far "have claimed a better sensitivity than that observed" in its independent evaluation (**Figure 2**) (31). It is worth noting that majority (>50%) of the samples in the cohort used for sensitivity evaluation by TGA are from early-stage infections ($\leq$ 14 days) which are expected to lead to lower sensitivity. Nevertheless, even when tested with all late-stage samples (> 14 days), only two out of the

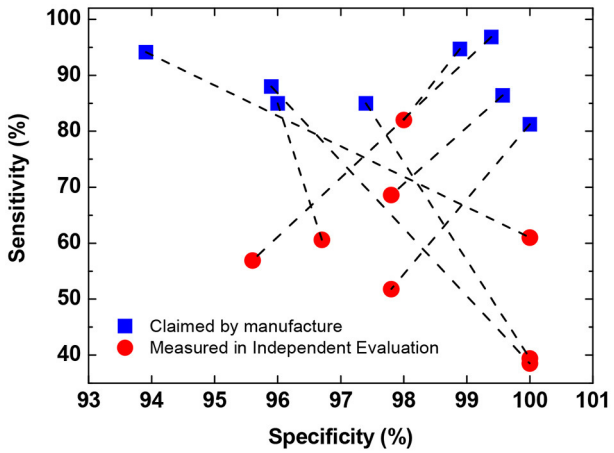

FIGURE 2 | Comparison of self-claimed and independently evaluated performance of eight POC immunodiagnostic tests. Data obtained from (31). Data in the plot show the mean sensitivity and specificity for total antibody detection (either IgM or IgG). In cases where the performance for IgM and IgG are reported separately by the manufacture, the lower values are used in the plot.

eight tests show a similar performance to that claimed by the manufacturers.

A meta-analysis of COVID-19 immunodiagnostic testing reveals that the sensitivity of LFA is significantly lower than that of ELISA and chemiluminescent immunoassays (CLIA) (32). Both ELISA and CLIA could provide quantitative immunodiagnostic measurements. Although CLIA has a higher sensitivity than ELISA, it also shows a significantly larger variability. Compared to CLIA, ELISA is less resource demanding and easier to translate into POC testing using simple microfluidic systems such as magnetic digital microfluidics. POC ELISA could improve the accuracy POC immunodiagnostic testing and offer quantitative antibody titer measurements to monitor immune response post-vaccination.

Existing immunodiagnostic tests are optimized for a high positive predictive value, which means they are designed to ensure the positive results are true positive. And most of these tests have a low negative predictive value, which means there could be a relatively large number of false negatives (31, 33). The framework appropriates vaccine doses based on negative immunodiagnostic results. Hence, POC immunodiagnostic tests with high negative predictive values should be selected for pre-vaccination screening. An orthogonal testing algorithm could be implemented in regions with a high COVID-19 prevalence where the tests are likely to have a low negative predictive value to ensure the negative results are true negative.

Lack of POC Testing for COVID-19 T-Cell Immune Response

Recent studies reveal that CD4 and CD8 T-cells respond to multiple SARS-CoV-2 proteins and "memorize" the immunity for a longer duration than antibodies (7, 8), suggesting that T-cell response could potentially serve as a more accurate biomarker for COVID-19 immunity than antibody titer. Effort has already been put into developing lab-based centralized T-cell testing for COVID-19 immunity check, and one has obtained emergency use authorization from US Food and Drug Administration (FDA) (34). Commonly used T-cell detection assays include flowcytometry and enzyme-linked immune absorbent spot (ELISpot). However, these assays are not readily translatable for POC testing. Sample preparation presents the greatest challenge for POC T-cell immunity testing. In the case of ELISpot, sample preparation could be accomplished by separating CD4 and CD8 T-cells from whole blood using immuno-conjugated magnetic particles. Assays that rely on magnetic particles can be readily translated to POC testing by using magnetic digital microfluidics. While these proposed approaches of POC T-cell immunity testing are feasible, it still presents a great challenge due to the complex assay format, and resource must be devoted to validate and optimize these testing for clinical use.

Considerations for Implementing POC Immunodiagnostic Testing

POC is an umbrella term that describes a wide variety of healthcare settings. Applicable scenarios for POC immunodiagnostic testing should be defined by answering "where is the point," "who to care," and "what and how to test" (35). In the context of POC immunodiagnostic testing for COVID-19 vaccine appropriation, we have categorized POC settings in three classes and summarized them in **Table 1** according to resource availability and testing requirements. While the framework already dictates "who to care" and "what to test," we need to examine "where" and "how" to conduct POC immunodiagnostic testing.

POC immunodiagnostic testing is recommended for community (Category II) and limited healthcare (Category III) settings. However, centralized testing should be given the priority if it is easily accessible in Category III settings. POC immunodiagnostic testing is not recommended for self-testing in home care settings (Category I) because a certain level of expertise is required to handle the sample, conduct the test and interpret the results. However, users may collect the testing on their own and send the samples for testing in community testing center or primary healthcare facilities.

To ensure the reliability of POC testing, training must be provided to local recruits, and routine inspection should be conducted to ensure test procedures are standardized, devices are calibrated and test kits are properly stored. Other considerations for POC immunodiagnostic testing include proper biohazard waste disposable protocol. Standard biohazard disposable protocol is usually already installed in Category III settings. Local sources and environment should be taken into consideration when establishing biohazard disposable protocol in Category II setting.

TABLE 1 | Settings and applicable scenarios for POC immunodiagnostic testing.

	Category I	Category II	Category III
POC settings (where is the point)	Self-testing in home care setting	IIa: Community with adequate resource IIb: Community with poor resource	IIIa: Primary healthcare setting IIIb: Bedside, examination room, emergency department in full-fledged hospital
Considerations for testing implementation (how to test)	- Certain level of expertise is required to conduct POC immunodiagnostic testing - Certain risks associated with handling potential contagious biosamples	- Relatively intensive training is required due to the general lack of medical background in local recruits - Select suitable POC immunodiagnostic testing according to local resource availability (e.g., electricity, cold chain, etc.) - Conduct Inspection at high frequency - Develop protocol for biohazard disposal according to local environment and resource	- Conduct necessary training - Integrate POC immunodiagnostic testing into existing medical managing systems
Recommendation	POC immunodiagnostic testing is not recommended for self-testing except for self-sample-collection	LFA is recommended for POC immunodiagnostic testing in Category IIa and IIb. Quantitative POC immunodiagnostic testing is recommended for IIa. POC testing for T-cell immune response is recommended for Category IIa if conditions permit.	Use POC immunodiagnostic testing as a supplement. Centralized testing should be given the priority if it is easily accessible. POC testing for T-cell immune response is recommended for Category IIa if conditions permit.

CONCLUSION AND OUTLOOK

The rapid pace of COVID-19 vaccine development brings hope to bring the pandemic under control. But the huge discrepancy between supply and demand means that difficult decision must be made on how to allocate this scarce resource in the way that is both fair and most efficacious (16). In this perspective, we propose an immuno-triaging framework for evidence-based COVID-19 vaccine appropriation. The implementation of the framework and the role of POC immuno-testing in the framework are described in detail, and the concerns with existing COVID-19 POC testing are also discussed. We hope the proposed framework could provide an objective approach to ensure fair and efficient utilization of the scarce vaccine resource at the individual level that also maximizes the collective societal benefit.

Accurate and precise POC immunodiagnostic testing could be a key tool in vaccine immuno-triage. Nonetheless, many existing POC immunodiagnostic tests only measure antibody response and are plagued by poor performance. Better POC tests and testing algorithms are needed to implement the proposed framework. New POC immunodiagnostic testing for T-cell immune response could further improve the identification of patients who do (and don't) need further booster vaccines. A cloud-based centralized information system to coordinate decentralized vaccination centers will provide the digital infrastructure to ensure the successful implementation of the proposed framework. We encourage both industry and academia to prioritize the development of POC immunodiagnostic tests for the second half of the battle against COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

YZ and SY conceptualized the framework and wrote the manuscript. AR, KN, and JG improved the framework and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: YZ declares equity interest in DropLab Scientific Ltd. DropLab Scientific is a startup company founded in Guangzhou, China in 2020. It is a spinoff of Sino-Singapore Joint International Research Institute and Nanyang Technology University Singapore. DropLab Scientific aims to commercialize magnetic droplet microfluidic technology for a wide range of applications such as energy harvest, in vitro diagnostics, and manufacturing.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Age-Adjusted Associations Between Comorbidity and Outcomes of COVID-19: A Review of the Evidence From the Early Stages of the Pandemic

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Objectives: Early in the COVID-19 pandemic, people with underlying comorbidities were overrepresented in hospitalised cases of COVID-19, but the relationship between comorbidity and COVID-19 outcomes was complicated by potential confounding by age. This review therefore sought to characterise the international evidence base available in the early stages of the pandemic on the association between comorbidities and progression to severe disease, critical care, or death, after accounting for age, among hospitalised patients with COVID-19.

Methods: We conducted a rapid, comprehensive review of the literature (to 14 May 2020), to assess the international evidence on the age-adjusted association between comorbidities and severe COVID-19 progression or death, among hospitalised COVID-19 patients – the only population for whom studies were available at that time.

Results: After screening 1,100 studies, we identified 14 eligible for inclusion. Overall, evidence for obesity and cancer increasing risk of severe disease or death was most consistent. Most studies found that having at least one of obesity, diabetes mellitus, hypertension, heart disease, cancer, or chronic lung disease was significantly associated with worse outcomes following hospitalisation. Associations were more consistent for mortality than other outcomes. Increasing numbers of comorbidities and obesity both showed a dose-response relationship. Quality and reporting were suboptimal in these rapidly conducted studies, and there was a clear need for additional studies using population-based samples.

Conclusions: This review summarises the most robust evidence on this topic that was available in the first few months of the pandemic. It was clear at this early stage that COVID-19 would go on to exacerbate existing health inequalities unless actions were taken to reduce pre-existing vulnerabilities and target control measures to protect groups with chronic health conditions.

Keywords: COVID-19, coronavirus, comorbidity, chronic disease, review

INTRODUCTION

After first emerging at the end of 2019, the novel coronavirus SARS-CoV-2 had infected at least 4.6 million people globally by mid-May 2020, with 0.3 million deaths. By July 2021, this had increased to at least 178.8 million people infected and 3.8 million deaths (1). The pandemic remains uncontained in many parts of the world, raising grave concerns about vaccine distribution keeping pace with subsequent waves and new variants. To minimise mortality and morbidity as the pandemic continues, and to direct scarce resources most appropriately, it is crucial to understand better the risk factors for progression to severe coronavirus disease 2019 (COVID-19) and death. It is also important to capture the nature of the evidence that was available to policymakers in the early months of the pandemic, especially given the many government enquiries that will be undertaken around the world into how well the early response tackled the crisis.

Initial reports from China and Italy suggested that people with underlying comorbidities were overrepresented in hospitalised cases and were at increased risk of progression to severe disease and death (2–4). Other countries subsequently reported similar findings (5, 6). Given that the prevalence of comorbidity increases with age, it was unclear whether and how comorbidity independently influences COVID-19 outcomes. Many early studies of COVID-19 epidemiology reported baseline comorbidities of hospitalised patients but not age-adjusted estimates of excess risk associated with comorbidities. Given the high prevalence of chronic disease globally (7), a better understanding of the age-adjusted relationship between comorbidity and COVID-19 outcomes would enhance health service planning and inform clinical management.

We conducted a rapid but comprehensive review of studies in the early stages of the pandemic when hospitalised patients were the population subgroup most readily accessible for research. We are mindful, however, of potential selection bias in these samples due to differential healthcare use, limited SARS-CoV-2 testing in the wider population, and under-ascertainment of asymptomatic and mild cases (8). This review therefore sought to characterise the international evidence base available in the early stages of the pandemic on the association between comorbidities and progression to severe disease, critical care, or death, after accounting for age, among hospitalised patients with COVID-19. We considered evidence to mid-May 2020, 5 months after the viral infection was first identified in Wuhan, China, and only 2 months after the World Health Organization (WHO) declared it a pandemic.

METHODS

Search Strategy and Selection Criteria

Our search was designed to address the question: according to the evidence base in the early stages of the COVID-19 pandemic, what was the age-adjusted association between co-morbidities and severe outcomes in hospital patients? We searched the literature to identify age-adjusted estimates of association between any comorbidity and in-hospital severe COVID-19 outcomes (**Table 1**), reported in peer-reviewed studies, pre-prints from repositories such as medRxiv, and several grey literature sources, published by 14 May 2020, in English, from any country (some searches preceded this date; see below). We defined comorbidity as a pre-existing health condition present at admission to hospital with COVID-19, including obesity but excluding health-related behaviours such as smoking.

The search strategy had five arms (**Figure 1**). First (7 April; updated 12 May 2020), we searched the MEDLINE full-text database (as title and abstract often omit age-adjustment) to identify analytical (rather than descriptive) studies that focused on comorbidities specifically or that reported multivariable analysis of risk factors (including comorbidities) for severe outcomes of COVID-19 (see Appendix 1 in **Supplementary Material** for full search terms). Second (on 14 May), we searched the medRxiv pre-print database. Third, we screened studies from our companion review on COVID-19 critical care outcomes (Pennington et al. unpublished, which by then had screened 2,665 items) for any meeting our narrower search criteria. Fourth, all studies identified in that companion review underwent Web of Science and Google Scholar forward-citation searches, with initial filtering for key terms relating to comorbidity and age (on 7 April). Fifth, additional sources searched (initially in April; updated 11 May) included: WHO; communicable disease centres of the USA, Europe, and China; and several COVID-19-specific evidence resources online ("other sources" in **Figure 1**; see Appendix 1 in **Supplementary Material** for details).

Screening for Inclusion

In Arms 1 and 2, title-abstract screening by one reviewer excluded studies clearly not meeting the inclusion criteria, followed by independent title-abstract screening of the remainder by two reviewers in EPPI Reviewer-4 (11). In each of Arms 3–5, title-abstract screening was followed by full-text screening by a single reviewer. Outputs of Arms 1–5 were combined, and remaining duplicates were excluded. To facilitate this *rapid* review, three reviewers shared searching and screening tasks, rather than repeating tasks independently, except where otherwise stated. Two reviewers independently screened the full text of the final set of potentially eligible studies. On 27 May, included pre-prints were checked for subsequent peer-reviewed publication (and again a year later for the post-script of this definitive article).

Data Extraction

One reviewer extracted age-adjusted estimates of excess risk (odds ratios or hazard ratios) associated with any comorbidity for the outcomes of interest. A second reviewer checked each extraction for accuracy. Where studies reported multiple estimates adjusted for different sets of covariates (e.g., age alone, age plus sex), one reviewer extracted all estimates then, with checking by others, selected the most appropriate estimate for reporting in the review, prioritising the most appropriate

TABLE 1 | Review inclusion and exclusion criteria: what was the age-adjusted association between comorbidities and severe or critical care outcomes in hospital patients with COVID-19 in the early stages of the pandemic?

	Include	**Exclude**
Population	Adult COVID-19 hospital patients. Studies with 10 or more patients.	Samples nested in clinical trials, samples from cruise ships, familial clusters. Community cases not receiving care in hospitals, including general population estimates of the spread of COVID-19. Studies focusing solely on infants and children (not part of a study including adults).
Outcomes	Relative risk, hazard ratio, odds ratio associated with comorbidity (pre-existing condition, chronic illness) status on admission, of: i. progression to severe disease ii. admission to critical or intensive care unit iii. invasive or non-invasive ventilation iv. death in hospital v. any adverse event (i.e., composite indicators of any of i–iv), for any reported comorbidity.	Other treatments inside and outside critical or intensive care departments, e.g., rates of patients receiving oxygen supplementation.
Comparison	Patients with and without any comorbidity at admission to hospital. Comorbidity was defined as pre-existing health conditions present at admission to hospital with COVID-19, including obesity.	Comparisons within a sample of patients who all have a comorbidity (e.g., studies of cancer patients only). Comparisons between groups of people based on their health-related behaviours (e.g., smoking), ethnicity, or socioeconomic circumstances.
Study design	All primary quantitative empirical observational studies that reported estimates of the independent relative hazard/odds of experiencing a severe outcome *according* to comorbidity status, adjusted for age only, or age and other plausible confounders of that association.	Any studies in which all estimates of excess risk associated with comorbidity were also adjusted for potential mediators between comorbidity and severe disease outcomes, such as clinically ascertained biomarkers (e.g., inflammatory response or organ function). Causal interpretation of hazard/odds ratios is inappropriate from models not designed to account for confounding of the exposure-outcome association of interest (9, 10), therefore in this review estimates would likely be biased towards the null if adjusted for clinical biomarkers. Qualitative studies. Intervention studies (e.g., clinical trials of new treatments for COVID-19). Projections or estimations of potential outcomes. Non-empirical studies, including editorials, opinions, or discussion pieces. Studies that do not report comorbidity-related risk estimates Review-level evidence
	Include	**Exclude**
Publication characteristics		
Publication stage, type	Pre-prints, peer-reviewed publications, grey literature on empirical evidence (e.g., official statistics).	Not applicable.
Language	English language publications.	Non-English language publications (not available for full text).
Date	Studies published between December 2019 and 14th May 2020.	

adjustment, e.g., for age and sex rather than age alone and not including potential mediators.

Synthesis

Evidence was synthesised narratively (12, 13) and, after piloting, study quality was assessed using a modified version of the Institute of Health Economics (14) quality appraisal checklist for case series studies (as recommended by the National Institute of Health and Care Excellence). Limited numbers of studies assessing each individual comorbidity, heterogeneity in key measures and statistical methods, and the inclusion of non-peer-reviewed pre-prints meant that meta-analysis was inappropriate. Extracted estimates are summarised in forest plots. Studies of mortality reported a mix of hazard ratios (HRs) and odds ratios (ORs), so we present both in the forest plots, but it should be noted that ORs and HRs are not directly comparable – ORs overestimate the relative risk of an outcome in studies (such as these) where the outcome is common (15).

RESULTS

Overall, 1,100 titles-abstracts and 118 full texts were screened (see **Figure 1** for exclusions). Of those 118 full texts, 101 were identified in the MEDLINE search, nine from medRxiv, three from the forward citation search from the companion review, and five from other sources. After full-text screening, 14 studies (16–29) met the inclusion criteria. Of these, seven were published in peer-reviewed journals and seven were identified in non-peer-reviewed pre-print form. Four of the pre-prints were included in the review as pre-prints (19, 25, 26, 29) while three were replaced with a peer-reviewed version before analysis (16, 18, 24).

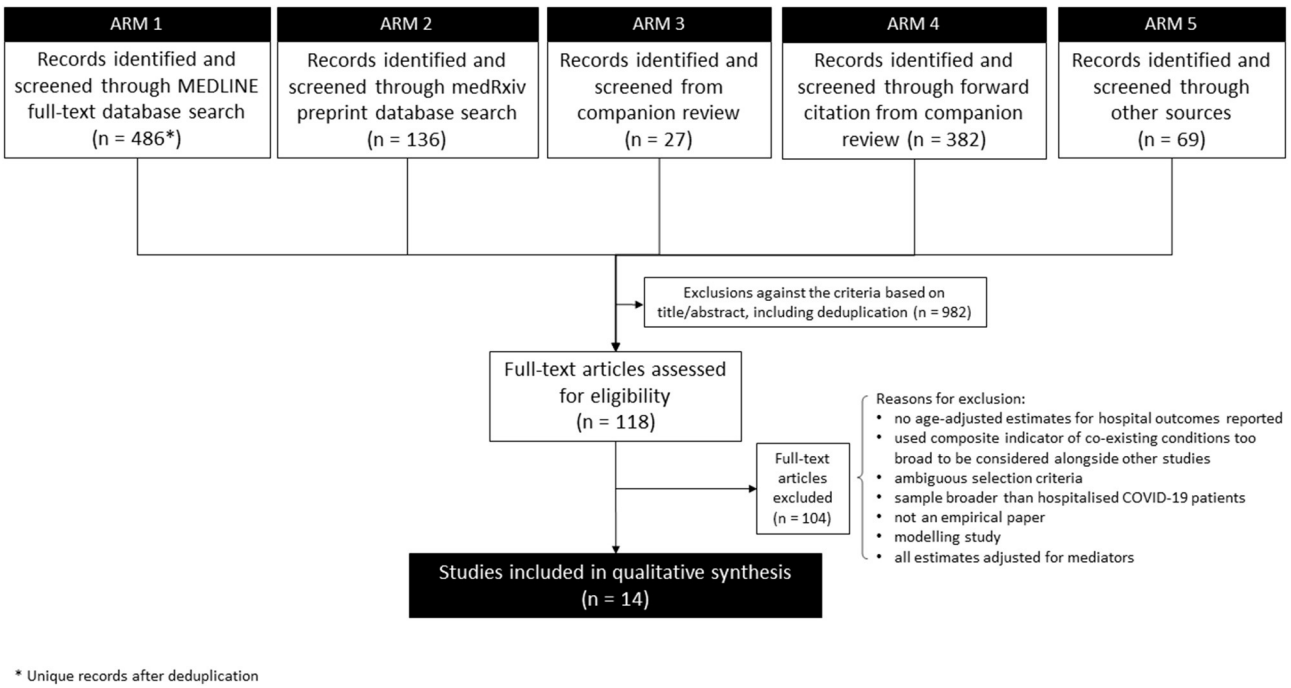

FIGURE 1 | Flowchart of progression of studies through the review of age-adjusted associations between comorbidity and outcomes of COVID-19 in the early stages of the pandemic.

Characteristics of the Included Studies

Six of the included studies were from China during the initial stages of the pandemic. Three of these used data from Wuhan and the Hubei province, the epicentre of the Chinese outbreak (17, 27, 29), while the others focused on cases hospitalised outside Hubei, using either national samples (20), city-wide reporting systems (28), or records from a single tertiary hospital in another province (16). Four studies used data from the USA (19, 21, 22, 24), three from the UK (18, 25, 26), and one from Iran (23). Eight were multi-centre studies. Sample sizes for the relevant estimates ranged from 103 (21) to 15,194 (18). Study quality varied from low (quality score 8/20) to moderately high (15/20) (**Table 2**; **Supplementary Figure 1**). The narrative synthesis of results focuses more on larger and higher quality studies.

Included studies mostly reported retrospective analyses of hospital records, usually with a case series or retrospective cohort study design and assessing associations between multiple risk factors, including comorbidities, and various outcomes. Although many were labelled as cohort studies, they did not generally recruit a random sample (convenience sampling was common), and many did not clarify whether the duration of follow-up allowed all participants to reach a study endpoint or recover. Detail of sample construction in many studies was scant.

For the outcome, three studies reported a composite severe endpoint (all including death or admission to ICU) (17, 20, 26), seven reported death (18, 22–25, 27, 29), and four reported severe disease, including ICU admission (16, 19, 21, 28). Three examined mechanical ventilation or intubation as a separate endpoint (19, 21, 24).

Comorbidities analysed by more than one study were overweight and obesity (five studies), diabetes mellitus (seven studies), hypertension and heart disease (seven studies), cancer (four studies), and chronic respiratory conditions such as chronic obstructive pulmonary disease (COPD) (five studies). The largest study also examined dementia, kidney disease, liver disease, and neurological conditions (not defined but giving stroke as an example). Additionally, four studies reported the association of one of the outcomes with the presence of any (or multiple) comorbid conditions rather than, or as well as, specific conditions (19, 20, 23, 25). Eight studies collated information on comorbidities from medical records, two studies included self-reported comorbidities, and four did not report the data collection method. Two studies reported robust methods of collating data on comorbidities involving cross-checking primary and secondary care records (25) or quality checks on extracted data (19). Only two studies mapped comorbidities to International Classification of Disease (ICD)-10 codes (19, 25).

All studies adjusted for age using multivariable regression models [Cox proportional hazards ($n = 5$), logistic ($n = 8$), both ($n = 1$)]. All but two studies also adjusted for one or more additional covariates, but these differed across studies. Additional covariates used in more than one study included sex, smoking, other specific comorbidities, and ethnicity. Although several papers acknowledged that missingness would probably be substantial in a pandemic context, only four formally reported missing data, and two imputed missing values.

TABLE 2 | Summary of included studies in review of age-adjusted associations between comorbidity and outcomes of COVID-19 in the early stages of the pandemic.

References	Comorbidities analysed	Setting	Single- or multi-centre study	Sample size	Quality score (max. 20)
Sapey et al. (25)	Any (of hypertension, diabetes mellitus, cancer, chronic lung disease, and others)	Birmingham, UK	Multi	2,217	15
Docherty et al. (18)	Obesity, hypertension, heart disease, diabetes, cancer, chronic lung disease (non-asthma), and others	UK (nationwide)	Multi	15,194	14
Cai et al. (16)	Obesity	Shenzhen, Guangdong Province, China	Single	387	14
Wang et al. (27)	Hypertension, heart disease	Wuhan, Hubei Province, China	Single	296	14
Palaiodimos et al. (24)	Obesity	New York, USA	Single	200	14
Guan et al. (20)	Hypertension, diabetes, cancer, chronic obstructive pulmonary disease; and any in combination	China (nationwide)	Multi	1,590	13
Zhang et al. (29)	Diabetes mellitus	Wuhan, Hubei Province, China	Single	258	13
Kalligeros et al. (21)	Obesity, hypertension, heart disease, diabetes, lung disease	Rhode Island, USA	Multi	103	13
Teo et al. (26)	Hypertension, ischaemic heart disease, diabetes mellitus	London, UK	Multi	437	12
Ebinger et al. (19)	Obesity, hypertension, heart disease, diabetes mellitus, chronic obstructive pulmonary disease or asthma; and any in combination	Los Angeles, USA	Multi	214	12
Dai et al. (17)	Cancer	Hubei Province, China	Multi	105 cancer patients, 536 controls	11
Nikpouraghdam et al. (23)	Any (of hypertension, cardiovascular disease, diabetes, cancer, chronic lung disease, and other)	Tehran, Iran	Single	2,964	10
Mehta et al. (22)	Cancer	New York, USA	Single	218 cancer patients, 1,090 controls	8
Yu et al. (28)	Hypertension, heart disease, diabetes, lung disease	Shanghai, China	Multi	333	8

Comorbidity and Severe COVID-19 Outcomes: Disease Progression, Critical Care, and Mortality

Across all comorbidities, the studies with the largest sample sizes and widest geographical coverage consistently showed evidence of increased risk of severe COVID-19 outcomes following hospitalisation associated with the presence of the comorbidity. These associations appeared to hold over studies of varying quality. Smaller studies tended to report similar point estimates to the larger studies but with wide confidence intervals, sometimes consistent with no association, possibly indicating insufficient statistical power. Evidence was most consistent for associations between comorbidities and death but more mixed for other outcomes (**Figures 2–5**; **Supplementary Table 1**).

Any Comorbidity and Multiple Comorbidities

Hospitalised patients with any comorbidity were more likely to be admitted to ICU, require invasive ventilation, or die from COVID-19, in three of the four studies that examined comorbidities collectively. In a study of 2,217 patients in a large UK city, Sapey et al. examined the effect of "any comorbidity" on mortality and found evidence of a dose response after adjusting for age, sex, ethnicity, and deprivation (25). Patients with one or two comorbidities had a 115% greater hazard of death than patients without comorbidities (95% CI: 1.50, 3.09), and those with three or more had 200% increased hazard of death (95% CI: 2.09, 4.31). Similarly, in a nationwide study of 1,590 patients, Guan et al. (20) also found a dose-response relationship: after adjustment for age and smoking status, patients with a single comorbidity had a 79% greater hazard of a severe outcome than patients without comorbidities, while those with multiple comorbidities had a 159% increased hazard (HR = 2.59, 95% CI: 1.61, 4.17). A study of mortality in 2,964 patients in Iran reported an odds ratio of 1.53 (95% CI: 1.04, 2.24) for any comorbidity compared with none (23). In contrast, a small study in the USA (19) reported on a validated comorbidity score and found no significant association between a standard deviation increase in the comorbidity score and either ICU admission (OR = 1.12, 95% CI: 0.86, 1.47) or intubation (OR = 0.86, 95% CI: 0.63, 1.18).

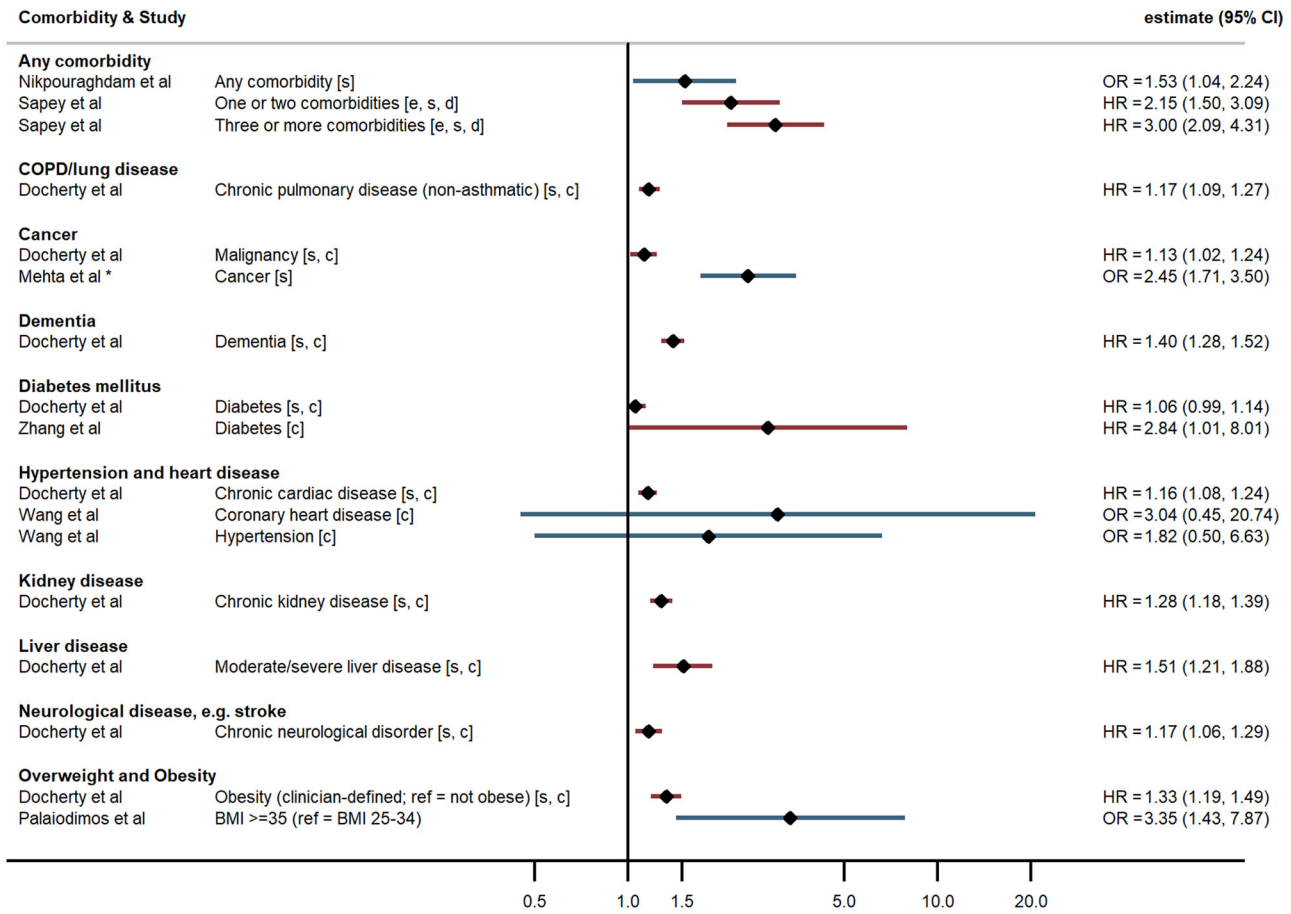

FIGURE 2 | Forest plot of study estimates of the association between comorbidities and COVID-19 mortality among hospitalized patients. Reference category for each comorbidity is the absence of that comorbidity, except where stated otherwise. HR, hazard ratio (red); OR, odds ratio (blue); CI, confidence interval; BMI, body mass index; COPD, chronic obstructive pulmonary disease. All estimates adjusted for age. Additionally adjusted for: sex [s]; other comorbidities [c]; ethnicity [e]; deprivation [d]. *95% CI not reported, but back-calculated from reported *p*-value.

Overweight and Obesity

In the largest study, Docherty et al. (18) reported hazard ratios for various comorbidities and other risk factors among 15,194 COVID-19 patients in UK hospitals. They found that obesity was associated with higher risk of COVID-19 mortality, after adjusting for age, sex, and other comorbidities (HR = 1.33, 95% CI: 1.19, 1.49). In two US studies, severe obesity (BMI $\geq$35 kg/m^2) was associated with higher odds of requiring invasive ventilation or dying from COVID-19 (24) compared with people who were overweight or moderately obese, and with higher odds of progressing to severe COVID-19 (21) compared with people of normal weight, independent of age, sex, and ethnicity. Cai et al. (25) examined the relationship between overweight and obesity and progression to severe pneumonia in 387 hospitalised COVID-19 cases in Shenzhen, a Chinese city in Guangdong province. They found that obesity significantly increased the age-adjusted risk of COVID-19 patients developing severe pneumonia (OR = 3.35, 95% CI: 1.47, 7.63). A dose-response relationship was observed, with overweight patients at intermediate risk relative to patients of healthy weight (OR = 1.78, 95% CI: 1.00, 3.21). This relationship was particularly pronounced in men (OR = 5.40, 95% CI 1.93, 15.09). In contrast, a study of 214 COVID-19 patients in Los Angeles, USA, found no evidence of an association between either overweight or obesity and severe disease, or the need for invasive ventilation, having adjusted for age and sex (19).

Hypertension and Heart Disease

Evidence of a relationship between pre-existing hypertension or heart disease and severe COVID-19 outcomes was mixed. In China, Guan et al. found that, after adjusting for age and smoking status, patients with hypertension at admission were 58% more likely to reach the composite severe endpoint (ICU admission, invasive ventilation, or death) than those without hypertension (HR = 1.58, 95% CI: 1.07, 2.32) (15). All other studies that

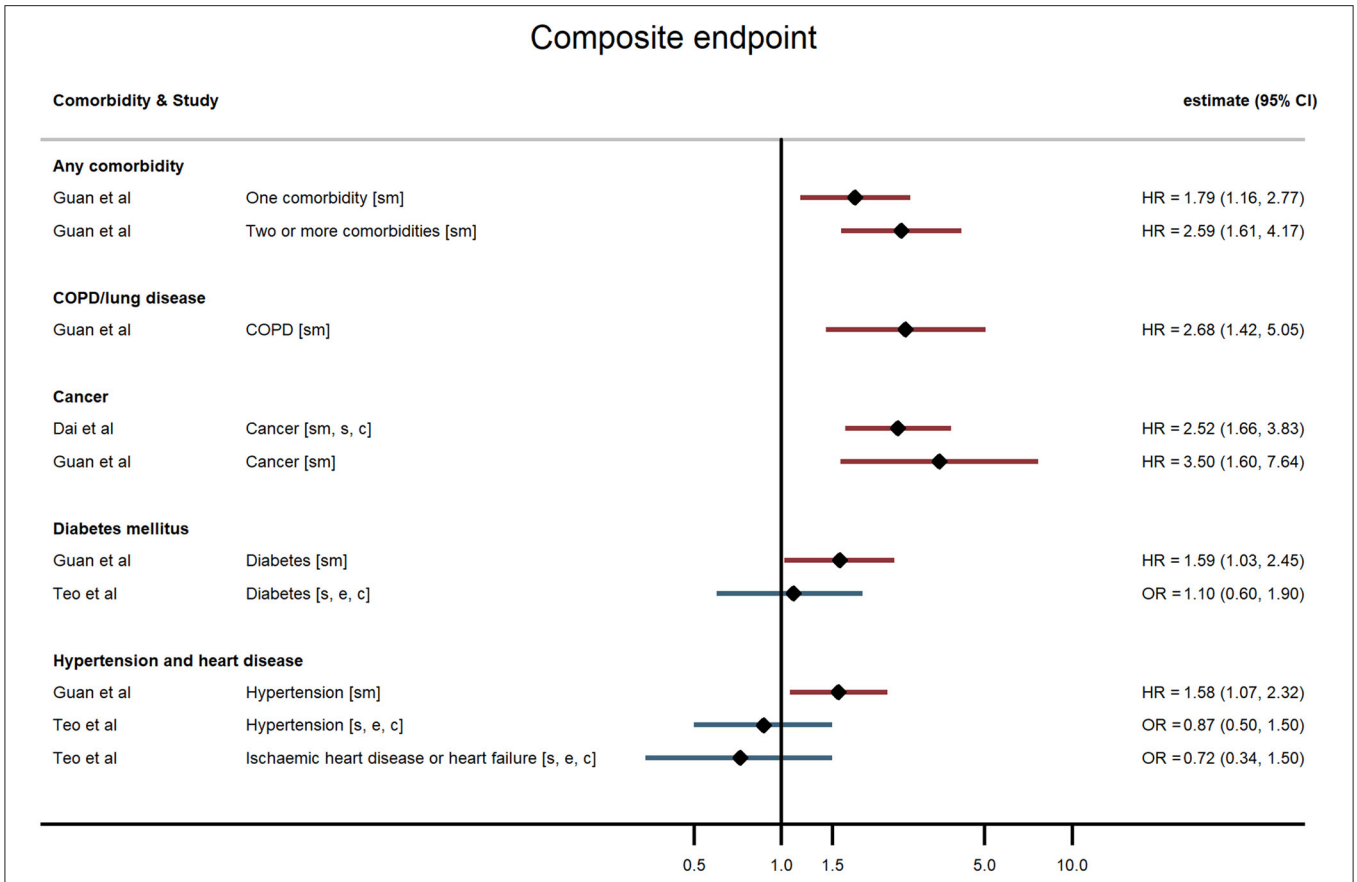

FIGURE 3 | Forest plot of study estimates of the association between comorbidities and composite severe endpoint (death, intensive care unit admission, severe or critical symptoms, or invasive mechanical ventilation) among hospitalized COVID-19 patients in the early stages of the pandemic. Reference category for each comorbidity is the absence of that comorbidity, except where stated otherwise. ICU, intensive care unit; HR, hazard ratio; OR, odds ratio; CI, confidence interval; COPD, chronic obstructive pulmonary disease. All estimates adjusted for age. Additionally adjusted for: smoking [sm]; sex [s]; other comorbidities [c]; ethnicity [e].

examined hypertension (in the UK, China, and the USA) found no evidence of an association with any outcome (19, 21, 26–28).

For chronic heart disease, Docherty et al. (18) found pre-existing disease was associated with higher risk of mortality after adjusting for age, sex, and other comorbidities (HR = 1.16, 95% CI: 1.08, 1.24). Evidence from other studies was mixed. Kalligeros et al. for example, found heart disease to be associated with greater odds of the need for invasive ventilation but not with admission to intensive care (21).

Diabetes Mellitus

In studies examining diabetes mellitus as a comorbidity, authors did not distinguish between Type 1 and Type 2 diabetes. The large UK study of 15,194 patients reported a small (6%) but non-significant increased hazard of death for people with compared with those without diabetes following hospitalisation with COVID-19 after adjusting for age, sex, and other comorbidities (HR = 1.06, 95% CI: 0.99, 1.14) (18). The nationwide study in China (20) found that hospitalised COVID-19 patients with diabetes had a 59% increased risk of the composite severe endpoint (ICU admission, invasive ventilation, or death) (HR = 1.59, 95% CI: 1.03, 2.45), independent of age and smoking status. Similarly, in a study of 258 COVID-19 patients at a Wuhan hospital, those patients with diabetes were more likely to die in hospital HR = 2.84, 95% CI: 1.01, 8.01) (29). In contrast though, four other studies found no evidence of an age-adjusted association between diabetes and any severe outcome (19, 21, 26, 28).

Cancer

All four studies examining cancer found it to be a risk factor for severe outcomes following hospitalisation with COVID-19. Docherty et al. (18) found a slightly elevated risk of death amongst UK-based COVID-19 patients with cancer (HR = 1.13, 95% CI: 1.02, 1.24). Guan et al. (20) found a substantially elevated risk of their composite severe endpoints – after adjusting for age and smoking status, patients with cancer had 3.5-fold the hazard of ICU admission, invasive ventilation, or death in hospital compared with patients without cancer (95% CI: 1.60, 7.64). Dai et al. (17) found a similar relationship in their sample of cancer patients and matched non-cancer patients in Hubei province, China (HR = 2.52, 95% CI: 1.66, 3.83) after additionally adjusting

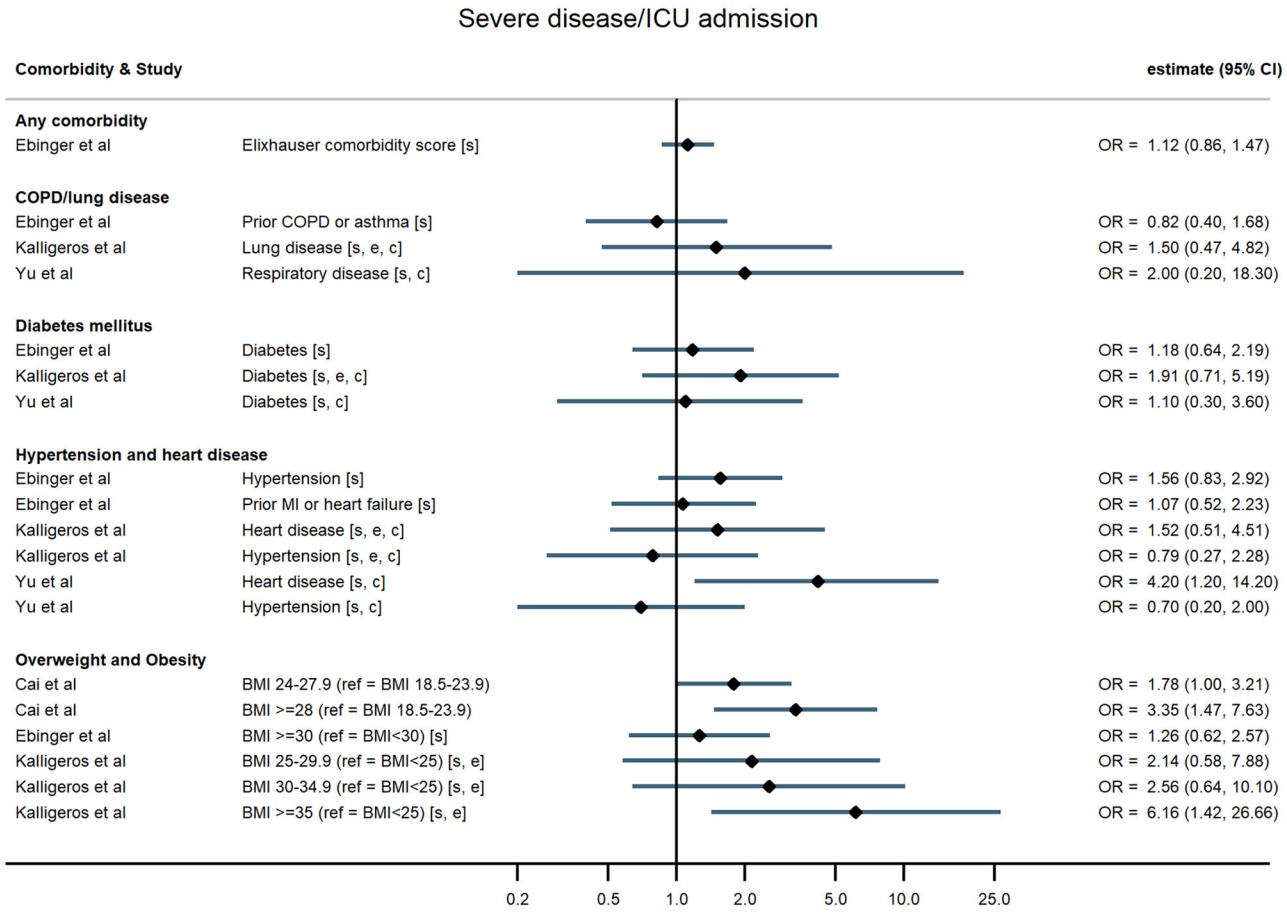

FIGURE 4 | Forest plot of study estimates of the association between comorbidities and progression to severe disease or intensive care unit admission among hospitalized COVID-19 patients in the early stages of the pandemic. Reference category for each comorbidity is the absence of that comorbidity, except where stated otherwise. ICU, intensive care unit; OR, odds ratio; CI, confidence interval; BMI, body mass index; COPD, chronic obstructive pulmonary disease; MI, myocardial infarction. All estimates adjusted for age. Additionally adjusted for: sex [s]; other comorbidities [c]; ethnicity [e].

for other comorbidities. They also reported relative hazards by cancer stage and type, finding that association with the composite severe endpoint was strongest for metastatic cancer and for lung and blood malignancies. Mehta et al. (22) used a similar study design and reported an age- and sex-adjusted odds ratio of 2.45 for cancer and in-hospital mortality.

Chronic Obstructive Pulmonary Disease

Five studies reported associations with pre-existing lung disease, either COPD specifically (20) or a broader set of pulmonary conditions (18, 21, 25, 28). One study excluded asthma, two included asthma, and one study did not state whether asthma was included. Docherty et al. reported 17% increased hospital mortality associated with non-asthmatic pulmonary disease (HR = 1.17, 95% CI: 1.09, 1.27), independent of age, sex, and other comorbidities (18). Guan et al. found that hospitalised patients with COPD had 168% higher risk of reaching that study's composite severe endpoint (ICU admission, invasive ventilation, or death) than patients without COPD (HR = 2.68, 95% CI: 1.42, 5.05), adjusted for age and smoking status (20). None of the three remaining studies – using endpoints of progression to severe disease and invasive ventilation – found an association with chronic lung disease, broadly defined (19, 21, 28).

Other Comorbidities

One large UK-wide study also found neurological disorders (giving the example of stroke), dementia, liver disease, and chronic kidney disease were all associated with increased risk of mortality after adjusting for age, sex, and other comorbidities (18).

Post-script: Of the four pre-prints included in the analysis (19, 25, 26, 29), a final check in May 2021 found that two (19, 25) now had a peer-reviewed publication (30, 31). There were no substantive changes in reported evidence in the published versions compared with the pre-print versions.

DISCUSSION

Summary of Findings

Five months after the first outbreak of COVID-19 in Wuhan, China, and 2 months after the WHO declared COVID-19 a pandemic, research was limited on comorbidities as

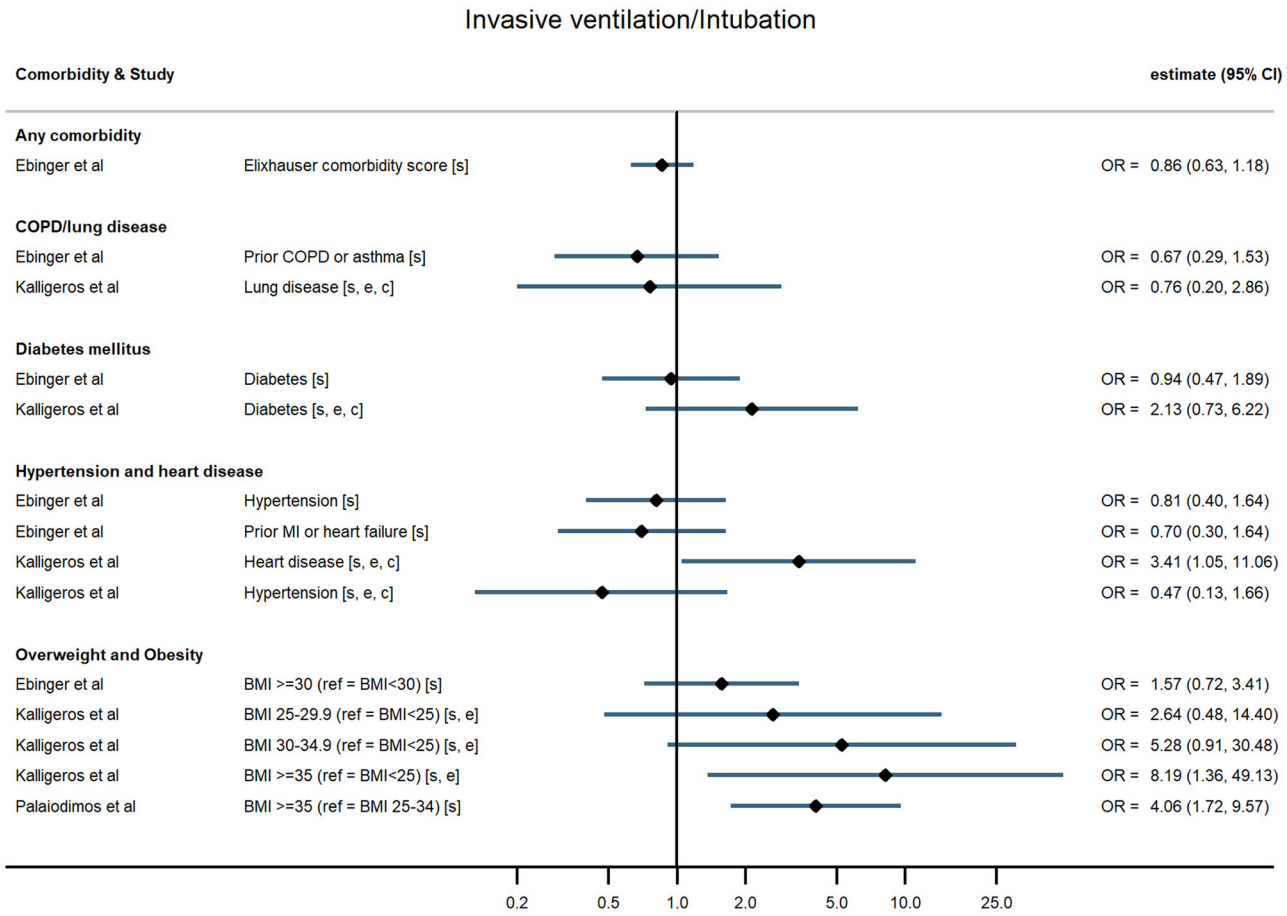

FIGURE 5 | Forest plot of study estimates of the association between comorbidities and invasive mechanical ventilation among hospitalized COVID-19 patients in the early stages of the pandemic. Reference category for each comorbidity is the absence of that comorbidity, except where stated otherwise. OR, odds ratio; CI, confidence interval; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease. All estimates adjusted for age. Additionally adjusted for: sex [s]; other comorbidities [c]; ethnicity [e].

independent risk factors for severe COVID-19. Our review of that emerging evidence base indicates that by mid-May 2020 there was broad support (32) for the hypothesis that many underlying health conditions confer additional risk of mortality among people hospitalised with COVID-19, independent of age. Evidence of increased risk of other severe COVID-19 outcomes was mixed. Most studies found that having at least one of obesity, diabetes, hypertension, heart disease, cancer, or COPD was significantly associated with worse outcomes following hospitalisation. A dose-response relationship was reported for increasing numbers of comorbidities, and evidence linking overweight and obesity to severe outcomes was strongest for more severe obesity. Overall, evidence for obesity and cancer increasing risk of severe disease or death was most consistent, with all but one of the numerous relevant studies reporting an increased risk associated with these conditions. Evidence was weakest for hypertension as an independent risk factor for severe outcomes. Two similar reviews that were published whilst our rapid review was under peer review – and included some more recent studies – found similar results (33, 34), although another found no association with obesity in meta-analysis (33–35).

Comorbidity has previously been shown to be associated with elevated risk of worse clinical outcomes in other severe acute respiratory outbreaks such as SARS (severe acute respiratory syndrome), MERS (Middle East respiratory syndrome), and avian influenza (36–38). This review suggests that comorbidity also pre-disposes individuals to poorer outcomes in the current COVID-19 pandemic. Whilst the mechanisms remain poorly understood, there are numerous biologically plausible explanations. The pathogenesis of severe COVID-19 is thought to involve dysregulated proinflammatory immune response and subsequent multi-system damage (39–41). Many underlying conditions may leave affected individuals more vulnerable to the effects of this. Obesity tends to reduce lung function and dysregulate the immune system (42). Similarly, diabetes mellitus can impair immune function (43), as do many cancer treatments. Patients with pre-existing cardiovascular disease may be at heightened risk of severe outcomes through various mechanisms, including therapeutic upregulation of ACE2 (the host receptor

for SARS-CoV-2) and greater vulnerability to hyperinflammatory immune responses and cardiac complications that are common with severe COVID-19 (9, 44).

Strengths and Limitations of the Evidence Base

This review includes two studies that are still to undergo peer-review. Such studies must be treated cautiously, but the need to summarise timely evidence in an emerging pandemic justifies including them, while requiring "a permanent critical attitude from the readers" (45). Of the other two studies originally included in pre-print form but subsequently peer-reviewed and published within a year of the analysis, we noted no substantive differences between the pre-print and published version. Quality appraisal revealed important limitations in most included studies: weaknesses in design or execution, inadequate detail, or lack of clarity in reporting, particularly around sampling, and inclusion and exclusion criteria.

Our review is limited to the hospitalised population of COVID-19 cases, a highly selected sample of the population. These studies are therefore at risk of selection (or collider) bias (46), which can induce spurious associations leading to flawed conclusions, particularly when the prevalence of a risk factor in the sample differs from its prevalence in the target population (8, 47). Viral load is an unmeasured factor that may differ between hospitalised and non-hospitalised COVID-19 patients and also be associated with risk of severe outcomes. In several included studies, obesity prevalence differed considerably in the study population from the wider population of the same country (16, 18, 19). The extent of missing data was also underreported in many studies, possibly adding further selection bias. The results therefore may not reflect causal effects, and there remains a need for confirmation with larger, population-based studies. Although some studies in this review acknowledged possible selection bias (and it was probably unavoidable early in an outbreak context) none included sensitivity analyses to assess this risk. Furthermore, patients in many studies had not yet reached their clinical endpoint. We now also note that similar reviews were published while ours was under peer review, and are also primarily focused on samples of hospitalised patients [e.g., (33–35)].

There was substantial heterogeneity in outcome and comorbidity definitions and the clarity of their reporting, compromising comparison of results and precluding pooling of estimates. Most studies used electronic health records, but many did not clearly specify data collection methods in any further detail, particularly for recording comorbidities. In some, lack of rigour in comorbidity ascertainment might have led to misclassification, but without more information it is difficult to assess how likely this is as a source of bias. Only two studies included ICD-10 criteria for comorbidities, no study distinguished between Type 1 and Type 2 diabetes, and BMI categorisations also differed between studies.

Across studies, models differed considerably in adjusting for obvious confounders, such as sex or smoking, making comparison challenging. By excluding studies reporting models that contain clinical predictors of disease progression, we excluded hazard ratios or odds ratios for comorbidity that were likely to overadjust for potential mediators (e.g., inflammation) of the possible effect of comorbidity on progression to a severe COVID-19 outcome. We did retain studies that adjusted estimates for one comorbidity by other comorbidities, although this may also lead to overadjustment if one comorbidity mediates another's effect on the outcome (e.g., Type 2 diabetes mediated by obesity). Some of the included estimates are potentially susceptible to residual confounding from omitted confounders such as ethnicity and socioeconomic disadvantage. The estimates in this review do tend, however, to be similar in magnitude across adjustment for various confounders.

Overall, the 14 studies varied considerably in both the quality of the design and reporting, and only a few were moderately high quality. Whilst hasty research and publication are understandable early in such a global emergency, rigour should not be compromised. As London and Kimmelman argued, "the moral mission of research remains the same: to reduce uncertainty and enable caregivers, health systems, and policy-makers to better address individual and public health" (48). Indeed, there is an ethical imperative to ensure that the conduct and reporting of research in a pandemic crisis maintains high standards of validity, reliability, and integrity to provide sufficiently robust evidence for these purposes.

Strengths and Limitations of the Review

Our review was rapid while also being as comprehensive as possible for that period of the pandemic. A companion review provided forward-citations, and our full-text searches included both pre-print archives and peer-reviewed literature, reflecting the fast-moving early stages of the pandemic and the increasing use of pre-print archives. Full texts were independently screened by two reviewers. To aid interpretation of the synthesised results, we systematically assessed study quality after modifying an existing tool to provide an appropriate appraisal framework for these studies.

The timing of the review meant that almost half the included studies came from China, and the others were restricted to a few other settings. The Chinese studies may have had a healthier case-mix because of different criteria for admission compared with other countries (e.g., the United Kingdom), where only relatively serious cases are hospitalised. The comorbidity profile of China also differs from many other countries (49–51). Our reliance on studies of hospitalised patients means the conclusions only indicate the increased risk associated with comorbidities in hospitalised COVID-19 patients rather than the effect that comorbidities may have on the initial risk of being infected with SARS-CoV-2 or on the outcomes of people with COVID19 outside hospital (e.g., in care homes). As noted above, selection bias may also affect these results. There is also a risk of publication bias - given the short time period, at least some studies published early in the pandemic are likely to have traded quality against the need for timely information. This demonstrates the importance of characterising the early evidence base, so it can later be contrasted will the evidence gathered over the longer run of the pandemic.

The primary focus of this review was to improve understanding of the independent relationships between comorbidities and COVID-19 outcomes, hence we collated evidence from studies where likely confounding by age had been appropriately controlled. With the exception of one meta-analysis that coarsely stratified by age (34), we note that subsequent similar reviews, published while ours was under peer review, have tended not to address explicitly the issue of heterogeneity across studies in terms of whether potential confounding by age was accounted for.

Implications for Future Data Collection and Research

Early studies will have informed policy decisions in this fast-moving pandemic. Speedy publication in a global emergency necessitated "loosening the critical parameters" (45) for the evidence base, and our quality appraisal suggests that reporting of methods has been adversely affected. Nevertheless, transparent and detailed reporting remains necessary for accurate interpretation. A lesson for future pandemics would be that having pre-agreed international guidelines on consistent methods and reporting of sample selection, description, and design (including variables to be measured and data collection tools) would facilitate more effective use and application of research efforts, by enabling pooling of results from different locations and settings to provide high-quality evidence quickly.

Caniglia et al. (10) argued that science during a pandemic must accommodate unavoidable uncertainty whilst maintaining science's social responsibility to the public good. This would include ensuring better skills, capacity, and theoretical frameworks are in place to enable better mobilisation of evidence under conditions of high uncertainty. Early in the pandemic, hospital-based studies were the most feasible, but population-based studies must now play a larger role in clarifying understanding of various independent risk factors for severe outcomes from COVID-19. Several such studies started emerging from May 2020 onwards [e.g., (52)], but making meaningful inferences can still be challenging (53). Regardless of study design, future studies should attempt to evaluate the robustness of conclusions to plausible sources of selection and other biases.

Implications for Policy and Practice

As early as May 2020, it was apparent that various comorbidities conferred an increased risk of severe disease progression and death after being hospitalised with COVID-19, independent of age. The evidence was slightly more consistent for obesity, although many other common chronic conditions across organ systems seemed to confer an elevated risk, and there is evidence that multimorbidity adds further risk. Given the relatively high population prevalence of most comorbidities covered in this review, the implications of elevated risk are substantial. It has been estimated from Global Burden of Disease prevalence data that one in five individuals globally may be at increased risk of severe COVID-19 due to underlying conditions (54), but this is likely to underestimate risk because obesity was omitted. Furthermore, this burden is not evenly distributed between countries, meaning COVID-19 is impacting healthcare systems already under pressure from high local burdens of non-communicable disease.

Whether COVID-19 accelerates the underlying condition, or weakened underlying organs or immune response increase vulnerability to severe COVID-19, or both, is subject to ongoing research globally. Nevertheless, even without a full explication of the mechanisms, early epidemiological evidence of an association between comorbidities and poor in-hospital outcomes supported action to protect these groups and mitigate their elevated risk.

The increased risk associated with many comorbidities supported strong, targeted primary prevention measures to "shield" people with comorbidities from SARS-CoV-2 and suggested a need for public health campaigns to promote awareness of these elevated risks and how people could protect themselves. Vaccines have been prioritised for those at higher risk. In terms of secondary prevention of COVID-19, it is important to detect it early in those with comorbidities, to reduce progression as treatments (such as dexamethasone for severe or critical disease) emerge. This evidence has implications for healthcare system demand in areas of high comorbidity prevalence. To address the greater burden of COVID-19 in communities with more pre-existing conditions, greater resources should be allocated according to this need. Approaches in the early stages of the pandemic were, however, not prioritising sufficiently these higher levels of need (55). There are also implications when preparing for subsequent waves of community transmission. In particular, the evidence presented here highlights greater urgency for reducing the prevalence and incidence of chronic disease, through support for non-communicable disease prevention efforts and addressing the wider determinants of health.

Finally, the intersection of underlying comorbidity with socioeconomic disadvantage, geography, and demographic factors, especially ethnicity, has proven to be a potent mix that will widen health inequalities, both within and between countries. In England, official statistics showed that COVID-19 age-standardised mortality rates in the most deprived parts of England are more than double the rate in the least deprived areas (56) and this is partly explained by inequalities in existing chronic health conditions. Furthermore, people from ethnic minority backgrounds are overrepresented among deaths from COVID-19 (57–59), with ethnicity apparently a risk factor independent of deprivation (60), probably partly due to higher prevalence of common comorbidities. In addition, there are numerous social and structural factors that increase risk of infection in these groups (such as overcrowded housing, greater reliance on public transport, and employment in essential and "frontline" occupations with much human contact where physical distancing is not feasible).

People with chronic health conditions are already disadvantaged and underrepresented in the workforce. They have been further disadvantaged by control measures such as

prolonged shielding – which can adversely affect their financial, social, and mental well-being. Further use of such measures – in this or another pandemic - will require tailored support and strategies to mitigate impacts.

Without concerted effort, reducing existing risk factors such as obesity and targeting support for people with pre-existing health conditions, the COVID-19 pandemic is likely to widen health inequalities between social, ethnic, and geographical groups. Pandemic responses must therefore prioritise and mitigate the unfair burden shouldered by disadvantaged and ethnic minority groups.

CONCLUSIONS

Building on evidence that people with comorbidities were overrepresented in hospitalised cases of COVID-19, this review compiled estimates from age-adjusted regression models across 14 studies from various settings globally in the early stages of the pandemic. It summarises for clinicians, policymakers, and academics the most robust evidence that was available in those first few months on this topic, to inform decision-making. Characterising this early evidence base helps to provide crucial context for the many enquiries, probes, and reflective exercises that will be performed around the world to scrutinise what should have been done better in the early response. Despite its limitations, the early evidence base showed that people with underlying chronic health conditions are at increased risk of severe disease progression and death and supported a range of public health and clinical approaches to protecting people with comorbidities. Given the distribution of comorbidities in the community, this evidence indicates that COVID-19 will exacerbate existing health inequalities, unless actions are taken to reduce these pre-existing vulnerabilities and target control measures to protect groups with chronic health conditions.

AUTHOR CONTRIBUTIONS

BB, AP, KM, and PM conceived of and designed the review. KM, PM, and AP undertook the searching and screening, with search support from the companion COVID-19 critical care review team at the University of Liverpool. KM did the initial data extraction, summarised study characteristics, and led the narrative synthesis. GM checked data extraction for accuracy and missing data. JD completed the quality assessment of the included studies. All authors contributed to the synthesis of evidence and writing of the manuscript.

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METHODS
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Triage Modeling for Differential Diagnosis Between COVID-19 and Human Influenza A Pneumonia: Classification and Regression Tree Analysis

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Background: The coronavirus disease 2019 (COVID-19) pandemic has lasted much longer than an influenza season, but the main signs, symptoms, and some imaging findings are similar in COVID-19 and influenza patients. The aim of the current study was to construct an accurate and robust model for initial screening and differential diagnosis of COVID-19 and influenza A.

Methods: All patients in the study were diagnosed at Fuyang No. 2 People's Hospital, and they included 151 with COVID-19 and 155 with influenza A. The patients were randomly assigned to training set or a testing set at a 4:1 ratio. Predictor variables were selected based on importance, assessed by random forest algorithms, and analyzed to develop classification and regression tree models.

Results: In the optimal model A, the best single predictor of COVID-19 patients was a normal or high level of low-density lipoprotein cholesterol, followed by low level of creatine kinase, then the presence of <3 respiratory symptoms, then a highest temperature on the first day of admission <38°C. In the suboptimal model B, the best single predictor of COVID-19 was a low eosinophil count, then a normal monocyte ratio, then a normal hematocrit value, then a highest temperature on the first day of admission of <37°C, then a complete lack of respiratory symptoms.

Conclusions: The two models provide clinicians with a rapid triage tool. The optimal model can be used to developed countries/regions and major hospitals, and the suboptimal model can be used in underdeveloped regions and small hospitals.

Keywords: COVID-19, influenza A, differential diagnosis, rapid triage tools, regression tree analysis

INTRODUCTION

On March 11, 2020, the World Health Organization (WHO) announced that coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection had become a global pandemic (1, 2). With the growing number of SARS-CoV-2 infection and associated fatalities, the early diagnosis of COVID-19 has become a priority (3). A positive SARS-CoV-2 nucleic acid test is currently the gold standard for the diagnosis of COVID-19 (4–6), but nucleic acid testing is subject to false-negative and false-positive results (7, 8). Therefore, both the World Health Organization and National Health Commission of the People's Republic of China recommend comprehensive consideration of historical epidemiology, imaging results, clinical signs and symptoms, and laboratory evidence such as etiology or serology indicators for diagnosis (9). These methods are labor-intensive, however, and require substantial material and medical resources.

Seasonal influenza viruses can cause acute respiratory infection and a high rate of morbidity and mortality (10, 11). They are classified into four types: A, B, C, and D. Among them, H1N1 influenza A viruses are quite common and are associated with a high mortality rate, for example the H1N1 "swine flu" which caused an influenza pandemic in 2009 (11).

Distinguishing between influenza and COVID-19 can be problematic because their main signs and symptoms are similar (12, 13). Although some differentiation between COVID-19 and influenza patients is possible *via* chest computed tomography features, different radiologists, scanning parameters, image quality, and stages of disease may affect interpretations of certain imaging details (14–16).

Given that often only limited diagnostic and treatment resources are available, triage tools that enable rapid differential identification of COVID-19 and seasonal influenza are crucial to facilitate the allocation of appropriate medical resources and the application of prevention and control measures.

The current study included 151 COVID-19 patients and 155 patients with influenza A pneumonia from a hospital in Anhui Province in China. Based on symptoms (especially in the first 3 days), clinical signs, and physical and chemical laboratory test indicators a new model for the initial screening and differential diagnosis of COVID-19 and seasonal influenza pneumonia was constructed.

MATERIALS AND METHODS

Patients

All patients were adults with COVID-19 or influenza A confirmed at the Fuyang No. 2 People's Hospital, Anhui Province, China. The study was approved by the Ethics Committee of the Fuyang No. 2 People's Hospital. The inclusion criteria were age equal and above 18 years, hospitalization with complete medical history, temperature records, complete blood count and serum biochemical indicators, and a confirmed diagnosis via SARS-CoV-2 or influenza A virus detection.

Research Procedures and Data Collection

Respiratory tract samples including oropharyngeal swab, sputum, bronchial lavage, and blood and fecal specimens were obtained from COVID-19 patients at hospital admission, stored in viral transport medium, then sent to the Disease Control and Prevention Center of Fuyang for laboratory verification of SARS-CoV-2. Bilateral tonsils and posterior pharyngeal swabs collected from patients with influenza A were sent to the Influenza Surveillance Laboratory (National Influenza Surveillance Network Laboratory) of the Disease Control and Prevention Center of Fuyang for pathogen determination. The admission examination of patients included complete blood count, and blood biochemistry including renal function, liver function, creatine kinase (CK), lactate dehydrogenase, and electrolytes.

Nursing records and laboratory examination results of adults confirmed COVID-19 and influenza A patients at Fuyang No. 2 People's Hospital were retrospectively collated. Admission data from COVID-19 patients ranged from 20 January 2020 to 17 February 2020. Admission data from influenza A patients ranged from 08 April 2013 to 18 April 2019. A standardized data collection form was used to record patients' demographic characteristics, clinical symptoms, and laboratory results. COVID-19 patient data were acquired from the hospital's electronic medical records, whereas influenza A patient data were acquired from both printed and electronic medical records. All data were recorded and reviewed by 5 researchers to ensure that the data collected were authentic and valid.

Laboratory Findings

Complete blood counts were acquired using an XE-2100 automatic hematology analyzer (Sysmex Corporation, Japan). Serum biochemical tests (including renal and liver function, CK, lactate dehydrogenase, and electrolytes), myocardial enzymes, and C-reactive protein were analyzed using a Hitachi 7180 automatic analyzer (Hitachi, Tokyo, Japan).

Coronavirus and Influenza A Virus Testing

The local Center for Disease Control and Prevention performed SARS-CoV-2 detection in respiratory specimens by real-time fluorescent RT-PCR. The local Center for Disease Control and Prevention of Influenza surveillance laboratory (National influenza surveillance network laboratory) performed influenza A virus detection in pharyngeal swabs via RT-PCR methods with commercial assay kits provided by Beijing Kinghawk Pharmaceutical CO., Ltd. (Beijing, China).

Modeling and Verification

Because reference ranges of some indicators in laboratory examination results vary with different kits and other factors, some results could not be directly compared, thus they were converted into the following groups of indicators: (1) Lower than the reference, (2) normal, (3) higher than the reference. Continuous variables are presented as medians and interquartile ranges (IQRs), and categorical variables are presented as numbers and percentages. Odds ratios (ORs) and confidence intervals (CIs) were calculated, and logistic regression was used to

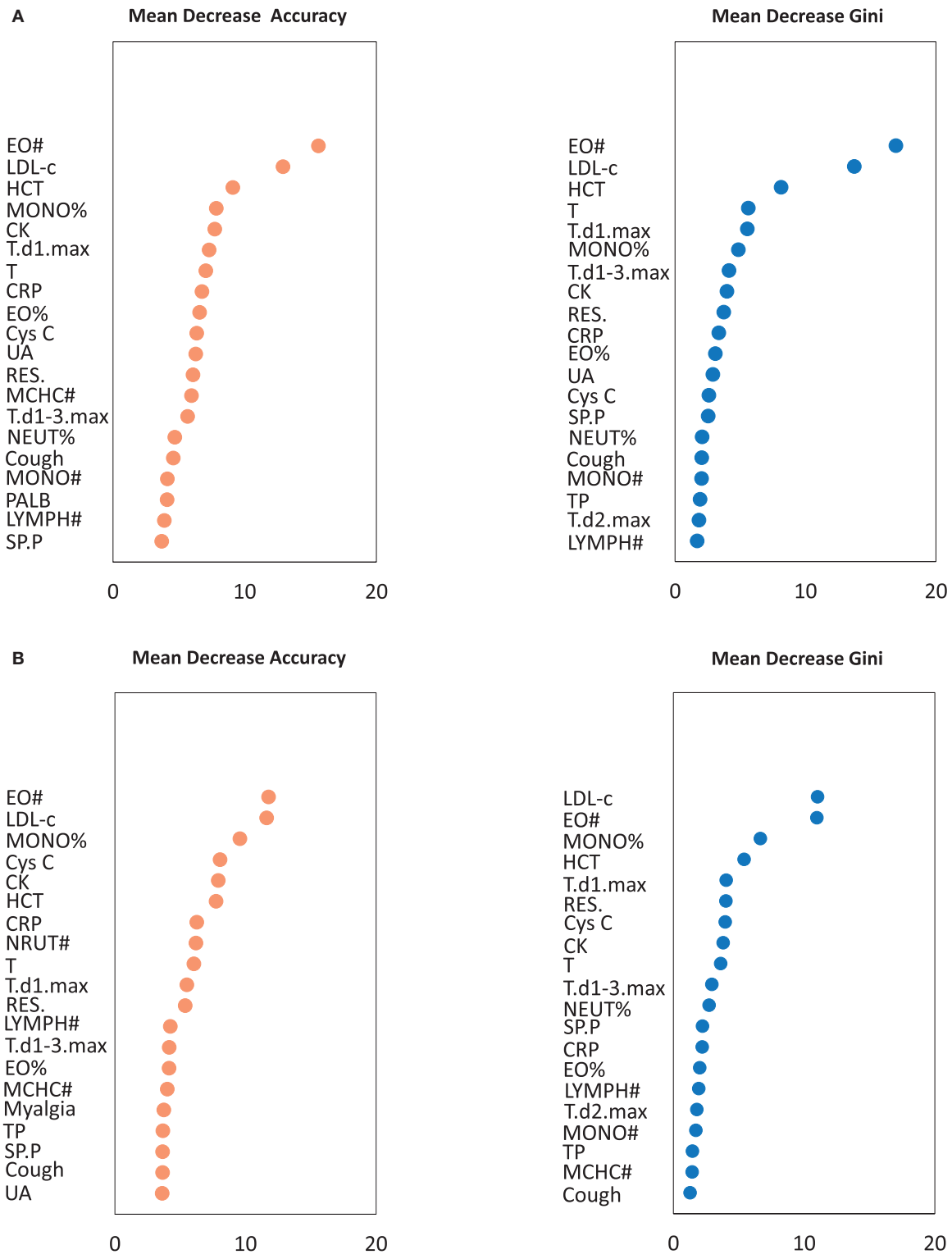


FIGURE 1 | The importance of variables in the random forest algorithm. **(A)** The importance of variables was calculated by all patients. **(B)** The importance of variables was calculated by training set. A multi-indicator model was constructed by combining 61 variables ($P < 0.05$ in logistic regression analysis). Only Top 20 was shown.

(Continued)

FIGURE 1 | EO#, eosinophil count; LDL-c, Low-density lipoprotein cholesterol; HCT, Hematocrit; MONO%, monocyte ratio; CK, creatine kinase; T, Body temperature of the admission day; T.d1.max, Highest temperature on the first day of admission; T.d2.max, Highest temperature on the second day of admission; T.d1-3.max, Highest body temperature during the first 3 days of admission; RES., The number of respiratory symptoms; SP.P, Sputum production; CRP, C-reactive protein; EO%, Eosinophil ratio; MONO#, Monocyte count; MCHC#, Mean corpuscular hemoglobin concentration; NEUT%, Neutrophil ratio; LYMPH#, Lymphocyte count; Cys C, Cystatin C; UA, Uric acid; PALB, Prealbumin; TP, Total protein.

compare the ORs of each variable in COVID-19 and influenza A patients. The random forest method was then used to determine the influence weighting of each variable and the risk factors with the greatest effects. Based on the results of random forest analysis three variables were selected, respectively, from the three groups of indicators, i.e., (1) demographic characteristics, clinical signs, and symptoms, (2) routine blood results, and (3) serum biochemistry results. A classification and regression tree (CART) model was then used to construct a decision tree. A training set (245 patients) and a testing set (61 patients) were created based on a ratio of 4:1. The training set was used for modeling and the testing set was used for verification.

Areas under the curve (AUCs) and a confusion matrix were used to evaluate the efficiency and robustness of the established models. Based on the characteristics of the models, sensitivity and specificity were calculated using the testing set. All statistical analyses were performed using R (version 3.6.3) with a significance level of $p < 0.05$.

RESULTS

Patient Characteristics

The demographic characteristics, clinical signs and symptoms, routine blood test results, and serum biochemistry results of the 306 patients included in the study are shown in **Supplementary Table 1**. There was no significant difference in age between the 151 COVID-19 patients (median 43 years, IQR 29–56 years) and the 155 influenza A patients (median 39 years, IQR 28–60). Men were at a 1.9 times greater risk of COVID-19 than women.

Both diseases tended to trigger fever at the onset of illness (94.2% of influenza A patients, 82.1% of COVID-19 patients), but the body temperature of influenza A patients on the first day of admission and the daily highest temperature in the first 3 days were higher than the corresponding medians in COVID-19 patients. COVID-19 patients were prone to diarrhea (OR 7.2, 95% CI 1.9–46.6), whereas influenza A patients showed more number of respiratory symptoms (OR 0.4, 95% CI 0.3–0.5); mainly coughing, expectoration, nasal discharge, pharyngalgia, chest congestion, and shortness of breath.

Complete blood count data on admission are shown in **Supplementary Table 1**. COVID-19 patients had lower white blood cell counts (OR 1.3, 95% CI 0.7–2.6), lymphocyte counts (OR 3.3, 95% CI 2.0–5.4), eosinophil counts (EO#s) (OR 79.0, 95% CI 28.2–330.3), and platelet counts (OR 5.4, 95% CI 2.2–16.4), and increased mean corpuscular hemoglobin concentration (OR 6.2, 95% CI 2.7–16.9).

Liver function remained normal in most patients, but elevated alanine aminotransferase (OR 2.5, 95% CI 1.2–5.5) and elevated aspartate aminotransferase (OR 2.6, 95% CI 1.4–5.3) were associated with a higher risk of COVID-19 infection. Increased low-density lipoprotein concentration was a protective factor in COVID-19 patients (OR 0.2, 95% CI 0.1–0.4). COVID-19 patients were more likely to exhibit abnormal cardiac enzymes than influenza A patients, as evidenced by a decrease in CK (OR 19.8, 95% CI 6.9–83.8) and an increase in lactate dehydrogenase (OR 1.7, 95% CI 1.1–2.7). Increased C-reactive protein concentration was evident in most patients (236/291, 81.1%), but it was more likely to be increased in influenza A patients (93.8%) than in COVID-19 patients (68.3%) (OR 0.1, 95% CI 0.0–0.3).

Random Forest Ranking

The results of random forest analysis are shown in **Figure 1**. The mean decrease accuracy plot and the mean decrease in Gini indicated that among clinical signs and symptoms, routine blood tests, and serum biochemistry results the most important variables were (1) highest temperature on the first day of admission, the number of respiratory symptoms, and coughing; (2) EO#, hematocrit, and monocyte ratio (MONO%); and (3) low-density lipoprotein cholesterol (LDL-c), C-reactive protein, and CK.

CART Model

The distributions of 9 important variables identified in the training set and the testing set were similar in the random forest plots (**Table 1**). CART modeling was then used to construct decision models (model A, **Figure 2**; model B, **Figure 3**) of clinical signs and symptoms and serum biochemistry, and clinical signs and symptoms and routine blood results of the 245 patients in the training set. Decision-making models C, D, and E were generated separately for the aforementioned three types of indicators, and an overall decision-making model (model F) was generated.

Figure 2A depicts decision tree model A constructed with 6 clinical signs and symptoms and serum biochemistry variables. The best indicator for distinguishing COVID-19 patients from influenza A patients was a decrease in LDL-c, which was associated with influenza A. When LDL-c was normal or elevated, a decrease in the secondary indicator CK contributed to the ability to identify COVID-19 patients. When LDL-c was normal or elevated and CK was normal or elevated, the third most important indicator was the number of respiratory symptoms present. When LDL-c was normal or elevated and CK was normal or elevated, the presence of $\geq$3 respiratory symptoms contributed to the ability to identify influenza A patients. When LDL-c was normal or elevated, CK was normal or elevated, and there were <3 respiratory symptoms, the highest body temperature on the first day of admission was the fourth most important indicator. When LDL-c was normal or elevated,

TABLE 1 | Characteristics of factors included in CART modeling, between training set and testing set.

	Training						Testing					
	Influenza A (*n* = 124)	**COVID-19 (*n* = 121)**	**All patients (*n* = 245)**	**Coef.**	**OR (95% CI)**	***P***	**Influenza A (*n* = 31)**	**COVID-19 (*n* = 30)**	**All patients (*n* = 61)**	**Coef.**	**OR (95% CI)**	***P***
The number of respiratory symptoms	2 (2, 3)	1 (0, 2)	2 (1, 3)	−1.1	0.3 (0.2, 0.5)	<0.001	2 (2, 3)	1 (0, 2)	2 (1, 2)	−1.0	0.4 (0.2, 0.7)	0.0029
Highest temperature on the first day of admission	37.9 (37.0, 38.5)	37.0 (36.7, 37.7)	37.3 (36.8, 38.0)	−0.8	0.4 (0.3, 0.6)	<0.001	38.3 (37.4, 38.7)	37.0 (36.6, 37.4)	37.4 (36.8, 38.3)	−1.8	0.2 (0.1, 0.4)	<0.001
Missing	1 (0.8%)	0 (0.0%)	1 (0.4%)									
Cough	111 (89.5%)	75 (62.0%)	186 (75.9%)	−1.7	0.2 (0.1, 0.4)	<0.001	28 (90.3%)	18 (60.0%)	46 (75.4%)	−1.8	0.2 (0.0, 0.6)	0.010
Eosinophil count, $\times 10^9$ per L	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)				0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)			
Normal	121 (97.6%)	47 (38.8%)	168 (68.6%)				31 (100%)	12 (40.0%)	43 (70.5%)			
Lower	3 (2.4%)	74 (61.2%)	77 (31.4%)	4.2	63.5 (22.2, 268.1)	<0.001	0 (0.0%)	18 (60.0%)	18 (29.5%)	20.5	812112207.5 (0.0, NA)	0.99
Hematocrit, %	36.8 (33.1, 40.1)	40.3 (37.7, 43.0)	38.8 (35.4, 42.1)				36.4 (29.4, 39.0)	41.8 (38.5, 44.0)	38.6 (34.8, 42.7)			
Normal	34 (27.4%)	92 (76.0%)	126 (51.4%)				7 (22.6%)	22 (73.3%)	29 (47.5%)			
Lower	89 (71.8%)	28 (23.1%)	117 (47.8%)	−2.2	0.1 (0.1, 0.2)	<0.001	24 (77.4%)	6 (20.0%)	30 (49.2%)	−2.5	0.1 (0.0, 0.2)	<0.001
Higher	1 (0.8%)	1 (0.8%)	2 (0.8%)	−1.0	0.4 (0.0, 9.5)	0.49	0 (0.0%)	2 (6.7%)	2 (3.3%)	15.4	4979978.4 (0.0, NA)	0.99
Monocyte ratio, %	8.9 (5.3, 13.3)	6.9 (5.5, 9.3)	7.5 (5.4, 11.9)				9.35 (6.10, 12.7)	7.60 (6.38, 9.75)	8.40 (6.28, 10.9)			
Normal	45 (36.3%)	99 (81.8%)	144 (58.8%)				12/30 (40.0%)	22 (73.3%)	34/60 (56.7%)			
Lower	12 (9.7%)	0 (0.0%)	12 (4.9%)	−17.4	0.0 (NA, Inf)	0.98	2/30 (6.7%)	1 (3.3%)	3/60 (5.0%)	−1.3	0.3 (0.0, 3.1)	0.31
Higher	67 (54.0%)	22 (18.2%)	89 (36.3%)	−1.9	0.1 (0.1, 0.2)	<0.001	16/30 (53.3%)	7 (23.3%)	23/60 (38.3%)	−1.4	0.2 (0.1, 0.7)	0.013
Low-density lipoprotein cholesterol, mmol/L	2.1 (1.6, 2.7)	2.1 (1.7, 2.8)	2.1 (1.7, 2.8)				1.71 (1.47, 2.12)	2.13 (1.91, 2.46)	2.01 (1.64, 2.41)			
Normal	44/120 (36.7%)	99/110 (90.0%)	143/230 (62.2%)				3/27 (11.1%)	28/28 (100%)	31/55 (56.4%)			
Lower	60/120 (50.0%)	0/110 (0%)	60/230 (26.1%)	−1.2	0.3 (0.1, 0.7)	0.0060	20/27 (74.1%)	0 (0.0%)	20/55 (36.4%)	−22.8	0.0 (NA, Inf)	1.00
Higher	16/120 (13.3%)	11/110 (10.0%)	27/230 (11.7%)	−19.4	0.0 (0.0, 477716.3)	0.98	4/27 (14.8%)	0 (0.0%)	4/55 (7.3%)	−22.8	0.0 (NA, Inf)	1.00
C-reactive protein, mg/L	39.1 (18.9, 77.1)	13.2 (3.2, 35.6)	24.4 (7.95, 54.9)				37.0 (22.7, 59.3)	17.3 (3.23, 36.9)	23.8 (10.5, 57.5)			

(Continued)

TABLE 1 | Continued

	Training						Testing					
	Influenza A (*n* = 124)	COVID-19 (*n* = 121)	All patients (*n* = 245)	Coef.	OR (95% CI)	*P*	Influenza A (*n* = 31)	COVID-19 (*n* = 30)	All patients (*n* = 61)	Coef.	OR (95% CI)	*P*
Normal	8/118 (6.8%)	38/117 (32.5%)	46/235 (19.6%)				1/28 (3.6%)	8/28 (28.6%)	9/56 (16.1%)			
Higher	110/118 (93.2%)	79/117 (67.5%)	189/235 (80.4%)	−1.9	0.2 (0.1, 0.3)	<0.001	27/28 (96.4%)	20/28 (71.4%)	47/56 (83.9%)	−2.4	0.1 (0.0, 0.6)	0.031
Creatine kinase, U/L	75.0 (46.0, 134.0)	62.0 (47.0, 88.0)	69.0 (46.5, 107.0)				63.5 (43.8, 156)	58.0 (38.8, 88.3)	58.0 (40.0, 123)			
Normal	105 (84.7%)	68/103 (66.0%)	173/227 (76.2%)				23/28 (82.1%)	16 (72.7%)	39 (75.0%)			
Lower	3 (2.4%)	32/103 (31.1%)	35/227 (15.4%)	2.8	16.5 (5.6, 70.4)	<0.001	0/28 (0.0%)	7 (31.8%)	7 (13.5%)	17.9	61158167.7 (0.0, NA)	0.99
Higher	16 (12.9%)	3/103 (2.9%)	19/227 (8.4%)	−1.2	0.3 (0.1, 0.9)	0.056	5/28 (17.9%)	1 (4.5%)	6 (11.5%)	−1.2	0.3 (0.0, 2.0)	0.28

Data are median (IQR), n (%), or n/N (%), where N is the total number of patients with available data. Comparing COVID-19 and influenza A are from logistic regression.
Coef., coefficient; OR, odds ratio; CI, confidence interval; Inf, infinity.

CK was normal or elevated, and there were <3 respiratory symptoms, a highest temperature on the first day of admission of <38°C contributed to the ability to identify COVID-19 patients, whereas a highest body temperature of ≥38°C on the first day of admission contributed to the ability to identify influenza A patients.

Figure 3A depicts decision tree model B constructed with 6 clinical signs and symptoms and routine blood test variables. The best indicator for distinguishing COVID-19 patients from influenza A patients was a low EO#. When the EO# was normal, an increase or decrease in the MONO% contributed to the ability to identify influenza A patients. When the EO# and MONO% were both normal, the third most important indicator was hematocrit (HCT). When the EO# and MONO% were both normal, low HCT contributed to the ability to identify influenza A patients. When the EO#, MONO%, and HCT were all normal, the fourth most important indicator was the highest body temperature on the first day of admission. When the EO#, MONO%, and HCT were all normal, a highest body temperature of <37°C on the first day of admission contributed to the ability to identify COVID-19 patients. When the EO#, MONO%, and HCT were all normal and the highest body temperature on the first day of admission was ≥37°C, the fifth most important indicator was the number of respiratory symptoms present. When the EO#, MONO%, and hematocrit were all normal and the highest temperature on the first day of admission was ≥37°C, a complete lack of respiratory symptoms contributed to the ability to identify COVID-19 patients, whereas the presence of ≥1 respiratory symptom contributed to the ability to identify influenza A patients.

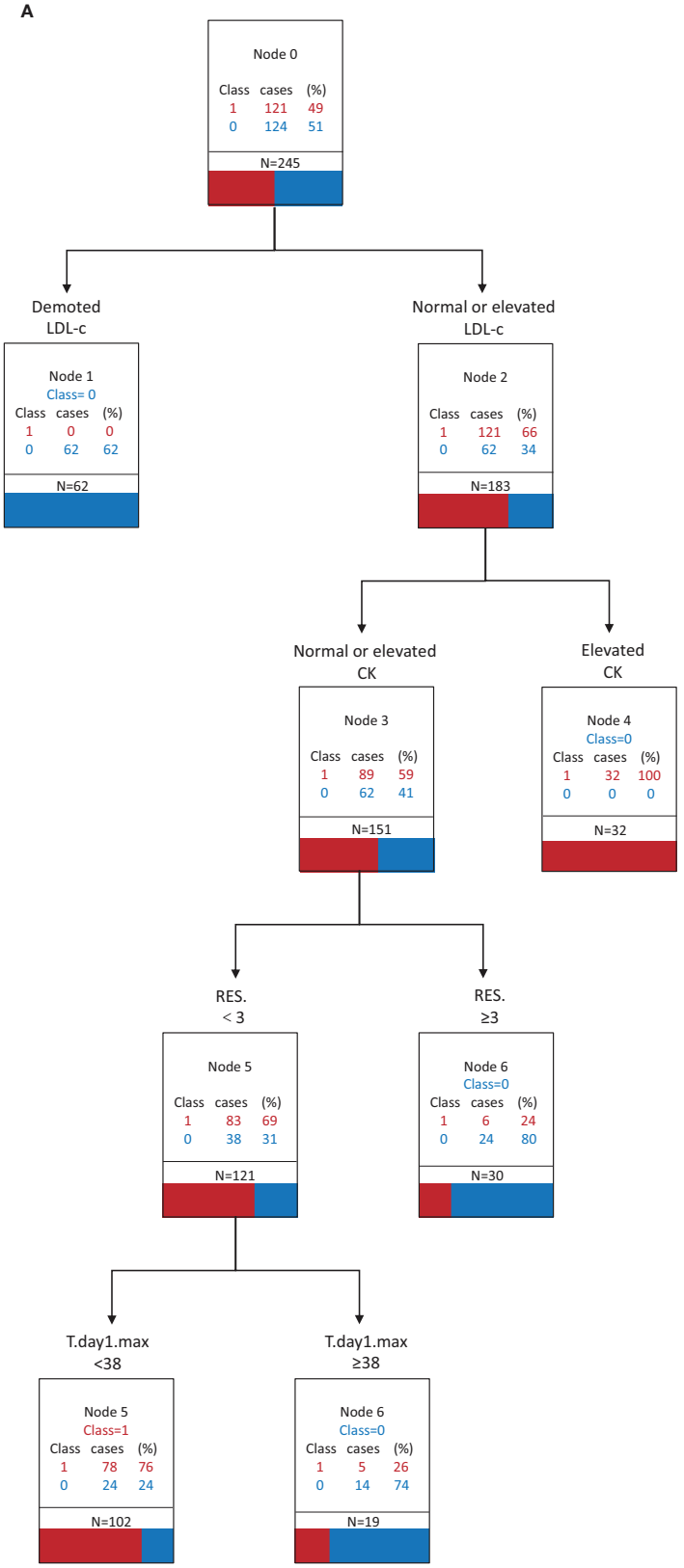
Model Validation

Model A (**Figure 2B**) correctly identified all COVID-19 patients, but it also incorrectly classified 4 influenza A patients as COVID-19 patients. Model A had a sensitivity of 1.00, a specificity of 0.87, a positive predictive value of 0.88, and a negative predictive value of 1.00. In a receiver operating characteristic curve of model A the AUC was 0.93.

Model B (**Figure 3B**) correctly identified all influenza A patients, but it also incorrectly classified 8 COVID-19 patients as influenza A patients. Model B had a sensitivity of 0.73, a specificity of 1.00, a positive predictive value of 1.00, and a negative predictive value of 0.79. In a receiver operating characteristic curve of model B the AUC was 0.87.

Confusion matrix analysis indicating the difference between the prediction results generated by models C–F and the real results among the testing set patients is shown in **Supplementary Figures 2–5**.

Model A demonstrated the best predictive capacity with respect to both COVID-19 patients and influenza A patients. Especially when it is necessary to predict and identify a COVID-19 patient, model A is able to minimize misdiagnosis and thus it is considered the optimal model. Model B also exhibited a favorable predictive performance in COVID-19 patients and influenza A patients, so it is regarded as a valid albeit suboptimal model.



B

Confusion matrix		Reference	
		0	1
Prediction	0	27	0
	1	4	30

Sensitivity	1.00
Specificity	0.87
Positive predictive value	0.88
Negative predictive value	1.00
Prevalence	0.49
Detection Rate	0.49
Detection Prevalence	0.56
Balanced Accuracy	0.94

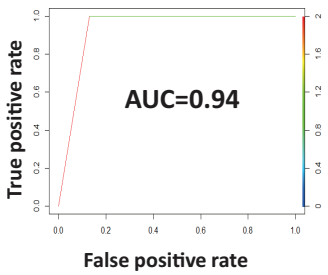
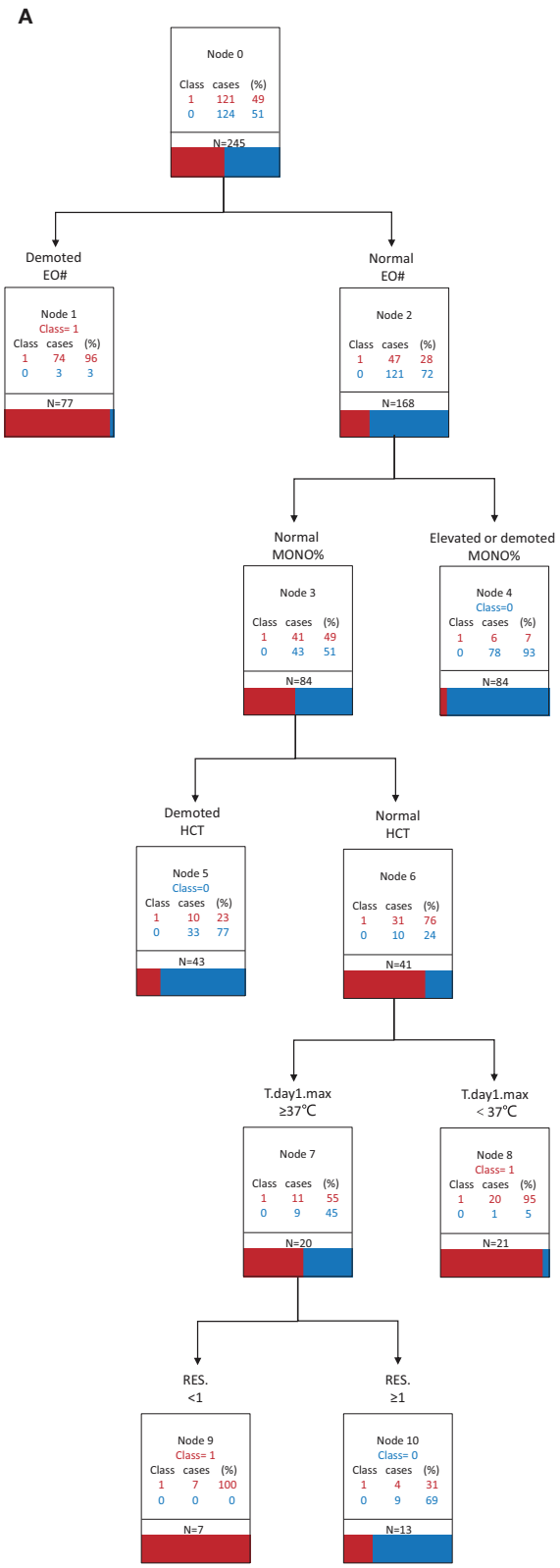


FIGURE 2 | Classification and regression tree analysis of variables that most distinguish COVID-19 from Influenza A in clinical signs and symptoms and in serum biochemistry (model A, optimal model). **(A)** 0, Influenza A; 1, COVID-19; N, the total number of patients; RES., The number of respiratory symptoms; T.day1.max, Highest temperature on the first day of admission; LDL-c, Low-density lipoprotein cholesterol; CK, creatine kinase. All factors are compared with the limit of the range of medical reference value. **(B)** Performance characteristics of the model validated by the testing set.



B

Confusion matrix		Reference	
		0	1
Prediction	0	31	8
	1	0	22

Sensitivity	0.73
Specificity	1.00
Positive predictive value	1.00
Negative predictive value	0.79
Prevalence	0.49
Detection Rate	0.36
Detection Prevalence	0.36
Balanced Accuracy	0.87

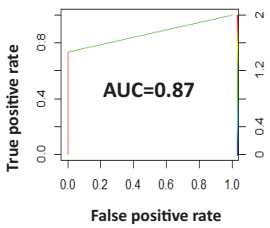


FIGURE 3 | Classification and regression tree analysis of variables that most distinguish COVID-19 from Influenza A in clinical signs and symptoms and in routine blood (model B, suboptimal model). **(A)** 0, Influenza A; 1, COVID-19; *N*, the total number of patients; RES., The number of respiratory symptoms; T.day1.max, Highest temperature on the first day of admission; EO#, eosinophil count; MONO%, monocyte ratio; HCT, Hematocrit. All factors are compared with the limit of the range of medical reference value. **(B)** Performance characteristics of the model validated by the testing set.

DISCUSSION

In the current study differential diagnosis models of COVID-19 and influenza A patients were generated based on individual signs and symptoms, routine blood tests, and serum biochemistry results. The models were classified as optimal (model A) or suboptimal (model B) with respect to their capacity for differential diagnosis. Given that the routine blood testing required in the suboptimal model is a more economical and common laboratory tool, that model is more suitable for underdeveloped areas. Both models A and B were accurate, sensitive, and robust, so that they can offer technical support for rapid clinical triage.

In the optimal model the frequency of respiratory symptoms and the highest temperature on the first day of admission were included as indicators. Patients with a greater number of respiratory symptoms were more likely to have influenza A than COVID-19. This is consistent with previous reports indicating that respiratory symptoms—especially upper respiratory symptoms—are not substantial in many COVID-19 patients (17). A high temperature on the first day of admission also indicated that influenza A was more likely than COVID-19. This is concordant with previous studies in which influenza A patients generally had high fever at the onset of illness (18, 19), whereas many COVID-19 patients exhibit no initial symptoms such as high fever (20).

With regard to routine blood tests, EO#, MONO%, and HCT were incorporated into model B (signs and symptoms + routine blood tests). Previous studies have not suggested changes or abnormalities in these three indicators in patients with influenza A or COVID-19 (21–25). However, in the present study model B indicated that combined with signs and symptoms and routine blood results, these three indicators can be used to distinguish between influenza A patients and COVID-19 patients. The pathophysiological basis underlying differences in these indicators in the two groups of patients warrants further research.

Although the incidence of decreased LDL-c did not differ significantly between COVID-19 patients and influenza A patients, most influenza A patients exhibited decreased LDL-c whereas no COVID-19 patients did. This indicator was well-distinguished in a subsequently generated CART algorithm. Lastly, in model A LDL-c was the most important indicator. It has previously been reported that C-reactive protein and CK may be elevated in influenza A patients (18, 26). Most COVID-19 patients have exhibited elevated C-reactive protein, and a few of them have exhibited elevated CK (22, 25, 27). In the current study model A indicated that increased CK suggested that influenza A was more likely than COVID-19, which was the same as previous studies (22, 28–30). C-reactive protein was excluded as an unimportant indicator. Although there is evidence that COVID-19 may lead to complications of heart disease (29), in the present study normal or reduced CK was more suggestive of COVID-19 than influenza A.

The initial classification model was not completely consistent with the preferred indicators applied alone for the diagnosis of COVID-19 or influenza A. According to the COVID-19 diagnosis and treatment plan published in China, in addition to a history of potential exposure the determination of suspected cases mainly involved the total number of white blood cells and lymphocyte counts with respect to clinical symptoms and laboratory examinations (12, 23, 28). Because the primary aim of the current study was to distinguish between influenza A and COVID-19 patients, there were differences between the laboratory indicators and the combinations of them used in the constructed models, and the indicators emphasized in the treatment plan. Notably, the model developed in the present study was designed to distinguish between patients with suspected influenza A or COVID-19 rather than simply identify COVID-19 patients.

The prevalence of COVID-19 in children is very low, and in one study a prevalence of just 2.1% among a group of people aged 0–18 years was reported (29). Conversely the prevalence of influenza A in children is higher, and can reportedly reach 25.7% (30). Therefore, inclusion of children in the current study could have introduced mixed effects caused by age. For this reason people under the age of 18 were excluded, and the diagnostic model tool was also constructed for adults. Diagnostic tools and models specifically designed for use in children can be developed in the future.

The current study had some limitations. The sample size was small. Although the models constructed were sensitive and robust, large numbers of COVID-19 and influenza A cases should be used in the future to further verify and develop the models. Another limitation was that due to yearly changes in influenza viral antigenic configuration, the conditions of historical cases may differ from those of current cases. With respect to influenza strains, the present study only involved H1N1. Lastly, the lack of anosmia data may affect the differentiation capacity of the model.

In the current study an optimal model for distinguishing between influenza A and COVID-19 patients was generated. Another tool for initial screening and identification based on individual signs and symptoms and routine blood indicators was also generated for use in underdeveloped areas where the economy, detection capacity, and medical resources may not be conducive to blood biochemistry examinations. In developing countries such as China, the cost of a routine blood test is only 1/6 of that of blood biochemistry examination, and in less developed regions it can cost merely 1/10. Therefore, a simplified identification tool is of high cost-benefit value, although it reduces the ability to identify COVID-19 patients, which may inevitably lead to a degree of misdiagnosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Fuyang No. 2 People's Hospital, Anhui Province. The patients/participants provided

their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

BY and WZhe had the idea for and designed the study and had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. AX collected the data of COVID-19 and human influenza patients. AX and HZ were in charge of the manuscript draft. AX, HZ, JX, LZ, KZ, BY, and WZhe contributed to writing the report. HZ, JX, CZ, ZR, NM, and XL contributed to data input, cleaning, and database establishment. HZ, LZ, WM, WZhu, and DT contributed to the statistical analysis. ZW, YH, and JL contributed to the data and results checking, review and revised the manuscript. All authors contributed to data acquisition, data analysis, or data interpretation, and reviewed and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.673253/full#supplementary-material

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Pan-Cancer Analysis of Genomic and Prognostic Characteristics Associated With Coronavirus Disease 2019 Regulators

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Background: Cancer patients are alleged to have poor coronavirus disease 2019 (COVID-19) outcomes. However, no systematic or comprehensive analyses of the role and mechanisms of COVID-19 receptor-related regulators in cancer are available.

Methods: We comprehensively evaluated the genomic alterations and their clinical relevance of six COVID-19 receptor-related regulators [transmembrane serine protease 2 (TMPRSS2), angiotensinogen (AGT), angiotensin-converting enzyme 1 (ACE1), solute carrier family 6 member 19 (SLC6A19), angiotensin-converting enzyme 2 (ACE2), and angiotensin II receptor type 2 (AGTR2)] across a broad spectrum of solid tumors. RNA-seq data, single nucleotide variation data, copy number variation data, methylation data, and miRNA–mRNA interaction network data from The Cancer Genome Atlas (TCGA) of 33 solid tumors were analyzed. We assessed the sensitivities of drugs targeting COVID-19 receptor-related regulators, using information from the Cancer Therapeutics Response Portal database.

Results: We found that there are widespread genetic alterations of COVID-19 regulators and that their expression levels were significantly correlated with the activity of cancer hallmark-related pathways. Moreover, COVID-19 receptor-related regulators may be used as prognostic biomarkers. By mining the genomics of drug sensitivities in cancer databases, we discovered a number of potential drugs that may target COVID-19 receptor-related regulators.

Conclusion: This study revealed the genomic alterations and clinical characteristics of COVID-19 receptor-related regulators across 33 cancers, which may clarify the potential mechanism between COVID-19 receptor-related regulators and tumorigenesis and provide a novel approach for cancer treatments.

Keywords: pan-cancer, COVID-19 regulators, genetic alterations, methylation, prognosis

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus has resulted in the ongoing coronavirus disease 2019 (COVID-19) pandemic. As of July 7, 2021, there are 184,820,132 confirmed cases and 4,002,209 deaths, with the numbers still surging worldwide (1). With the continued increase in cases and affected regions, patients with chronic conditions, such as cancer, have been disproportionately affected (2–5). The COVID-19 pandemic has been identified as a global health emergency by the World Health Organization (WHO).

Respiratory inflammation is activated by the renin–angiotensin–aldosterone system (RAS), which maintains the blood pressure by angiotensin II (Ang II) and is catalyzed by the angiotensin-converting enzyme (ACE). Angiotensinogen (AGT) is the protein precursor of Ang II (6, 7). Ang II receptor type 2 (AGTR2), a member of the G-protein coupled receptor 1 family, functions as a receptor for Ang II. ACE2 degrades Ang II, counteracting its chronic effects, and serves as the SARS-CoV-2 receptor. ACE2 is also a molecule present on the surface of various cell types, including type II alveolar cells, bronchial transient epithelial secretory cells, endothelial cells, intestinal epithelium cells, and uterine epithelial cells (8). The spike protein (S protein) of SARS-CoV binds to cell surface ACE2 receptors (9). ACE1, homologous to the ACE2 gene, may be involved in the progression of diseases caused by several human coronaviruses (10, 11). Transmembrane serine protease 2 (TMPRSS2), a member of the serine protease family, facilitates human coronavirus infections (SARS-CoV and SARS-CoV-2) *via* proteolytic cleavage of the ACE2 receptor, which promotes viral uptake and cleavage of coronavirus spike glycoproteins, activating glycoproteins for host cell entry (12–14). Solute carrier family 6 member 19 (SLC6A19), a SARS-CoV-2 co-receptor, is a neutral amino acid transporter and forms a heterodimer with ACE2 (15). However, the genomic alterations and prognostic characteristics of COVID-19 receptor-related regulators in cancer are still unclear.

The clinical symptoms of COVID-19 range from asymptomatic to severe cardiopulmonary disease (16–18). Enhanced expression of ACE2 and immunosuppressive states caused by malignancies and anticancer treatments, such as chemotherapy or surgery, contribute to more severe disease in older patients with COVID-19 (19, 20). Recent studies also identified that aberrant expression of ACE2 receptor-related regulators is associated with the activation of several cancer-associated pathways (21–23). Therefore, it is of great clinical significance to clarify the genomic and clinical characteristics of the six ACE2 receptor-related regulators among 33 solid tumors for the management and treatment of tumor patients with COVID-19.

METHODS

Dataset Acquisition and Preprocessing

The Genotype-Tissue Expression (GTEx) dataset (V7.0) (https://commonfund.nih.gov/GTEx/) was used for gene expression analysis in normal tissues from healthy individuals. The tumor-associated data are composed of mRNA Seq data, clinical data, single nucleotide variation (SNV) data, copy number variation (CNV) data, and methylation data, which were collected from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/). Reverse phase protein array (RPPA) data were obtained from The Cancer Proteome Atlas (TCPA) (https://tcpaportal.org/tcpa/index.html). The Genomics of Drug Sensitivity in Cancer (GDSC) database (www.cancerrxgene.org) was used to investigate the correlation between gene expression and drug sensitivity.

Samples from 33 solid cancer types were investigated in the final analysis, namely, adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), acute myeloid leukemia (LAML), brain low-grade glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), mesothelioma (MESO), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TGCT), thyroid carcinoma (THCA), thymoma (THYM), uterine corpus endometrial carcinoma (UCEC), uterine carcinosarcoma (UCS), and uveal melanoma (UVM).

mRNA Expression Analysis

For mRNA differential expression analysis between paired tumor and normal samples, TCGA mRNA expression was normalized using RNA-Seq by Expectation-Maximization (RSEM). The number of samples for each cancer type ranged from 48 to 1,098, where only 14 cancer types that harbored over 10 pairs of tumor and normal samples were incorporated into analyses, namely, BLCA, BRCA, COAD, ESCA, HNSC, KICH, KIRC, KIRP, LIHC, LUAD, LUSC, PRAD, STAD, and THCA. Gene expression values were represented as RNA-Seq by Expectation-Maximization (RSEM) normalized data (24). The genes with a fold change (FC) <2 and significance false discovery rate (FDR) <0.05 underwent further analysis.

Subtype Analysis

Expression subtype analysis was used to find clinically relevant genes that may affect cancer subtype. To make the analysis feasible, the number of subgroups in a given subtype was at least 10, leaving 11 cancer types for gene analysis. We analyzed 11 cancer types for ACE2 receptor-relevant genes using a Student's *t*-test (n_subtype $= 2$) and ANOVA test (n_subtype > 2). The method used for the clinically relevant analysis depends on the number of subgroups in each cancer subtype.

Survival Analysis

For expression survival analysis, mRNA expression and clinical survival data were merged by the sample barcode. Tumor samples were divided into "high" and "low" gene expression groups using the median RSEM value. The R package "survival" was used to fit the survival time and survival status for the two groups. A Cox Proportional-Hazards model was calculated for each gene using the R package. Genes that had a Kaplan–Meier log-rank test p-value <0.05 were retained.

SNV Analysis

SNV data of 33 cancer types ($N = 8663$) were investigated. SNV oncoplot (or waterfall plot) was generated by maftools (25). The TCGA SNV data includes the following variant type values: Missense_Mutation, Silent, 5' Flank, 3' UTR, RNA, In_Frame_Del, Nonsense_Mutation, Splice_Site, Intron, 5' UTR, In_Frame_Ins, Frame_Shift_Del, Nonstop_Mutation, 3' Flank, Frame_Shift_Ins, and Translation_Start_Site. The Silent, Intron, IGR, 3' UTR, 5' UTR, 3' Flank, and 5' Flank were filtered out for SNV percentage calculation. The percentage of SNVs in each gene's coding region was calculated by the number of mutated samples divided by the number of cancer samples. SNV data and clinical overall survival data were combined, and the R package was used to estimate the survival difference between mutated and non-mutated genes.

CNV Analysis

CNV raw data from 33 cancer types ($N = 11{,}495$) were investigated and processed with GISTICS2.0 (26). The CNV was divided into heterozygous and homozygous CNV subtypes, which represented the occurrence of CNV on one chromosome or two chromosomes, respectively. The homozygous or heterozygous CNV profile showed the percentage of homozygous or heterozygous CNV, including CNV amplification and deletion percentages for each gene in each cancer. The percentage of CNV subtypes was calculated using GISTIC-processed CNV data. Only genes with >5% CNV were considered significant. As the method has been employed by Schlattl et al. (27), the mRNA expression and CNV data were merged by a sample's TCGA barcodes. The association between paired mRNA expression and CNV percentage were detected based on a Pearson product–moment correlation coefficient and t-distribution.

Methylation Analysis

Methylation data of paired tumor and normal samples across 14 cancer types ($N = 10{,}129$) were investigated. The mRNA expression and methylation data were merged by a sample's TCGA barcode. The association between paired mRNA expression and methylation was tested based on a Pearson product–moment correlation coefficient and t-distribution. The mRNA expression and methylation data of the regulators were merged *via* the TCGA barcode. The association between paired mRNA expression and methylation data was calculated using the Pearson's product–moment correlation coefficient, followed by a t-distribution test. p-values were adjusted by the FDR, and genes with an FDR ≤ 0.05 were retained. Further analysis was carried out on genes that were significantly influenced by genome methylation. Methylation data and clinical overall survival data were combined, and the methylation level of a gene was divided into two groups by median methylation. Cox regression was performed to estimate the hazard (risk of death). If the Cox coefficient was <0, the high methylation group showed a poorer survival, the Hyper_worse defined as High risk, otherwise defined as Low risk.

Pathway Activity Analysis

Following the method used by Ye et al. (28), RPPA data from TCPA were used to calculate a score for 7,876 samples. RPPA data of replicates-based normalization (RBN) were median-centered and normalized by the standard deviation across all samples for each component to obtain the relative protein level. The pathway score is the sum of the relative protein levels of all positive regulatory components minus the sum of the relative protein levels of all negative regulatory components in a given pathway. Gene expression was divided into two groups (upregulation group or downregulation group) by the median expression. The difference in the pathway activity score (PAS) between the two groups was determined. When PAS (gene A, upregulation group) was greater than the PAS (gene A, downregulation group), we considered gene A as having an activating effect on a pathway; otherwise, gene A had an inhibitory effect on a pathway.

miRNA Regulation Network Analysis

miRNA regulation data ($N = 9{,}105$) was collected from TCGA across 33 cancer types. miRNA expression and gene expression were merged by TCGA barcode. The association between paired mRNA and miRNA expression was tested based on a Pearson product–moment correlation coefficient and t-distribution. The p-value was adjusted by the FDR, and genes with an FDR of ≤ 0.05 ($R < 0$) were retained. The correlation was calculated for all paired samples. In addition, with consideration to the presence of positive regulators (including transcription factors), an miRNA–gene pair with negative correlation was considered as a potential negative regulation pair. Network was generated by visNetwork R packages.

Drug Sensitivity Analysis

Following the method used by Rees et al. (29), 481 small molecules from the Cancer Therapeutics Response Portal (CTRP) were collected. To analyze the correlation between gene expression and drug sensitivity, the values from the area under the dose–response curve (AUC) for drug and gene expression profiles for all cancer cell lines were downloaded. The Pearson correlation coefficients of transcription levels and AUCs were normalized using Fisher's z transformation. The Pearson correlation coefficients of the transcript levels and AUCs were normalized using Fisher's z transformation. A Bonferroni-corrected, two-tailed distribution test, with a family-wise error rate of <0.025 in each tail, was used for the z-score calculation. Pearson correlation coefficients of annotated drug–target pairs were compared with the same number of correlation pairs generated by random sampling of the correlations. The gene set drug resistance analysis was performed on IC_{50} drug data.

Statistical Analysis

Correlations between gene expression were evaluated using the Spearman's correlation test. The prognostic significance of the indexes was estimated using Kaplan–Meier survival curves and compared by a log-rank test. The Cox proportional hazards model was used to calculate the adjusted hazard ratio (AHR). All statistical analyses were performed with SPSS version 23.0 (SPSS Inc, Chicago, IL, USA) and R version 3.4.4 (http://www.r-project.org). $p < 0.05$ was considered as statistical significant.

RESULTS

mRNA Expression and Subtypes of ACE2 Receptor Regulators

Six ACE2 receptor-related regulators, namely, TMPRSS2, AGT, ACE1, SLC6A19, ACE2, and AGTR2, were identified and analyzed in this study. We first explored the differential expression of the six receptor-related regulators across cancers based on the TCGA expression data. As shown in **Figure 1A**, the regulators were identified as having significantly abnormal expression in 14 solid cancers. Expression of TMPRSS2 in KIRC, LUAD, BRCA, COAD, KIRP, LIHC, LUSC, and HNSC; ACE1 in LUAD and LUSC; AGT in KICH and HNSC; ACE2 in KICH; SLC6A19 in KIRC, KICH, COAD, KIRP, and LIHC; and AGTR2 in KIRC, KICH, LUAD, BRCA, KIRP, LUSC, and THCA was significantly downregulated ($p < 0.001$). However, expression of AGT in KIRC, LUAD, BRCA, COAD, THCA, and STAD; ACE2 in KIRC and LUAD; ACE1 in KIRC and LIHC; SLC6A19 in BRCA; and TMPRSS2 in KICH was significantly upregulated ($p < 0.001$). To further identify the expression of clinically relevant genes that affect cancer subtype, regulator gene expression was explored. The regulator expression subtypes were significantly associated with the tumorigenesis of BRCA, LUSC, KIRC, STAD, LUAD, HNSC, and BLCA (**Figure 1B**; $p < 0.05$). ACE2 in BRCA, ACE1 in LUSC and BLCA, ACE2 and SLC6A19 in KIRC, AGT and AGTR2 in STAD, ACE2 and TMPRSS2 in LUAD, and TMPRSS2 in HNSC were the main regulator subtypes. The results indicated that COVID-19 may be more infectious in BRCA, LUSC, KIRC, STAD, LUAD, HNSC, and BLCA patients than in the normal population.

We further explored the effect of regulator expression on cancer survival and found that high expression of TMPRSS2 in ACC; ACE1 in UVM and LUSC; AGTR2 in KIRP, KICH, and LUSC; ACE2 in LGG; and AGT in UVM were associated with poor survival of cancer patients, while expression of TMPRSS2 in KIRP, KICH, PAAD, and LIHC; ACE1 in KIRC, LIHC, and OV; SLC6A19 in KIRC, KIRP, and ESCA; ACE2 in KIRC and UVM; and AGT in PAAD were associated with good survival in cancer patients (**Figure 1C** and **Supplementary Figure 1**; $p < 0.05$). As shown in **Figure 1D**, the low expression of ACE2 was significantly associated with poor survival in KIRC (HR = 0.54; $p = 6.4e{-}05$). These results indicated that the expression of COVID-19 receptor-related regulators may play an important role in the progression and deterioration of cancer with COVID-19.

Somatic Mutations of ACE2 Receptor Regulators

We analyzed ACE2 receptor regulator-related SNP data to detect frequency and variant types in each cancer subtype. As shown in the oncoplot in **Figure 2A**, the main variant type of the regulators in different cancer subtypes were missense_mutation, in_frame_del, nonsense_mutation, splice_site, in_frame_ins, frame_shift_del, frame_shift_ins, and multi-hit. Regulator SNV frequency was increased in SKCM, UCEC, LUAD, and LUSC. The SNV frequency of the regulators in pan-cancers was 100% (520 out of 520 tumors). The SNV frequency of ACE1, SLC6A19, ACE2, AGTR2, AGT, and TMPRSS2 were 37, 26, 20, 16, 14, and 12%, respectively. SNV percentage analysis indicated that ACE1, SLC6A19, ACE2, AGTR2, AGT, and TMPRSS2 were 42, 26, 34, 26, 26, and 22%, respectively, in UCEC; 46, 23, 15, 29, 14, and 18%, respectively, in SKCM; 11, 18, 7, 8, 5, and 0%, respectively, in LUSC; and 18, 15, 9, 6, 6, and 3%, respectively, in LUAD (**Figure 2B**). The most frequent mutations were X971_splice/R971W in ACE1, H195Y/X195_splice in ACE2, F430Lfs*25 in AGT, R182* in AGTR2, D334N in SLC6A19, and G492S/C in TMPRSS2 (**Supplementary Figure 2**). In addition, ACE1 mutations found in malignancies were distributed across all exons of ACE1, with several hot spot mutation sites, such as R487H in GBM; R508Q, E510K, and R487C in UCEC; and E510K in UVM (**Supplementary Table 1**). Pan-cancer mutation prognosis analysis showed that ACE1 and TMPRSS2 mutations were associated with better survival in cancer patients (**Supplementary Figure 3**; $p = 0.0273$ and $1.18e{-}10$), whereas mutated SLC6A19 was associated poor survival in cancer patients (**Supplementary Figure 3**; $p = 1.47e{-}4$). These results indicated that mutations in ACE2 receptor regulators are involved in tumorigenesis and associated with clinical survival.

CNV of ACE2 Receptor Regulators

To identify the CNV change of ACE2 receptor regulators at the chromosome arm level, we analyzed the CNV data of ACE2 receptors from the TCGA database. We found that TMPRSS2, SLC6A19, ATGR2, AGT, ACE2, and ACE1 had >5% CNV amplification or deletion in 33 cancers. As shown in the CNV pie distribution in **Figure 3A**, TMPRSS2 had 80% heterozygous amplification in TGCT but 63% heterozygous deletion in ESCA; ACE2 had 51% heterozygous amplification in ACC and >50% heterozygous deletion in OV and KICH; and AGT in LUAD, UCS, BRCA, LIHC, CESC, LUSC, SKCM, ESCA, and CHOL; ACE1 in KIRP; and SLC6A19 in ACC and LUSC had almost 50% heterozygous amplification, whereas ACE1 in KICH; SLC6A19 in TGCT; and AGT in KICH had almost 50% heterozygous deletion. To identify the heterozygous/homozygous CNV profile in each cancer, we further analyzed heterozygous/homozygous amplification and heterozygous/homozygous deletion. As shown in **Figure 3B**, all regulators had heterozygous amplification and deletion. However, homozygous CNV analysis showed that SLC6A19 had homozygous amplification in 12 solid cancers,

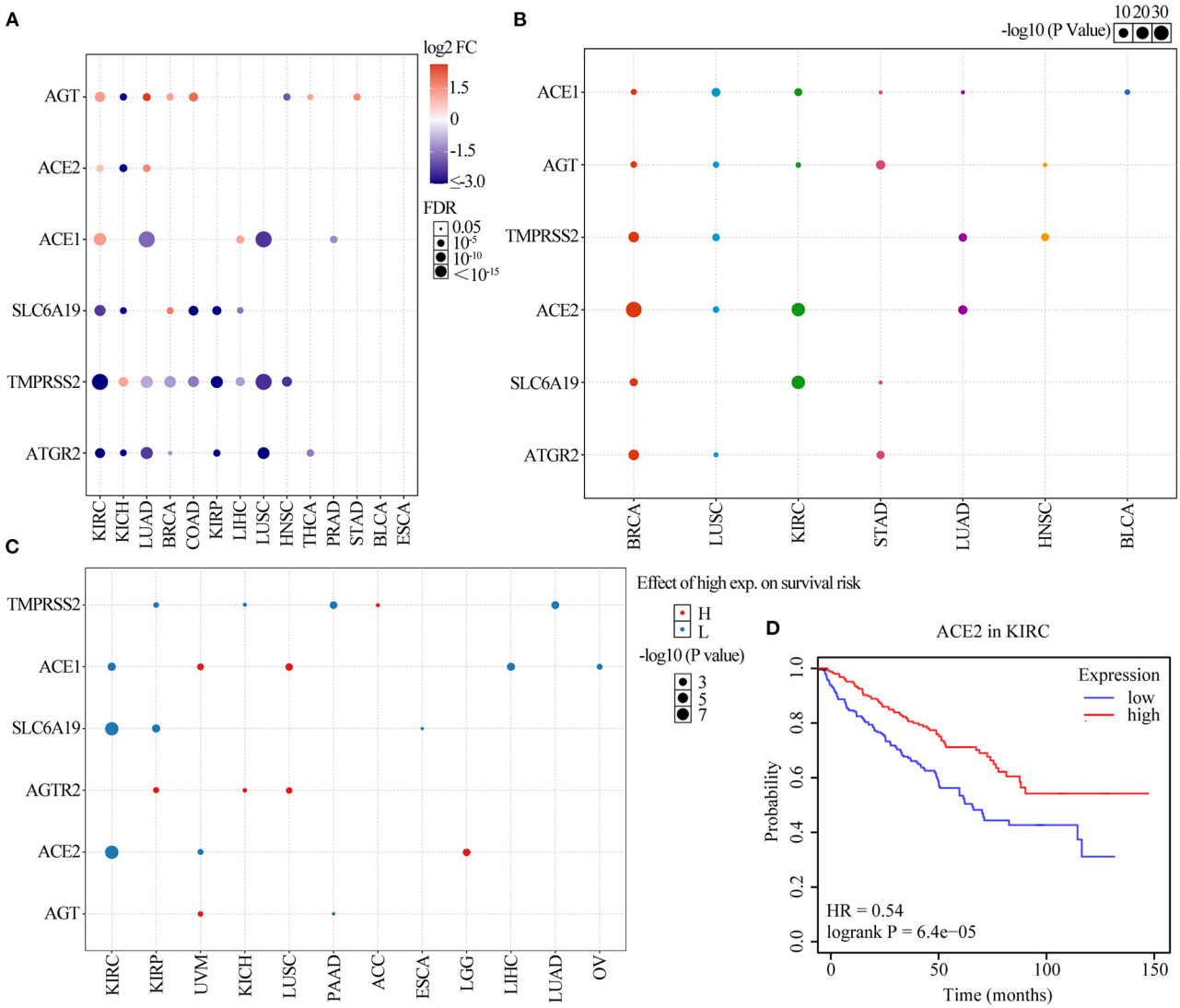

FIGURE 1 | Gene set expression of ACE2 receptor-related regulators. **(A)** Differential expression in 14 solid tumors. The *y*-axis refers to the gene and the *x*-axis refers to the cancer type. The colors range from purple to red, representing the increasing fold change between tumor vs. normal sample, respectively. The size of the dot indicates the degree of significance. **(B)** A graph showing how each subtype is affected by ACE2 receptor-related regulator mRNA expression in seven solid tumors, where each gene can have differential expression in each regulator subtype. **(C)** Survival analysis of ACE2 receptor-related regulators. The dot size represents the significance of a gene affecting survival in each cancer type, and the *p*-value is obtained from a Kaplan–Meier analysis. The red dot color indicated the worse of the high or low expression in the cancer types and the blue dot indicates low expression. **(D)** The survival of ACE2 gene in KIRC.

with TMPRSS2 homozygous deletion only found in PRAD (**Figure 3C**).

Comparing the relationship between CNV and mRNA expression, the correlation analysis indicated that mRNA expression of each regulator was positively correlated with its CNV in most cancers ($p < 0.05$). However, the expression of TMPRSS2 in KIRC; SLC6A19 in ESCA and PAAD; and ACE1 in TGCT, SKCM, and LIHC were negatively correlated with the CNV ($p < 0.05$) (**Figure 3D**). These results indicated that the CNV of ACE2 receptor-related regulators mediated their abnormal expression, which may play an important role in cancer patients with COVID-19.

Methylation Analysis of ACE2 Receptor Regulators

We explored the methylation analysis of ACE2 receptor regulators to identify the corresponding epigenetic methylation levels. As shown in **Figure 4A**, ACE2 in COAD, BLCA, KIRC, LUSC, KIRP, LUAD, and ESCA; AGTR2 in HNSC, UCEC, COAD, KIRC, LUSC, PRAD, and LUAD; SLC6A19 in HNSC; UCEC, BLCA, KIRC, LUSC, and KIRP; ACE1 in HNSC, BLCA, KIRC, and ESCA; AGT in HNSC and KIRC; and TMPRSS2 in PRAD were hypomethylated ($p < 0.05$); TMPRSS2 in COAD, KIRC, LUSC, KIRP, LUAD, ESCA, LIHC, and BRCA; AGT in BLCA, LUAD, and BRCA; ACE1 in PRAD; and SLC6A19 in

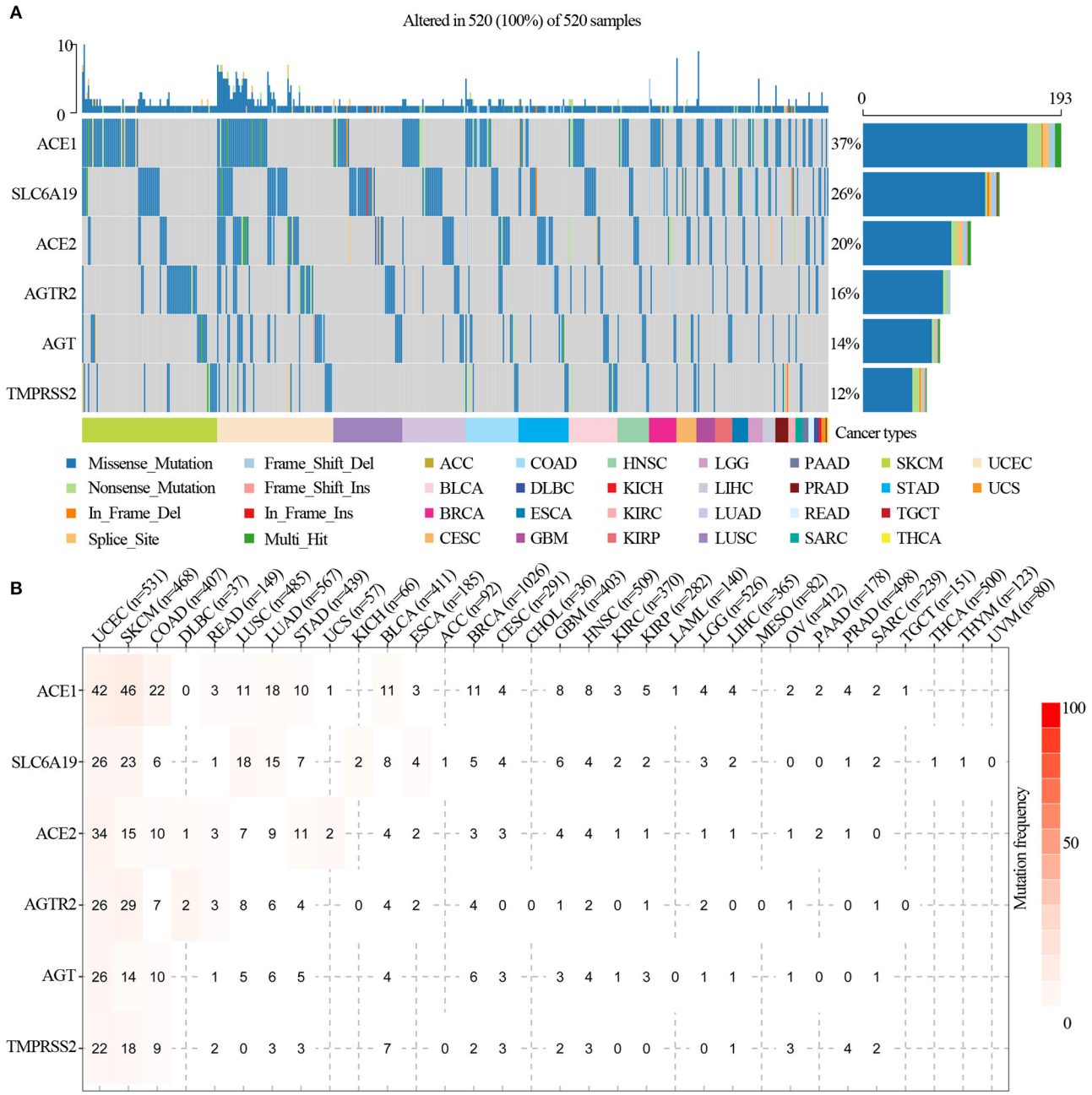

FIGURE 2 | SNV frequency and variant types of ACE2 receptor-related regulators. **(A)** SNV oncoplot. An oncoplot showed the mutation distribution of ACE2 receptor-related regulators and a SNV classification of SNV types (such as missense mutation, frame shift deletion, and non-sense mutation). All selected cancer samples are shown together. Side bar plot and top bar plots show the number of variants in each sample or gene. **(B)** The SNV frequency of genes in cancers. The darker the color, the higher the mutation frequency. Numbers represent the number of samples that have the corresponding mutated gene for a given cancer. "0" indicates that there was no mutation in the gene coding region, and no number indicates that there was no mutation in any region of the gene.

COAD, PRAD, and PAAD were significantly hypermethylated ($p < 0.05$). We assessed regulator methylation and mRNA expression through correlation analysis and found that the mRNA expression of AGT in KICH and HNSC; ACE2 in KICH; ACE1 in LUAD, LUSC, and PRAD; SLC6A19 in KIRC, KICH, COAD, KIRP, and LIHC; TMPRSS2 in KIRC, LUAD, BRCA, COAD, KIRP, LIHC, LUSC, and HNSC; and AGTR2 in KIRC, KICH, LUAD, BRCA, KIRP, LUSC, and THCA were negatively correlated with their methylation ($p < 0.05$; **Figure 4B** and **Supplementary Figure 4**). The mRNA expression of ACE1 in KIRC and LIHC; AGT in KIRC, LUAD, BRCA, COAD, THCA, and STAD; ACE2 in KIRC and LUAD; AGT in KIRC,

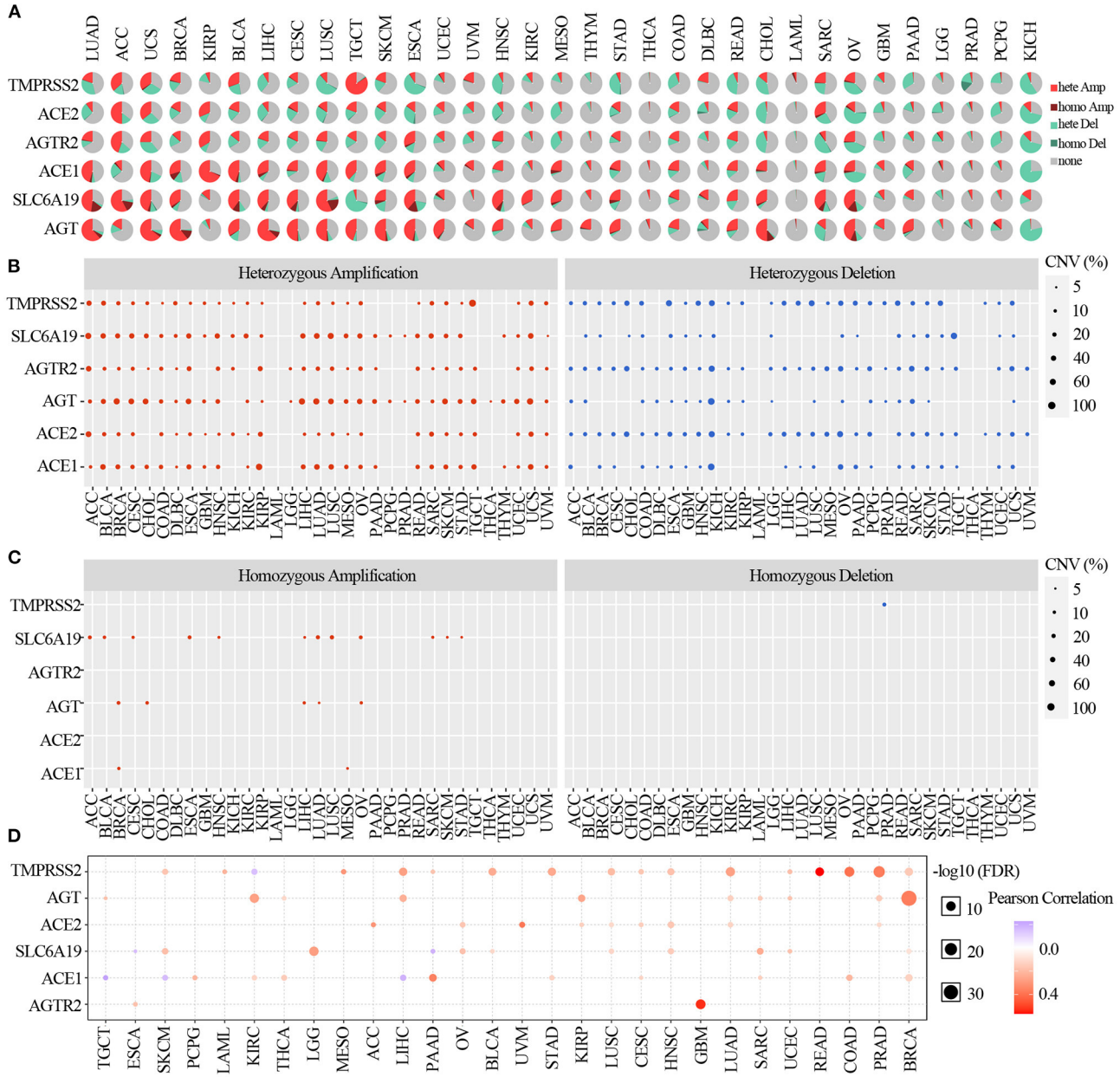

FIGURE 3 | Copy number variation of ACE2 receptor-related regulators. **(A)** CNV pie distribution in 33 cancers. CNV pie plot showed the combined heterozygous/homozygous CNV of each gene in each cancer. A pie represented the proportion of different types of CNV of one gene in one cancer, and different colors represented different types of CNV. Hete Amp, heterozygous amplification; Hete Del, heterozygous deletion; Homo Amp, homozygous amplification; Homo Del, homozygous deletion; None, no CNV. **(B)** Heterozygous CNV profile showing the percentage of heterozygous CNV, including the percentage of amplification and deletion of heterozygous CNV for each gene in each cancer. Only genes with >5% CNV in a given cancer are shown as a point on the figure. **(C)** Homozygous CNV profile showing the percentage of homozygous CNV, including the percentage of amplification and deletion of homozygous CNV for each gene in each cancer. Only genes with >5% CNV in a given cancer are shown as a point on the figure. **(D)** CNV correlation with mRNA. The association between paired mRNA expression and CNV percentage in samples was based on a Pearson product–moment correlation coefficient. The size of the point represents the statistical significance, where the bigger the dot size, the higher the statistical significance.

LUAD, BRCA, COAD, THCA, and STAD; SLC6A19 in BRCA; and TMPRSS2 in KICH were positively correlated with their methylation ($p < 0.05$; **Figure 4B** and **Supplementary Figure 4**). Prognosis analysis showed that hypermethylation of AGTR2 in BRCA; AGT in SKCM, CESC, and LAML; TMPRSS2 in KIRP, LUAD, READ, ACC, and HNSC; SLC6A19 in KIRP; ACE2 in ACC; and ACE1 in HNSC were associated with poor survival. Hypermethylation of TMPSS2 in GBM and UVM; AGT in THCA and KIRP; ACE2 in ESCA; SLC6A19 in BRCA; AGTR2 in LGG; and ACE1 in LGG and SKCM were associated with

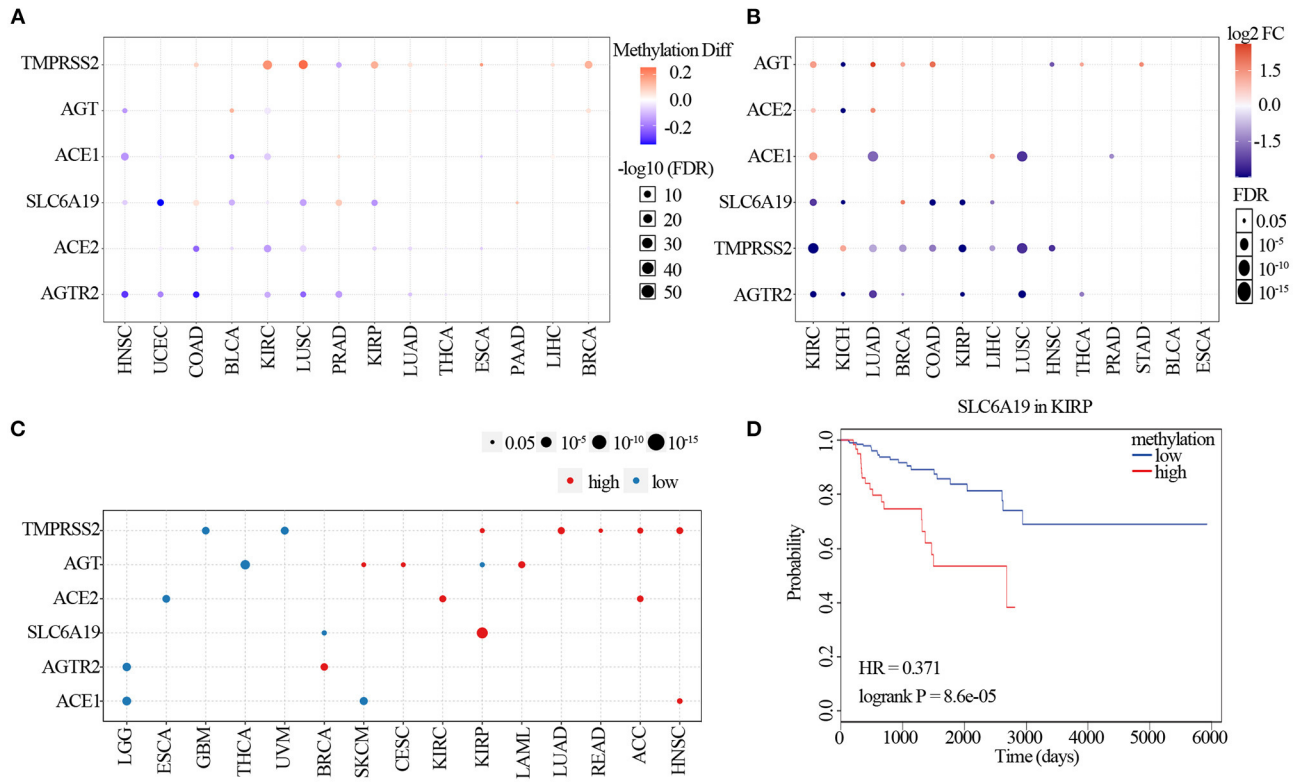

FIGURE 4 | Methylation of ACE2 receptor-related regulators. **(A)** Differential methylation changes in ACE2 receptor-related regulators between tumor and normal samples in each cancer. Blue points represent decreased methylation in tumors and red points represent increased methylation in tumors, where the darker the color, the larger the difference in methylation levels. **(B)** Correlation between methylation and mRNA gene expression. Blue points represent a negative correlation and red points represent a positive correlation, where the darker the color, the higher the correlation. **(C)** Survival difference between samples with ACE2 receptor-related regulators with high and low methylation. Red points represent poorer survival in high methylation groups; blue points were just the opposite. The size of the point represents the statistical significance, where the larger the dot size, the higher the statistical significance. **(D)** Survival analysis of SLC6A19 methylation in KIRP.

good survival ($p < 0.05$; **Figure 4C**). As shown in **Figure 4D**, the hypermethylation of SLC6A19 was significantly associated with poor survival in KIRP ($p = 8.6e{-}05$; **Figure 4D**).

Pathway Activity Analysis

The pathway relation network indicated that ACE2 receptor-related regulators were involved in TSC/mTOR, RTK, RAS/MAPK, PI3K/AKT, hormone ER, hormone AR, EMT, DNA damage response, cell cycle, and apoptosis pathways (**Figure 5A**). The global percentage of cancers in which regulators have an effect on a pathway showed that ACE1 was involved in the activation of apoptosis, DNA damage, epithelial–mesenchymal transition (EMT), hormone ER, hormone AR, RAS/MAPK, and RTK pathways and the inactivation of the cell cycle and TSC/mTOR pathways. ACE2 was associated with the activation of PI3K/AKT, RAS/MAPK, and TSC/mTOR pathways, and with the inactivation of the cell cycle, DNA damage, EMT, and hormone AR pathways. AGT was associated with the activation of the EMT pathway and the inactivation of apoptosis. AGTR2 was associated with the activation of RAS/MAPK, RTK, and TSC/mTOR pathways and the inactivation of apoptotic, cell cycle, DNA damage, hormone ER, and hormone AR pathways. SLC6A19 was involved in the activation of RTK and hormone AR pathways and the inactivation of hormone ER and TSC/mTOR pathways. TMPRSS2 was associated with the activation of the RTK pathway and inactivation of EMT (**Figure 5B**). As ACE2 receptor-related regulators are often mutated in UCEC, we further analyzed the global percentage of pathway activity in UCEC. We found that ACE1 was mostly involved in the inhibition of the cell cycle (21% inhibition vs. 7% activation) and activation of RAS/MAPK (9% inhibition vs. 13% activation). ACE2 was mainly involved in the inhibition of hormone AR (12% inhibition vs. 7% activation) and activation of the RTK pathway (0% inhibition vs. 19% activation). AGT was associated with inhibition of apoptosis (18% inhibition vs. 0% activation) and activation of EMT (6% inhibition vs. 16% activation). TMPRSS2 was mainly involved in the inhibition of the DNA damage response (12% inhibition vs. 7% activation) and EMT (34% inhibition vs. 4% activation), while it was associated with the activation of hormone AR (9% inhibition vs. 13% activation), hormone ER (9% inhibition vs. 13% activation), and RTK (6% inhibition vs. 22% activation) pathways (**Supplementary Figure 5**). These results indicated that the abnormal expression of ACE2 receptor-related regulators

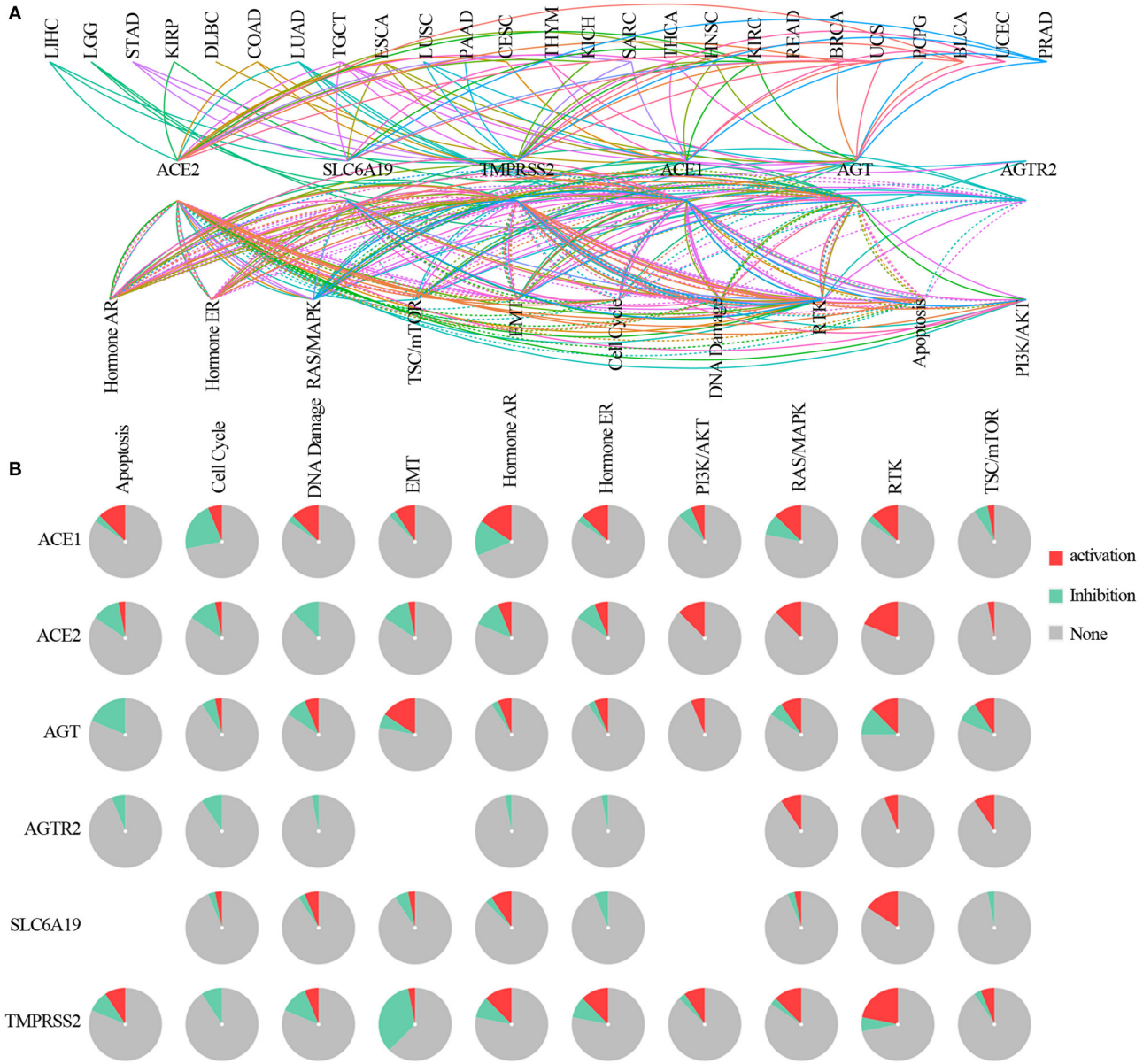

FIGURE 5 | The pathway network between ACE2 receptor-related regulators. **(A)** A line represents a connection between different pathways, where a solid line represents activation and a dashed line represents inhibition. Color of line represented different cancer types. **(B)** Global percentage of cancers in which ACE2 receptor-related regulators had an effect on the pathway among 32 cancer types, obtained as follows: number of activation or inhibition cancer types/32 × 100%.

mediated the abnormal activation of cancer-related signaling pathway, which played different roles in regulating tumorigenesis and progression.

miRNA Regulation Analysis

To clarify any miRNA regulation of ACE2 receptor-related regulators, visNetwork was used to generate miRNA regulation networks. As shown in **Figure 6**, hsa-miR-98-5P, hsa-let-7a-5P, hsa-miR-665, hsa-miR-432-5P, hsa-let-7b-5P, hsa-let-7d-5p, hsa-let-7g-5p, hsa-miR-545-3P, hsa-miR-452-5P, hsa-miR-939-5P, hsa-miR-7-5P, hsa-miR-513c-5P, hsa-miR-514-5P, hsa-miR-664a-3P, and hsa-let-7i-5p, hsa-let-7f-5P, hsa-let-7e-5P, hsa-miR-214-3P, hsa-miR-3154, hsa-miR-573, and hsa-miR-183-5P were negatively correlated with TMPRSS2 expression ($p < 0.05$); hsa-miR-31-5p, hsa-miR-181a-5p, hsa-miR-181b-5p, hsa-miR-181C-5p, hsa-miR-636, and hsa-miR-320e were negatively correlated with AGT; and hsa-miR-632, hsa-miR-330-5p, hsa-miR-200c-3p, hsa-miR-141-3p, hsa-miR-632, hsa-miR-26b-5p, hsa-miR-149-5p, hsa-miR-3125, hsa-miR-3143, hsa-miR-3187-3p, hsa-miR-200c-3p, and hsa-miR-3065-5p were negatively regulated with

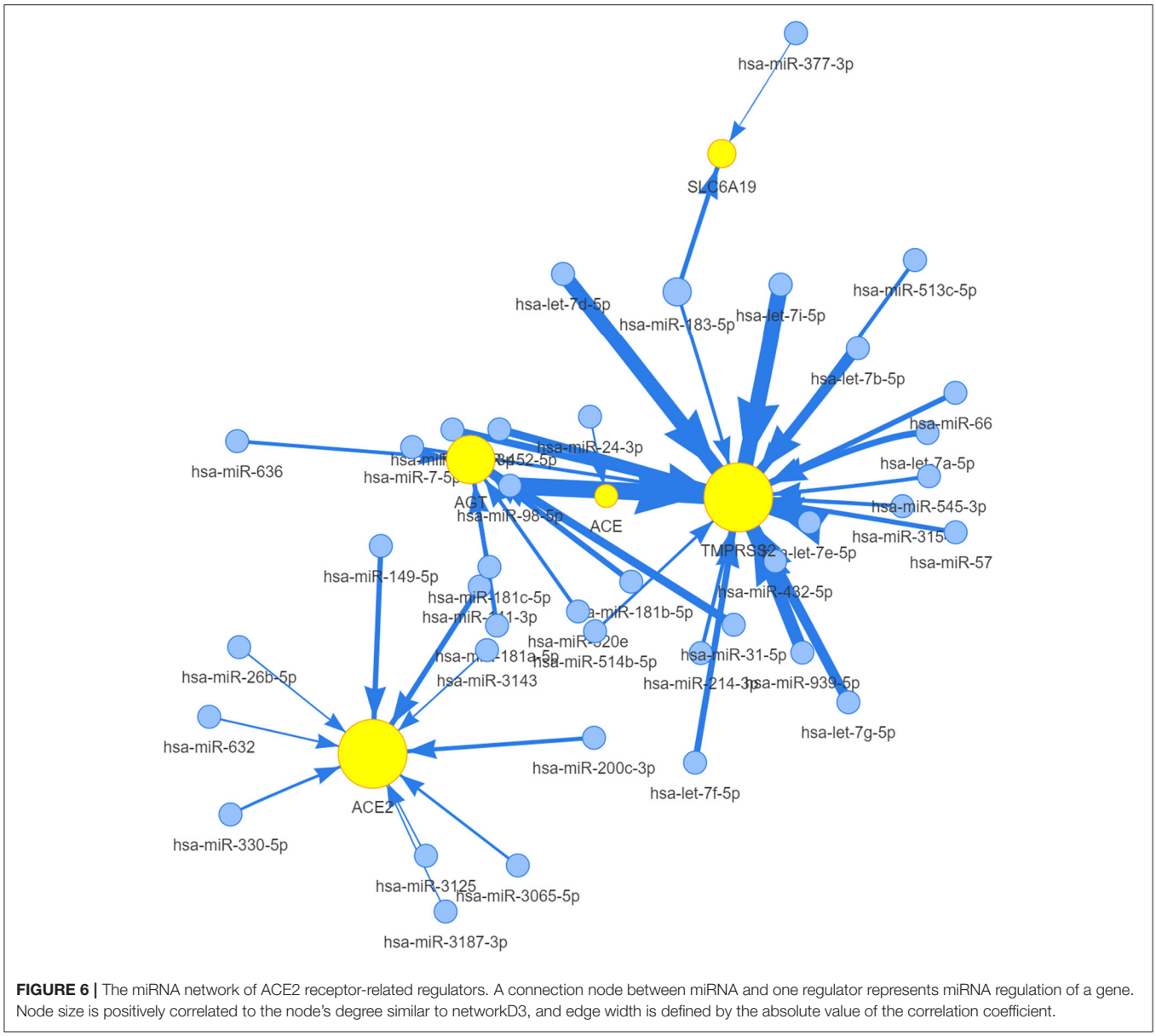

FIGURE 6 | The miRNA network of ACE2 receptor-related regulators. A connection node between miRNA and one regulator represents miRNA regulation of a gene. Node size is positively correlated to the node's degree similar to networkD3, and edge width is defined by the absolute value of the correlation coefficient.

the expression of ACE2 ($p < 0.05$); hsa-miR-183-5p and hsa-miR-377-3p were negatively regulated with the expression of SLC6A19 ($p < 0.05$); and hsa-miR-24-3p were negatively regulated with the expression of ACE1 ($p < 0.05$). These results indicated that the miRNA regulation network mediated ACE2 receptor-related regulators, which may be involved in the progression of cancer in patients with COVID-19.

Drug Sensitivity Analysis

Genomic aberrations influenced clinical response to treatment and are potential biomarkers for drug screening in cancer. To know the role of ACE2 receptor-related regulators on chemotherapy or targeted therapy, drug sensitivity and gene expression profiling data of cancer cell lines from the CTRP were integrated. Spearman's correlation analysis showed that drug sensitivity toward vincristine, teniposide, ouabain, docetaxel, doxorubicin, erlotinib, afatinib, AZD7762, and AT13387 correlated with the expression of AGTR2, SLC6A19, ACE2, and TMPRSS2 (negative correlation with IC_{50}). Drug resistance toward staurosporine correlated with the expression of TMPRSS2, JW55, FGIN-1-27, BRD-K96431673, BRD-K86535717, BRD-K75293299, and BRD-K49290616 (positive correlation with IC_{50}) (**Figure 7**). These results indicated that the abnormal expression of ACE2 receptor-related regulators may mediate sensitivity to chemotherapy and targeted drug therapy.

DISCUSSION

COVID-19 is a global health emergency problem with a large number of confirmed cases and deaths that are much greater than any infection in recent decades. Condition severity and mortality have been identified as being significantly higher in patients with

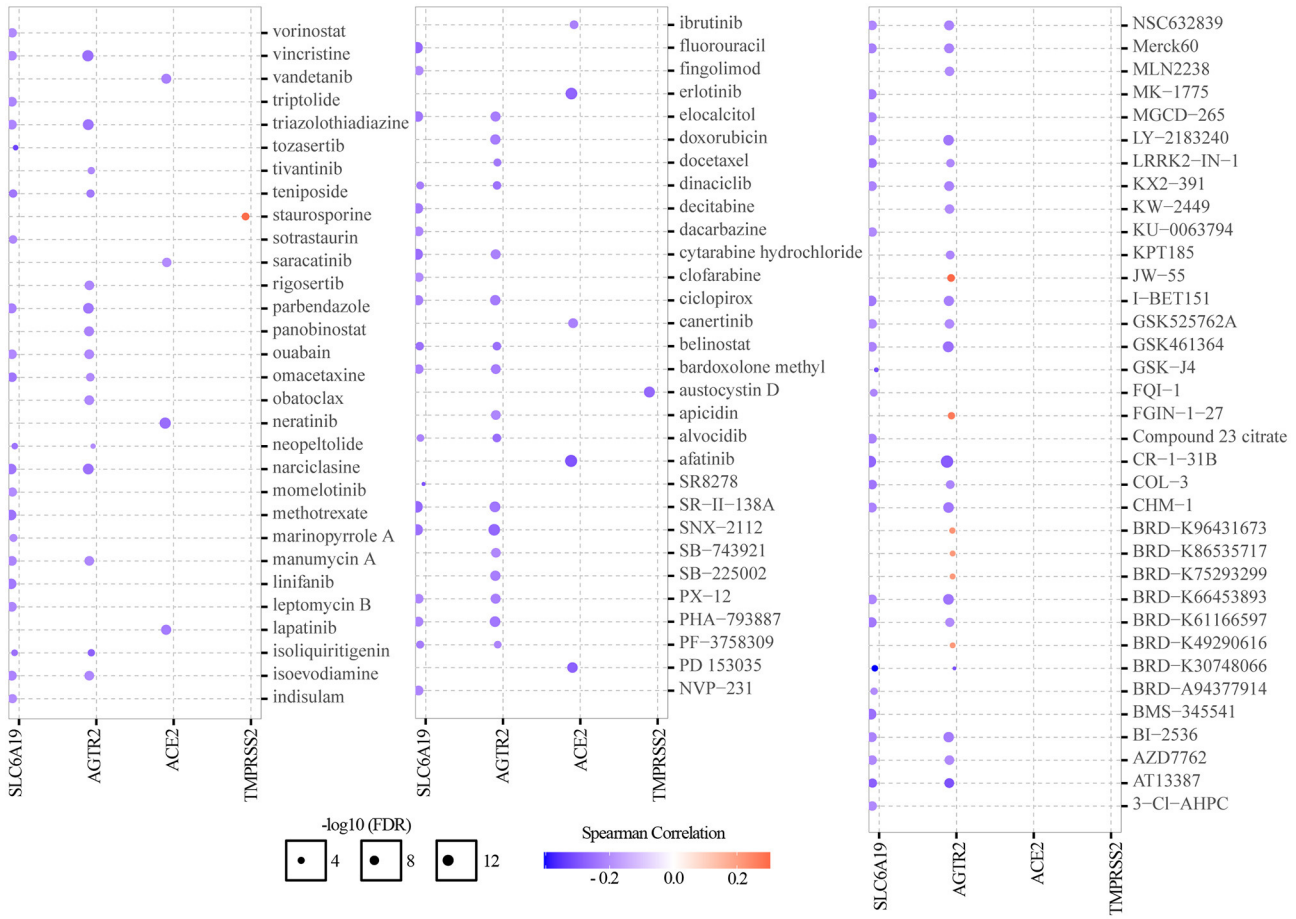

FIGURE 7 | Drug sensitivity analysis of ACE2 receptor-related regulators. The gene set drug resistance analysis from CTRP IC_{50} drug data. Spearman's correlation represented how the gene expression correlates with a drug. A positive correlation means that a gene with high expression was resistant to a drug, and *vice versa*.

other comorbidities, such as cancer (30–32). As care for chronic conditions, such as cancer, still needs to continue during the pandemic, it is necessary for healthcare providers to determine which type of cancer will put patients at a higher risk of exhibiting severe forms of the COVID-19 infection. Patients have also had to balance the risks and benefits of cancer-directed interventions within the context of the added risk of contracting COVID-19. In this study, we comprehensively analyzed the genomic and prognostic characteristics of six COVID-19 receptor-related regulators, where we found that genetic and epigenetic alterations, and an miRNA network of COVID-19 receptor-related regulators, led to their abnormal expression, which correlated significantly with the activation of cancer hallmark-related pathways and clinical survival. Targeting these COVID-19 receptor-related regulators may be an important method to treat cancer patients with COVID-19.

We firstly explored the genetic alterations and prognoses of these regulators and found that the abnormal expression was associated with clinical prognosis. Our results indicated that there were 14 tumor types that differentially expressed one or more of these regulators. The regulators were highly expressed in normal mucosal epithelial tissue, such as kidney, urinary bladder, and mucocutaneous and gastrointestinal tract, which was consistent with ACE2 protein distribution. This co-expression pattern further validated that the SARS-CoV-2 entry process requires the interaction of these regulators. By tracking the genetic differences in these six regulators, we found that missense mutations were the main mutation type in SNV, with ACE1 having the highest mutation frequency in cancer. In addition, ACE1 mutations in malignancies were distributed across all exons of ACE1 with several hot spot mutation sites. ACE mutations have been reported to be involved in a number of lymph node metastases of gastric cancer (33) and associated with a worse prognosis in prostate cancer (34). However, there was also non-conformity between genomics alternation and clinical prognosis. Thus, we speculated that genetic and epigenetic alteration of the regulators may cause gene dysfunction and promote tumorigenesis in certain contexts.

Further investigation into the biological function of the regulators identified several pathways, including TSC/mTOR, RTK, RAS/MAPK, PI3K/AKT, hormone ER, hormone AR, EMT, DNA damage response, cell cycle, and apoptosis pathways, that were significantly enriched in cancers. In UCEC, different ACE2 receptor-related regulators were associated with different

cancer-related signaling pathways. For example, TMPRSS2 was involved in the activation of the RTK pathway and AGTR2 was associated with the inhibition of the cell cycle and the apoptosis pathway. Recent studies identified that Ang II can also promote cell growth and proliferation *via* the transforming growth factor-beta (35), RTK (36), and mTOR pathways (37). Activation of Ang II receptor in cancer cell lines resulted in increased MAPK activation, JAK-STAT signaling, and cell proliferation (38, 39). Thus, activation and inhibition of cancer-related signaling pathways mediated by ACE2 receptor-related regulator molecular networks played different roles in tumorigenesis and prognosis.

In clinical applications, dexamethasone, which can reduce inflammation, and remdesivir, which can inhibit viral replication, have been widely used to decrease the mortality in cancer patients with COVID-19 (40, 41). There are currently no effective drugs for COVID-19. There is an urgent need for therapeutic interventions, especially for cancer patients with weakened immune systems. Our drug sensitivity analysis identified that ACE2 receptor-related regulator expression levels were also involved in drug sensitivity. Vorinostat is an anticancer histone deacetylase (HDAC) inhibitor and has previously been shown to have anti-fibrotic effects and can reduce the risk of acute respiratory deterioration by upregulating ACE2 expression (42, 43). Erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, has been reported to inhibit endocytosis and intracellular trafficking of multiple viruses, including hepatitis C, dengue, and Ebola, exerting broad-spectrum antiviral effects by increasing ACE2 expression (44). Thus, we speculate that targeting ACE2 receptor-related regulators will become an ideal approach in cancer treatment. However, variations of ACE2 receptor-related regulators exist at all regulation levels, including genetics and epigenetic alterations, mRNA expression, miRNA networks, and pathway correlations. These variations may alter drug effects, treatment responses, and patient survival. Thus, the potential mechanisms of each drug's effect on ACE2 receptor-related regulator expression and cancer progression require further investigation.

CONCLUSION

In conclusion, our findings indicate the need for precautions for and protection of cancer patients during the COVID-19 pandemic. However, the balance between the risks and benefits of cancer-directed interventions should be reassessed. Thus, targeting ACE2 receptor-related regulators could be a promising strategy against cancer patients with COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JZ, HJ, and KD performed all experiments, prepared the figures, and drafted the manuscript. JZ, TX, BW, and BC participated in the data analysis and results interpretation. JZ, KD, CC, YY, and JY designed the study and participated in the data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.662460/full#supplementary-material

Supplementary Figure 1 | Overall survival of ACE2 receptor related regulators in cancers.

Supplementary Figure 2 | Mutational map of ACE2 receptor related regulators in cancers.

Supplementary Figure 3 | Overall survival of the mutations of ACE2 receptor related regulators in cancers.

Supplementary Figure 4 | Correlation analysis of methylation and mRNA expression of ACE2 receptor related regulators.

Supplementary Table 1 | Mutations of ACE2 receptor related regulators in cancers.

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Clinical Characteristics and the Long-Term Post-recovery Manifestations of the COVID-19 Patients—A Prospective Multicenter Cross-Sectional Study

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Objective: Coronavirus disease 2019 (COVID-19) or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is a global issue. In addition to managing acute cases, post-COVID-19 persisting symptoms/complaints and different hematological values are of great concern. These have an impact on the patient's well-being and are yet to be evaluated. Therefore, clinical and primary diagnosis based on routine laboratory findings bears high importance during the initial period of COVID-19, especially in regions with fewer diagnostic facilities.

Methods: Clinical information and associated complaints of the COVID-19 illness confirmed by reverse transcription-polymerase chain reaction (RT-PCR) were collected directly from the patients. Regular follow-ups were obtained on the phone every 2 weeks following recovery for 20 weeks. Initial hematological and radiology findings of the hospitalized patients except for intensive care unit (ICU) and high dependency units (HDUs) and a follow-up evaluation after 4 weeks following recovery were analyzed.

Results: The post-COVID-19 persisting symptoms/complaints were found among 21.4% of symptomatic patients, which persisted for ≥20 weeks and had a significant relationship with the duration of COVID-19 illness and the existing comorbidity ($p < 0.05$). Post-COVID-19 primary type 2 diabetes mellitus (DM, 0.64%) and hypertension (HTN, 1.28%) and unstable DM (54.55%) and HTN (34.78%) to the pre-existing diabetic and hypertensive patients were observed. Post-recovery remarkable changes in the laboratory values included leukocytosis (16.1%), lymphocytosis (14.5%), and an increased prothrombin time (PT, 25.8%). Abnormalities in the D-dimer, serum ferritin, hemoglobin, and erythrocyte sedimentation rate (ESR) levels were present to an extent. Laboratory findings like chest X-ray, ESR, white blood cell (WBC) count, lymphocyte count, C-reactive protein (CRP), serum glutamic pyruvic transaminase (SGPT), serum ferritin, PT, D-dimer, and serum creatinine are important markers for the diagnosis and prognosis of COVID-19 illness ($p < 0.05$).

Conclusion: Post-COVID-19 persisting symptoms and the changes in the laboratory values need to be considered with importance and as a routine clinical measure. Post-COVID-19 periodic follow-up for evaluating the patient's physical condition and the biochemical values should be scheduled with care and managed accordingly to prevent future comorbidity in patients with the post-COVID-19 syndrome.

Keywords: COVID-19, SARS-CoV-2, clinical characteristics, post-recovery manifestations, Bangladesh, post-COVID-19 syndrome

INTRODUCTION

Coronavirus disease 2019 (COVID-19) or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is a current global pandemic caused by an RNA virus of the beta Coronavirus family (1). After the first report in the Wuhan province of China, the disease quickly spread all over the globe. However, the presentation of COVID-19 varies depending on the region of origin. Thus, in a region with inadequate facilities, primary identification of SARS-CoV-2 infection remains a challenge. Furthermore, the overlapping clinical presentation of COVID-19 with local viral flu often misleads the diagnosis, thus causing morbidity, mortality, and further spread of the infection.

Additionally, like an emerging disease, managing the persisting symptoms/complaints and the abnormality of the laboratory values following the post-COVID-19 recovery period is complex. Proper understanding of these complaints is necessary for both the healthcare professionals and the patients, as it has a broad impact on patient well-being and health status. The changes in the laboratory values following COVID-19 recovery are yet to be evaluated. This manuscript is intended to investigate the acute and post-recovery manifestations of COVID-19 illness, including the post-COVID-19 persisting symptoms/complaints and changes in the laboratory parameter, namely, post-COVID-19 syndrome among the patients in Bangladesh.

METHODS

Study Population and Data Collection

The list of SARS-CoV-2 confirmed cases by reverse transcription-polymerase chain reaction (RT-PCR) was identified and collected from the local health facilities of the Chattogram division in Bangladesh. All the patients took treatments in different COVID-19 designated hospitals. Preliminary information of the symptomatic cases that required treatments regarding the COVID-19 illnesses was collected directly from the outpatient department (OPD) and the hospitalized patients. These included disease symptoms, history, comorbid condition, and associated complaints. In addition, initial radiology and laboratory tests such as chest computed tomography (CT), X-ray chest P/A view, hemoglobin level, erythrocyte sedimentation rate (ESR), total and differential counts of white blood cell (WBC), red blood cell (RBC), platelet, C-reactive protein (CRP), serum glutamic pyruvic transaminase (SGPT), serum ferritin, prothrombin time (PT), D-dimer, and serum creatinine were performed, and the values were noted. In addition, febrile patients were tested for dengue NS1 antigen, dengue immunoglobulin G (IgG) and immunoglobulin M (IgM) antibody, Salmonella Typhi/Para-Typhi IgM and IgG antibody, immunochromatographic test (ICT) for malaria, Widal test to exclude dengue, malaria, and enteric fever.

Ethical Consideration and Consent

Ethical committee approval was taken from Xi'an Jiaotong University, China. Informed written consent was taken in every case. In the case of the below 16 years old participants, written informed consent was obtained from a parent or guardian.

Exclusion Criteria

Critically ill COVID-19 patients in the intensive care unit (ICU) and high dependency units (HDUs); patients who had a preexisting uncontrolled comorbid condition with compromised organ function such as severe chronic obstructive pulmonary disease (COPD) exacerbation, advanced ischemic heart disease, severe uncontrolled diabetes mellitus (DM), advanced renal and hepatic disease, and advanced stage of carcinoma; and prehospitalized (not due to COVID-19), immunocompromised, and pregnant patients were not included in this study. In addition, those who had a recent history of hematological, biochemical, or chest radiograph abnormality within the last 30 days were excluded. Expired patients during this study who missed the regular follow-up schedule twice and who missed the follow-up laboratory evaluation and those who were unwilling to continue participation were also excluded from the study.

Post-COVID-19 Persisting Symptoms and Hematological Manifestations

COVID-19 recovery was defined by two negative PCR 7 days apart. The recovery time was calculated from the date of the first negative PCR. COVID-19 symptoms or newer symptoms, which persisted or appeared following the COVID-19 recovery, were identified as persisting symptoms. Regular

Abbreviations: COVID-19, coronavirus disease 2019; CDC, Centers for Disease Control and Prevention; CRP, C-reactive protein; CT, computed tomography; DM, diabetes mellitus; ESR, erythrocyte sedimentation rate; HTN, hypertension; IgG, immunoglobulin G; IgM, immunoglobulin M; IEDCR, Institute of Epidemiology, Disease Control, and Research; LMWH, low-molecular weight heparin; MERS, Middle East respiratory syndrome; OPD, outpatient department; PT, prothrombin time; RBC, red blood cell; RT-PCR, reverse transcription-polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SGPT, serum glutamic pyruvic transaminase; T2DM, Type 2 diabetes mellitus; WBC, white blood cell.

TABLE 1 | General characteristics of the study population.

Variables		Number of cases (*n*)	Percentage %
Number of patients ($n = 313$)	M	251	80.2
	FM	62	19.8
Age (in years)	Minimum	3 years	
	Maximum	75 years	
	Mean ± SD	37.74 ± 13.70	
	Median	35	
	1–10 years	3	1.0
	11–20 years	19	6.1
	21–30 years	91	29.1
	31–40 years	82	26.2
	41–50 years	56	17.9
	51–60 years	40	12.8
	60+ years	22	7
Hospitalization ($n = 62$, 19.80%)	M	55	88
	FM	7	11.29
	0–10 days	34 (M 29, FM 5)	54.8
	>10 days	28 (M 26, FM 2)	45.2
Duration of COVID-19 symptoms	0–10 days	239 (M 185, FM 54)	76.4
	>10 days	74 (Male 66, FM 8)	23.6
Recovery duration (confirmation of case to negative PCR)	1–10 days	157 (M 120, FM 37)	50.2
	11–20 days	133 (M 112, FM 21)	42.5
	>21 days	23 (M 19, FM 4)	7.3
Post-COVID-19 persisting symptoms/complaints	None	246 (M 198, FM 48)	78.6
	Present	67 (M 53, FM 14)	21.4
Comorbidity 64 (20.4%) None 249 (79.6%)	Cancer	1	0.3
	Gastritis	1	0.3
	Hypotension	1	0.3
	Bronchial asthma	13	4.2
	T2DM	20	6.4
	HTN	21	6.7
	Ischemic heart disease	2	0.6
	Liver disease	1	0.3
	Skin Allergy	1	0.3
	HBs Ag (+Ve)	1	0.3
	HTN and T2DM	2	0.6

COVID-19, coronavirus disease 2019; FM, female; HTN, hypertension; M, male; T2DM, Type 2 diabetes mellitus.

follow-ups were obtained on the phone every 2 weeks from each participant to identify and analyze the post-COVID-19 persisting symptoms/complaints. This process continued for 20 weeks. The symptoms that may have been attributed to other illnesses during this study by any current or chronic condition were carefully examined and not included in the study.

An initial laboratory assessment while hospitalized and a post-COVID-19 follow-up evaluation after 4 weeks were done to understand the changes. The follow-up laboratory evaluations included X-ray chest P/A view, a complete blood count (hemoglobin level, ESR, total and differential count of WBC, RBC, platelet), CRP, SGPT, serum ferritin, PT, D-dimer, and serum creatinine. In addition, patients with persisting fever were tested for dengue, malaria, and enteric fever. In the cases of laboratory abnormalities, treatments were given to the patients; therefore, no further laboratory follow-up data were collected.

Data and Statistical Analysis

Data were presented as mean ± standard deviation, and statistical analysis was carried out using SPSS V25.0. Column statistics, chi-square test, and a *t*-test with a 95% confidence interval were done to see the significance of the necessary values. $p < 0.5$ was considered statistically significant.

RESULTS

Demographics and Mortality Rate Among the Study Population

Initially, 512 symptomatic COVID-19 cases were included. Among them, 53 patients refused to participate in the study. Out of the rest, 83 were hospitalized, and 376 were OPD cases. While continuing treatment, 12 OPD patients required hospitalization.

Later, they were included among the hospitalized patients. Therefore, 95 hospitalized and 364 OPD cases were enrolled. After enrollment, 33 hospitalized patients were excluded during the study; 11 had preexisting comorbid conditions that fit the exclusion criteria, 10 died during the treatment, and 12 were lost during follow-up. Among the OPD participants, 113 were excluded from the study; 45 patients had preexisting conditions, 48 were unable to maintain regular follow-up, and 20 were unwilling to continue participating. Finally, 313 patients were included in the study for analysis, among whom 62 were hospitalized and 251 were OPD patients. The mortality rate among the COVID-19 patients in our study was 1.96%. The hospitalization rate among the OPD-treated patients was 3.2%.

General Characteristics of the Study Population

The total number of patients was 313; 251 (80.2%) male and 62 (19.8%) female. The mean age was 37.74 years, range from 3 to 75 years. Patients according to the age-groups were 3 (1–10 years), 19 (11–20 years), 91 (21–30 years), 82 (31–40 years), 56 (41–50 years), 40 (51–60 years), and 22 (60+ years). The age-group 21–30 years was the most affected, consisting of 91 (29.1%) patients. Among the hospitalized patients ($n = 62$), (19.80%), 55 (88%) were male and 7 (11.29%) were female. In addition, 239 (74%) patients experienced <10 days and 74 (23.6%) experienced >10 days of COVID-19-related symptoms. Sixty-seven (21.4%) of the total study population had post-COVID-19 persisting symptoms/complaints. Moreover, 20.4% of the patients had comorbidities such as hypertension (HTN) [21 (6.7%)] and Type 2 diabetes mellitus (T2DM) [20 (6.4%)] (**Table 1**).

Clinical Characteristics of COVID-19 Patients

Most COVID-19 patients presented with fever [274 (87.5%)] and cough [103 (32.9%)]. Other symptoms were myalgia [54 (17.25%)], loss of appetite [51 (16.30%)], headache [43 (13.74%)], abdominal cramps/pain [34 (11.50%)], difficulty in breathing [19 (6.07%)], loss of smell [15 (5.11%)], fatigue [16 (5.11%)], chest tightness [11 (3.11%)], vomiting [9 (2.86%)], non-specific discomfort–unable to describe [9 (2.86%)], rhinorrhea [9 (2.86%)], sore throat [7 (2.24%)], insomnia [6 (1.91%)], diarrhea [5 (1.60%)], sneezing [5 (1.60%)], burning sensation in the body [4 (1.28%)], partial loss of hearing [2 (0.64%)], neck pain [1 (0.32%)], joint pain [1 (0.32%)], chest pain [1 (0.32%)], and itching [1 (0.32%)]. Nonetheless, anxiety was experienced by all of these patients at a certain level (**Table 2**).

A significant difference was found in a *t*-test among initial radiological and hematological parameters with the post-COVID-19 recovery follow-ups. These were chest X-ray consolidated, ESR, the total count of WBC, lymphocyte, CRP, serum ferritin, PT, D-dimer, and serum creatinine levels ($p < 0.05$). However, no significant differences were found in the neutrophil, RBC, platelet, and hemoglobin levels ($p > 0.05$; **Table 3**).

TABLE 2 | COVID-19 symptoms among the study population.

Name of the symptoms	Number of patients (*n*)	Percentage %
Fever	274	87.5
Cough	103	32.9
Myalgia	54	17.25
Loss of appetite	51	16.30
Headache	43	13.74
Abdominal cramp/pain	36	11.50
Difficulty in breathing	19	6.07
Loss of smell	16	5.11
Fatigue	16	5.11
Chest tightness	11	3.51
Vomiting	9	2.86
Non-specific discomfort	9	2.86
Rhinorrhea	9	2.86
Sore throat	7	2.24
Insomnia	6	1.91
Diarrhea	5	1.60
Sneezing	5	1.60
Burning sensation in the body	4	1.28
Partial loss of hearing	2	0.64
Loss of taste	1	0.32
Neck pain	1	0.32
Joint pain	1	0.32
Chest pain	1	0.32
Itching	1	0.32

COVID-19, coronavirus disease 2019.

Post-COVID-19 Persisting Symptoms/Complaints and Their Characteristics

Out of the total, 78.6% (246) patients had no complaints following recovery. The post-COVID-19 persisting symptoms were lethargy [23 (7.4%)], cough [16 (5.1%)], chest discomfort and pain [12 (3.8%)], breathlessness on activity [12 (3.8%)], fatigue [12 (3.8%)], significant anxiety with lack of concentration and occasional amnesia that impaired daily activity [5 (1.6%)], headache [4 (1.2%)], low-grade fever [2 (0.6%)], and joint pain [2 (0.6%)]; mild body ache/chill/back pain was experienced by one (0.3%) each. Following recovery from COVID-19, 2 (0.64%) patients developed T2DM and 4 (1.28%) developed HTN. Nine (55.55%) of the total diabetic patients faced difficulties controlling the glycemic levels and had to take additional hypoglycemic therapy. Eight (34.78%) of the total hypertensive patients had to increase the dose or take additional antihypertensive therapy to achieve proper blood pressure control (**Table 4**).

Furthermore, 0.6% of participants had the earliest relief from post-COVID-19 persisting symptoms on the third week from the recovery date. Fifteen (4.8%) recovered on the 10th week. Seven (2.2%) patients experienced 20 weeks or more duration of the persisting complaints (**Table 5**). However, the correlations between the duration of COVID-19 symptoms of 0–10 days and

TABLE 3 | Comparison between the initial on admission laboratory and radiology finding and post-COVID-19 recovery (following negative PCR) 4-week follow-up.

Parameters	Initial findings during admission	Post-COVID-19 4-week follow-up	*t*-test (95% CI)
Chest X-ray consolidation	13.36 ± 11.35	0	$p < 0.0001$****
Hemoglobin	13.28 ± 1.55	13.35 ± 1.75	$p = 0.824$
ESR	31.42 ± 19.41	18.89 ± 9.83	$p < 0.0001$****
WBC total count	7,162 ± 3,005	1,035 ± 1,999	$p = 0.036$**
RBC	4.94 ± 6.78	5.16 ± 0.70	$p = 0.0837$
Platelet	230,859 ± 66,213	232,016 ± 75,655	$p = 0.93$
Neutrophil	65.97 ± 12.71	62.75 ± 8.21	$p = 0.0741$
Lymphocyte	28.42 ± 12.52	36.24 ± 9.56	$p < 0.0001$****
Monocyte	3.78 ± 2.34	3.33 ± 2.32	$p = 0.289$
Eosinophil	1.75 ± 1.24	2.08 ± 1.16	$p = 0.57$
Basophil	0.067 ± 0.25	0.080 ± 0.27	$p = 0.77$
CRP	29.86 ± 23.65	5.3 ± 9.07	$p < 0.0001$****
SGPT	62.71 ± 45.18	49.13 ± 23.35	$p = 0.0385$*
Serum ferritin	543.3 ± 460.6	162 ± 105.2	$p < 0.0001$****
Prothrombin time	11.67 ± 1.92	12.59 ± 1.74	$p = 0.0063$**
D-dimer	1.05 ± 1.24	0.08 ± 0.18	$p < 0.0001$****
Serum creatinine	1.028 ± 0.27	0.85 ± 0.26	$p = 0.0003$***

*Data are presented as mean ± SD. For the reference values, please see **Table 7**.*
*COVID-19, coronavirus disease 2019; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; RBC, red blood cell; SGPT, serum glutamic pyruvic transaminase; WBC, white blood cell. Level of significance of the p-value *, **, ***, ****.*

>10 days and post-COVID-19 persisting symptoms of 0–8 weeks and >8 weeks were statistically significant (**$p = 0.00$; **Table 6**).

Initial and Post-recovery Laboratory Manifestations of COVID-19 Cases

The mean age was 43.32 years (21–75 years). Forty-three (69.35%) patients had consolidations in the chest X-ray during admission, while at 4-week follow-up, three (0.48%) patients had mild features of pneumonitis. Lymphocytopenia was detected among 19 (30.6%). CRP was increased in all the patients during admission but in 11 (17.7%) during the follow-up. ESR, WBC, neutrophil, serum ferritin, D-dimer, and PT were increased in 43 (69.4%), 8 (12.9%), 11 (17.7%), 28 (45.1%), 31 (50%), and 14 (22.6%) patients during admission, while during follow-up, they were 34 (54.8%), 10 (16.1%), 4 (33.8%), 6 (9.7%), 4 (6.4%), and 16 (25.8%) subsequently. Males had a higher percentage (52.7%) of increased D-dimer during admission. Erythrocytopenia was found only in females. Moreover, females had a greater presentation of an increase in the CRP level (42.8%), serum ferritin (14.2%), PT (42.8%), lymphocyte (28.6%), and X-ray (mild features of pneumonitis) (14.2%) during the follow-up. However, D-dimer (7.3%) and total WBC (16.3%) levels were increased in the males (**Table 7**).

Subgroup Analysis of the Persisting Symptoms of COVID-19 Cases

Lethargy mainly was seen in age-group 21–30 years (nine patients) and cough (four patients each) in age-groups 21–30, 31–40, 41–50, 51–60 years. Chest pain and breathlessness on activity were dominant in 31–40 years (six patients) and anxiety in 11–20 years (four patients). Four patients 51–60 years of age complained of headache, and two patients each among those aged 41–50 and 31–40 years complained of breathlessness on activity and joint pain. Back pain and myalgia were experienced by two patients of the age-group 21–30 years. In addition, 53 (11.2%) males and 14 (4.5%) females had post-COVID-19 persisting symptoms. Chest pain (12), breathlessness on activity (2), and joint pain (2) were solely complained about by the male participants. On the other hand, six female participants suffered from persisting fever with headache and lethargy. The difference between patients having persisting symptoms according to gender was not statistically significant ($p = 0.277$) (**Supplementary Table 1**).

According to age-group, subgroup analyses of patients with persisting symptoms and the duration of the persisting symptoms were done. This revealed that 1–10 years had no complaints, and the 41–50 years age-group had post-COVID-19 complaints that persisted for >20 weeks. The age-groups 21–30 and 41–50 years started to recover the earliest at 3 weeks and the complain persisted up to 16 weeks, and 8 weeks post-recovery respectively; and those were 41–50 years complained up to 20+ weeks (**Supplementary Table 2**).

Subgroup Analysis of Duration of the Persisting Symptoms Among the Comorbid Patients

A total of 26 patients who experienced post-COVID-19 persisting symptoms had comorbidity. HTN was found in 11 patients; among these patients, four patients experienced a delayed recovery from the post-COVID-19 symptoms/complaints about ≥20 weeks. T2DM and bronchial asthma were found among 12 patients (six each). They had an earlier recovery from the post-COVID-19 persisting symptoms on the 15th and 12th

TABLE 4 | Post-COVID-19 persisting symptoms/complaints and comorbidity.

Name of the persisting symptom	Number of patients (*n*)	Percentage of the patients %
No complaint	246	78.6
Lethargy	23	7.4
Cough	16	5.1
Chest discomfort and pain	12	3.8
Fatigue	12	3.8
Breathlessness on activity	12	3.8
Anxiety, lack of concentration, and occasional amnesia	5	1.6
Headache	4	1.2
Fever (persisting fever)	2	0.6
Joint pain	2	0.6
Mild body ache	1	0.3
Chill	1	0.3
Enteric fever	1	0.3
Back pain	1	0.3
T2DM (following COVID-19)	2	0.64
HTN (following COVID-19)	4	1.28
Post-COVID uncontrolled DM	9	54.55
Post-COVID uncontrolled HTN	8	34.78

COVID-19, coronavirus disease 2019; HTN, hypertension; T2DM, Type 2 diabetes mellitus.

TABLE 5 | Duration of the post-COVID-19 complaints or symptoms.

Duration (in weeks)	Number of patients	Percentage of the patients %
None	246	78.6
3rd	2	0.6
4th	3	1.0
5th	9	2.9
6th	3	1.0
7th	11	3.5
8th	6	1.9
9th	2	0.6
10th	15	4.8
15th	5	1.6
16th	4	1.3
20 or more	7	2.2
Total	313	100.0

week. Among the persisting symptoms, fever was experienced by hypertensive patients. Lethargy was complained about by 12 patients; among these 12 patients, five had HTN, four had T2DM, two had bronchial asthma, and one had skin allergy. Persisting cough was complained about by five patients, and four who had bronchial asthma suffered from chest pain and breathlessness on activity until the 10th week following a negative PCR. A significant relationship was found between the comorbidity and the duration of the post-COVID-19 persisting symptoms $p = 0.000$ in the chi-square test (**Supplementary Table 3**).

DISCUSSION

SARS-CoV-2 is a systemic infection causing a cytotoxic storm that exerts an effect on the homeostatic mechanism (2). The incubation period of COVID-19 can be up to 14 days. Presenting symptoms of COVID-19 may differ from mild to a severe illness expressed by respiratory distress, chest tightness, or pain. COVID-19 symptoms might present with fever, cough, myalgia, chest tightness, breathing difficulty, sore throat, and loss of taste or smell. A deteriorating sign of SARS-CoV-2 infection includes central cyanosis, confusion, respiratory difficulty, persistent chest pain, heaviness in the chest, and inability to stay awake (3). Studies have reported differences in presenting symptoms of COVID-19 illness, depending on regions. However, several symptoms are common among all the reports. Hematological manifestations of COVID-19 are also different depending on the geographical locations and socioeconomic conditions. Moreover, post-COVID-19 persisting symptoms/complaints and associated laboratory manifestations are yet to be evaluated.

The impact of the patient's clinical characteristics and post-recovery manifestations of the COVID-19 patients will shed light on this disease's systematic management (4). Initial investigations from China have shown that clinical symptoms such as fever, cough, dyspnea, and hematological findings of COVID-19 patients are like those in SARS-CoV (5–8). Heterogeneous clinical manifestations have been discovered in COVID-19 patients from different areas of China (9–16), Europe (17, 18), US (19, 20), Malaysia (21), Jordan (22), Kuwait (23), South Korea (24), and Africa (25). Thus, understanding the clinical features can help clinicians predict the disease's progression and apply possible intervention approaches.

TABLE 6 | Relation between the duration of the COVID-19 symptoms and the duration of post-COVID-19 persisting complaints.

Duration of the COVID-19 symptoms	Duration of the post-COVID-19 complaints			Number of cases (*n*)	
	0–8 weeks	>8 weeks	None		
0–10 days	21	31	187	239	
>10 days	13	02	59	74	Chi-square (X^2) $p = 0.00$**
Total	34	33	246	313	

*COVID-19, coronavirus disease 2019. **Level of significance.*

TABLE 7 | Primary (on admission) hematological findings and the post-COVID-19 recovery (following negative PCR) 4-week follow-up of the hospitalized patients.

Parameters	Initial findings (admission day)			Post-COVID-19 4-week follow-up		
	Patients (*n*)	Male	Female	Patients	Male	Female
Chest X ray	62	55	7	3 (0.48%)	2 (3.6%)	1 (14.2%)
Decreased Hb	31 (50%)	28 (50.9%)	4 (57.1%)	26 (41.9%)	23 (41.8%)	3 (42.8%)
Increased ESR	43 (69.4%)	39 (70.9%)	4 (57.1%)	34 (54.8%)	30 (54.5%)	4 (57.1%)
Leukocytosis	8 (12.9%)	6 (10.9%)	2 (28.6%)	10 (16.1%)	9 (16.3%)	1 (14.2%)
Decreased RBC	4 (6.5%)	0	4 (57.1%)	3 (4.84%)	0	3 (42.8%)
Platelet	0	0	0	0	0	0
Neutrophil	↑11 (17.7%)	↑10 (18.2%)	↑1 (14.3%)	↑4 (33.8%)	↑4 (7.2%)	0
Lymphocyte	↓11 (17.7%)	↓10 (18.2%)	↓1 (14.3%)	↑9 (14.5%)	↑7 (12.7%)	↑2 (28.6%)
Monocyte ↑↓	0	0	0	0	0	0
Esinophil ↑↓	0	0	0	0	0	0
Basophil ↑↓	0	0	0	0	0	0
Increased CRP	62 (100%)	55 (100%)	7 (100%)	11 (17.7%)	8 (14.5%)	3 (42.8%)
Increased SGPT	18 (29%)	16 (29%)	2 (28.6%)	3 (4.8%)	3 (5.4%)	0
Increased ferritin	28 (45.1%)	25 (45.5%)	3 (42.9%)	6 (9.7%)	5 (9.1%)	1 (14.2%)
Increased PT	14 (22.6%)	11 (20%)	3 (42.9%)	16 (25.8%)	13 (23.6%)	3 (42.8%)
Increased D-dimer	31 (50%)	29 (52.7%)	2 (28.6%)	4 (6.4%)	4 (7.3%)	0
Increased creatinine	2 (3.2%)	2 (3.6%)	0	0	0	0

The total number of patients (n = 62); Male: 55, Female: 7. Reference value: Hb (g/dl): Female: 12–16; Male: 14–18; ESR (mm in first hour): Female: 0–15; Male: 0–10; WBC/CC: 4,000–11,000; RBC (Million/CC): 4.0–6.2; Platelet/CC: 150,000–450,000; Neutrophil: 40%−75%; Lymphocyte: 20–45%; Monocyte: 2–10%; Eosinophil: 1–6%; Basophil: 0–1%; CRP (mg/dl): <3; SGPT (Units/L) Male: 16–63 Female: 14–59; Serum ferritin (ng/ml): Male: 13–370, Female: 9–253; PT (s): 0–13. COVID-19, coronavirus disease 2019; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; PT, prothrombin time; RBC, red blood cell; SGPT, serum glutamic pyruvic transaminase; WBC, white blood cell. ↑increase value, ↓decrease value.

This study evaluated the clinical characteristics of COVID-19 illness, post-COVID-19 persisting symptoms/complaints, and changes in the laboratory findings following COVID-19 recovery among the patients in Bangladesh. Our study contains 313 symptomatic patients 3–75 years of age. Among them, 251 (80.2%) were male and 62 (19.8%) were female. The age-groups 20–30 years and 31–40 years were the most affected by SARS-CoV-2 infection, 91 (29.1%) and 82 (26.2%). Overall, 50.2% of the patients recovered within 10 days, 42.5% recovered within 10–20 days, and 7.3% required >21 days to recover. Sixty-seven (21.4%) of the total participants experienced varying degrees of persisting symptoms/complaints following COVID-19 recovery that persisted up to 20 weeks and more. Fifty-three (21.11%) of the males and 14 (22.5%) of the female participants had experienced post-COVID-19 persisting complaints. The distribution of these symptoms was almost similar in both genders. Furthermore, 21.6% of the COVID-19 patients had comorbid conditions, e.g., T2DM, HTN, ischemic heart disease, and bronchial asthma. Diabetic and hypertensive patients were 6.4 and 6.7% (**Table 1**).

Moreover, in our study, the duration of COVID-19 symptoms and the duration of the post-COVID-19 persisting symptoms/complaints had a significant correlation (****$p = 0.00$; **Table 6**). This signifies a longer duration of the illness caused by the prolongation of the post-recovery symptoms.

According to recent studies, COVID-19 symptoms can occasionally continue for months. Thus, the SARS-CoV-2 virus can increase the incidence of long-standing health complications. Carfi et al. (26), in their study, found that in patients who had recovered from COVID-19, 87.4% reported persistence of at least one symptom, especially fatigue and dyspnea. Balachandar et al. (27) also conducted a longitudinal study to evaluate the COVID-19 recovered patient's health status, which revealed that COVID-19 might have the potential impact to cause multiorgan damage.

Another study on post-COVID-19 recovery in patients with or without preexisting diabetes by Akter et al. (28) found that people with diabetes have faced severe COVID-19 manifestation and post disease problems. Greenhalgh et al. (29) also reported that nearly 10% of individuals experience persistent illness after COVID-19 infection. Subsequently, another study also reported persisting symptoms of COVID-19 (30). However, studies on persisting symptoms of COVID-19 are scanty. Therefore, it is suggested that follow-up study of COVID-19 recovered patients would be useful to assess any changes in the other organs.

According to this study, COVID-19 patients in this region presented with fever, cough, myalgia, loss of appetite, headache, abdominal cramps, breathing difficulty, loss of smell, fatigue, chest tightness, vomiting, rhinorrhea, sore throat, insomnia, diarrhea, and sneezing (**Table 2**). However, all participants complained about anxiety, though it was not reported as a symptom in this study. A total of 78% of the participants had no complaints following recovery. Though all the persisting symptoms found in our study caused impairment of routine activity, 2 (0.64%) had developed T2DM and 4 (1.28%) patients developed HTN following recovery. Many preexisting diabetic and hypertensive patients experienced difficulties controlling their normal level and had to receive additional therapy.

The extent of the post-COVID-19 persisting symptoms was at least 3 weeks up to 20 weeks and more. The greatest number of recoveries from post-COVID-19 persisting complaints was on the 10th week, followed by the seventh and fifth weeks among the participants (**Table 5**). However, the very early age-group of 0–10 years had no report of persisting symptoms (**Supplementary Tables 1, 2**). According to the initial hematological findings (**Table 7**), CRP was increased in all the patients. A notable percentage of patients had an increased ESR, WBC, CRP, SGPT, serum ferritin, PT, D-dimer, and serum creatinine level. Approximately 50% of patients had decreased hemoglobin levels, and 6.5% had decreased RBC levels.

An increased percentage of lymphocytosis, leukocytosis, and high PT was observed in the 4-week post-recovery follow-up (**Tables 3**, **7**). No patient had consolidation on chest X-ray during post-COVID-19 4-week follow-up, except three patients who had mild pneumonitis features. However, the new increase in WBC level and PT is a matter of deep concern. There were significant differences in several of these parameters between initial findings during admission and post-COVID-19 4-week follow-up. These parameters are chest X-ray consolidated, ESR, WBC, lymphocyte, CRP, SGPT, serum ferritin, PT, D-dimer, and serum creatinine level ($p < 0.05$; **Table 3**). This proves the reliability and high accuracy of these parameters in the diagnosis and prognosis of COVID-19 illness. Evaluation of the underlying cause and management of increased PT, lymphocyte, and WBC demand close attention in the case of post-COVID-19 patients. Though it might be a positive feedback response to hemodilution therapy, further study is required for a better understanding of the cause and management of this condition.

Our study had several limitations. The sample size was relatively small; the follow-up radiology and laboratory values were evaluated only once after 4 weeks; critically ill patients in the ICU were not included. Recovery analysis based on the different drug treatments was not done, and the death cases were not accessed. Despite the limitations, we tried to analyze the clinical features and post-recovery manifestations of the COVID-19 cases without any existing comorbid conditions.

CONCLUSION

COVID-19 epidemic is a global concern. Primary diagnosis of SARS-CoV2 infection is crucial for the proper management and control of the epidemic, especially in the low facility regions where a PCR and CT chest are not readily available. In our study, 21.4% of the symptomatic COVID-19 patients suffered from persisting symptoms/complaints following recovery, which might persist for more than 20 weeks. They included lethargy, cough, chest discomfort and pain, breathlessness on activity, fatigue, anxiety with lack of concentration and occasional amnesia, headache, persistent low-grade fever, joint pain, body ache, chill, and back pain. Post-COVID-19 development of T2DM (0.64%) and HTN (1.28%) and also the development of unstable DM (54.55%) and HTN (34.78%) to the pre-existing DM and hypertensive patients are a matter of immediate concern. The post-COVID-19 significant changes in the laboratory values included leukocytosis (16.1%), lymphocytosis (14.5%), and an increased PT (25.8%). Besides, an abnormality in the D-dimer, serum ferritin, hemoglobin, and ESR levels was also present to an extent. Moreover, existing comorbidity among the COVID-19 patients increases the risk and duration of the post-COVID-19 persisting symptoms. Management of the post-COVID-19 persisting symptoms and the abnormal laboratory values appeared to be a challenge for healthcare professionals. Better understanding of these conditions is equally important for the patients. A periodic follow-up and evaluation of the patient's physical condition and the biochemical values will help prevent future comorbidity in patients with the post-COVID-19 syndrome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical Committee approval was taken from Xi'an Jiaotong University, China. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AM: research concept, data collection, data interpretation, statistical analysis, manuscript writing, and editing. MK: data collection, manuscript writing, and editing. MA: statistical analysis. JI: data collection. YL: interpreted the data of COVID-19

patients. SH: review of the manuscript, maintained supervision, and ensured the quality of the research. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.663670/full#supplementary-material

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Questionnaire Survey on the Current Situation and Experience in Prevention and Control Measures at Urology Clinics During the COVID-19 Epidemic in China

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COVID-19, the coronavirus disease 2019; SARS-CoV-2, the coronavirus 2; ACE2, angiotensin converting enzyme 2; S protein, spiked glycoprotein; TMPRSS2, transmembrane serine protease 2; WHO, World Health Organization.

Purpose: Although the coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2, has been viably controlled in China, a new normal in healthcare strategies has become standard in China and worldwide. We conducted a questionnaire study to disseminate the experience from China in terms of urology outpatient prevention and control measures under standardized prevention policies against COVID-19.

Participants and Methods: From May 3, 2020 to June 25, 2020, we conducted an anonymous cross-sectional questionnaire study, focused on the status of and experiences with outpatient urology prevention and control measures during the COVID-19 pandemic. The targeted respondents were urologists in mainland China, covering all levels of hospitals and clinics.

Results: A total of 216 (97%) valid responses were collected. We found that 183 (85%) respondents were from outside of Hubei province in China. One-hundred-and-fifty-eight (73%) respondents believed that SARS-CoV-2 could be detected in urine, and that protection against urine exposure was needed. Over 80% of respondents recommended WeChat application or similar online video meetings for virtual outpatient consultations. The suggested flowcharts and recommendations to prevent new cases were easy to understand and approved by most physicians, which could provide reference for outpatient prevention and control. We still need to make adequate preparations under the new normal of the COVID-19 Epidemic, especially for those suspected of being infected.

Conclusions: Although the scientific validation of the questionnaire is limited, it provides a first snapshot of the experiences relating to the prevention and control measures in urology clinics in China, and can inform future policies in this field.

Keywords: COVID-19, experience, SARS-CoV-2, questionnaire, urological outpatient, epidemic

INTRODUCTION

The coronavirus disease 2019 (COVID-19) began at the end of 2019 and has rapidly become an ongoing global pandemic. The COVID-19 pandemic has major ramifications for global health and the economy (1, 2). To control the spread of the epidemic, the Chinese Government and people have put forth unremitting efforts, which proved to be effective so far (3). COVID-19 prevention and control measures have become standard in Chinese healthcare providers practice due to the difficult situation of both an ongoing global pandemic and sporadic domestic epidemics. The recommended preventive measures include social distancing, wearing face masks, hand washing, disinfecting surfaces, and isolation for people exposed (4). The COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has highly variable symptoms ranging from almost none to life-threateningly severe (5, 6). It has been confirmed that SARS-CoV-2 can be transmitted by droplets and close contact. Additionally, SARS-CoV-2 is detectable in urine, as it becomes concentrated in the urinary system and in fecal matter, remaining positive in the excretion of some patients who have recovered from COVID-19 (7). A systematic review of COVID-19 and its potential urological manifestations revealed that 5.74% urine samples from COVID-19 patients were positive for viral RNA, but the duration of viral shedding in urine was unknown (8). A global survey on the impact of COVID-19 on urological services showed that 41% of respondents reported that their hospital staff had been diagnosed with COVID-19 infection (2). To this end, we conducted a national questionnaire survey to disseminate the Chinese urology outpatient experience with prevention and control measures under the new normality of COVID-19. We hope our results and experiences can help guide our future outpatient work, and can also help international counterparts with anti-COVID-19 efforts in urological outpatient practice.

PARTICIPANTS AND METHODS

Study Design and Population

To best represent the current situation in prevention and control measures in urology outpatient practice in China, during the COVID-19 pandemic, the targeted respondents were urologists in mainland China, covering five levels of hospitals and clinics from 22 provinces, two autonomous regions and four municipalities (**Table 1**). We conducted an anonymous cross-sectional questionnaire study targeting domestic urologists in China. Questionnaires were distributed by email and the WeChat application from May 3, 2020 to June 25, 2020. WeChat is one of most popular social applications, with over one billion users and over 400 million active users daily.

TABLE 1 | Demographics of survey respondents.

Variables	*n*	%
	(N=216)	
1. Title (years in practice)		
Chief physician	69	32.0
Associate chief physician	78	36.0
Attending physician	42	19.5
Resident physician	19	8.8
Others	8	3.7
2. Types of hospital/institution		
Level III-A hospital	138	64.0
Level III-B hospital	27	12.5
Level II hospital	43	19.9
Level I hospital	5	2.3
Others	3	1.3
3. Region		
ZheJiang province	47	21.8
HuBei province	33	15.3
Beijing municipal	13	6.0
GuangDong province	12	5.6
HeBei province	10	4.6
ShanDong province	9	4.2
ShanXi province	8	3.7
HeNan province	8	3.7
HuNan province	8	3.7
AnHui province	7	3.2
HeiLongJiang province	6	2.8
SiChuan province	6	2.8
LiaoNing province	5	2.3
JiLin province	5	2.3
JiangSu province	5	2.3
FuJian province	5	2.3
ChongQing	5	2.3
JiangXi province	3	1.4
HaiNan province,	3	1.4
GuiZhou province	3	1.4
Inner Mongolia autonomous Region	3	1.4
YunNan province,	2	0.9
ShanXi province	2	0.9
GanSu province	2	0.9
TianJin	2	0.9
ShangHai	2	0.9
QingHai province	1	0.5
Ningxia Hui Autonomous Region	1	0.5

The Design and Contents of the Questionnaire

The questionnaire comprised a total of 34 questions, with single-choice and multiple-choice response items. The content of the questionnaire was mainly based on the seventh edition of the "Chinese Novel Coronavirus Diagnosis and Treatment Guide for Pneumonia," which was relatively authoritative in China at the beginning of the COVID-19 outbreak (9). The questionnaire was also referred to published scientific articles of the latest research on COVID-19 at the beginning of the COVID-19 outbreak (10–12). Considering that our understanding of this disease was limited, most of the questions designed were in non-scale form. Although the questionnaires have had passed the reliability and validity test according to The Statistical Program for Social

Sciences software analysis, the scientific evidence upon which the survey was based was still limited. The entire questionnaire is presented in **Supplementary Tables 1–6**.

Data Analysis

We randomly distributed 250 questionnaires *via* email and the WeChat application to urologists who had "urology" listed as their specialty. A total of 222 (89%) questionnaires were returned. The number of 250 questionnaires was expected to provide adequate data with appropriate statistical power and workload with optimized representative domestic professional scale. After excluding questionnaires from non-urologist and incomplete responses, 216 (97%) responses were confirmed as valid. For each question in the questionnaire, the number of participants and the corresponding percentage was calculated for each of the response items.

RESULTS

Demographics

The results of this questionnaire represented the views of physicians mainly outside Hubei Province in China (**Table 1**). The respondents comprised of urological doctors working at various hospital levels, from senior urologists to junior urological specialists. Physicians from various provinces of China completed the questionnaire, including physicians from the Hubei province and its capital city, Wuhan, where the first reported and confirmed COVID-19 cluster cases were location (9).

SARS-CoV-2 and the Genitourinary System

Most respondents indicated that they believed that SARS-CoV-2 could be detected in urine, while only some believed that SARS-CoV-2 was detectable in semen (**Table 2.1.1,2.1.2**). Regarding transmission methods, only 35 (16%) respondents believed that SARS-CoV-2 could be spread through the urogenital system (**Table 2.1.3**). Over 152 (70%) respondents believed that the kidney, bladder, and testes could be affected by SARS-CoV-2 (**Table 2.1.4**).

Impact of COVID-19 on Outpatient Clinics

One-hundred-and-seventy-five (81%) respondents recommended WeChat or similar internet video meetings as virtual outpatient consultations during the epidemic (**Table 2.2.1**). One-hundred-and-eighteen (55%) respondents believed that these virtual visits were better than routine service for the prevention of new COVID-19 cases (**Table 2.2.2**).

Impact of COVID-19 on Psychological Status

According to the results of the questionnaire, both the respondents and their patients have been significantly affected by the COVID-19 epidemic, mainly in terms of weariness and anxiety (**Table 2.3.1, 2.3.2**). One-hundred-and-seventy-eight (82%) respondents believed that appropriate psychological consultations are essential for supporting their outpatient practice (**Table 2.3.4**). In addition, 110 (51%) respondents indicated that their patients experienced significant frequent urination symptoms during the epidemic (**Table 2.3.3**). In addition to medical issues, changes in mental health status may affect patients' lower urinary tract symptoms, especially in terms of frequency.

Experience With COVID-19 Screening in Outpatient Clinics

According to the results of the questionnaire, epidemiological history and chest computed tomography (CT) examination were considered the most important screening methods (**Table 2.4.1**). The majority of respondents used the following preventive measures in their outpatient practice: wearing a surgical mask [199(93%)], wearing gloves and performing hand sterilization [149(69%)], and environmental disinfection [149(69%)] (**Table 2.4.2**). The majority of respondents believed that catheterization [135(63%)] and intravesical installation [111(51%)] were the main procedures requiring precautions to be taken during outpatient treatments (**Table 2.4.3**). During outpatient surgery, cystoscopy, ureteral stent removal or replacement, and extracorporeal shock wave lithotripsy were the primary concerns (**Table 2.4.4**). In these procedures, the operator is likely to be in close contact with patients and the patients' urine.

The flowcharts we provided were overwhelmingly recognized and approved by the respondents, and included an outpatient procedures flowchart for screening patients with COVID-19 (**Figure 1**), a flowchart for wearing protection in outpatient treatment rooms (**Figure 2**), a protection flowchart for wearing protection in outpatient operating rooms (**Figure 3**), and a table of recommendations for protective options in urological outpatient clinics (**Table 3**).

Experience With Patients With a History of COVID-19 in Outpatient Clinics

When treating patients who have recovered from COVID-19, the majority of respondents stated that it is still necessary to examine such patients separately, and that their medical waste should be separated in outpatient clinics (**Table 2.5.1**). Most respondents recommended testicular and semen screening for fertility in men of reproductive age who have recovered from COVID-19 (**Table 2.5.2**).

DISCUSSION

SARS-CoV-2 and the Genitourinary System

COVID-19 has been found to infect human respiratory epithelial cells *via* a molecular mechanism of spiked glycoprotein (S protein) interaction with human angiotensin converting enzyme 2 (ACE2) receptor, causing lung tissue damage (13). Transmembrane serine protease 2 (TMPRSS2) appears to be the progenitor of the S protein that enhances ACE2 receptor-mediated viral entry (14). Utilizing the latest single-cell RNA sequencing data, a research team from Shanghai Jiaotong University analyzed ACE2 receptor expression in relevant organs and cell types of major human physiological systems and

TABLE 2 | The main results of the questionnaire survey.

1. SARS-CoV-2 and the genitourinary system	***n* (%)**
1.1 Did you think the SARS-CoV-2 can be detected in the urine?	
Yes	158 (73%)
No	34 (16%)
Not sure	24 (11%)
1.2 Did you think the SARS-CoV-2 can be detected in the semen?	
Yes	19 (9%)
No	56 (26%)
Not sure	141 (65%)
1.3 Did you know how the SARS-CoV-2 may be transmitted? (multiple-choice response items)	
Droplet transmission	214 (99%)
Close contact transmission	210 (97%)
Aerosol transmission	184 (85%)
Blood transmission	39 (18%)
Semen transmission	35 (16%)
1.4 What organs in the genitourinary system did you think may be affected by SARS-CoV-2? (multiple-choice response items)	
Kidney	163 (75%)
Testis	182 (84%)
Epididymis	123 (57%)
Bladder	167 (77%)
Seminal vesicle	117 (54%)
Prostate gland	87 (40%)
2. Impact of COVID-19 on outpatient clinics	
2.1 Which were the recommended virtual outpatient consultation ways during the epidemic? (multiple-choice response items)	
Telephone	118 (55%)
WeChat or video	175 (81%)
Third Party APP	72 (33%)
2.2 During the epidemic, did you think the online virtual outpatient service was better than the routine outpatient service?	
Yes	118 (55%)
No	66 (30%)
Not sure	32 (15%)
3. Impact of COVID-19 on psychological status	
3.1 During the epidemic, how did you feel when you came in close contact with outpatient patients, especially those suspected of COVID-19? (multiple-choice response items)	
Tired	130 (60%)
Anxious	91 (42%)
Delayed or resisted	75 (35%)
Confused	29 (13%)
Easily excited	53 (25%)
Depressed	45 (21%)
Loss of attention and memory	49 (23%)
Poor sleep and even insomnia	49 (23%)
Poor or increased appetite	14 (6%)
Nothing changed	10 (5%)
3.2 During the epidemic, did any patients experience any of the following negative emotions as a result of the epidemic? (multiple-choice response items)	
Tired	109 (50%)
Anxious	118 (55%)
Delayed or resisted	46 (21%)
Confused	115 (53%)
Easily excited	47 (22%)

(Continued)

TABLE 2 | Continued

Depressed	68 (31%)
Loss of attention and memory	24 (11%)
Poor sleep and even insomnia	96 (44%)
Poor or increased appetite	18 (8%)
Nothing changed	15 (7%)
3.3 Did you observe any urinary symptoms or exacerbation of urinary symptoms due to mood changes caused by the epidemic during the outpatient? (multiple-choice response items)	
Frequent micturition	110 (51%)
Nocturia	65 (29%)
Pain in the penis or burning sensation in the urethra	24 (11%)
Erectile dysfunction	31 (14%)
Premature ejaculation	17 (8%)
Pain and discomfort in the perineum	42 (19%)
Discomfort and pain in the testicles or perineum	29 (13%)
Nothing changed	71 (33%)
3.4 During the epidemic, was there a need for psychosocial support for outpatient health workers or patients?	
Yes	178 (82%)
No	10 (5%)
Not sure	28 (13%)
4. Experience with COVID-19 screening in outpatient clinics	
4.1 During the epidemic, what did you consider to be the main bases of importance for the initial determination of patients with suspected COVID-19 in outpatient clinic? (multiple-choice response items)	
Consultation of epidemiological history	176 (81%)
Measurement of body temperature	110 (51%)
Coughs and other respiratory symptoms	76 (35%)
Examination of chest CT	152 (70%)
Blood routine, CRP, blood pressure test and so on	68 (31%)
Antibody or nucleic acid test for SARS-CoV-2 virus	84 (40%)
4.2 What preventive and control measures did your urology outpatient take during the epidemic? (multiple-choice response items)	
wore a surgical mask	199 (93%)
Wore gloves and disinfected hands	149 (69%)
Disinfected the clinic environment	129 (60%)
Wore protective goggles	119 (55%)
Wore a protective surface screen	96 (44%)
Wore simple protective clothing	84 (39%)
Wore standard full-body protective clothing	32 (15%)
Patients were accompanied by family members	84 (39%)
Patients were unaccompanied by family members and alone	94 (42%)
4.3 In light of the current prevention policy, which groups of individuals should be cautious in urological outpatient? (multiple-choice response items)	
Urinary catheterization	135 (63%)
Prostate palpation and treatment	85 (39%)
Collection of patient secretions	96 (44%)
Bladder perfusion	111 (51%)
Urodynamics-related tests	51 (24%)
Replacement of stoma fistula	97 (45%)
Replacement of stoma pockets	42 (19%)
4.4 What were the main important outpatient procedures that required increased protection during the epidemic? (multiple-choice response items)	
Circumcision	86 (40%)
Cystoscopy	169 (78%)
Urethral stent removal or replacement	145 (67%)

(Continued)

TABLE 2 | Continued

Extracorporeal vibration lithotripsy	133 (62%)
Urethral dilation	93 (43%)
Resection of local superficial lump	40 (19%)
5. Experience of patients with a history of COVID-19 in outpatient clinics	
5.1 Did patients with history of COVID-19 need to be examined separately? And did the medical waste need to be disposed separately?	
Yes	178 (82%)
No	28 (13%)
Not sure	10 (5%)
5.2 What else should be done in the urology outpatient clinic for the patients who recovered from COVID-19? (multiple-choice response items)	
Detection of SARS-CoV-2 in urine and semen	119 (55%)
Testicular and semen screening for fertility in men of reproductive age	126 (58%)
Ultrasound of the urinary system	58 (27%)
CT examination of the urinary system	44 (20%)
There was no need to specifically check the urinary system	50 (23%)

found that the heart, esophagus, kidney, bladder, and ileum all have ACE2 receptor expression similar to or higher than that in alveoli (15). Additionally, a research team from Suzhou Hospital affiliated with Nanjing Medical University analyzed the expression of ACE2 receptor in different human organs using existing datasets and found that ACE2 receptor is highly expressed in renal tubular cells, testicular mesangial cells, and seminiferous tubular cells of the testis (16). The above findings imply that, at the RNA level, the kidneys, bladder, and testes of the urinary system are potential target organs for SARS-CoV-2, concordant with the beliefs of the respondents in the current study.

Furthermore, Guan et al. (10) confirmed that SARS-CoV-2 can be detected in excreta such as urine and feces. Consequently, whether sexual contact is one of the potential routes of SARS-CoV-2 transmission has been raised (17). Li et al. (18) found that SARS-CoV-2 was detected in the semen of 6 out of 38 patients diagnosed with COVID-19. Interestingly, Pan et al. (19) did not detect SARS-CoV-2 in the semen of 34 patients diagnosed with COVID-19 in Wuhan, China. Therefore, we suspect that the detectability in semen might be related to viral load, virus survival time, and the blood-testis barrier. Currently, there is no direct evidence of this mode of transmission. Accordingly, our questionnaire study found that less than half of respondents believed that SARS-CoV-2 is detectable in semen. However, Corman et al. (20) suggested that RNAemia is not equivalent to infectiousness. Additionally, there is a lack of direct evidence that SARS-CoV-2 can be transmitted by blood transfusion (21).

Acute kidney injury has been reported to be a common complication among hospitalized patients with COVID-19 (22). It is considered a marker of disease severity and a negative prognostic factor for survival in patients with COVID-19, based on experience in the United States (23). It was still unclear whether SARS-CoV-2 directly affect the kidney, although renal histopathological analyses of 26 Chinese COVID-19 patients suggested varying degrees of acute tubular injury (24). The prognosis of COVID-19 patients with acute kidney injury was poor, especially those patients with other underlying diseases (25, 26).

Impact of COVID-19 on Outpatient Clinics

According to the local public health department advice, most people chose to stay at home during the epidemic, especially at the beginning of the outbreak. In order to effectively maintain close contact and communication with patients, physicians and patients stayed in touch *via* WeChat, video calls, and telephone. A global internet survey revealed that the use of telemedicine related devices by urologists nearly tripled during the COVID-19 epidemic (27). This was indeed a pragmatic approach to reducing the risk of transmission, and was worth promoting. Most patients have already benefited from virtual outpatient consultations. However, treatment during the epidemic was compromised for patients with primary symptoms of urinary tumors and post-operative patients with urinary tumors, which would affect their survival over time.

Impact of COVID-19 on Psychological Status

Psychological factors, such as psychological stress and depression, clearly affect the human endocrine and immune systems (28). Psychological factors can cause metabolic dysregulation of neurotransmitters, such as monoamines and peptides, resulting in hypothalamic-pituitary-gonadal axis and hypothalamic-pituitary-adrenal axis dysfunction, which in turn affects the endocrine and immune reproductive system functions in human, resulting in reproductive dysfunction (29). The psychological states of stress and anxiety experienced by patients with chronic prostatitis are considered an important factor in the development or exacerbation of inflammation. A global survey of psychological impact among surgical providers showed that the

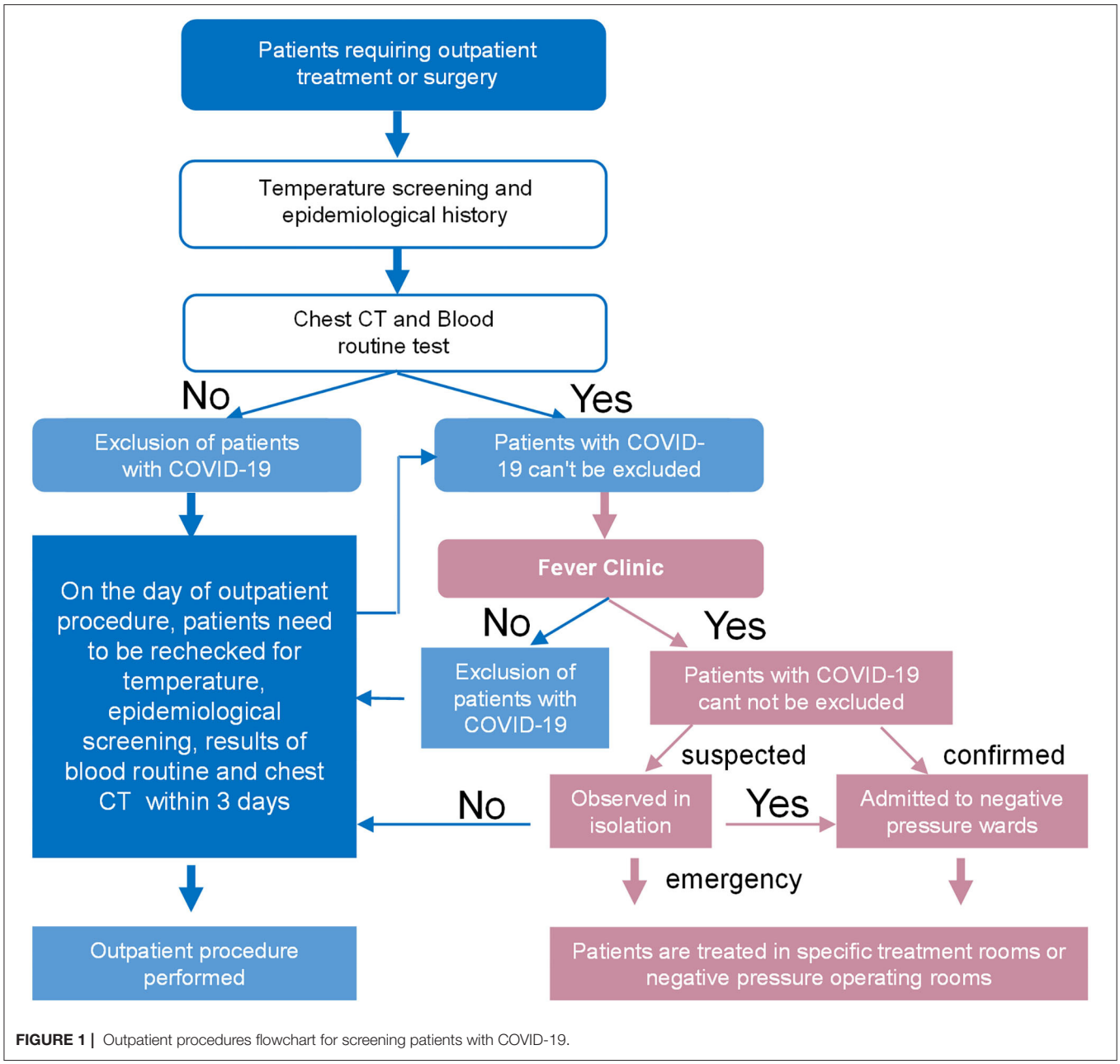

FIGURE 1 | Outpatient procedures flowchart for screening patients with COVID-19.

COVID-19 pandemic may have a mental health legacy outlasting its course (30). Based on the results of our questionnaire study, the psychological status of both clinical staff and patients have been impacted by the epidemic to varying degrees, and both clinical staff and patients need opportune mental help. Above all else, patients should be effectively managed to obtain accurate healthcare information from the World Health Organization (WHO) internationally and the domestic National Health Care Commission (31, 32), so as to not be impacted by rumors and to not spread inaccurate data. These recommendations can still benefit those patients who need assistance during the COVID-19 pandemic.

Experience With COVID-19 Screening in Outpatient Clinics

With the continuous importation of COVID-19 cases from outside the country (33–35), and the increasingly subtle epidemiological history and atypical clinical manifestations (36), additional cases will appear in outpatient clinics for two or three generations (37). The performance of PCR-based diagnostic depended upon several factors such as sample type, different stage of infection in patient, the skill of the collection, and the quality and consistency of the PCR-based diagnosis assays being used (38, 39). Therefore, the performance of PCR-based diagnosis may be false negative, resulting in clinical underdiagnosis. Our

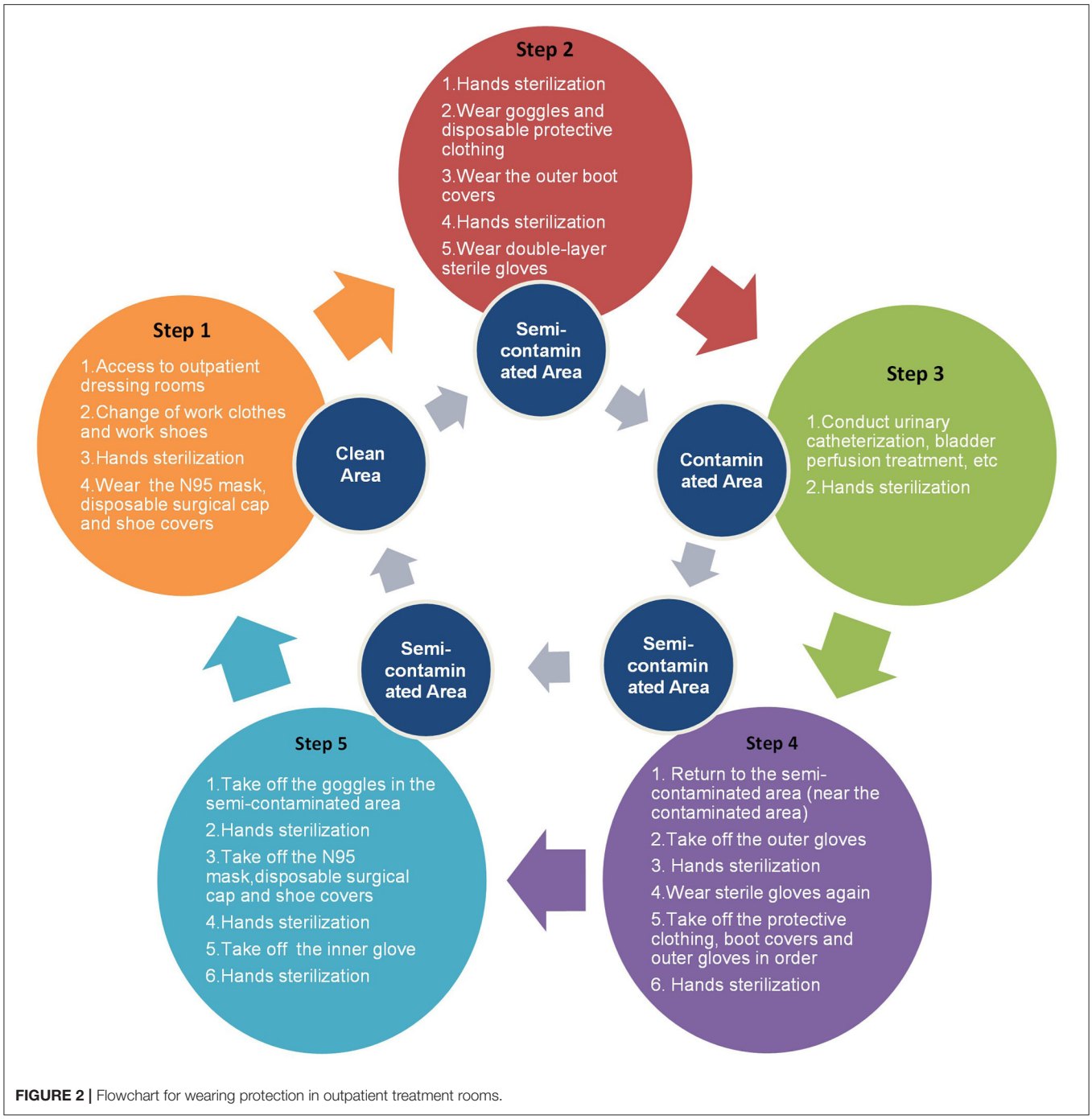

FIGURE 2 | Flowchart for wearing protection in outpatient treatment rooms.

survey results showed that CT and epidemiological history are accepted by the majority of respondents. CT examination is not only convenient and quick, but also provides strong evidence, and is especially suitable for emergency and scheduled surgeries in inpatients and outpatients with suspected COVID-19. Chen et al. (40) considered hand sanitization as one of the most important measures to prevent epidemic-associated viral infections. Additionally, the (WHO) recommends hand decontamination by rubbing the hands with alcohol, for example, after glove removal (41). The above recommendations were generally endorsed by the respondents in the current study.

Aerosols are common in medical work environments, such as with nebulized inhalation and tracheal intubation, and samples of blood, urine, feces, etc. SARS-CoV-2 can be detected in aerosols for up to 3 h, on copper surfaces for up to 4 h, on cardboard for up to 24 h, and on plastics and stainless steel for up to 2 to 3 days (42). Guo et al. (43) analyzed workspaces in Wuhan Vulcan Hill Hospital (in the epidemic area) and found that SARS-CoV-2

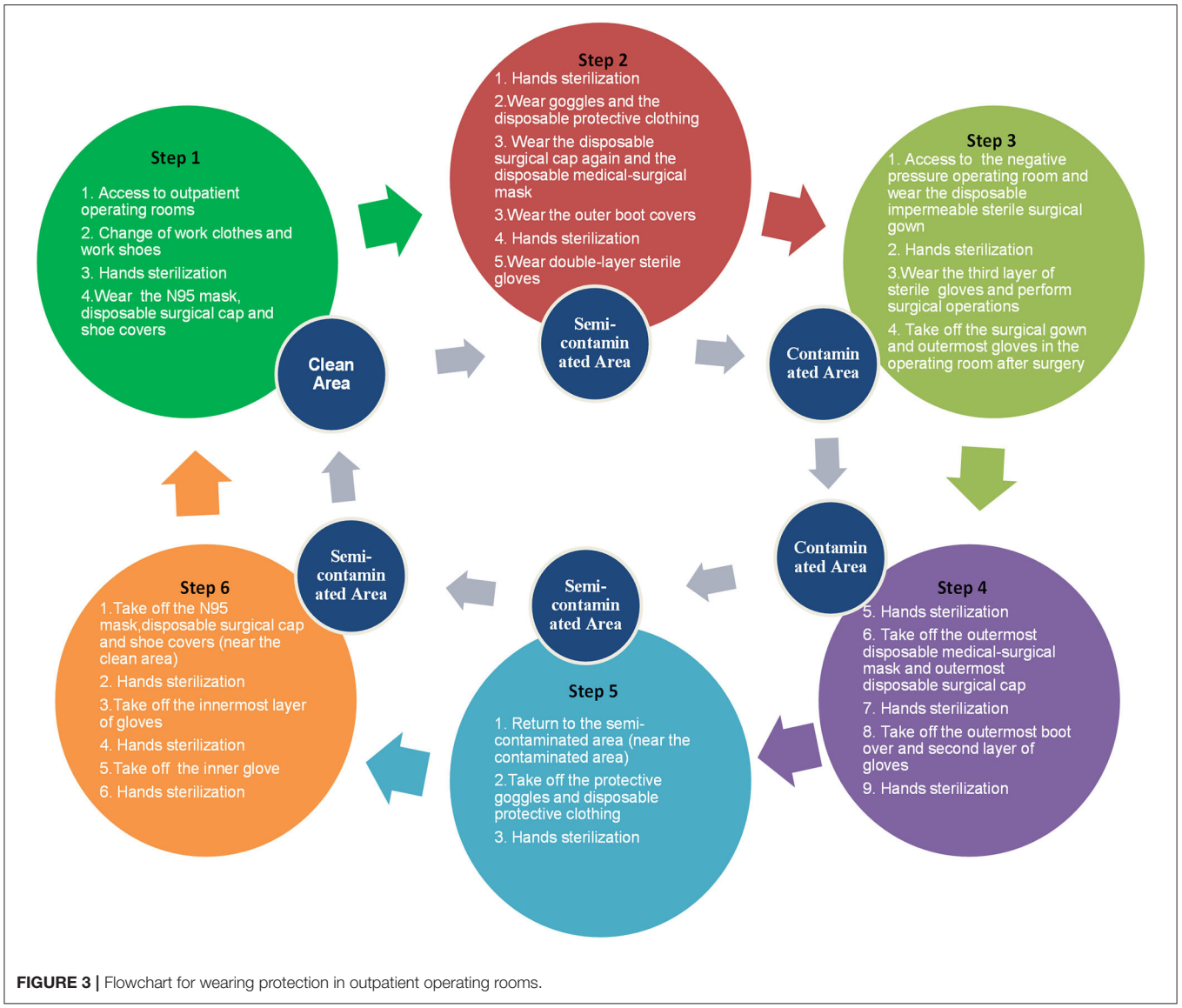

FIGURE 3 | Flowchart for wearing protection in outpatient operating rooms.

was widely distributed in the air and on the surface of objects in the intensive care unit and general COVID-19 ward. Since the outpatient clinic room is an enclosed environment, staff and patients are susceptible to SARS-CoV-2 transmission by aerosols. In particular, physicians should raise the level of protection when contacting suspected or confirmed patients with COVID-19.

Xu et al. (44) found that some patients had a positive rectal swab even after a negative nasopharyngeal swab test. Therefore, when conducting digital rectal examinations in patients with suspected COVID-19 or recovering from COVID-19, it is recommended to do so gently and to wear double gloves, minimizing the occurrence of glove breakage. Some cases of post-operative death due to nosocomial infection of COVID-19 had been reported (45). Some studies had shown that pre-operative CT examination and nucleic acid testing are helpful to reduce the incidence of postoperative COVID-19 (12, 46). Electrosurgical and energetic devices should be used appropriately during outpatient procedures to promptly aspirate smoke and reduce aerosol injuries. During outpatient treatments or surgical procedures, physicians should use disposable instruments and other items as much as possible. For patients with suspected or confirmed COVID-19, devices used during the procedure, such as disposable instruments, sharp boxes, and catheters, should be clearly identified with a confirmed or suspected COVID-19 label and removed *via* the special infection channel (45, 46). Furthermore, Kuang et al. (46) suggested that clinical staff disinfect their soles before stepping out of the room of a patient with COVID-19.

The included flowcharts were standard procedures in our hospital, mainly based on the diagnosis and treatment protocol and technical guidelines of COVID-19 in China (9, 47). Although not officially standard, they were simple and easy to understand and could provide reference for outpatient protection work. If occupational exposure occurs during the above process, prompt

TABLE 3 | Recommendations for protective options in urological outpatient clinics.

—	Hands sterilization	Disposable medical-surgical mask	N95 mask	Protective goggles	Latex gloves	Disposable isolation garment	Protective clothing	Disposable medical cap	Disposable medical shoe covers	Work clothing
Protection of outpatient staff	√	√	S	S	√	N	N	√	N	√
Outpatient surgical procedure protection against non-confirmed or suspected COVID-19 patients	√	√	S	S	√	S	N	√	N	√
Outpatient surgical protection against confirmed or suspected COVID-19 patients	√	N	√	√	√	N	√	√	√	√
Protection against the transfer of confirmed or suspected patients' contamination	√	N	√	S	√	√	S	√	√	√

√ indicates personal protective equipment is recommended. S indicates personal protective equipment is a selective option; N indicates no recommendation.

symptomatic treatment is required. If body fluid exposure occurs, it is recommended that the operator should immediately remove the contaminant and repeatedly apply a large amount of saline rinse or 0.05% iodine for skin or mucous membrane rinsing and disinfection, respectively. In cases of blood exposure, the operator should gently squeeze the blood near the wound from the proximal end to the distal end, squeezing out as much blood as possible at the wound, followed by a rinse with flowing water, and disinfection with 75% alcohol or 0.5% iodine (12, 41). In the event of respiratory exposure, it is recommended to gently wipe the nasal cavity with a cotton ball containing 75% alcohol, followed by flushing with plenty of normal saline and medical isolation.

Experience With Patients With a History of COVID-19 in Outpatient Clinics

Ma et al. (48) found significant changes in sex hormone levels in male patients with COVID-19 of reproductive age. Changes in sex hormones may affect future fertility (49). Our questionnaire recommended testicular and semen screening for fertility in men of reproductive age who have recovered from COVID-19. It may also provide new evidence on whether SARS-CoV-2 attacks the testes in the future. The majority of respondents in the current study indicated that outpatient urological control measures still need to be continued in the future in response to the domestic and international situation of the ongoing pandemic.

Limitations

The survey and experiences were achieved from one specialty (urology) in one country (China), therefore the main results and conclusions may not apply to different settings or other regions. Considering that our knowledge of the disease was limited at the time of questionnaire formulation, this questionnaire study had some limitations. On some questions we limited the answer options, so that respondents might not be able to respond completely objectively. There were even more uncertainties when designing the questionnaire and responses, such as whether SARS-CoV-2 could be detected in semen and the possibility of transmission of SARS-CoV-2 through urine or semen. In addition, there were certain limitations in the sample size and survey distribution method of the questionnaire that may have affected the final dataset.

CONCLUSIONS

Through the questionnaire survey, we learned about the current situation, experience and measures of epidemic prevention and control of COVID-19 in urology outpatient department in China. Although the scientific evidence of the survey was low, it could still provide us with a lot of helpful measure. The majority of physicians believed that SARS-CoV-2 could be detected in urine, and that protection against urine exposure was needed. These suggested flowcharts and recommendations to prevent new cases were accepted by most urologists in this survey. In terms of protective measures, it is still recommended to wear protection, especially when in contact with suspected samples during outpatient practice under the new normal of COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The First Affiliated Hospital of Wenzhou Medical University and Taizhou Hospital of Zhejiang Province

affiliated to Wenzhou Medical University, China. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

H-HJ and MW had roles in conception and design. X-LZ, M-HJ, W-LL, J-HL, and Z-LS had roles in the questionnaire design, data collection, data analysis, data interpretation, and literature search. H-HJ and X-LZ had roles in writing of the manuscript. H-HJ, X-LZ, and MW contributed to the critical revision and data interpretation of the manuscript. MW had roles in supervision. All authors contributed to data acquisition, analysis, and interpretation, and reviewed and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.670889/full#supplementary-material

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COVID-19 and Tuberculosis Coinfection: An Overview of Case Reports/Case Series and Meta-Analysis

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Background: Coronavirus disease 2019 (COVID-19) and tuberculosis (TB) are two major infectious diseases posing significant public health threats, and their coinfection (aptly abbreviated COVID-TB) makes the situation worse. This study aimed to investigate the clinical features and prognosis of COVID-TB cases.

Methods: The PubMed, Embase, Cochrane, CNKI, and Wanfang databases were searched for relevant studies published through December 18, 2020. An overview of COVID-TB case reports/case series was prepared that described their clinical characteristics and differences between survivors and deceased patients. Pooled odds ratios (ORs) with 95% confidence intervals (CIs) for death or severe COVID-19 were calculated. The quality of outcomes was assessed using GRADEpro.

Results: Thirty-six studies were included. Of 89 COVID-TB patients, 19 (23.46%) died, and 72 (80.90%) were male. The median age of non-survivors (53.95 ± 19.78 years) was greater than that of survivors (37.76 ± 15.54 years) ($p < 0.001$). Non-survivors were more likely to have hypertension (47.06 vs. 17.95%) or symptoms of dyspnea (72.73% vs. 30%) or bilateral lesions (73.68 vs. 47.14%), infiltrates (57.89 vs. 24.29%), tree in bud (10.53% vs. 0%), or a higher leucocyte count (12.9 [10.5–16.73] vs. 8.015 [4.8–8.97] $\times 10^9$/L) than survivors ($p < 0.05$). In terms of treatment, 88.52% received anti-TB therapy, 50.82% received antibiotics, 22.95% received antiviral therapy, 26.23% received hydroxychloroquine, and 11.48% received corticosteroids. The pooled ORs of death or severe disease in the COVID-TB group and the non-TB group were 2.21 (95% CI: 1.80, 2.70) and 2.77 (95% CI: 1.33, 5.74) ($P < 0.01$), respectively.

Conclusion: In summary, there appear to be some predictors of worse prognosis among COVID-TB cases. A moderate level of evidence suggests that COVID-TB patients

are more likely to suffer severe disease or death than COVID-19 patients. Finally, routine screening for TB may be recommended among suspected or confirmed cases of COVID-19 in countries with high TB burden.

Keywords: COVID-19, tuberculosis, co-infection, clinical features, risk factors

INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by a novel beta-coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread worldwide since December 2019, causing significant global public health and economic problems (1, 2). The World Health Organization declared COVID-19 a pandemic on March 11, 2020 (2). As of December 19, 2020, there have been over 75.7 million cases and 1.68 million deaths associated with COVID-19 worldwide (3). Nearly half of these cases involved four COVID-19 high-burden countries, including the United States (23.1%), India (13.2%), Brazil (9.5%), and Russia (3.7%) (3). Evidence to date suggests that COVID-19 patients with preexisting comorbidities such as hypertension, diabetes, and cardiovascular disease are at greater risk of death, but few studies have involved COVID-19 patients coinfected with other respiratory infectious diseases (4).

The initial signs and symptoms of COVID-19 are similar to other respiratory infections, such as tuberculosis (TB) and influenza. However, coinfections with common viral, bacterial, and fungal pathogens among COVID-19 patients are not unusual (5–7), which can interfere with the diagnosis and treatment of COVID-19. Before the COVID-19 outbreak, TB had been the most fatal infectious disease in the world for many years (8). Globally, an estimated 10 million people contracted TB and 1.4 million died from TB in 2019 (8). At present, evidence suggests that the main transmission route of both COVID-19 and TB is via respiratory droplets, and their main target are the lungs, which can lead to a worse outcome among COVID-19 and TB coinfection patients (aptly abbreviated COVID-TB) (7, 8). Therefore, due to the high prevalence of both of these infectious diseases and the potential worse prognosis of coinfection, an intensive investigation of COVID-TB cases may be of great clinical significance (3, 4, 8). However, few studies have focused on COVID-TB cases to date, and most of these are case reports involving only one patient, thus precluding systematic summaries of the clinical characteristics of coinfection cases (7, 9, 10). In addition, it is unclear whether COVID-TB patients have a worse prognosis or are more likely to develop severe disease, thus necessitating further study.

In this study, we aimed to more fully assess the impact of TB coinfection on COVID-19 patients using the following approach: (1) we present an overview of COVID-TB case reports or case series published through December 18, 2020 and describe the demographic characteristics, clinical symptoms, comorbidities, imaging features, laboratory indicators, type of TB coinfection, and treatment strategies for both COVID-TB survivors and non-survivors; (2) we performed a pooled analysis of published data regarding the odds ratios (ORs) of death or severe disease, comparing the COVID-TB and non-TB groups; and (3) we assessed the quality of outcomes using GRADEpro.

METHODS

Search Strategy

An extensive search of the literature was conducted using the PubMed, Embase, Cochrane Central Register of Controlled Trials (CENTRAL), and two Chinese databases—the China National Knowledge Infrastructure (CNKI) and Wanfang databases—for articles published through December 18, 2020. The following keywords and Medical Subject Headings in partial or complete combinations were used in the search strategy of this review: "Coronavirus 2019," "COVID-19," "SARS CoV-2," "COVID," "novel coronavirus," "2019-nCoV," "severe acute respiratory syndrome," "nCoV," "CoV-2," "SARS-2," "new coronavirus," and "tuberculosis." The Chinese translations of these terms were searched in the CNKI and Wanfang databases (**Table A1**).

Study Selection

We identified 6,919 publications, which were imported into EndNote X9 (Clarivate Analytics, Philadelphia, PA, USA), and 508 duplicate reports were excluded. First, three authors (WM. S., YF. L., and SJ. L.) reviewed the titles and abstracts for selection. Inclusion criteria were as follows: case reports or cases series of COVID-19 and TB coinfection; or original studies (retrospective or prospective clinical studies) that described the number/percentage of TB patients among confirmed COVID-19 cases. After preliminary screening, 149 full-text records were reviewed. Exclusion criteria were as follows: (1) case report or cases series without outcomes of COVID-TB cases (discharge or death); (2) original articles without the number/percentage of death/non-death or severe/non-severe cases among COVID-TB and COVID-19 subgroups; (3) sample size < 10 patients in the cohort study; and 4) publication overlap. The definition of severe COVID-19 is as follows: SpO_2 <94% on room air at sea level; ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2) <300 mm Hg; respiratory frequency > 30 breaths/min; or lung infiltrates >50%.

Data Extraction and Quality Evaluation

The following relevant data were extracted and collected in Excel: (1) baseline data, including the first author, country, publication date, type of study, number of patients with COVID-19, number of COVID-19 patients with TB, mean age, male/female ratio, outcome (survival or death), or clinical classification (severe or non-severe); (2) for case reports or cases series, we also collected detailed data on clinical symptoms, comorbidities, imaging features, laboratory indicators, and treatment strategies; and (3)

for retrospective or prospective clinical studies, we collected the number/percentage regarding death/survival and severe/non-severe disease for the COVID-TB and COVID-19 subgroups.

The methodological quality of case reports or case series was evaluated using the Mayo Evidence-Based Practice Centre tool according to four domains (selection, ascertainment, causality, and reporting) (11). In addition, the methodological quality of other retrospective or cohort studies was assessed using the modified Newcastle–Ottawa scale (12). Two investigators (WM. S. and TT. X.) performed the analyses and summarized the scores of each study. Publications with a score ≥ 6 were considered of high quality and included in our analysis.

Statistical Analysis

Eighty-nine COVID-TB cases were divided into survival and non-survival groups. Continuous variables including hematological and biochemical indicators of each group were described as the median (P_{25}, P_{75}) due to their skewed distribution. The Mann–Whitney *U* test was used for comparing two groups of continuous data (13). The mean and standard deviation of the age variable were described. Categorical variables, including age subgroup, sex, country, symptoms at admission, type of TB, computed tomography (CT) findings, and therapy, were expressed as frequencies and proportions. Categorical variables were compared using the chi-squared test or Fisher's exact test. Forest plots were prepared to show the pooled estimated ORs and associated 95% confidence intervals (CIs) for death from COVID-TB or severe disease, respectively. The heterogeneity of studies included in the Forest plots was assessed using Cochran's *Q* test and the I^2 statistic. A fixed-effect model (inverse variance) was used when I^2 was <50%. Otherwise, a random-effect model (DerSimonian–Laird) was used (14). A visual inspection of funnel plots was conducted to evaluate publication bias (**Figures A1**, **A2**), in which an asymmetric, inverted funnel shape usually indicates publication bias. Finally, we assessed the quality of outcomes using the GRADEpro software. A two-sided $p < 0.05$ was considered statistically significant. All statistical analyses were carried out using Review Manager (RevMan; version 5.3), SPSS (version 22.0), and GRADEpro (version 3.6.1).

RESULTS

Characteristics of Included Studies

A total of 6,919 articles were retrieved from the literature, including 599 from PubMed, 1,034 from Embase, 3,523 from Cochrane, 1,660 from CNKI, and 103 from Wanfang. After removing 508 duplicates, we evaluated the eligibility of 6,411 articles by screening the title and abstract, resulting in 149 studies being enrolled for full-text screening. Ultimately, we identified 36 eligible studies for our final analysis, of which 26 studies were used for an overview of COVID-TB cases, and 10 studies were used for the estimation of pooled ORs (**Figure 1**, **Table 1**).

Clinical Features of COVID-TB Cases

Table 2 describes the demographic characteristics, clinical symptoms, comorbidities, imaging features, laboratory indicators, treatment strategies, and type of TB among the overall group and the COVID-TB non-survivors and survivors. A total of 89 COVID-TB patients were included in the overview of case reports, of which 19 (23.46%) died, and 72 (80.9%) were male. The number and proportion of COVID-TB patients in the 0–14, 15–24, 25–44, 45–64, and 65+ years age groups were 4 (4.49%), 9 (10.11%), 40 (44.94%), 24 (26.97%), and 12 (13.48%), respectively, with an average age of 41.21 ± 17.84 years. The median age of the non-survivor group (53.95 ± 19.78 years) was older than that of the survivor group (37.76 ± 15.54 years) ($p < 0.001$). The non-survivors were less likely to be 25–44 years old compared with survivors (10.53 vs. 54.29%) but more likely than survivors to be in the 65+ years age group (47.37 vs. 4.29%) ($p < 0.01$). More than 85% of these cases were from India ($n = 31$, 4.83%), Italy ($n = 26$, 29.21%), and China ($n = 12$, 13.48%).

Among all 89 COVID-TB cases examined, 88.76% involved active TB, 8.99% had previous TB, and 2.25% had latent tuberculosis infection (LTBI). The proportions of pulmonary TB only, extrapulmonary TB only, and pulmonary TB/extrapulmonary TB (>1 site possible) were 79.78, 8.99, and 8.99%, respectively. Moreover, 5.62% were classified as central nervous system TB, 4.49% pleural TB, and 2.25% lymphadenitis. A total of 56.41% (44/78) of the COVID-TB patients had comorbidities, the most common of which was diabetes (24.36%, 19/78), followed by hypertension (17.95%, 14/78), HIV infection (6.41%, 5/78), hepatitis (3.85%, 3/78), epilepsy (3.85%, 3/78), chronic kidney disease (2.56%, 2/78), cerebrovascular disease (2.56%, 2/78), chronic obstructive pulmonary disease (2.56%, 2/78), asthma (2.56%, 2/78), or cancer (2.56%, 2/78). The non-survivors had more complications, such as hypertension (47.06 vs. 17.95%), hepatitis (17.65 vs. 0%), and cancer (11.76% vs. 0%), than survivors ($p < 0.05$). The 10 most common symptoms of COVID-TB at admission were fever (77.78%), cough (64.2%), dyspnea (35.8%), weight loss (16.05%), fatigue (11.11%), expectoration (9.88%), chest pain (9.88%), headache (8.64%), myalgia (8.64%), and vomiting (7.41%). The non-survivors had a higher percentage of dyspnea than survivors (72.73 vs. 30%) ($p = 0.014$).

In terms of treatment, 88.52% of the 61 COVID-TB patients received anti-TB therapy, 50.82% received antibiotics, 22.95% received antiviral therapy, 26.23% received hydroxychloroquine, 16.39% received oxygen therapy, 11.48% received corticosteroids, 8.2% received interferon-α, 6.56% received traditional Chinese medicine, and 4.92% received intravenous immunoglobulin. The most widely used antibiotic was azithromycin (21.31%), followed by ceftriaxone (14.75%), moxifloxacin (4.92%), amikacin (3.28%), and meropenem (1.64%). The antiviral drugs used included lopinavir/ritonavir (11.48%), umifenovir hydrochloride (9.84%), tenofovir (6.56%), remdesivir (1.64%), lamivudine (1.64%), dolutegravir (1.64%), and favipiravir (1.64%). These treatments did not differ significantly between survivors and non-survivors.

Features of lung imaging among the 89 patients were as follows: 52.81% had bilateral lesions, and 20.22% had unilateral lesions. The 10 most common imaging features included cavities (32.58%), infiltrates (31.46%), ground-glass opacity (19.1%), nodules (16.85%), pleural effusion (11.24%), fibrosis (12.36%),

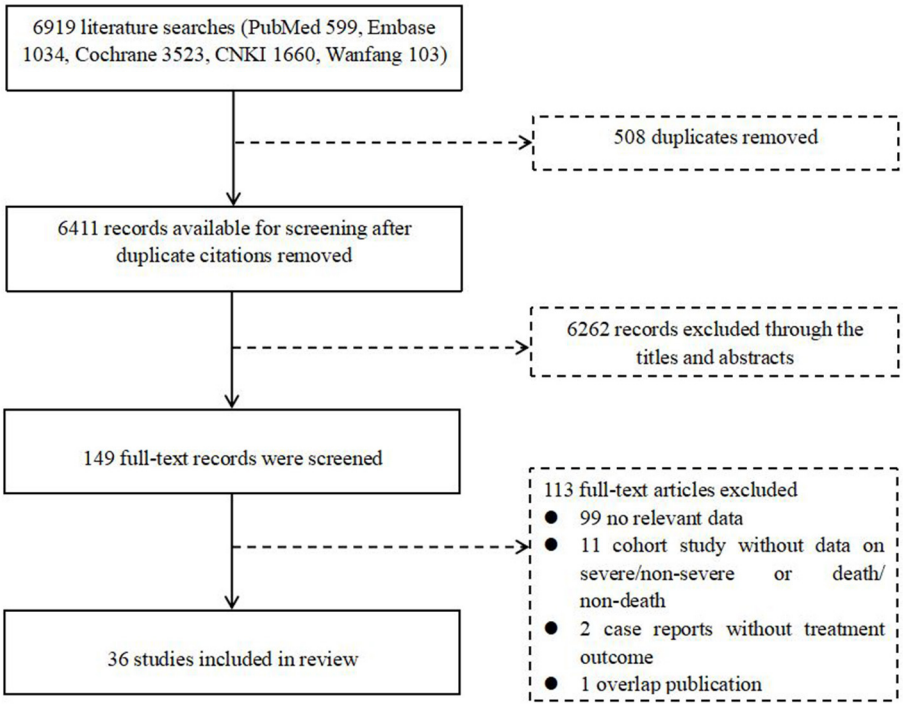

FIGURE 1 | Flow diagram for the inclusion of studies. CNKI, Chinese National Knowledge Infrastructure.

patchy shadows (8.99%), consolidation (8.99%), military lesions (5.62%), and reticules (5.62%). In addition, we found that non-survivors were more likely than survivors to have bilateral lesions (73.68 vs. 47.14%), infiltrates (57.89 vs. 24.29%), or tree in bud (10.53 vs. 0%) features ($p < 0.05$).

Elevated laboratory findings in COVID-TB patients included neutrophil count (7.60 [6.93–7.60] $\times$ 10^9/L), D-dimer (1.407 [1.09–2.65] μg/ml), C-reactive protein (CRP, 77.10 [29.20–184.70] mg/L), erythrocyte sedimentation rate (ESR, 75.50 [55.75–88.50] mm/h), procalcitonin (PCT, 2.57 [0.50–5.76] ng/ml), ferroprotein (FER, 739.50 [511.50–952.50] ng/ml), aspartate transaminase (46 [27.55–78.50] U/L), lactate dehydrogenase (LDH, 384 [290.25–471.75] U/L), and creatinine (335.92 [189.51–396.92] μmol/L). Reduced laboratory indicators included lymphocyte count (0.99 [0.73–1.31] $\times$ 10^9/L) and hemoglobin (99 [83.5–114] g/L). Leucocyte count, platelet count, and alanine transaminase levels were in the normal range. The nonsurvivors had a higher leucocyte count than the survivors (12.9 [10.5–16.73] vs. 8.015 [4.8–8.97] $\times$ 10^9/L, $P = 0.007$). There were no significant differences between the survivors and non-survivors regarding the abovementioned laboratory examinations, except for the leucocyte count.

Pooled ORs for Death or Severe COVID-TB vs. COVID-19

As shown in **Figures 2**, **3**, **Table 3**, the pooled ORs for death or severe disease in the COVID-TB group compared with the non-TB group were 2.21 (95% CI: 1.80, 2.70; heterogeneity: chi-squared = 3.82, df = 3, $p = 0.28$; $I^2 = 21\%$; Test for overall effect: $Z = 7.72$, $p < 0.00001$) and 2.77 (95% CI: 1.33, 5.74; heterogeneity: chi-squared = 8.29, df = 5, $p = 0.14$; $I^2 = 40\%$; Test for overall effect: $Z = 2.73$, $p < 0.006$), with a moderate level of evidence. The percentages of deaths and cases of severe disease, respectively, were 5.69% (123/2,161) and 51.43% (18/35) in the COVID-TB group, 3.24% (699/21,571) and 28.04% (675/2,407) in the non-TB group, and 3.46% (822/23,732) and 28.38% (693/2,442) in the overall group. The proportion of TB among the non-survivor, survivor, and overall group was 14.96% (123/822), 8.90% (2038/22,910), and 9.11% (2,161/23,732), respectively. Moreover, the proportion of TB among the severe, non-severe, and overall patient groups was 2.60% (18/693), 0.97% (17/1,749), and 1.43% (35/2,442), respectively.

DISCUSSION

This meta-analysis of 36 studies provided a summary of demographic, radiological, and laboratory characteristics as well as symptoms at admission, comorbidities, therapy, and outcomes of COVID-TB cases and investigated the impact of TB on the prognosis of COVID-19 patients. Our study showed that non-survivors were older and had more complications associated with hypertension, hepatitis, and cancer, had more symptoms of dyspnea, and were more likely to have CT imaging features of bilateral lesions, infiltrates, tree in bud, and higher leucocyte count than survivors. We also found a moderate level of evidence

TABLE 1 | Summary of 36 studies included in the systematic review.

References	Country	Publication time	Type of study	Number of patients with COVID-19	Number of COVID-TB patients	Mean age	Male/Female	Death or Severe
Ata et al. (15)	India	2020 Aug	Case report	1	1	28	M	OD
Yousaf et al. (16)	Nepal/India/ Bangladesh	2020 Sep	Case series	6	6	35.5	6 M	OD
Yao et al. (17)	China	2020 Jul	Case series	3	3	50.33	3 M	1D
Vilbrun et al. (18)	Haiti	2020 Nov	Case report	1	1	26	M	OD
Tham et al. (19)	India/Bangladesh	2020 Jul	Case series	4	4	31.75	4 M	OD
Stochino et al. (20)	Italy	2020 Jul	Retrospective study	20	20	34.5	12 M/8 F	1D
Gupta et al. (21)	India	2020 Nov	Retrospective study	22	22	40.59	20 M/2 F	6D
Rivas et al. (22)	Panama	2020 Oct	Case series	2	2	41	2 M	OD
Luciani et al. (23)	Italy	2020 Oct	Case report	1	1	31	F	OD
Lopinto et al. (24)	France	2020 Sep	Case report	1	1	58	M	OD
Liu et al. (25)	China	2020 Jul	Case series	3	3	40	3 M	OD
He et al. (26)	China	2020 Oct	Case series	3	3	56.33	3 M	OD
Goussard et al. (27)	South Africa	2020 Sep	Case report	1	1	2	M	OD
Garg and Lee (28)	America	2020 Aug	Case report	1	1	44	M	OD
Gadelha Farias et al. (29)	Brazil	2020 Oct	Case series	2	2	41	2 M	OD
Freij et al. (30)	America	2020 Sep	Case report	1	1	5	F	1D
Faqihi et al. (31)	Saudi Arabia	2020 Jul	Case report	1	1	3	F	OD
Essajee et al. (32)	South Africa	2020 Sep	Case report	1	1	60	M	OD
Çinar et al. (33)	Turkey	2020 Oct	Case report	1	1	55	M	OD
Wang et al. (34)	China	2020 May	Case report	1	1	45	M	1D
Cao et al. (35)	China	2020 Sep	Case report	1	1	47	F	OD
Kumar et al. (36)	India	2020 Sep	Case report	1	1	38	M	1D
Motta et al. (37)	Italy/Spain	2020 May	Retrospective study	8	8	69.38	7 M/1 F	8D
Yadav and Rawal (38)	India	2020 Aug	Case report	1	1	43	M	OD
Sarma et al. (39)	India	2020 Nov	Case report	1	1	53	F	OD
Cao et al. (9)	China	2020 Oct	Case report	1	1	47	F	OD
Boulle et al. (40)	South Africa	2020 Aug	Population cohort study	22,308	2,128	— —	15,256 M/7,052 F	625D (2.80%)
Liu et al. (41)	China	2020 Jul	Cohort study	1,190	24	57 (47, 67)	635 M/555 F	157D (13.19%)
Chen et al. (42)	China	2020 Sep	Retrospective study	55	1	74 (65–91)	34 M/22 F	19D (34.54%)
Du et al. (43)	China	2020 May	Prospective cohort study	179	8	57.6 ± 13.7	97 M/82 F	21D (11.73%)
Li et al. (44)	China	2020 Apr	Retrospective study	548	9	60 (48–69)	279 M/269 F	269S (49.09%)
Liu et al. (45)	China	2020 Jun	Retrospective study	342	2	56(45–67)	183 M/159 F	146S (42.69%)
Xiao et al. (46)	China	2020 Feb	Retrospective study	143	4	45.13 ± 1.04	73 M/70 F	36S (25.17%)
Zhang et al. (47)	China	2020 Apr	Retrospective study	1,350	5	44.1 ± 17.9	664 M/686 F	229S (16.96%)
Xu et al. (48)	China	2020 Apr	Retrospective study	23	2	46.0 (40.5, 52.0)	15 M/8 F	4S (17.39%)
Liu et al. (49)	China	2020 Mar	Retrospective study	36	13	47 ± 14	18 M/18 F	9S (25.00%)

COVID-19, coronavirus disease 2019; COVID-TB, COVID-19 and tuberculosis coinfection.

indicating that COVID-TB patients are at higher risk of death or serious illness than COVID-19 patients without TB.

Older age, especially >65 years, may be a risk factor for death from COVID-TB, consistent with previous findings indicating that the mortality rate from COVID-19 increases exponentially with age (50, 51). According to a model-based analysis, the estimated overall death rate for COVID-19 was 0.66%, but increasing to 7.8% among patients aged >80 years and decreasing to 0.0016% among children aged <9 years (52). There are several primary reasons for these differences, including more preexisting comorbidities, dysregulation in the immune response, and chronic subclinical systemic inflammation (inflammaging) among older adults than younger persons (53). Thus, the elderly should be the primary focus of both COVID-19 and COVID-TB mitigation efforts due to its much higher mortality risk in that group.

COVID-TB patients had a much higher rate of comorbidities than COVID-19 patients (56.41 vs. 25.1%) (54). The most prevalent comorbidities among COVID-19 patients were hypertension (21.1, 95% CI: 13.0–27.2%), diabetes (9.7%, 95 CI: 7.2–12.2%), cardiovascular disease (8.4%, 95% CI: 3.8–13.8%), and respiratory system disease (1.5%, 95% CI: 0.9–2.1%),

TABLE 2 | Clinical characteristics of 89 patients with COVID-19 disease and tuberculosis.

Clinical characteristics	All patients ($n = 89$)	Survivor ($n = 70$)	Non-survivor ($n = 19$)	p-value
Age, years ($n = 89/70/19$)				
Average	41.21 ± 17.84	37.76 ± 15.54	53.95 ± 19.78	$P < 0.001$***
0–14	4 (4.49%)	3 (4.29%)	1 (5.26%)	1.000
15–24	9 (10.11%)	8 (11.43%)	1 (5.26%)	0.677
25–44	40 (44.94%)	38 (54.29%)	2 (10.53%)	0.001**
45–64	24 (26.97%)	18 (25.71%)	6 (31.58%)	0.609
65+	12 (13.48%)	3 (4.29%)	9 (47.37%)	$P < 0.001$***
Sex ($n = 89/70/19$)				
Female	17 (19.1%)	13 (18.57%)	4 (21.05%)	0.809
Male	72 (80.9%)	57 (81.43%)	15 (78.95%)	0.809
Country ($n = 89/70/19$)				
India	31 (34.83%)	24 (34.29%)	7 (36.84%)	0.836
Italy	26 (29.21%)	20 (28.57%)	6 (31.58%)	0.798
China	12 (13.48%)	10 (14.29%)	2 (10.53%)	1.000
Bangladesh	3 (3.37%)	3 (4.29%)	0 (0%)	1.000
Spain	3 (3.37%)	0 (0%)	3 (15.79%)	0.009**
Brazil	2 (2.25%)	2 (2.86%)	0 (0%)	1.000
South Africa	2 (2.25%)	2 (2.86%)	0 (0%)	1.000
America	2 (2.25%)	1 (1.43%)	1 (5.26%)	0.383
Panama	2 (2.25%)	2 (2.86%)	0 (0%)	1.000
Nepal	2 (2.25%)	2 (2.86%)	0 (0%)	1.000
Turkey	1 (1.12%)	1 (1.43%)	0 (0%)	1.000
France	1 (1.12%)	1 (1.43%)	0 (0%)	1.000
Haiti	1 (1.12%)	1 (1.43%)	0 (0%)	1.000
Saudi Arabia	1 (1.12%)	1 (1.43%)	0 (0%)	1.000
TB ($n = 89/70/19$)				
Previous TB	8 (8.99%)	7 (10.00%)	1 (5.26%)	1.000
LTBI	2 (2.25%)	2 (2.86%)	0 (0%)	1.000
Site				
Pulmonary TB only	71 (79.78%)	55 (78.57%)	16 (84.21%)	0.753
Extrapulmonary TB only	8 (8.99%)	5 (7.14%)	3 (15.79%)	0.360
Pulmonary TB/extrapulmonary TB (>1 site possible)	8 (8.99%)	8 (11.43%)	0 (0%)	0.194
Site of extrapulmonary TB				
Central nervous system	5 (5.62%)	3 (4.29%)	2 (10.53%)	0.289
Pleural	4 (4.49%)	4 (5.71%)	0 (0%)	0.574
lymphadenitis	2 (2.25%)	1 (1.43%)	1 (5.26%)	0.383
Gastrointestinal	1 (1.12%)	1 (1.43%)	0 (0%)	1.000
renal+ brain+ meningeal	2 (2.25%)	2 (2.86%)	0 (0%)	1.000
pericardial+ pleural+ splenic+ bone	1 (1.12%)	1 (1.43%)	0 (0%)	1.000
disseminated systemic tuberculosis	1 (1.12%)	1 (1.43%)	0 (0%)	1.000
Comorbidities ($n = 78/61/17$)				
Any	44 (56.41%)	32 (52.46%)	12 (70.59%)	0.183
Hypertension	14 (17.95%)	6 (9.84%)	8 (47.06%)	$P < 0.001$***
Diabetes	19 (24.36%)	15 (24.59%)	4 (23.53%)	1.000
Hepatitis	3 (3.85%)	0 (0%)	3 (17.65%)	0.009**
Chronic kidney disease	3 (3.85%)	1 (1.64%)	2 (11.76%)	0.118
Cerebrovascular disease	2 (2.56%)	2 (3.28%)	0 (0%)	1.000
COPD	2 (2.56%)	1 (1.64%)	1 (5.88%)	0.391
Asthma	2 (2.56%)	2 (3.28%)	0 (0%)	1.000
Bronchiectasis	1 (1.28%)	1 (1.64%)	0 (0%)	1.000
Glioma	1 (1.28%)	1 (1.64%)	0 (0%)	1.000

(Continued)

TABLE 2 | Continued

Clinical characteristics	All patients ($n = 89$)	Survivor ($n = 70$)	Non-survivor ($n = 19$)	p-value
Epilepsy	3 (3.85%)	3 (4.92%)	0 (0%)	1.000
HIV	5 (6.41%)	4 (6.56%)	1 (5.88%)	1.000
Cancer	2 (2.56%)	0 (0%)	2 (11.76%)	0.045*
Others	15 (19.23%)	11 (18.03%)	4 (23.53%)	0.729
Symptoms at admission ($n = 81/70/11$)				
Fever	63 (77.78%)	52 (74.29%)	11 (100%)	0.111
Cough	52 (64.2%)	45 (64.29%)	7 (63.64%)	1.000
Dyspnea	29 (35.8%)	21 (30%)	8 (72.73%)	0.014*
Weight loss	13 (16.05%)	11 (15.71%)	2 (18.18%)	1.000
Fatigue	9 (11.11%)	8 (11.43%)	1 (9.09%)	1.000
Expectoration	8 (9.88%)	6 (8.57%)	2 (18.18%)	0.297
Chest pain	8 (9.88%)	8 (11.43%)	0 (0%)	0.590
Headache	7 (8.64%)	6 (8.57%)	1 (9.09%)	1.000
Myalgias	7 (8.64%)	7 (10%)	0 (0%)	0.585
Vomiting	6 (7.41%)	5 (7.14%)	1 (9.09%)	1.000
Chest tightness	2 (2.47%)	2 (2.86%)	0 (0%)	1.000
Diarrhea	2 (2.47%)	1 (1.43%)	1 (9.09%)	0.255
Reduced appetite	3 (3.7%)	3 (4.29%)	0 (0%)	1.000
Hemoptysis	4 (4.94%)	4 (5.71%)	0 (0%)	1.000
Sore throat	1 (1.23%)	0 (0%)	1 (9.09%)	0.136
Night sweats	2 (2.47%)	2 (2.86%)	0 (0%)	1.000
Chills	2 (2.47%)	2 (2.86%)	0 (0%)	1.000
Asymptomatic	4 (4.94%)	4 (5.71%)	0 (0%)	1.000
CT findings ($n = 89/70/19$)				
Cavities	29 (32.58%)	23 (32.86%)	6 (31.58%)	0.916
Infiltrates	28 (31.46%)	17 (24.29%)	11 (57.89%)	0.005**
Ground-glass opacity	17 (19.1%)	15 (21.43%)	2 (10.53%)	0.347
Nodules	15 (16.85%)	14 (20%)	1 (5.26%)	0.177
Pleural effusion	10 (11.24%)	9 (12.86%)	1 (5.26%)	0.683
Fibrosis	11 (12.36%)	8 (11.43%)	3 (15.79%)	0.695
Patchy shadows	8 (8.99%)	8 (11.43%)	0 (0%)	1.000
Consolidation	8 (8.99%)	7 (10%)	1 (5.26%)	1.000
Miliary	5 (5.62%)	3 (4.29%)	2 (10.53%)	0.289
Reticules	5 (5.62%)	5 (7.14%)	0 (0%)	0.580
Calcific lesions	4 (4.49%)	4 (5.71%)	0 (0%)	0.574
Pleural thickening	3 (3.37%)	2 (2.86%)	1 (5.26%)	0.518
Lymphadenopathy	3 (3.37%)	3 (4.29%)	0 (0%)	1.000
Minimal signs of interstitial thickening	3 (3.37%)	2 (2.86%)	1 (5.26%)	0.518
Tree in bud	2 (2.25%)	0 (0%)	2 (10.53%)	0.044*
Air bronchogram	2 (2.25%)	2 (2.86%)	0 (0%)	1.000
Mediastinal emphysema	2 (2.25%)	1 (1.43%)	1 (5.26%)	0.383
Pleural empyema	2 (2.25%)	1 (1.43%)	1 (5.26%)	0.383
Atelectasis	2 (2.25%)	2 (2.86%)	0 (0%)	1.000
Fibrous stripes	1 (1.12%)	1 (1.43%)	0 (0%)	1.000
Bilateral	47 (52.81%)	33 (47.14%)	14 (73.68%)	0.040*
Unilateral	18 (20.22%)	17 (24.29%)	1 (5.26%)	0.105
Therapy ($n = 61/51/10$)				
Antibiotics	31 (50.82%)	28 (54.9%)	4 (40%)	0.496
Anti-TB therapy	54 (88.52%)	45 (88.24%)	9 (90%)	1.000
Antiviral treatment	14 (22.95%)	13 (25.49%)	1 (10%)	0.429
Hydroxychloroquine	16 (26.23%)	14 (27.45%)	2 (20%)	1.000

(Continued)

TABLE 2 | Continued

Clinical characteristics	All patients ($n = 89$)	Survivor ($n = 70$)	Non-survivor ($n = 19$)	p-value
Corticosteroids	7 (11.48%)	6 (11.76%)	1 (10%)	1.000
Traditional Chinese medicine	4 (6.56%)	4 (7.84%)	0 (0%)	1.000
Intravenous immunoglobulin	3 (4.92%)	3 (5.88%)	0 (0%)	1.000
Tocilizumab	2 (3.28%)	2 (3.92%)	0 (0%)	1.000
High-flow nasal cannula oxygen therapy	10 (16.39%)	8 (15.69%)	2 (20%)	0.663
Non-invasive mechanical ventilation	2 (3.28%)	2 (3.92%)	0 (0%)	1.000
ECMO	1 (1.64%)	1 (1.96%)	0 (0%)	1.000
Hemoperfusion	1 (1.64%)	1 (1.96%)	0 (0%)	1.000
Azithromycin	13 (21.31%)	12 (23.53%)	1 (10%)	0.674
Ceftriaxone	9 (14.75%)	9 (17.65%)	0 (0%)	0.332
Moxifloxacin	3 (4.92%)	2 (3.92%)	1 (10%)	0.421
Amikacin	2 (3.28%)	1 (1.96%)	1 (10%)	0.303
Meropenem	1 (1.64%)	1 (1.96%)	0 (0%)	1.000
Lopinavir/ritonavir	7 (11.48%)	7 (13.73%)	0 (0%)	0.587
Umifenovir hydrochloride	6 (9.84%)	6 (11.76%)	0 (0%)	0.577
Tenofovir	4 (6.56%)	4 (7.84%)	0 (0%)	1.000
Remdesivir	1 (1.64%)	0 (0%)	1 (10%)	0.164
Lamivudine	1 (1.64%)	1 (1.96%)	0 (0%)	1.000
Dolutegravir	1 (1.64%)	1 (1.96%)	0 (0%)	1.000
Favipiravir	1 (1.64%)	1 (1.96%)	0 (0%)	1.000
Interferon–α	5 (8.2%)	5 (9.8%)	0 (0%)	0.580
Low molecular weight heparin	2 (3.28%)	2 (3.92%)	0 (0%)	1.000
Aspirin	2 (3.28%)	1 (1.96%)	1 (10%)	0.303
Laboratory examinations				
Leucocyte count (reference range 3.5–9.5 × 10^9/L) ($n = 36/30/6$)	8.25 (5.18–9.88)	8.015 (4.8–8.97)	12.9 (10.5–16.73)	0.007**
Neutrophil count (reference range 1.8–6.3 × 10^9/L) ($n = 14/13/1$)	7.6 (6.93–7.6)	7.6 (6.77–7.6)	8.74	0.155
Lymphocyte count (reference range 1.1–3.2 × 10^9/L) ($n = 32/29/3$)	0.99 (0.73–1.31)	1 (0.72–1.3)	0.9 (0.83–1.36)	0.721
Hemoglobin (reference range 115–150 g/L) ($n = 16/14/2$)	99 (83.5–114)	99 (85.5–120,75)	93.5 (88.25–98.75)	0.634
Platelet count (reference range 125–350 × 10^9/L) ($n = 7/4/3$)	253 (201–323)	235.5 (209.5–259.75)	366 (273–398)	0.480
D-dimer (reference range 0–0.5 μg/mL) ($n = 20/18/2$)	1.41 (1.09–2.65)	1.41 (1.12–2.51)	3.11 (2.04–4.18)	0.801
CRP (reference range 0–8 mg/L) ($n = 21/20/1$)	77.1 (29.2–184.7)	67.05 (26.60–181.18)	293.8	0.137
ESR (reference range 0–20 mm/h) ($n = 6/5/1$)	75.5 (55.75–88.5)	70 (51–81)	123	0.143
PCT (reference range 0–0.05 ng/mL ($n = 5/5/0$)	2.57 (0.5–5.76)	2.57 (0.5–5.76)	—	—
FER (reference range 11.0–306.8 ng/mL) ($n = 18/17/1$)	739.5 (511.5–952.5)	768 (513–978)	137	0.102
ALT (reference range 13–35 U/L) ($n = 14/13/1$)	28.1 (25.05–36)	28.1 (28.1–33)	178	0.093
AST (reference range 7–40 U/L) ($n = 3/3/0$)	46 (27.55–78.5)	46 (27.55–78.5)	—	—
LDH (reference range 120–150 U/L) ($n = 14/12/2$)	384 (290.25–471.75)	350 (283–500.25)	433.5 (422.25–444.75)	0.465
Creatinine (reference range 62–106 umol/L) ($n = 7/4/3$)	335.92 (189.51–396.92)	189.51 (70.34–337.25)	335.92 (335.92–455.26)	0.154

*COVID-19, coronavirus disease 2019; TB, tuberculosis; LTBI, latent tuberculosis infection; COPD, chronic obstructive pulmonary disease; ECMO, extracorporeal membrane oxygenation; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; PCT, procalcitonin; FER, ferroprotein; ALT, alanine transaminase; AST, aspartate transaminase; LDH, lactate dehydrogenase; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.*

whereas the most common comorbidities among COVID-TB patients were diabetes (24.36%), hypertension (17.95%), HIV infection (6.41%), hepatitis (3.85%), epilepsy (3.85%), and cancer (2.56%) (54). Indeed, both HIV infection and diabetes are important risk factors for TB infection (55). Interestingly, we found that COVID-TB patients who died had a much higher proportion of hypertension (47.06 vs. 9.84%) and cancer (11.76 vs. 0%) than those who survived. A previous study also

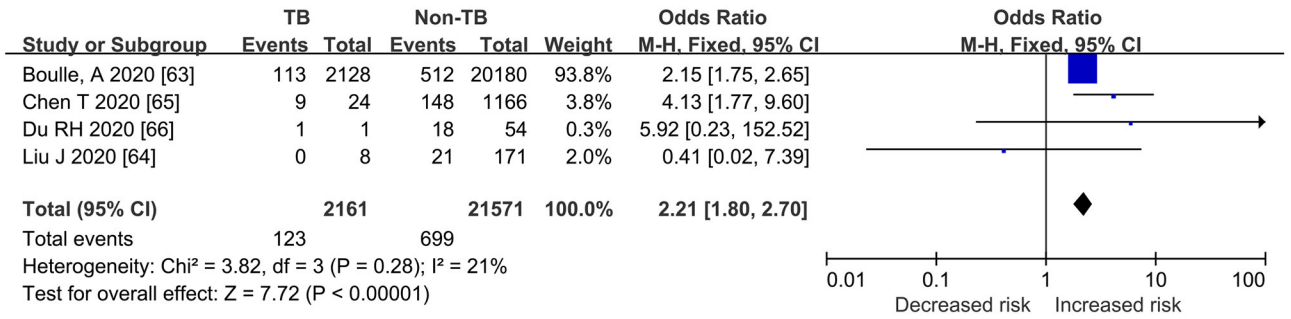

FIGURE 2 | Forrest plot demonstrating the pooled ORs of death in COVID-19 patients with tuberculosis. ORs, odds ratios; TB, tuberculosis; CI, confidence interval; events refer to the occurrence of death.

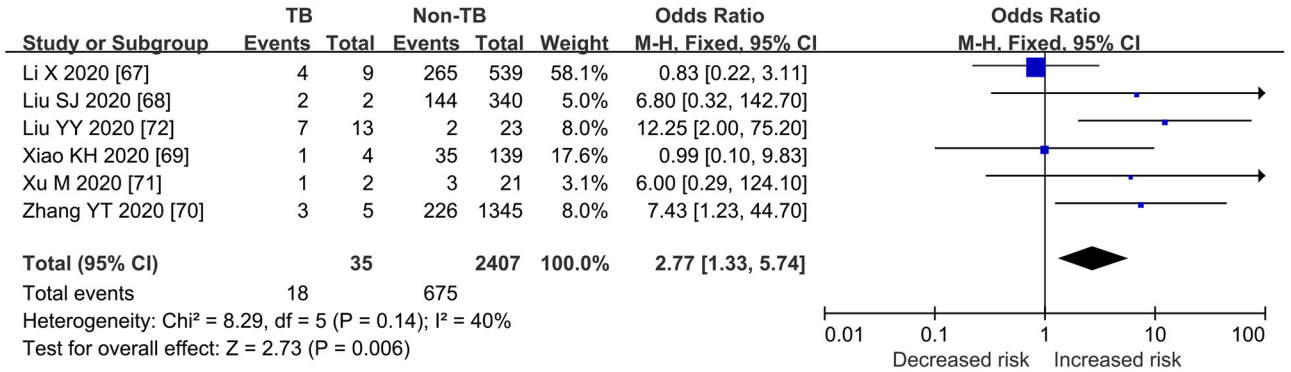

FIGURE 3 | Forrest plot demonstrating the pooled ORs of severe cases in COVID-19 patients with tuberculosis. ORs, odds ratios; TB, tuberculosis; CI, confidence interval; events refer to the occurrence of severe cases.

indicated that underlying diseases such as hypertension (OR = 2.72, 95% CI: 1.60, 4.64) and diabetes (OR = 3.68, 95% CI: 2.68, 5.03) are risk factors for critical disease/mortality (56). Early publications reported a "harmful hypothesis" that SARS-CoV-2 binds to target cells via angiotensin-converting enzyme 2 (ACE2), and patients with hypertension usually have increased expression of ACE2 due to the use of renin angiotensin system inhibitors (57). Although some studies indicated that ACE inhibitors (angiotensin converting enzyme inhibitors) and ARB (angiotensin-receptor blockers) therapy was harmful in COVID-19 patients, an updated meta-analysis concluded that ACEI/ARB therapy does not contribute to increased risk of mortality or severe manifestations among COVID-19 patients (58, 59). It was recommended that ACEI/ARB therapy be continued among patients with coexisting hypertension (60). However, whether the guidelines regarding ACEI/ARB therapy among COVID-19 patients are equally applicable to COVID-TB patients remains to be determined.

The most common clinical manifestations of COVID-TB are fever, cough, dyspnea, weight loss, fatigue, and expectoration (13, 61). Existing evidence indicates that the features of lung imaging among COVID-19 patients include bilateral involvement, peripheral distribution, mixed ground-glass opacity and consolidation, and vascular thickening (62), whereas the most common CT findings of COVID-TB include bilateral lesions, cavities, infiltrates, ground-glass opacity, nodules, pleural effusion, and fibrosis. Thus, clinicians should take COVID-TB coinfection into consideration upon encountering the above CT imaging features in the future instead of just focusing on one disease. The increased prevalence of dyspnea and CT findings including bilateral lesions, infiltrates, and tree in bud among COVID-TB patients who died suggests that they may be good predictors of disease severity, in line with the findings of previous studies (13, 61).

Markedly elevated levels of inflammatory markers, including CRP, ESR, PCT, FER, and LDH, slightly increased neutrophil count and D-dimer level, and decreased lymphocyte count and hemoglobin level were observed in COVID-TB patients. However, we did not find any significant differences in these indexes (except the leucocyte count) between the survivors and non-survivors, which was inconsistent with previous findings that inflammatory markers were elevated in severe disease and critically ill groups (63, 64). The findings regarding the characteristics of COVID-TB biomarkers may provide references for conventional hematological and inflammatory examinations for disease severity classification, and early warning of progression (65).

TABLE 3 | GRADEpro assessment of methodologic quality of included studies examining the ability of TB to increase the risk of death or severe cases among COVID-19.

Risk of death or severe cases among COVID-TB compared to COVID-19 **Patient or population: [COVID-19]** **Settings:** **Intervention: TB** **Comparison: non-TB**						
Outcomes	**Illustrative comparative risks* (95% CI)**		**Relative effect (95% CI)**	**No of Participants (studies)**	**Quality of the evidence (GRADE)**	**Comments**
	Assumed risk	**Corresponding risk**				
	Non-TB	**TB**				
Death	Study population		**OR 2.21** (1.8 to 2.7)	23,732 (4 studies)	⊕⊕⊕⊖ **moderate**[a]	
	32 per 1,000	69 per 1,000 (57 to 83)				
	Moderate					
	125 per 1,000	240 per 1,000 (205 to 278)				
Severe	Study population		**OR 2.77** (1.33 to 5.74)	2,442 (6 studies)	⊕⊕⊕⊖ **moderate**[a]	
	280 per 1,000	519 per 1,000 (341 to 691)				
	Moderate					
	210 per 1,000	424 per 1,000 (261 to 604)				

The basis for the **assumed risk (e.g., the median control group risk across studies) is provided in footnotes. The **corresponding risk** (and its 95% confidence interval) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI). **CI,** Confidence interval; **OR:** Odds ratio.*

*GRADE Working Group grades of evidence **High quality:** Further research is very unlikely to change our confidence in the estimate of effect. **Moderate quality:** Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate. **Low quality:** Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate. **Very low quality:** We are very uncertain about the estimate.*

[a] *Relative risk > 2.*

COVID-19, coronavirus disease 2019; TB, tuberculosis. Bold indicates the pooled ORs of for death or severe disease in the cases in COVID-TB group compared with the non-TB group were as 2.21 and 2.77 respectively, with a moderate level of evidence.

According to the COVID-19 treatment guidelines, the main treatments include antiviral therapy, immune-based therapy, and adjunctive therapy (66). Antiviral therapies may have a greater effect in the early course of COVID-19, whereas immunosuppressive/anti-inflammatory therapies will be more beneficial in the later stages (66, 67). Although previous studies indicated that corticosteroids are associated with a reduction in short-term mortality and the need for mechanical ventilation in COVID-19 patients, whether immunosuppressive therapies such as dexamethasone, a corticosteroid, can be used in COVID-TB patients as well has not been investigated (68). In our study, although there was no statistically significant difference in the proportion of corticosteroid therapy among COVID-TB survivors and non-survivors, we still recommend a more cautious use of corticosteroids in COVID-TB patients because of the potential increased risk of active or severe TB infection associated with corticosteroid use. Studies involving larger samples are needed to explore the impact of corticosteroid therapy on the prognosis of COVID-TB patients (69). It is also worthwhile to explore whether COVID-19 patients with active TB, LTBI, or previous TB should receive standard anti-TB treatment.

Based on this meta-analysis, we found that COVID-TB patients were 2.21 and 2.27 times more likely to die or develop severe COVID-19, respectively. In many countries, the ongoing COVID-19 pandemic coincides with other major public health problems, especially TB, and the impact of the COVID-19 pandemic may be ameliorated if we continue to implement health-care services and key prevention measures (70, 71). COVID-TB infection is a novel disease that remains to be further explored and needs more attention in high-TB burden countries such as India, Indonesia, and China (8). Encouragingly, it has been reported that use of the GeneXpert MTB/RIF platform for the surveillance of COVID-19 is relevant and achievable, especially in low-income and middle-income countries without sufficient classical real-time PCR capabilities but with an already existing GeneXpert MTB/RIF network (72).

Our study has some strengths. First, detailed information was collected in our study, including data regarding demographic characteristics, imaging findings, symptoms at admission, comorbidities, therapies, and outcomes, and the synthesis of these characteristics may provide further guidance for clinicians in terms of diagnosis and treatment of COVID-TB. Second, a comprehensive literature search of both Chinese and English language databases were performed, resulting in a more accurate evaluation of summary estimates with higher precision. Third, the studies included in our meta-analysis had relatively low levels of heterogeneity.

Our study also has several limitations. First, although we performed an extensive search of the literature, most of the eligible studies included in the Forest plots were Chinese. Second, some detailed patient information was not available due to publication bias or no relevant laboratory tests having been performed. Finally, the sample size of our case overview was

still limited; thus, further large cohort studies of COVID-TB are needed.

CONCLUSION

In summary, older age, complications including hypertension, hepatitis, cancer, symptoms of dyspnea at admission, CT imaging features of bilateral lesions, infiltrates, tree in bud, and higher leucocyte count may be predictors for poor prognosis of COVID-TB patients. Furthermore, a moderate level of evidence suggests that people with COVID-TB are 2.21 and 2.27 times more likely to die or develop severe disease, respectively, than COVID-19 patients. Finally, routine screening for *Mycobacterium tuberculosis* is recommended among suspected or confirmed cases of COVID-19 in high–TB burden countries due to the worse prognosis of COVID-TB and the confounding clinical symptoms of these two diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

H-cL, W-mS, and Y-fL conceived and designed the study. H-cL, X-hZ, Q-qA, S-qL, and YL directed its implementation including the data analysis and writing of the paper. W-mS and Y-fL analyzed the data. W-mS, YL, Q-yZ, J-yL, T-tX, S-jL, X-hZ, and N-nT contributed to data collection, materials, and analytic tools. W-mS and H-cL wrote and revised the manuscript. All authors revised it critically for important intellectual content, gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.657006/full#supplementary-material

Table A1 | Search strategies for PubMed, Embase, Cochrane, CNKI, and Wanfang. CNKI, Chinese National Knowledge Infrastructure.

Figure A1 | Funnel plot to detect publication bias.

Figure A2 | Funnel plot to detect publication bias.

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Rapid and Laboratory SARS-CoV-2 Antibody Testing in High-Risk Hospital Associated Cohorts of Unknown COVID-19 Exposure, a Validation and Epidemiological Study After the First Wave of the Pandemic

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Objective: We aimed to use SARS-CoV-2 antibody tests to assess the asymptomatic seroprevalence of individuals in high-risk hospital cohorts who's previous COVID-19 exposure is unknown; staff, and patients requiring haemodialysis or chemotherapy after the first wave.

Methods: In a single Center, study participants had five SARS-CoV-2 antibody tests done simultaneously; one rapid diagnostic test (RDT) (Superbio Colloidal Gold IgM/IgG), and four laboratory tests (Roche Elecsys® Anti-SARS-CoV-2 IgG [RE], Abbott Architect i2000SR IgG [AAr], Abbott Alinity IgG [AAl], and Abbott Architect IgM CMIA). To determine seroprevalence, only positive test results on laboratory assay were considered true positives.

Results: There were 157 participants, of whom 103 (65.6%) were female with a median age of 50 years (range 19–90). The IgG component of the RDT showed a high number of false positives ($n = 18$), was inferior to the laboratory assays ($p < 0.001$ RDT vs. AAl/AAr, $p < 0.001$ RDT vs. RE), and had reduced specificity (85.5% vs. AAl/AAr, 87.2% vs. RE). Sero-concordance was 97.5% between IgG laboratory assays (RE vs. AAl/AAr). Specificity of the IgM component of the RDT compared to Abbott IgM CMIA was 95.4%. Ten participants had positivity in at least one laboratory assay, seven (9.9%) of which were seen in HCWs. Two (4.1%) hematology/oncology (H/O) patients and a single (2.7%) haemodialysis (HD) were asymptomatically seropositive. Asymptomatic seroprevalence of HCWs compared to patients was not significant ($p = 0.105$).

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Conclusion: HCWs (9.9%) had higher, although non-significant asymptomatic seroprevalence of SARS-CoV-2 antibodies compared to high-risk patients (H/O 4.1%, HD 2.7%). An IgM/IgG rapid diagnostic test was inferior to laboratory assays. Sero-concordance of 97.5% was found between IgG laboratory assays, RE vs. AAI/AAr.

Keywords: COVID-19, SARS-CoV-2, rapid antibody test, seroprevalence SARS-CoV-2, high-risk hospital cohorts, first wave

INTRODUCTION

Asymptomatic carriage of SARS-CoV-2 virus was identified early in the course of the pandemic and the potential infectivity of these patients has been speculated upon since that time. Early studies suggested asymptomatic COVID-19 may be highly transmissible (1). Quantification studies show this is not the case (2), but asymptomatic infection is still likely to be an important factor in the transmission dynamics of the virus with reported secondary infection between 5 and 18% (3–5).

Large seroprevalence studies of populations which included asymptomatic and symptomatic individuals during and after the first wave were done across the globe and showed low levels of seropositivity. Seroprevalence in Wuhan varied from 3.2 to 3.8% from 9th March to 10th April (6), 4.65% on April 10–11 2020 in Los Angeles County California (7), and 1.79% in Idaho in testing of 4,856 individuals over 1 week in April (8). Seropositivity was between 1.0 and 6.9% across 10 U.S. sites between March and April 2020 (9). The SEROCoV-POP study in Geneva, Switzerland of 2,766 individuals showed seroprevalence as high as 10.9% (10). The national seroprevalence rate in Ireland was estimated to be 1.7% in June/July 2020 at the same time of this study (11).

Serial hospital attendance is a necessary endeavor for some high-risk patients including those attending hematology and oncology (H/O) out-patient services and patients receiving haemodialysis (HD). These attendances may increase exposure to COVID-19 in these immunocompromised cohorts. In studies performed prior to the availability of vaccination for COVID-19, oncology patients were shown to have both higher seroprevalence of SARS-CoV-2 antibodies (12) and worse outcomes compared to the standard population (13, 14). Similarly, HD patients had worse outcomes than the standard population (15, 16), prevalence of infection in a HD unit was reported as high as 41.1% in one center, 40.5% of whom had no symptoms at the time of virus detection (17). Healthcare workers (HCWs) also represent a cohort with a higher incidence of COVID-19, occupational exposure to asymptomatic patients may be a factor in this (18).

The aim of this study was to assess the asymptomatic seroprevalence of SARS-CoV-2 infection in high-risk patient cohorts that have unavoidable hospital attendances using a rapid SARS-CoV-2 antibody test and laboratory serological assays. Additionally, the seroprevalence of HCWs was also investigated. A secondary aim was to determine the agreement of the rapid diagnostic test (RDT) with laboratory testing in both groups. Assessing the accuracy of antibody tests in real world studies is critical to determining their clinical utility (19).

METHODS

This study was designed as a single center 3-day prospective cohort study. The study was done in the Mater Misericordiae University Hospital (MMUH), Dublin, a 580-bed tertiary referral center which contains the National Isolation Unit (NIU) for Ireland. MMUH has treated over 450 in-patients with COVID-19 since its first case on the 3rd March 2020.

Inclusion and Exclusion Criteria

Candidates were required to be patients or staff of the H/O directorate, be dialysis patients attending MMUH, be over 18 years of age and have capacity to consent to be included in the study. Individuals were excluded from the study if they had ever had a diagnosis of COVID-19 with confirmatory nasopharyngeal (N-P) polymerase chain reaction (PCR) testing, if they had typical symptoms of COVID-19 at the time of recruitment, or if they were currently receiving intravenous immunoglobulin (IVIG) as reports of reactive antibodies to SARS-CoV-2 in commercially available IVIG have been reported (20).

Although participants were asymptomatic for typical symptoms of COVID-19 at the time of the study, this cohort represents individuals whose prior exposure to COVID-19 since the onset of the first wave in March 2020 is unknown and unconfirmed, as routine antigen testing was not available at that time.

Antibody Tests

Four SARS-CoV-2 commercially available antibody detection tools were used. A rapid antibody IgM/IgG colloidal gold test produced by Superbio, Jiangsu. This RDT is CE approved in Europe and pending FDA approval in the United States. Literature provided by the company report sensitivity of 95.3% and specificity 98.2% and consistency value between serum, plasma and whole blood at 100%. No cross reactivity was reported in samples with antibody positivity for influenza A/B, coronavirus (CoV), respiratory syncytial virus (RSV), *Haemophilus influenzae*, and anti-nuclear antibody (ANA). The comparative laboratory based IgG automated serological assays used were; Abbott Architect i2000SR (AAr) chemiluminescent microparticle immunoassay (CMIA) (Abbott Diagnostics, Chicago, USA) which has demonstrated 8.6% (<6 days) to 100% (>14 days) sensitivity, and specificity of 99.9% (8, 21), and Abbott Alinity (AAl) i SARS-CoV-2 IgG CMIA, negative percent agreement (NPA) of 99.63%, and a positive percent agreement (PPA) of 100% (in those 14 days post symptoms) (22), and Roche Elecsys® IgG (RE) electrochemiluminescence

immunoassay analyser (ECLIA), sensitivity 100% and specificity 99.81% (23). The Abbott Architect i2000SR CMIA IgM was used as a standard to compare the IgM component of the IgM/IgG RDT, the laboratory assay demonstrates 99.56% specificity and 95% sensitivity in those tested 14 days post infection (24). All assays target the viral nucleocapsid. The assays were performed at the University College Dublin (UCD) Clinical Research Center (CRC) School of Medicine and Medical Science.

Study Design

Consenting participants had a finger prick RDT and a serum sample taken at the same time for comparative laboratory serological assays. Day one of the study was conducted on the 24th June 2020 in the H/O directorate and included all patients on 31-single bed ward, all patients attending the H/O day-ward on that day and all associated staff members including staff nurses, nurse managers, research nurses, doctors, phlebotomists, healthcare assistants (HCAs), allied health professionals, and administrative staff. Days 2–3 of the study were conducted on the 5th−6th July 2020 in the 22 bed HD unit, where 72 patients encompassing the complete dialysis cohort of MMUH were considered for eligibility.

Interpreting Test Results

The RDT test results were interpreted by two study team members together and were categorized as strongly positive, weakly positive, equivocal or negative, as follows: band intensity similar to the control band were deemed strongly positive, faint bands seen only in direct light were equivocal, while the range of band intensities between these two classifications were considered weakly positive. No quantifiable techniques apart from direct visualization were used to determine band intensity in order to replicate the qualitative visual assessment of real-world use. This scoring system was applied to bands at both the IgM and IgG positions of the RDT cassette. Equivocal bands were deemed positive when compiling RDT results, as SARS-CoV-2 cannot be entirely excluded in these cases and no guidance of an "exclusion threshold" of band intensity was offered with the literature provided by the manufacturers.

For the laboratory assays, manufacturer recommended indices of positivity were applied; cut-off index (COI) for AAI was $\geq$1.40, AAr $\geq$ 1.40 (both IgM and IgG), and RE $\geq$ 1.0. The IgG AAI and AAr are Abbott tests on different systems, and are the same test (AAI/AAr). In essence for IgG, three individual platforms were used the RDT, RE, AAI/AAr. A positive test on any laboratory assay was considered a true positive result.

Participants with IgM positive/equivocal test results on the RDT, had GeneXpert® N-P PCR testing performed at the time of antibody testing. Laboratory IgM testing using Abbott Architect CMIA was done on the 9th October 2020, once the test was validated and FDA approved.

Managing Test Results

Results of the RDT were given to participants with the understanding that testing was done in the context of a research study. No decisions to isolate participants were made on the basis of the results of the rapid antibody test. Participants with an IgM positive band on the RDT who were subsequently found to be positive on the validated N-P PCR positive would then proceed through established COVID-19 pathways within the hospital. It was explained to patients during enrolment that if a positive RDT IgM is found, they may be precluded from treatment on the day of the study or offered admission to the hospital in their best interest, depending on the results of the subsequent N-P PCR test.

Statistical Analysis

Data was compiled in Microsoft Excel® 2019. Descriptive analysis was done on collected data. McNemar's test was used to compare the differences in proportions of positive tests between the RDT and IgG serological assays. This was done separately for both RE and AAI/AAr. McNemar's test was also used to compare the differences in proportions for positive tests from the IgM component of the RDT to Abbott's IgM assay. The specificities of the IgM and IgG components of the RDT were calculated compared to the laboratory assays. Fisher's exact test was used to determine if seropositivity in HCW's compared to patient cohorts was significant. The statistical software used was SPSS V26.0.

The cumulative prevalence of COVID-19 was retrospectively calculated for each cohort (H/O patients, H/O staff, HD patients) by adding seropositive results of the study to the previously confirmed COVID-19 cases that were excluded and deemed not eligible for antibody testing.

Ethics

Ethical approval was sought and approved by the local research ethics committee at MMUH. All participants provided written informed consent prior to testing.

RESULTS

In total 221 individuals were assessed for eligibility. Of 64 individuals excluded, 14 had previously confirmed COVID-19 on N-P PCR swab, **Figure 1**. No participants with typical COVID-19 symptoms were identified during recruitment. The total number of participants included was 157. The majority were female (65.4%), median age was 50 years old (Range 19–90), and predominate ethnicity was Caucasian (87.3%). Participants included 71 (45.2%) H/O staff, 49 (31.2%) H/O patients, and 37 (23.6%) HD patients, **Table 1**. Diagnosis and treatment of H/O patients can be seen in the **Supplementary Appendix 1**.

Rapid Test IgG Results

In total 27 participants had a positive IgG on the RDT, 18 of whom were only positive on the RDT IgG and neither of the validated laboratory platforms (RE, AAI/AAr) (**Figure 2**). These 18 were deemed false positives. Using McNemar's test to compare the RDT to the laboratory tests it was found there was a statistically significant difference in both instances due to the high false positive rate (n = 157, RDT vs. RE p <

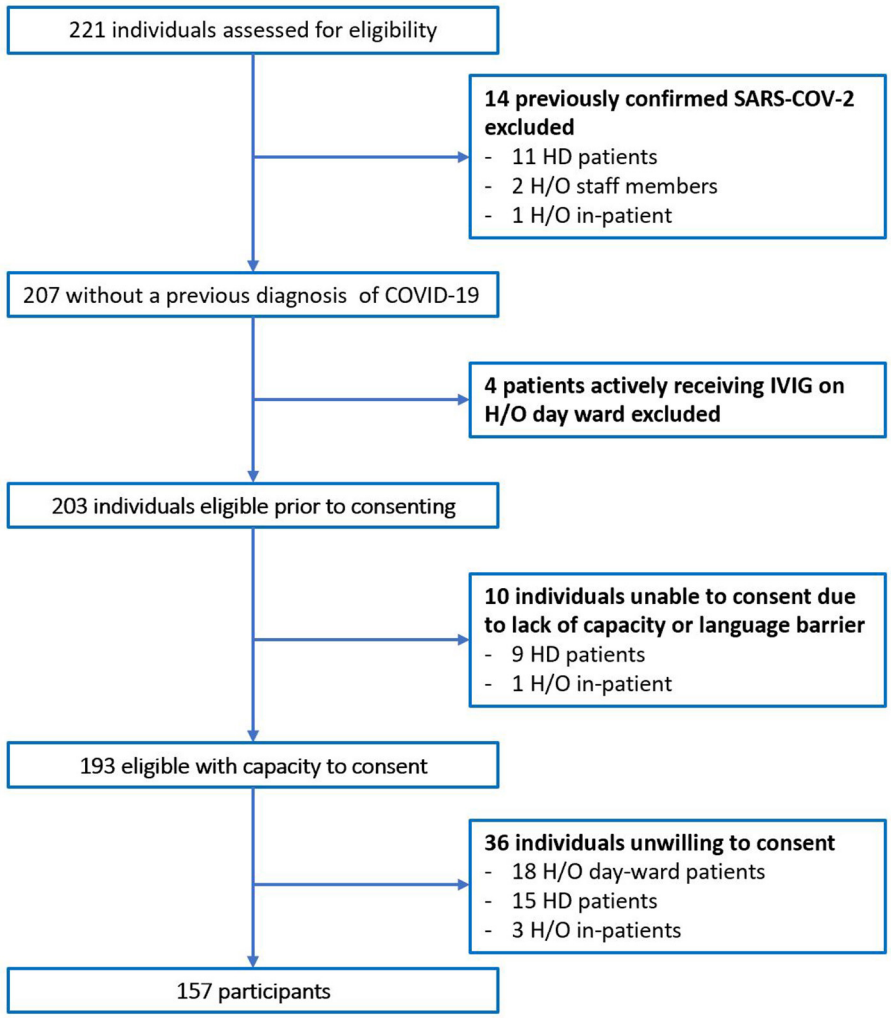

FIGURE 1 | Flow diagram of excluded patients.

0.001 and RDT vs. AAl/AAr $p < 0.001$). A breakdown of positive IgG tests within staff and patient subgroups can be seen in **Table 2**.

Rapid Test IgM Results

Seven participants had a positive IgM on the RDT and four participants were IgM positive on the Abbott CMIA platform. No participants were positive in both. All individuals who had positive RDTs at the time of the study had same-day N-P PCR swabs done on GeneXpert® platform and all were negative for SARS-CoV-2. McNemar's test indicate no significant difference between the samples ($p = 0.549$), the specificity of the IgM component of the RDT is 95.4%.

Sero-Concordance of Laboratory Tests

In total ten (6.4%) of study participants had at least one positive validated laboratory test, **Table 3**. Three IgM/IgG positive, six IgG positive, and one IgM positive. Regarding the IgG laboratory-based assays; 100% sero-concordance was seen between AAl and AAr as expected. Five participants had a positive IgG in both RE and AAl/AAr. The overall sero-concordance between RE and AAl/AAr was 97.5%. Of the four discordant IgG participant results, three of these cases were positive in RE and negative in AAl/AAr, and one case was positive in AAl/AAr and negative in RE.

Of these ten seropositive findings on laboratory platforms seven were HCWs, conferring a 9.9% seroprevalence in asymptomatic staff. Two (4%) of H/O patients tested IgG positive and a single (2.7%) HD patient was seropositive. The difference between healthcare workers and patients was not found to be statistically significant, $p = 0.103$. Four of the ten seropositive participants had symptoms in the preceding weeks when asked retrospectively, all were negative on N-P PCR for SARS-CoV-2 when tested at the time of those symptoms.

Cumulative Prevalence of COVID-19

Taking individuals with historical COVID-19 that were excluded prior to enrolment and adding them to the seropositive asymptomatic participants identified using laboratory serology tests, the cumulative prevalence of COVID-19 in these cohorts was calculated retrospectively; 6% (3/50) in H/O patients, 12.3% (9/73) of all staff members in the H/O directorate and 25% (12/48) of dialysis patients. The overall cumulative prevalence for all 157 participants was 14%.

TABLE 1 | Demographics of participants tested.

Total (*N* =157)	
Male	54 (35.4%)
Female	103 (65.6%)
Age-years (median)	50 (range 19–90)
Ethnicity	
- Caucasian	137 (87.3%)
- Non-Caucasian	20 (12.7%)
H/O directorate patients total	49 (31.2%)
- In-patients	26 (16.6%)
- Day-ward patients	23 (14.6%)
- On chemotherapy	45 (92%)
H/O directorate staff total	71 (45.2%)
- Nursing staff	30 (42.3%)
- Medical staff	22 (31%)
- Other staff	19 (26.8%)
Dialysis patients	37 (23.6%)
- Charlson Comorbidity Index (CCI) median(range)	6 (range 2–11)

DISCUSSION

In this study of 157 staff and patients associated with the hospital environment whose previous exposure to COVID-19 is unknown and who were asymptomatic at the time of the study, we found a SARS-CoV-2 seroprevalence of 6.4%. There was some variation in the seroprevalence of the individual cohorts although this did not reach significance ($p = 0.103$); 4% in H/O patient cohort, 2.7% HD cohort an 9.9% in staff members. When examining frontline workers with most patient contact i.e., nursing staff/medical staff in this study, we find an asymptomatic seroprevalence of 13.5% (7/52). Prevalence studies of COVID-19 in HCWs during and after the first wave have been done with variable findings. One study using both RT-PCR and antibody testing (includes asymptomatic and symptomatic staff at the time of the study) against the Spike protein (Euroimmun SARS-CoV-2 IgG) have found overall infection rates of 12.6%, although being a nurse/physician was not a risk factor for this (25). A study in a specialist infectious diseases directorate in Italy, found prevalence (RT-PCR plus serology, MAGLUMI 2019-nCoV IgM/IgG, spike and nucleocapsid) in asymptomatic staff as low as 3.4%, three of the four positive participants were either a nurse or physician (26). Another study of 249 HCWs in a single center in Kentucky reported 7.6% seroprevalence using serological testing targeting the SARS-CoV-2 spike protein in

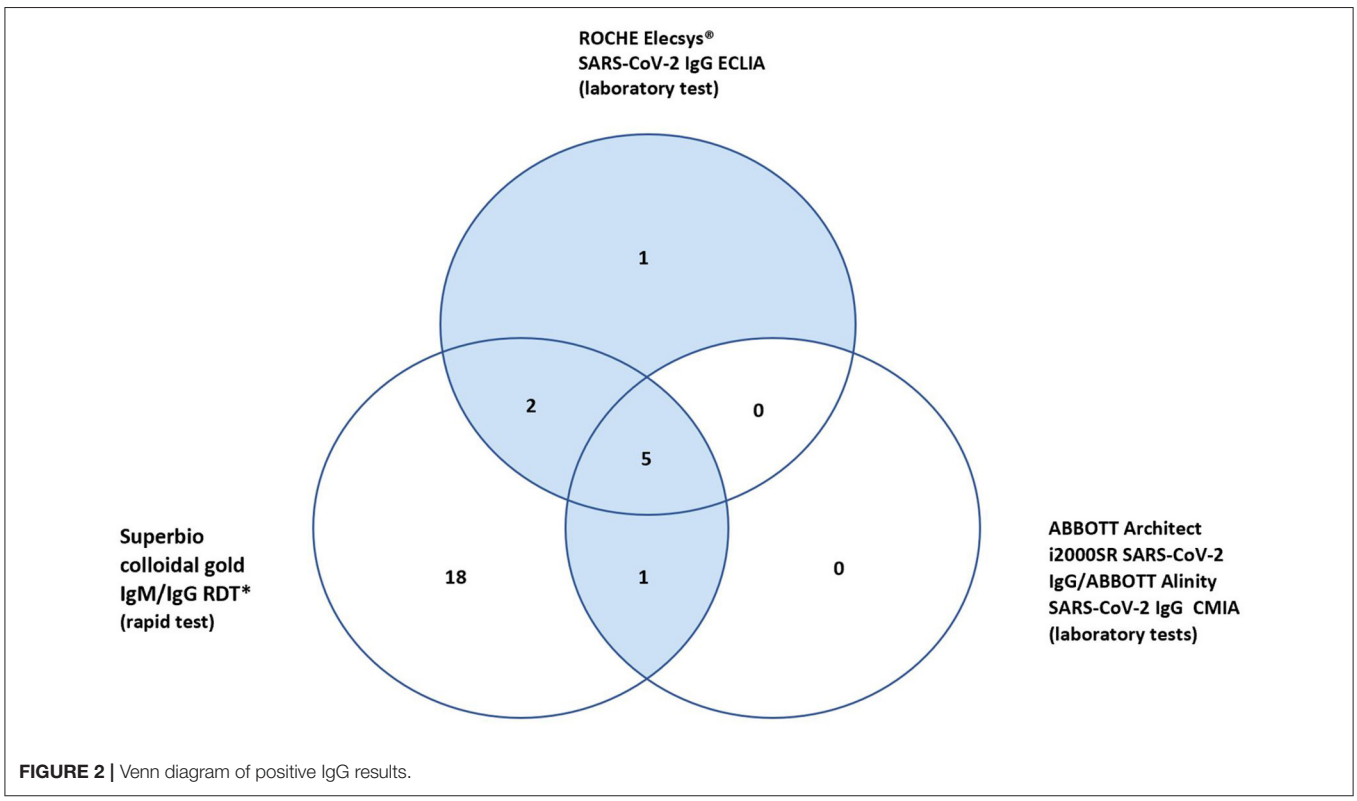

FIGURE 2 | Venn diagram of positive IgG results.

TABLE 2 | Positive results in each cohort and results of McNemar's test between RDT and laboratory assays.

		Superbio Colloidal GOLD IgG	Superbio Colloidal GOLD IgM	Roche Elecsys® IgG ECLIA	Abbott Alinity/ Architect i2000 IgG CMIA	Abbott Architect IgM CMIA	McNemar's test IgG RE vs. RDT *p*	McNemar's test IgG AAI/AAr vs. RDT *p*	McNemar's test IgM Abbott vs. RDT *p*
Total *n* (%)	157	27 (17.2%)	7 (4.5%)	8 (5.1%)	6 (3.8%)	4 (2.6%)	<0.001	<0.001	0.549
Sub-group analysis									
H/O patients	49	7 (14.2%)	4 (8.2%)	1 (2%)	0 (0%)	1 (2%)			
H/O staff	71	15 (21.1%)	2 (2.8%)	6 (8.5%)	5 (7%)	3 (4.2%)			
HD patients	37	5 (13.5%)	1 (2.7%)	1 (2.7%)	1 (2.7%)	0 (0%)			

symptomatic and asymptomatic staff. Of the 19 positives, 11 (68.4%) were a physician or a nurse (27). A study of staff in a H/O directorate in Milan in April 2020 showed 6.9% (7/101) seropositivity amongst doctors and 11.3% (15/133) amongst nurses/paramedics/other staff members using PRISMA IgM/IgG targeting the SARS-CoV-2 nucleocapsid in asymptomatic, pauci-symptomatic, and symptomatic patients (28). Based on these limited studies there does appear to be a trend toward higher seroprevalence in staff members with the most exposure to patients i.e., physicians/nurses.

Regarding the types of antibody tests used in seroprevalence studies, some studies examine antibodies to either the viral nucleocapsid, Spike protein or both. In this study the antibody tests target the nucleocapsid alone. Anti-nucleocapsid serological testing has been found to be highly sensitive and specific; Muench et al. show that in a sample size of 10,453 patients at ≥14 days post PCR positivity, RE assay shows sensitivity of 99.5% and specificity of 99.8% (29). Large studies have adopted these tests for assessing seroprevalence. The PRECISE study examining antibodies in symptomatic and asymptomatic HCWs across two sites in Ireland; St James's Hospital and University Hospital Galway have used both RE and AAr in 5,787 staff members and found seroprevalence of 15% and 4.1%, respectively (30). The SCOPI study was also a national seroprevalence study in Ireland that used AAr for symptomatic and asymptomatic participants. Of 1,733 participants aged 12–69 an overall seroprevalence of 1.7% was found (11). It could be argued that in our study and the studies outlined above where antibodies against the nucleocapsid alone were used to identify seropositive patients there may be participants that have acquired COVID-19 and mounted antibodies to the Spike protein alone, therefore their antibody status would go unrecognized using anti-nucleocapsid assays. Conversely, very high agreement has been shown between antibody tests that target either the nucleocapsid or Spike protein; Prince et al. compare AAr with three assays targeting the Spike protein (DiaSorin Liaison, Ortho Vitros, and Euroimmun) and show a consensus negative interpretation from 96.7 to 100% and a consensus positive interpretation from 94.3 to 100% (31). In essence this effect may be small and using anti-nucleocapsid assays for seroprevalence studies is likely valid.

The RDT performed poorly due to its high false positive rate compared to RE and AAI/AAr. The RDT IgG component showed a lack of specificity ranging between 85.5 and 87.2% depending on which validated laboratory test it was compared to **Table 4**. Similar findings were also found in an FDA report, describing a specificity of 85% for the Superbio Colloidal Gold RDT (32). One factor attributing to this may be misinterpretation of the RDT results as a spectrum of band intensities were found. Although an association of stronger bands with true positives ($n = 7$ of 10) was found, this does not necessarily aid the user in real-world settings, where bands of any intensity cannot fully exclude presence of SARS-CoV-2 antibodies.

The RDT IgM component showed more specificity (95.4%) when compared to the Abbott IgM test. Although none of the four laboratory IgM positives were positive on the RDT, inferring there may be a lack of sensitivity with the test.

There was good agreement between laboratory serology tests (RE, AAI/AAr) for SARS-CoV-2 IgG (97.5%). Harley and Gunsolus also show 98.7% agreement in a cohort of 667 ($n =$ 103 COVID-19 confirmed, $n = 564$ pre COVID-19 samples) comparing RE and AAI (33). With only 10 seropositive results in our study, it is not sufficiently powered to determine the sensitivity of the serology tests.

In participants deemed positive without full consensus across laboratory tests (participants 2,5,6,9 **Table 3**), the results of negative tests in this group are below COIs for positivity (RE ≥ 1.0, AAI/AAr ≥ 1.40) but have values higher than truly negative individuals with no positive results. False positive results, cross-reactivity, and waning antibody levels may be explanations for this lack of consensus. A Cochrane review of 38 studies of antibody tests found false positive results in just 2% of cases, some variability was found depending on prevalence of COVID-19 within populations (34). Regarding waning antibody levels, a study examining levels of 34 mildly symptomatic individuals found an exponential decay of antibodies levels greater than that seen in SARS-CoV-1, with a half-life of 73 days over the study period (35). The first case of community acquired COVID-19 reported in the Republic of Ireland was on February 29th 2020 (36), by the date of enrolment for this study on 24th of June, antibody levels hypothetically could have fallen below the threshold of positivity for commercially available tests for participants infected early during the first wave. If this is the case, there may be under-reporting of truly positive participants in this study or any seroprevalence study.

There may also be some variability in the mounting of antibody responses between symptomatic and asymptomatic

TABLE 3 | Table of individuals with a positive test in at least one laboratory-based assay.

Participant	Colloidal gold IgG (RDT)*	Roche Elecsys® ECLIA IgG COI$^{\alpha}$ ≥ 1.0	ABBOTT Architect i2000SR IgG COI ≥ 1.40	ABBOTT Alinity IgG COI ≥ 1.40	Colloidal GOLD IgM	ABBOTT IgM CMIA COI ≥ 1.40	Participant	Sex	Previous N-P swab (±)	Previous symptom severity
1	**Strong positive**	**120.8**	**4.52**	**5.68**	Negative	**2.69**	Staff nurse	F	No	asymptomatic
2	**Weak positive**	**3.58**	0.63	0.62	Negative	0.07	Doctor	M	Yes (–)	moderate: cough, sore throat, anosmia
3	**Strong positive**	**93.94**	**4.31**	**5.61**	Negative	**3.0**	Staff nurse	F	Yes (–)	moderate
4	**Strong positive**	**86.24**	**4.35**	**5.08**	Negative	**6.32**	Staff nurse	F	No	asymptomatic
5	**Strong positive**	0.064	**1.56**	**1.60**	Negative	0.04	Staff nurse	F	No	asymptomatic
6	**Weak positive**	**8.62**	0.16	0.14	Negative	0.03	Staff nurse	M	Yes (–)	moderate: general malaise 3 weeks
7	**Strong positive**	**43.01**	**2.26**	**2.30**	Negative	0.02	Staff nurse	F	Yes (–)	mild
8	**Strong positive**	**110.6**	**5.00**	**6.04**	Negative	0.21	Dialysis patient	M	No	asymptomatic
9	Negative	**1.6**	0.26	0.24	Negative	0.03	H/O in-patient	F	No	asymptomatic
10	**Weak Positive**	0.21	0.69	0.58	Negative	**1.46**	H/O out-patient	F	No	asymptomatic

**The results of rapid antibody testing are included here but were not a contributing factor in deeming a participant positive. All results highlighted in bold were a positive result in that particular test.*

$^{\alpha}$*Cut-off Index.*

TABLE 4 | Performance of the rapid test vs. laboratory tests.

	N = 157	True positive	False positive	True negative	False negative	Specificity
Superbio Colloidal GOLD IgM	(vs. Abbott IgM)	0	7	150	4	95.4%
Superbio Colloidal GOLD	(vs. RE)	7	19	138	1	87.2%
IgG	(vs. AAI/AAr)	6	20	137	0	85.5%

patients. In the case of The Diamond Princess cruise ship, of 215 individuals that were asymptomatic and initially N-P PCR negative, nine individuals subsequently swabbed positive and all nine went on to develop antibodies by day 8 (37). Another study did not find any association between IgG plateau levels and clinical severity of the disease (38). Conversely, it has been shown mild disease may be associated with reduced antibody response compared to severe disease (39). One study comparing six symptomatic with eight asymptomatic/mild infections found all six symptomatic individuals mounted IgG response, four of whom also mounted IgM. No asymptomatic/mildly symptomatic patients developed IgM and five of eight developed IgG antibodies (40). Interestingly it appears that even if antibody levels have fallen below the threshold of positivity there is some neutralization ability at least up to 6 months as was seen in a large study of 12,666 HCWs in the UK using that presence of anti-Spike IgG and/or anti- nucleocapsid IgG where very low levels of re-infection were found (41).

Overall, the cumulative prevalence (seroprevalence of asymptomatic study participants + PCR positive individuals excluded, **Figure 1**) of COVID-19 for this cohort of individuals with high-risk exposure was 14% (24/172). This value is vastly higher than the seroprevalence of the "background population" in Ireland at the time of 1.7% elucidated by the SCOPI study and suggests high risk hospital associated cohorts may be at increased risk of acquiring COVID-19 by a number of fold due to their needs to frequently engage with the hospital environment (11).

Although low seroprevalence levels were seen in both patient groups (H/O 4%, HD 2.7%) the cumulative prevalence of COVID-19 in these patient groups was calculated to be 6 and 25%, respectively. This does appear to be quite a difference for patients attending the same hospital. The COVID-19 management strategies were similar for both directorates. The single biggest difference in care was the transposition of the H/O day-ward to a repurposed nurse training center adjacent to the hospital; a step not feasible for the HD unit. Another likely important factor in the higher prevalence of COVID-19 in the HD group is the much higher frequency of visits HD patients require, i.e., three times per week. Also 92% (45/49) of H/O patients were receiving some form of chemotherapy, it is unclear to what extent immunosuppressants impact antibody levels in COVID-19 infection.

This study has a number of weaknesses. With a sample size of 157 from a single center, the study is not significantly powered to determine sensitivity of the serology tests used. Also, only assays targeting the nucleocapsid were used, there may have been

participants who had acquired COVID-19 and had antibodies to the Spike protein alone that were not identified in this study. The patient cohorts in this study were immunocompromised and may not have mounted detectable antibody responses with the assays used and would therefore be false negative results. Testing was also done at a single time-point, taking serial serological samples through time would give a better understanding of how antibody levels change over time and a better understanding of the transmission dynamics of SARS-CoV-2 infection in hospitals. Enrolling participants from a uniform population i.e., sampling of asymptomatic staff members only, or high-risk H/O or HD patients across multiple sites would have provided more uniformity and robustness to the study. The results of this study are specific to the high-risk cohorts sampled (H/O patients/staff and HD patients) are not generalisable to all HCWs. We also highlight that although participants were asymptomatic at the time of the study the prior exposure to COVID-19 is unknown. We assume that patients and staff with typical symptoms would have been identified through screening pathways already in place within the H/O and HD directorates and would have had PCR testing. Those individuals identified as having a positive test would then be excluded as part of the exclusion criteria of this study.

CONCLUSION

In conclusion, asymptomatic seroprevalence in high-risk hospital associated cohorts where previous COVID-19 exposure was unknown was 6.4% ($n = 10$ participants). Highest seroprevalence was in HCWs (9.9%). There was strong agreement in laboratory IgG antibody testing for SARS-CoV-2 with 97.5% sero-concordance between RE and AAl/AAr. The RDT had a high number of false positives, $n = 18$ (11.5%), and their clinical use cannot be supported by this particular study.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Mater Misericordiae University Hospital local research ethics committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BO'K, RMc, RO'D, AC, TM, GA, WC, PO'G, PD, and JL: study design. BO'K, RMc, RO'D, HC, RMu, RD, LC, PO'G, RI, and PD: execution of study. BO'K, SH, and JL: statistical analysis. BO'K, RMc, RO'D, AC, TM, EM, WC, DS, MH, PO'G, PD, RI, SH, YO'M, SE, and JL: writing the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.642318/full#supplementary-material

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REVIEW
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Cyclosporin A: A Repurposable Drug in the Treatment of COVID-19?

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Coronavirus disease 2019 (COVID-19) is now at the forefront of major health challenge faced globally, creating an urgent need for safe and efficient therapeutic strategies. Given the high attrition rates, high costs, and quite slow development of drug discovery, repurposing of known FDA-approved molecules is increasingly becoming an attractive issue in order to quickly find molecules capable of preventing and/or curing COVID-19 patients. Cyclosporin A (CsA), a common anti-rejection drug widely used in transplantation, has recently been shown to exhibit substantial anti-SARS-CoV-2 antiviral activity and anti-COVID-19 effect. Here, we review the molecular mechanisms of action of CsA in order to highlight why this molecule seems to be an interesting candidate for the therapeutic management of COVID-19 patients. We conclude that CsA could have at least three major targets in COVID-19 patients: (i) an anti-inflammatory effect reducing the production of proinflammatory cytokines, (ii) an antiviral effect preventing the formation of the viral RNA synthesis complex, and (iii) an effect on tissue damage and thrombosis by acting against the deleterious action of angiotensin II. Several preliminary CsA clinical trials performed on COVID-19 patients report lower incidence of death and suggest that this strategy should be investigated further in order to assess in which context the benefit/risk ratio of repurposing CsA as first-line therapy in COVID-19 is the most favorable.

Keywords: SARS-CoV-2, COVID-19, cyclosporin A, cyclophilin, angiotensin converting enzyme-2

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INTRODUCTION

The first outbreak of coronavirus disease 2019 (COVID-19) was reported by China at the end of 2019 (1–3). Evidence was rapidly reported that patients were infected by a novel betacoronavirus lineage 2b/sarbecovirus tentatively named 2019 novel coronavirus (2019-nCoV) before being known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) with respect to its phylogenetic relationship (80% nucleotide identity) with the SARS-CoV (4). To date, it is the seventh characterized coronavirus described as capable of causing a respiratory infection in human. From the start of 2020, COVID-19 has become a global pandemic and has been declared a global health emergency by the World Health Organization (WHO). In 1 year, more than 75 million people were infected worldwide and this virus has caused more than 1.6 million deaths (https://coronavirus.jhu.edu/map.html, December 18, 2020). Depending on the health status, age, and

comorbidities (hypertension, coronary heart diseases, cerebrovascular diseases, diabetes, chronic kidney diseases) of the infected individuals, SARS-CoV-2 may either be asymptomatic, give a picture of influenza infection, or induce severe forms of COVID-19 with acute respiratory distress syndrome and multiple organ failure syndrome which can lead to death in about 2.27% of infected individuals (2, 5, 6).

The SARS-CoV-2 is an enveloped RNA^+ virus surrounded by spike (S) glycoproteins. The genomic length of SARS-CoV-2 is about 30 kb and encodes as many as 14 open reading frames (ORFs) leading to the synthesis of 29 proteins (7, 8). Coronaviruses have the largest viral RNA genomes known to date (e.g., human immunodeficiency virus genome is only 10 kb), and it was hypothesized that their expansion and selection was likely enabled by acquiring enzyme functions that counter the high error frequency of RNA polymerases (9). During the early infection process, the trimeric SARS-CoV-2 S1 spike first binds to the N-terminal portion of the angiotensin I-converting enzyme 2 (ACE2) which acts as viral receptor at the surface of susceptible cells (10). In addition to ACE2, molecules such as neuropilin-1 (11), chaperone GRP78 (12), and CD209/DC-SIGN (13) can act as SARS-CoV-2 receptors or co-receptors. Furthermore, the cellular transmembrane protease serine 2 (TMPRSS2) contributes to enhance the S-protein-driven viral entry (14). After cleavage at the S1/S2 junction, the S2 takes the conformation required for insertion of the fusion peptide into the cellular lipid bilayers. The viral nucleocapsid is thus delivered into the cytoplasm through the endocytic vesicle. After acidification of the late endosome, the action of cathepsin enables the uncoating of the genomic RNA. SARS-CoV-2 like other pathogenic CoVs possesses a linear plus-sense strand RNA genome (gRNA) that has a $5'$ methylated cap and $3'$ poly-A tail, allowing its anchorage to ribosomes for the synthesis of polyprotein precursor. The two-thirds of this gRNA (about 20 Kb) is occupied by the ORF1a (expressed by genome translation) and ORF1ab (expressed by genome translation and ribosomal frameshift) and encodes the polyproteins precursors pp1a and pp1ab, respectively, giving rise to the production of 16 non-structural proteins (Nsps) by auto-proteolytic processing (15–17). The $3'$-proximal third sequence of the gRNA serves as template for several subgenomic mRNAs having common $3'$ UTRs (18) that encode the viral structural (the spike/S, the envelope/E, the membrane/M, and the nucleocapsid/N) and accessory proteins. The S, E, and M proteins are synthesized and anchored on the endoplasmic reticulum (ER) with the N protein translated in the cytosol. Post-translational modifications of viral proteins occur within the endoplasmic reticulum and trans-Golgi network vesicles. After assembly in the ER–Golgi intermediate compartment (ERGIC), the E protein plays an essential role in virus assembly and the mature M protein shapes the virus. Mature virions are released from smooth-walled vesicles by exocytosis. The accumulation of knowledge relating to the intracellular cycle of replication of the virus as well as the nature of the interactions between the viral and cellular proteins is essential to choose in the large panel of FDA-approved therapeutic compound the molecules capable of blocking the deleterious effects of this virus in infected individuals or to design new antiviral drugs.

Because of the urgent need for safe and efficient therapeutic drugs able to lower morbidity and mortality of COVID-19, multiple clinical trials have been conducted including repurposing of antiviral drugs, anti-inflammatory molecules, and also all kinds of low-cost old drugs known for their *in vitro* antiviral properties. Several independent studies reported in the literature had revealed the *in vitro* antiviral properties of cyclosporin A (CsA), a well-characterized immunosuppressant largely used in the prevention of graft rejection. *In vitro*, this drug was shown to be active against different viruses and to inhibit the replication of coronaviruses, including that of HCoV-229E and SARS-CoV-1 (19, 20). Unsurprisingly, when tested *in vitro* on SARS-CoV-2, CsA was also found to inhibit the replication of this new virus (21). Moreover, the CsA analog alisporivir (called Debio-025) was also shown to block SARS-CoV-2 replication *in vitro* (22, 23). The question of CsA or CsA analog use in the treatment of COVID-19 is now more pressing (**Table 1**).

DISCOVERY OF CYCLOSPORIN A, A CYCLOPHILIN INHIBITOR, AND FK506, AN FKBP INHIBITOR

The cyclosporin story started in the 1969–1970 at the Sandoz Laboratories in Basel (Switzerland). The 11-amino-acid lipophilic cyclic peptide cyclosporin (CsA, also known as ciclosporin) of 1.2 kDa molecular weight, produced from the fungus *Tolypocladium inflatum* and other microorganisms such as *Fusarium solani*, *Neocosmospora varinfecta*, and *Aspergillus terreus* (39), was found to exhibit immunosuppressive properties offering new hope to transplant surgeons to avoid transplant rejection of the patients. The CsA cyclic peptide is insoluble in water and soluble in ethanol or in olive or sesame oil at 60°C and next can be kept in a solution at room temperature. The olive oil-soluble form of the peptide supplemented with 12.5% ethanol was the first form of manufactured CsA for oral administration, which must be dispersed in juice or milk for ingestion (40). CsA was introduced in clinical practice in 1978 (41). The bioavailability of the original corn oil-based preparation of cylosporine (Sandimmune®, Novartis Pharma, Basel, Switzerland) largely varied in cyclosporine blood levels among patients leading to the development of microemulsion formulation (Neoral®, Novartis Pharma) (42, 43). Usually, a dose of 20 mg CsA/kg daily is recommended after solid organ transplant with progressive decrease every week down to 5 mg/kg daily, while a dose of 1 mg/kg daily is recommended after hematopoietic stem cell transplantation (44). Upon administration, CsA is absorbed at the intestinal level by the epithelial cells and the efficiency of this process is influenced by different factors such as dietary composition or bile flow. In the plasma, CsA is found bound to lipoproteins and spreads in the extravascular space (45). CsA is metabolized by liver cells through the P450 3A4 (CYP3A4) leading to the generation of a number of metabolites (46). After a single dose of CsA, there is

TABLE 1 | *In vitro* activity of cyclosporine A against viruses.

Virus	Cyclophilin inhibitor	Read out	Dose of action	Effect	References
SARS-CoV-2	Cysclosporin A	Vero E6 cells model of SARS-CoV-2 infection	IC_{50}: 3 μM	Reduce viral production	(21)
SARS-CoV-2	Debio-025	Vero E6 cells	0.46 ± 0.04 μM	Reduced SARS-CoV-2 RNA production in a dose-dependent manner	(22)
SARS-CoV-2	Debio-025	Vero E6 cells	4.3 μM	Reduced SARS-CoV-2 progeny virions production	(23)
SARS-CoV-1	Cysclosporin A	Vero E6 cells and 293/ACE2 cells.	16 μM	Reduced viral replication and reporter gene expression of SARS-CoV–GFP; inhibition of SARS-CoV RNA synthesis; the protein synthesis was almost undetectable	(19)
SARS-CoV-1	Debio-025	Vero E6 cells	4.3 μM	Reduced SARS-CoV progeny virions production	(23)
SARS-CoV-1	FK506	VeroFM cells	EC_{50}: 6.9 μM	Decreased viral infection and inhibition of SARS-CoV-1 replication	(24)
HCoV- 229	Cysclosporin A	Huh7 cells	32 μ	Reduced reporter gene expression and the production of infectious progeny were also significantly decreased	(19)
HCoV-229E	FK506	HuH7 cells	EC_{50}: 5.4 μM	Decreased viral infection and inhibition of HCoV-229E replication	(24)
HCoV-NL63	FK506	CaCo2 cells	EC_{50} of about 13.4 M	Decrease viral infection and inhibition of HcoV-NL63replication	(24)
Human immunodeficiency virus type 1 (HIV-1)	Cysclosporin A	Human $CD4^+$ T cells Jurkat target cells	2.5 μM 2.5 μM	Reduced viral infectivity	(25)
HIV-1	Cysclosporin A	Jurkat T cells	10 μM	Decreases $gp120^{env}$ and $gp41^{env}$ incorporation into HIV-1 virions and impaired fusion of these virions with susceptible target cells	(26)
HIV-1 ($HIV\text{-}1_{NL4-3}$)	Cysclosporin A	HIV Rev-dependent indicator cell line and Peripheral blood mononuclear cells (PBMCs)	All dosage s from 100 to 600 nM	Inhibits HIV-1 replication (including subtherapeutic concentrations)	(27)
HIV-1	SDZ NIM 811	MT4 cell line (human T-cell leukemia virus-transformed T4 cell line)	IC_{50}: 0.084 g/ml	Inhibits HIV-1 replication	(28)
HIV-1	STG-175	Peripheral blood mononuclear cells (PBMCs)	0.5 and 5 μM	Inhibits HIV-1 replication	(29)
HIV-1 ($HIV\text{-}1_{LAI}$)	FK506-modified HIV-protease inhibitor	T cells	IC_{50} of 4.2 nM	The FK506-modified HIV-protease inhibitor retains anti-HIV-1 protease Activity *in vitro* and is partitioned into the cellular component of whole blood via binding to FKBP	(30)
HIV-1	Cyclophilin Inhibitor CPI-431-32	Blood-derived $CD4^+$ T-lymphocytes	2 μM	Inhibits HIV-1 replication	(31)
Hepatitis B virus (HBV)	Cysclosporin A	HepaRG; HepAD38; primary human hepatocytes primary human hepato-cytes	4 μM	Inhibits HBV entry into cultured hepatocytes decreased HBs and HBe secreted from the infected cells in a dose-dependent manner decreased HBs and HBe secreted from the infected cells in a dose-dependent manner CsA decreased HBs and HBe secreted from the infected cells in a dose-dependent manner (Inhibits the transporter activity of sodium taurocholate cotransporting polypeptide, NTCP)	(32)

(Continued)

TABLE 1 | Continued

Virus	Cyclophilin inhibitor	Read out	Dose of action	Effect	References
HBV	STG-175	Human hepatoma Huh7.5.1 cells	0.5 and 5 μM	Decreased HBV replication	(29)
Hepatitis C virus (HCV)	Cysclosporin A	Huh 5-2 cells	EC_{50}: 2.8 ± 0.4 μg/mL	Inhibition of HCV subgenomic replicons	(33)
HCV	Debio-025 in combination with other antiviral drugs	Hepatoma cells	0.1 or 0.5 μM	Antiviral activity in short-term antiviral assays	(23)
HCV	NIM811	Huh7 cells	1–3 μg/ml	Reduction of HCV RNA levels	(34)
HCV	NIM811	Huh 21-5 cells	IC_{50}: 0.66 μM	Reduction of HCV RNA levels	(35)
HCV	SCY-635	MDCKII-hMDR1 cells	IC 50: 0.20 μM	Inhibition of HCV replication	(36)
HCV	STG-175	Human hepatoma Huh7.5.1 cells	0.5 and 5 μM	HCV cell clearance	(29)
HCV	Cyclophilin inhibitor CPI-431-32	Human hepatoma Huh7.5.1 cells	2 nM	Inhibition of HCV replication	(31)
Mouse hepatitis virus (MHV)–GFP	Cysclosporin A	17CL1 cells	16 μM	Reduction of reporter gene expression and progeny virions	(19)
Vesicular stomatitis virus (VSV)	Cysclosporin A	BHK cells	25 mM	Inhibition of VSV-NJ infectivity	(37)
Flaviviruses (including West Nile virus, dengue virus, yellow fever virus)	Cysclosporin A	Huh-7.5 cells	8-20 μM	Reduced viral RNA synthesis and flavivirus production	(38)

a peak of drug blood concentrations (Cmax) during the first 2 h followed by elimination (C0), and the drug bioavailability should be carefully monitored in clinical settings using the Cmax and a measure of drug concentration every 2 h (C0, C2, C4, C6, C8) to determine when an additional dose should be administered (47).

The mechanism of action of CsA was elucidated in 1984 with isolation from thymocytes of cyclophilin (CyP), an 18-kDa highly basic charged cytosolic protein that binds CsA with high affinity (48). Next, a structurally different immunosuppressant, a macrolide named FK506 isolated from *Streptomyces tsukubaensis*, emerged and was found to interfere with T-cell activation through a similar mode of action than CsA leading to suppression of mixed lymphocyte reaction (MLR), interleukin (IL)-2 and IL-2 receptor, IL-3, and γ-interferon (49). Like CsA, FK506 binds to a member of peptidylproline cis-trans isomerase (PPIase) family, but instead of binding cyclophilin (also called rotamase), it binds the FK506-binding protein (FKBP) (50). Similarly, rapamycin, another immunosuppressant synthesized by *Streptomyces hygroscopicus* (a macrolid originally described in 1975 as an antifungal agent), also binds FKBP and more likely the FKBP12 and FKBP52 isoforms (51, 52). The immunosuppressive effects of FK506 as well as of rapamycin are considered independent of the chaperone function of FKBP. When complexed with ligands, FKBP adopts a conformation allowing its binding to calcineurin and the mammalian target of rapamycin (mTOR). FKBP can also bind the inositol 1,4,5-triphosphate receptor (IP3R) Ca^{2+} channel, which is activated through phosphorylation by the protein kinase A (PKA), while its inactivation is induced through dephosphorylation by calcineurin (53, 54). FKBP also binds to the ryanodine receptor (RyR) channel and the type 1 transforming growth factor beta (TGFβ) receptor (55). Both CsA, FK506 (also known as fujimycin or tacrolimus) and rapamycin (or sirolimus) inhibit the phosphatase activity of calcineurin, thereby preventing the dephosphorylation of the nuclear factor of activated T cells (NF-AT). NF-AT is usually induced after Ca^{2+} binds to calmodulin, leading to the binding of calmodulin to calcineurin, a calcium–calmodulin-activated serine/threonine-specific phosphatase, which in turn is activated (52). In a model of liver fibrosis in rats, rapamycin was reported to inhibit mTOR, to demonstrate potent antifibrotic activity, and to improve portal pressure (56).

FUNCTION OF CYCLOPHILINS

The main function of peptidylproline cis-trans isomerase, PPIases, is that of chaperone proteins involved in folding, assembly, and trafficking of other proteins (57, 58). The human genome encodes 17 cyclophilins: the peptidyl-prolyl isomerase A (PPIA or CyPA also called Cyp-18a a cytosolic protein of molecular mass 18 kDa) encoded by a gene located on chromosome 7, PPIB (CypB also called Cyp-22/p, an endoplasmic reticulum and Golgi protein of molecular mass 22 kDa) encoded by a gene on chromosome 15, PPIC (CypC an endoplasmic reticulum and Golgi protein of molecular mass 33 kDa), PPID (CypD a mitochondrial protein of molecular mass 20 kDa; the cytosolic CyPD and CyPF are named CyP40), PPIE (CypE, a component of the spliceosomal apparatus), PPIF (CypF is a component of the mitochondrial permeability transition pore involved in apoptosis regulation), PPIG (CypG or SR–cyclophilin or matrix–cyclophilin a nuclear matrix protein which interacts with RNA polymerase II is

a component of the spliceosomal apparatus), PPIH (CypH), NKTR (Cypp), PPIL1 (encoded by the X-chromosome), PPIL2, PPIL3, PPIL4, PPIL6, PPWD1, RANBP2, and SDCCAG-10, respectively (59, 60). The CyPA exhibits multiple functions including folding of the procollagen I and transferrin, nuclear translocation of ERK1/2 kinases, transport of molecules to the plasma membrane through interaction with the Ig-like CD147 receptor, cholesterol transport, nuclear export of zinc-finger protein-1, and stimulation of apoptosis (61, 62). Although CyPA is mainly a cytosolic protein, there is also a secreted form of this molecule which is produced in response to different inflammatory stimuli, particularly infection (63). The secretion of CyPA is mediated *via* a vesicular transport pathway that depends on the Rho-kinase activation (64). The secreted form of CyPA acts as a chemoattractant for monocytes and leukocytes (63, 65, 66). To date, although several functions of most cyclophilin isoforms remain unknown, the different isoforms of cyclophilins exhibit domain-specific properties apart from their function as chaperones. For example, PPIA was found to bind the non-receptor tyrosine kinase Itk, playing a role in the maturation of thymocytes; PPIH and PPIL1, respectively, interact with the hPRP4 and SKIP proteins in the spliceosome, and PPIE shows a RNA-specific isomerase activity. Besides encoding 17 cyclophilins, the human genome encodes 18 FKBPs and three parvulins, the smallest PPIases (67).

It was reported that CsA can bind PPIA, PPIB, PPIC, PPID, PPIE, PPIF, PPIG, PPIH, PPIL1, NKTR, and PPWD1, while PPIL2, PPIL6, RANBP2, and SDCCAG-10 are incompetent to ligate CsA (60). Special attention was given to the CsA/CypA interaction and a quantitative transcriptomics analysis (RNA-Seq) was performed to determine the tissue-specific expression of the CypA gene. This study indicated that CypA is ubiquitously expressed (68) (**Figure 1**).

CsA REPURPOSING IN AIDS THERAPY: A PRECEDENT IN THE TREATMENT OF A VIRAL DISEASE WITH CsA

Based on the hypothesis according to which the multiplication of the human immunodeficiency virus type 1 (HIV-1) in the organism is all the more important as the CD4 cells are activated, 25 years ago, CsA was considered as a possible drug to treat AIDS. During a press conference, the results of a preliminary CsA clinical trial carried out on AIDS patients by a team of medical doctors from the Laënnec Hospital (Paris, France) in October 1985 were reported (69). Unfortunately, after the death of two HIV patients under CsA therapy, a campaign fueled by media tended to discredit this work (70, 71). Among the criticisms that had been expressed, it was emphasized that using an immunosuppressant to treat a disease characterized by an immunosuppression (e.g., HIV-1-induced progressive depleted of $CD4^+$ lymphocytes being at the origin of AIDS) was surprising. Despite the media attacks, the pilot phase was continued by the team of Andrieu who reported on the CsA treatment of eight patients who were given 7.5 mg CsA/kg daily and concluded, based on their observation, that clinical trials with CsA would be worth pursuing (72). However, adverse effects of this experimental treatment were reported by another team, which published the results of a CsA pilot study on nine patients with AIDS (six presented with *Pneumocystis carinii* pneumonia and three had Kaposi's sarcoma) who experienced severe toxic symptoms: one developed massive intravascular hemolysis and was withdrawn from the study after 13 days of treatment, the other also experienced severe symptoms which necessitated discontinuation of CsA therapy in six of them, and the condition of all patients improved after therapy was stopped (73). Although the results from these last clinical studies were disappointing, another study that enrolled 53 patients with renal transplantation, the HIV infection of whom was caused by an infected transplant or by blood transfusion, indicated that after 5 years the cumulative incidence of AIDS was lower in 40 patients who received CsA than in 13 transplant patients receiving immunosuppressive treatment without CsA (74). Several other reports highlighted a possible positive impact of CsA treatment on the progression of AIDS (75–79). Coming back to the animal model to explore pathophysiology without putting patients at risk, it was shown by the team of Fauci that administration of CsA to monkeys inoculated with the simian immunodeficiency virus (SIV) was beneficial relatively to the kinetics of CD4 cell depletion (80). This result revived the scientific debate on the use of CsA in the treatment of AIDS, but rather than using it as monotherapy on patients with declared AIDS (low $CD4^+$ cell count), the choice fell on the use of CsA in combination with highly active antiretroviral therapy (HAART) during primary infection. This new therapeutic strategy was based on the hypothesis that rapid shutdown of T-cell activation in the early phase of primary infection could have long-term beneficial effect on the outcome of the disease. The team of Pantaleo reported that during a 64-week follow-up, patients receiving CsA in combination with HAART consistently maintained significantly higher levels of $CD4^+$ T cells than those taking HAART alone (81). This promising result relaunched the investigation on the use of CsA in AIDS (27, 82–86) (**Table 2**). In 2014, De Iaco and Luban reported that CypA binds HIV-1 capsid (CA) and influences early steps in the HIV-1 replication cycle and that disruption of CypA binding to CA by CsA reduces the efficiency of HIV-1 transduction in some cells but not in others (90). More recently, Nicolas and colleagues reported the results of a clinical investigation, which concluded that unintegrated DNA forms of viral genome increased in the CsA-treated group compared with controls, suggesting an anti-integration effect of the drug (89) (**Figure 2**). This is consistent with earlier data demonstrating that cell activation is dispensable for viral entry but is required for the HIV-1 provirus integration (91–93). It will therefore have taken more than 30 years of research to begin to understand in which specific therapeutic conditions CsA can be beneficial in the treatment of AIDS. Finally, it was recently reported that CsA decreases HIV-1 infectivity by blocking CypA interaction with HIV-1 CA protein and incorporation of HIV-1 envelope glycoproteins (gp120 and gp41) into virions thereby impairing fusion with target cells (26). Altogether, these results suggest that treatment with CsA can be beneficial in the prevention of AIDS but that the window of action of this treatment is narrow,

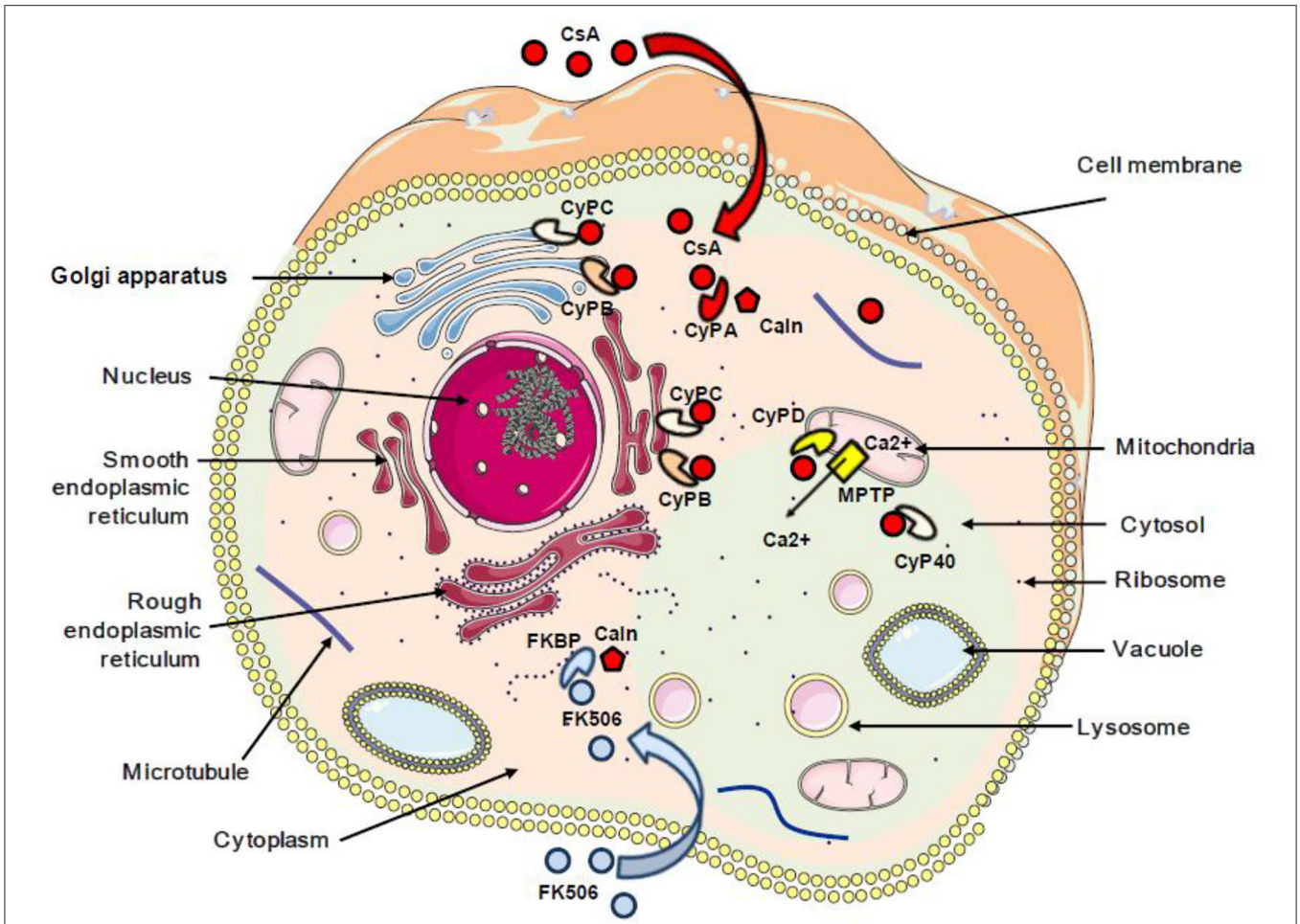

FIGURE 1 | Schematic representation of the subcellular localization of cyclophilins and FKBP proteins. The red arrow indicates the interaction between cyclosporin A and cyclophilins. The blue arrow indicates the interaction between FK506 and FKBP. CsA, cyclosporin A; CyPA, CyPB, CyPC, CyPD, and CyP40, cyclophilins A, B, C, D, and 40; FKBP, FK506-binding protein; Caln, calcineurin; MPTP, mitochondrial permeability transition pore; Ca^{2+}, calcium.

limited to primary infection to prevent the integration of the viral genome, while it is no longer efficient on chronic infection once the provirus is already integrated into the DNA of infected cells.

IS THERE A PERSPECTIVE FOR CsA REPURPOSING IN COVID-19?

Immunocompromised patients including patients with HIV, those receiving immunomodulatory therapy for autoimmune disease, patients with cancer, and solid organ transplant recipients who are immunosuppressed to prevent complication associated to alloimmune responses are generally considered at risk for more severe viral infection because of their poor immune response. In transplant recipients, CsA and tacrolimus calcineurin inhibitors are the most prescribed drugs for the prevention of alloimmune responses (41, 94). Therefore, the question of using CsA in COVID-19 recently comes into debate since it remains unclear if immunosuppression in transplanted patients alters the predisposition to acquiring COVID-19 and/or modifies the disease outcome for better or worse (95). Today, solid organ transplant recipients are listed as high-risk individuals for the development of severe forms of COVID-19 (96), and there is a specific follow-up of transplanted patients to evaluate their outcome when they become infected with SARS-CoV-2. It is generally admitted that immunosuppressive therapy in transplanted patients modulates humoral and cell-mediated immunity increasing the risk of severe infection when exposed to viruses (97). In regard to this idea, some authors suggested pausing immunosuppressant drugs as a precaution in transplanted patients found positive for SARS-CoV-2 (98). Yet, it was also reported that transplanted patients have not been found more susceptible to viral infections and severe forms of COVID-19 than the general population (99–101), which begs questions about the relationship between CsA treatment and COVID-19. An observational clinical study from Spain which followed 29 kidney transplant recipients with COVID-19 reported a mortality of 12.5% in the group of patients under

TABLE 2 | *In vitro* effect of CsA on HIV replication and on disease progression in HIV-infected patients.

Date	Type of study	Results	References
In vitro			
1988	HIV *in vitro* infection and replication H-9 T-cell leukemic line human peripheral blood-derived lymphocytes	Pretreatment of cells and human lymphocytes with CsA over 24 h prevented viral infection over a 21-day period, whereas the addition of drug at 2 h postinfection with HIV-1 had a significant inhibitory effect on viral replication and expression of the virus-specific antigens p17 and $p24^{gag}$	(87)
1992	HIV and CD4 T cells	CsA induced a 100-fold reduction in the yield of HIV infection CsA inhibited the growth of HIV infected cells	(75)
1994	HIV T4 lymphoid cell lines, in a monocytic cell line, and in HeLa T4 cells	SDZ NIM 811 selectively inhibited HIV-1 replication in CD4+ lymphoid cell lines, in a monocytic cell line, and in HeLa T4 cells	(88)
2010	HIV and Human CD4+-T cells	CsA inhibited HIV infectivity	(26)
2013	HIV and T cell line or peripheral blood mononuclear cells	CsA inhibited HIV-1 replication in a GFP indicator T cell line and peripheral blood mononuclear cells	(27)
In patients			
1978	Transplanted patients ($n = 7$)	CsA was effective in inhibiting rejection (adverse effect: nephrotoxicity and hepatotoxicity.)	(41)
1988	AIDS patients ($n = 8$)	CsA (7.5 mg/kg daily) Sustained and increased > 600 CD4+ cells/mm3, decreased CD8+ cell count. Lymphadenopathy disappeared. Reversibility once CsA was stopped	(72)
1989	AIDS patients ($n = 8$)	Severe toxic syndrome requiring discontinuation of CsA Decreased lymphocyte count, CD4+ and CD8+ T- cells, and no resolution of symptoms	(73)
1993	Transplanted kidney patients & HIV-1 ($n = 53$)	5-year cumulative risk of AIDS: 31% in CsA group vs. 90% in non CsA group, $P = 0.001$	(74)
2002	9 early HIV patients treated HAART + CsA	Significantly higher CD4+ T cells in patients treated with CsA	(81)
2004	3 HIV patients treated HAART + CsA	Pharmacological adjustment of CsA in association with HAART	(83)
2010	54 early HIV (ART + CsA vs. ART)	No apparent immunological and virological benefit	(86)
2017	20 early HIV (ART + CsA vs. ART)	Increased non-integrated DNA in the CsA arm between weeks 0 and 36 weeks CsA has unintegrated effect	(89)

CsA therapy ($n = 23$) compared with 50% mortality in the control group with reduced doses in CsA ($n = 6$), supporting the hypothesis that CsA therapy is safe and might be beneficial to transplanted patients with COVID-19 (102). However, this study should be interpreted with caution due to variability of other drugs used in these patients. Observational studies have shown that patients receiving CsA for the prevention of graft vs. host (GVH) disease have a lower risk of developing a COVID-19 infection than patients receiving basic treatment with tacrolimus or corticosteroids (**Table 3**). Interestingly, in a recent study including 40 kidney-transplanted patients, Demir and colleagues identified by using a multivariable analysis that the use of CsA was associated with a lower incidence of death [0.077 (95% CI, 0.018–0.324; $P \leq 0.001$)] (105). The question currently being raised is whether the background immunosuppressive therapy in transplanted patients should be modified, when possible, by CsA to prevent the occurrence of COVID-19 (100).

At least eight FDA-approved clinical trials of CsA and FK506 are currently underway in patients with severe COVID-19 (**Table 4**). The majority of the clinical trials presented in **Table 4** are still ongoing and no results have been disclosed. Preliminary results (not certified by peer review) made available recently indicate that CsA (9 mg/kg/day) in short courses of treatment for COVID-19 patients requiring oxygen (clinical trials NCT04412785; first posted February 6, 2020) is safe and associated with significant reductions of hyperinflammation (108). An open-label, non-randomized pilot clinical study on 209 adult patients confirmed positive for SARS-CoV-2 receiving enoxaparin, methylprednisolone, or prednisone compared the clinical outcome of 105 patients who received CsA (oral CsA at a dose of 1–2 mg/kg daily) plus steroids to that of 104 patients treated with steroids alone; this study concluded that CsA used as adjuvant to steroid treatment improves the outcomes of patients with moderate to severe forms of COVID-19 and reduces mortality (109).

Altogether, these results suggest that CsA could have a beneficial effect in the treatment of COVID-19 patients and that such repurposing strategy should be further investigated while being aware of possible side effects. In addition, these data also raise questions about the mechanisms by which CsA might influence the outcome of COVID-19.

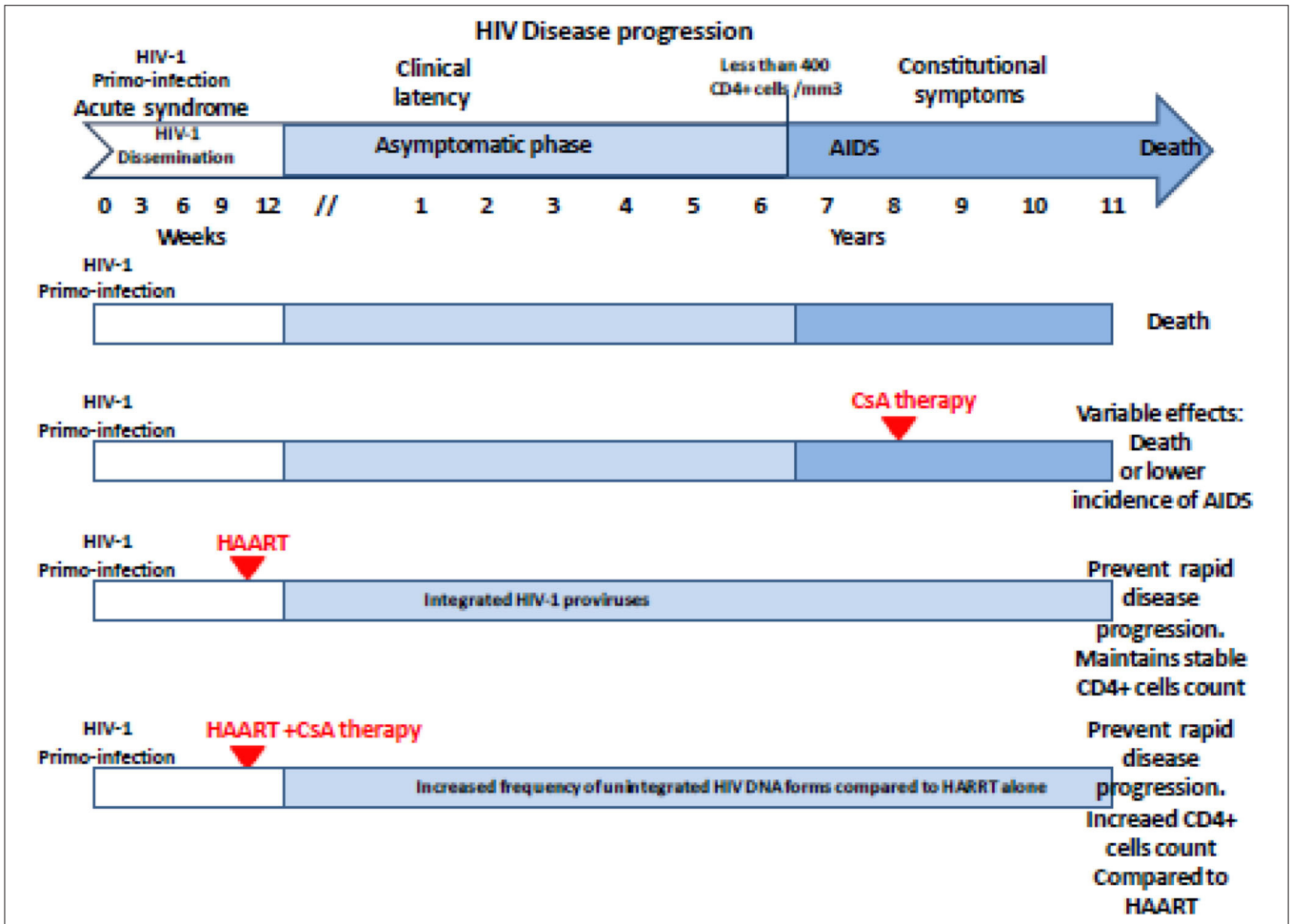

FIGURE 2 | Schematic representation of the antiviral effect of CsA treatment on the HIV-1 disease progression regarding the clinical trials reported in the literature. The effectiveness and beneficial effects of CsA depend on the stage of the disease at which the treatment is given. Unintegrated DNA forms of viral genome increased in the CsA-treated group compared with controls when CsA is given post-primo-infection in association with HAART. AIDS, acquired immunodeficiency syndrome; HAART, highly active antiretroviral therapy; CsA, cyclosporine A.

CsA AND CYCLOPHILIN IN PROINFLAMMATION PROCESSES: IMPLICATION FOR COVID-19

Upon entering the cell, the immunosuppressants CsA and FK506 bind with high affinity to CyPs (also named immunophilins) and inhibit their peptidyl prolyl cis-trans isomerase activities. The CyP–CsA (or FKP–FK506) complex binds to calcineurin and inhibits its phosphatase activity. Many of the suppressive actions of CsA on T cells appear to be due to an inhibition of T-cell receptor (TCR)-induced activation signals with minimal effects on already activated $CD8^+$ cytotoxic T cells (110). Although CSA affects T-cell differentiation and proliferation and cytokine production, these cells still express the interleukin-2 receptor (IL-2R) and proliferate under IL-2 stimulation (111, 112). However, CsA can apparently also trigger a status on T-cell-mediated autoimmunity (113). CsA inhibits the development of both $CD4^+CD8^{neg}$ T-cell and $CD4^{neg}CD8^+$ T-cell lineages (114). CsA inhibits a T-cell receptor-dependent and calcium-dependent signal transduction pathway and blocks T-cell proliferation by inhibition of the IL-2 synthesis, and this is achieved after forming a complex with CyPA. In the absence of CsA, TCR-induced activation signal triggers Ca^{2+} binding to calmodulin that leads calmodulin to form a complex with calcineurin, a calcium/calmodulin-dependent serine threonine phosphatase. The activation of calcineurin triggers dephosphorylation of the cytoplasmic nuclear factor of activated T cells (NF-ATcP). Once dephosphorylated, NF-ATc translocates from the cell cytoplasm into the cell nucleus and activates the transcription of the IL-2 gene (115). Under CsA treatment, the CsA/CyPA complex specifically binds to calcineurin and inhibits its phosphatase function (116, 117). Due to a lack of phosphatase activity, the nuclear factor of activated T cells (NF-AT) remain under its inactive cytoplasmic phosphorylated form (NF-ATcP). *In vivo* studies have highlighted that CsA promotes the expansion of $Foxp3^+$ T regulator cells (Treg) (118). Indeed, the result of CsA

TABLE 3 | Cyclosporin A based treatment in transplanted patients.

No. of transplanted patients	Cysclosporin A	Corticoids	Intensive care unit (ICU)	Death	References
Heart					
6 transplanted patients	6/6 patients received Cysclosporin A (70–200 mg/d)	NA	2/6 patients admitted inICU (2 and 16 days)	2 died: 1 with acute respiratory distress syndrome. 1 with sepsis. Their Cysclosporin A therapy was reduced in both cases (100 and 40%, respectively)	(103)
Kidney transplantation					
2 patients	1 patients	NA	1 patient not treated with Cysclosporin A	1 patient not treated with Cysclosporin A	(104)
40 patients	5 patients (12%)	40 (100%)	SEVERITY Cysclosporin A associated reduction risk of mortality multivariate analysis OR: 0.077 (IC0, 018–0.32) $p < 0.001$		(105)
19/2,493 kidney transplant recipient	9/19 patients (47.4%)	NA	NA	2 patients (22%) died in the cyclosporin A treated group vs. 7 patients alive (70%) $p = 0.03$	(106)
23 patients	6 patients already treated with Cysclosporin A 19 patients switched to Cysclosporin A therapy	NA	NA	Mortality was higher in the immunosuppression minimization strategy group, 3/6 patients (50%), as compared to the Cysclosporin A strategy group 3/23 patients (13%)	(102)
Liver transplantation					
151 reports SARS CoV 2 with liver transplantation	8 patients	67 (44%)	NA	4/28 died patients received Cysclosporin A vs. 4/123 alive patients (non-significative)	(107)

treatment is a change in the balance between T helper cells and Treg cells that favor the Treg population. The CypA is regulated by inflammatory stimuli, and several cell types secrete CypA in response to oxidative stress. Zhang and colleagues also reported that serum CypA concentration correlates with serum interleukin-6 (IL-6), matrix metalloproteinase-9 (MMP-9), and C-reactive protein expression (119). It was recently reported that the secreted CypA can be used as a potential inflammatory biomarker of chronic obstructive pulmonary disease (COPD), as its expression levels are elevated in serum of COPD patients and reflects the severity of inflammation (119).

PATHOLOGICAL SIMILARITIES BETWEEN TRANSPLANTED PATIENTS AND COVID-19 PATIENTS: TISSUES INJURED WITH PICTURE OF CHRONIC VASCULAR REJECTION

Significant parallels are observed between SARS-CoV-2 tissue injury (120, 121) and allograft rejection and especially with chronic vascular rejection (122, 123). In tissues of patients who died from COVID-19, similar lesions to those observed in chronic vascular rejection grade D were observed (122). Vascular rejection is characterized by concentric thickened arteries and/or veins, due to fibrointimal connective tissue. These lesions usually start with intimal proliferation, then fragmented and discontinuous internal elastic lamina (120, 121), as illustrated in **Figure 3**. Concurrent endovasculitis has also been observed (123). In patients suffering from GVH disease, lung histological lesions are characterized by alveolar changes (intra-alveolar fibrin, organizing pneumonia, and chronic interstitial pneumonia), atypical pneumocytes, intra-epithelial bronchiolar T cells, and perivenular cuffing (124–127).

Lung analysis of patients who died from COVID-19 showed an inflammatory perivascular lymphocyte infiltration (120, 121), as illustrated in **Figure 4**, that presents some similarities to those observed in GVH, although non-specific (128). Perivascular inflammation was reported to be patchy and scattered, composed mainly of lymphocytes, with thrombi in the branches of the pulmonary artery and focal areas of congestion in the alveolar septal capillaries, as well as septal capillary lesions with wall and luminal fibrin deposition (128).

In these diseases, critical epithelial stem cell populations are preferentially targeted: in one instance by cytotoxic immune pathways, in the other by a viral protein–receptor interaction. Moreover, in both diseases again, severe injuries are mediated by cytokine deregulation named the "cytokine storm syndrome" which leads to cell apoptosis. Cytokine dysregulation has historically been reported in the early phase of acute GVH

TABLE 4 | FDA approved clinical trial proposing cyclosporine A to treat SARS-CoV-2 infection.

	Clinical trial	Study title	Intervention	Countries
1	NCT04412785	Cysclosporin in Patients With Moderate COVID-19	Phase 1 safety study to determine the tolerability, clinical effects, and changes in laboratory parameters of short course oral or IV Cysclosporin (CSA) administration in patients with COVID-19 disease requiring oxygen supplementation but not requiring ventilator support.	University of Pennsylvania Philadelphia, Pennsylvania, United States
2	NCT04392531	Clinical Trial to Assess Efficacy of cYclosporine Plus Standard of Care in Hospitalized Patients With COVID19	Open, Controlled, Randomized Clinical Trial to Evaluate the Efficacy and Safety of Cysclosporin Plus Standard Treatment vs. Standard Treatment Only in Hospitalized Patients With COVID-19 Infection	Complejo Hospitalario Universitario La Coruña La Coruña, Galicia, Spain Hospital Quiron La Coruña La Coruña, Galicia, Spain Hospital Rey Juan Carlos Mostoles, Madrid, Spain
3	NCT04540926	Cysclosporin A Plus Low-steroid Treatment in COVID-19 Pneumonia	Consecutive patients with suspected or confirmed diagnosis of COVID-19 were assigned, in an unblinded and non-randomized fashion, to receive either steroids plus CsA (intervention group) or steroids only (standard of treatment in this hospital, control group), as per individual clinical judgment	Jose Luis Jl Galvez-Romero Puebla, Mexico
4	NCT04492891	Cysclosporin For The Treatment Of COVID-19(+)	Phase IIa clinical trial in which 75 non-ICU hospital inpatients will be randomized 2:1 to 7 days of Neoral (2.5 mg/kg PO BID) + standard of care (SOC) or no CSA + SOC.	Baylor College of Medicine Houston, Texas, United States
5	NCT04451239	Topical Steroids and Cyclosporin-A for COVID-19 Keratoconjunctivitis	Single Group Assignment All patient will be treated with Topical 1% prednisolone acetate for 7 days as initial treatment + non-preserved artificial tears and Cysclosporin A 0.5% four times daily.	Farawanyia hospital Kuwait, Farawanyia, Kuwait
6	NCT04341038	Clinical Trial to Evaluate Methylprednisolone Pulses and Tacrolimus in Patients With COVID-19 Lung Injury	Open Randomized Single Centre Clinical Trial to Evaluate Methylprednisolone Pulses and Tacrolimus in Patients With Severe Lung Injury Secondary to COVID-19	Hospital Universitari de Bellvitge L'Hospitalet de Llobregat, Barcelona, Spain
7	NCT04420364	Maintenance vs. Reduction of Immunosuppression for Renal Transplant Patients Hospitalized With COVID-19 Disease	Maintenance or reduction of immunosuppression, phase II-III Single-blind, parallel-group, randomized, active-controlled trial	Birgham and Women's Hospital, Boston, Massachusetts
8	NCT04569851	Clinical Characteristics and Prognostic Factors of Patients With COVID-19 (Coronavirus Disease 2019)	Retrospective, observationnal Clinical Characteristics and Prognostic Factors of Patients With COVID-19 Using Big Data and Artificial Intelligence Techniques (BigCoviData)	Hospital Universitario de Guadalajara Guadalajara, Spain Hospital Universitario La Princesa Madrid, Spain

disease described by Ferrara as a "cytokine storm" (129) and subsequently used to describe the exacerbated immune response observed in severe COVID-19 infection (130, 131). Thus, it could explain some of the histological similarities observed, even chronic, since physiological mechanisms involved in these lesions are in part common. Stem cell death by apoptosis is associated with activation of the p53–p73 "suicide pathway" observed in GVH disease, and perivascular lymphocyte infiltrates were identified in case of GVH disease (132–135).

COVID-19 INFECTION IN TRANSPLANTED PATIENTS

Recipients of allogeneic hematopoietic stem cell transplant (HSCT) are generally considered at particular risk of developing severe forms of COVID-19 when infected with SARS-CoV-2 due to the profound immunosuppression related to transplant-associated anti-rejection therapy expected to reduce the immune defense of the host thereby favoring *in vivo* viral replication. It was reported that treatment with the selective JAK1/2 inhibitor ruxolitinib has shown promising results in the context of COVID-19 patients with GVH disease (136). In COVID-19, tissue injury observed in patients with severe forms of the disease appears to be related to a massive increase of inflammatory cytokine level and increase of $CD15^{+}CD16^{+}$ neutrophils known for being involved in proinflammatory processes (137, 138). It is currently admitted that severe forms of COVID-19 are associated with a release of cytokines and chemokines such as IL-2, IL-6, IL-7, IL-10, tumor necrosis factor (TNF), and granulocyte colony-stimulating factor (GCSF) (2, 139).

Among these cytokines, therapeutic approaches targeting excessive inflammation caused by IL-6 interaction with its

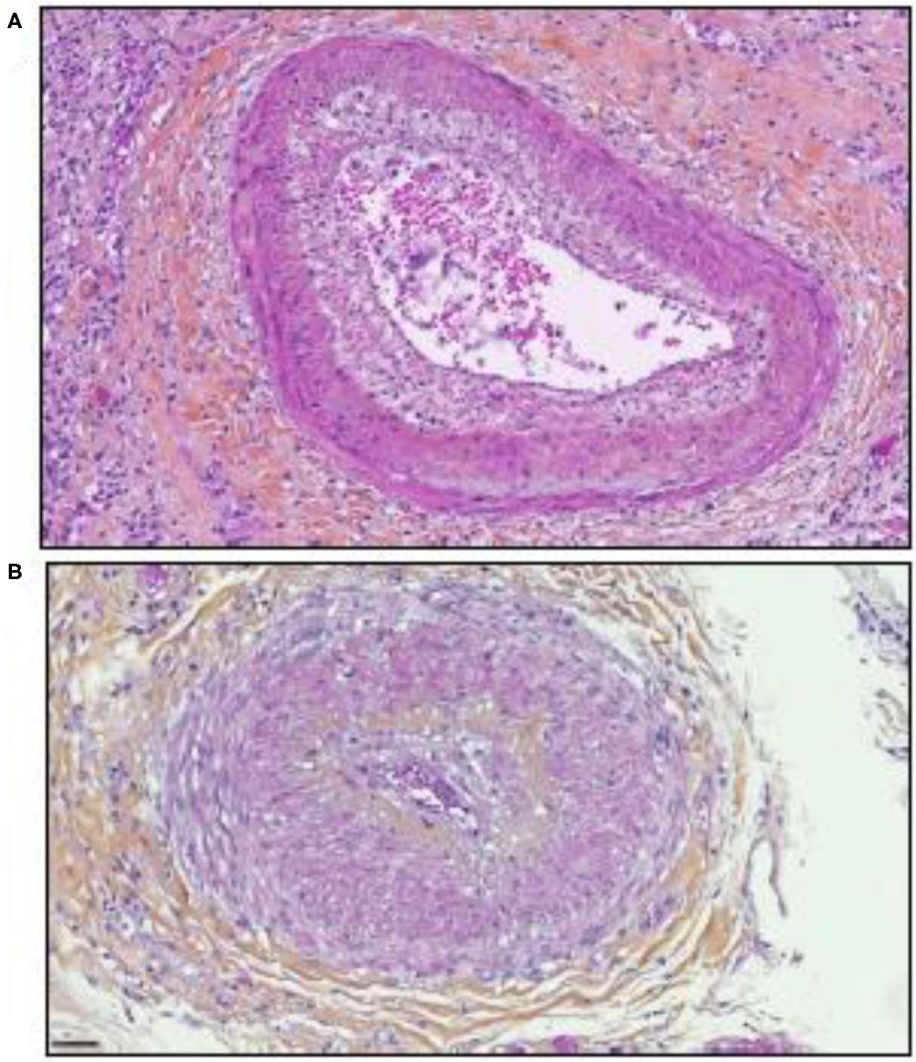

FIGURE 3 | Illustration of the microscopic examination of histological sections of tissues from patients who died of COVID-19 (postmortem formalin lung sample from medical autopsy performed in the forensic medicine department of Marseille Hospital). The histological sections were stained using hematoxylin, eosin, and saffron (hematoxylin stains the cell nuclei blue, eosin stains the extracellular matrix and cytoplasm pink, the saffron stain in orange the conjunctive matrix). **(A)** Vascular rejection is characterized by concentric thickened artery secondary to intimal proliferation and endovasculitis. Original magnification × 150. **(B)** Concentric thickened artery secondary to fibrointimal proliferation. Original magnification × 200 μm.

cellular receptor IL-6R have been under investigation using IL-6 antagonists such as tocilizumab and sarilumab used in the treatment of autoimmunity (140–143). It was recently shown that the total number of $CD4^+$ T cells, $CD8^+$ T cells, B cells, and NK cells in patients was markedly decreased in the most severe forms of COVID-19 and that there is an increase of IL-2, IL-6, IL-10, and IFN-γ (131, 144–146). There is likely space for investigating the possible beneficial effect of immunosuppressant CsA therapy in COVID-19, since this molecule is known to reduce IL-2 production that contributes to the cytokine storm reported in the severe forms of COVID-19 (**Figure 5**). It is also worth noting that the Nsp1 protein found to have multiple functions (e.g., binds to 40S ribosomal subunit and inhibits translation, triggers host mRNA degradation by endonucleolytic cleavage, induces cell cycle arrest, inhibits IFN signaling) was reported in SARS-CoV to enhance IL-2 production when overexpressed and that SARS-CoV infection increases signaling through the calcineurin/NF-AT (147). Such Nsp1 induction of IL-2 production is probably also occurring with SARS-CoV-2.

CsA AND CYCLOPHILIN IN VIRAL INFECTIOUS PROCESSES: IMPLICATION FOR COVID-19

Different isoforms of cyclophilins CyPA and CypB were reported to specifically bind a proline-containing sequence in the polyprotein $Pr55^{gag}$ and the $p24^{gag}$ capsid protein of the HIV-1,

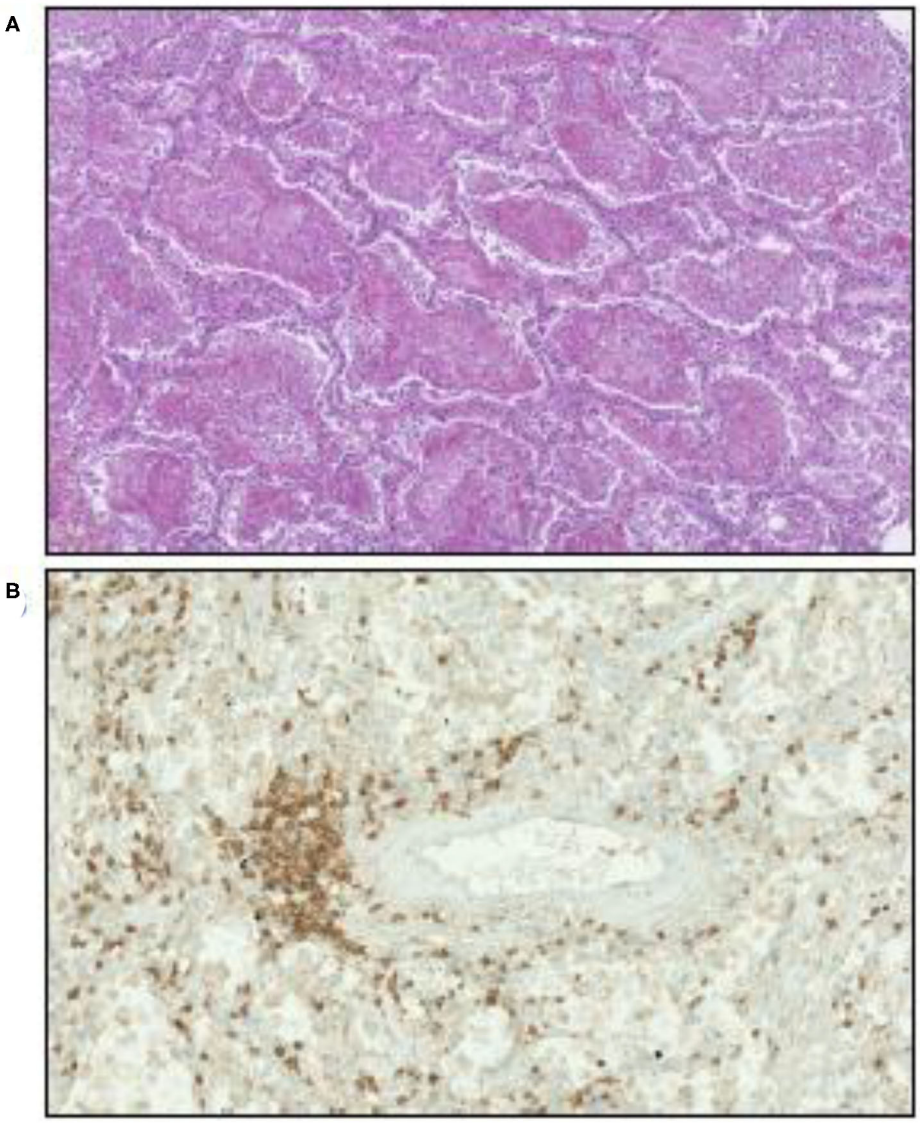

FIGURE 4 | Illustration of microscopic examination of tissues from patients who died of COVID-19 (postmortem formalin lung sample from medical autopsy performed in the forensic medicine department of Marseille Hospital). **(A)** Hematoxylin, eosin, and saffron staining showing intra-alveolar fibrin. Original magnification × 70. **(B)** Inflammatory perivascular lymphocytes T infiltration evidenced by anti-CD3 monoclonal antibody immunostaining. Original magnification × 170.

and CsA disrupts the interaction of these proteins with CyPA and also with CyPB although with less efficiency (148). *In vitro*, CsA was reported to inhibit the replication of HIV-1 (149). The non-immunosuppressant analog of CsA, SDZ NIM 811 (Sandoz), was also found to inhibit HIV-1 *in vitro* (150).

Besides HIV-1, CsA was reported to inhibit the vesicular stomatitis virus (37), the hepatitis C virus (HCV) (151, 152), the human papillomavirus (HPV)-16 (153), the influenza A virus (154), and the Rift Valley fever virus (155). Regarding the HCV, the RNA-dependent RNA polymerase NS5B from the virus binds the human CypA and CypB proteins (156, 157), and CypA was also found to interact with the NS2 protein of HCV (158), while CypB appeared to regulate the HCV polymerase and CyP40 seemed to also be involved in HCV replication (159). First, a 3.5 log reduction of HCV load was demonstrated with the CsA analog DEBIO-025 (160). In light of these results, clinical trials of Cyp inhibitors (DEBIO-025, SCY635, and NIM811) have started against HCV, and a very elegant *in vitro* work evidenced that NIM811 reduces HCV replication by inhibiting CyPs, including CyPA, CypH, and CyPE, and identified many cellular compounds interacting with these CyPs (161).

Similarly, in flaviviruses, it was reported that CsA blocks the West Nile virus, dengue-2 virus, and yellow fever virus replication. CsA was found to inhibit the interaction between CypA and the NS5 protein (and also CyPA and viral RNA) of the West Nile virus (38), while CyPB was found to interact

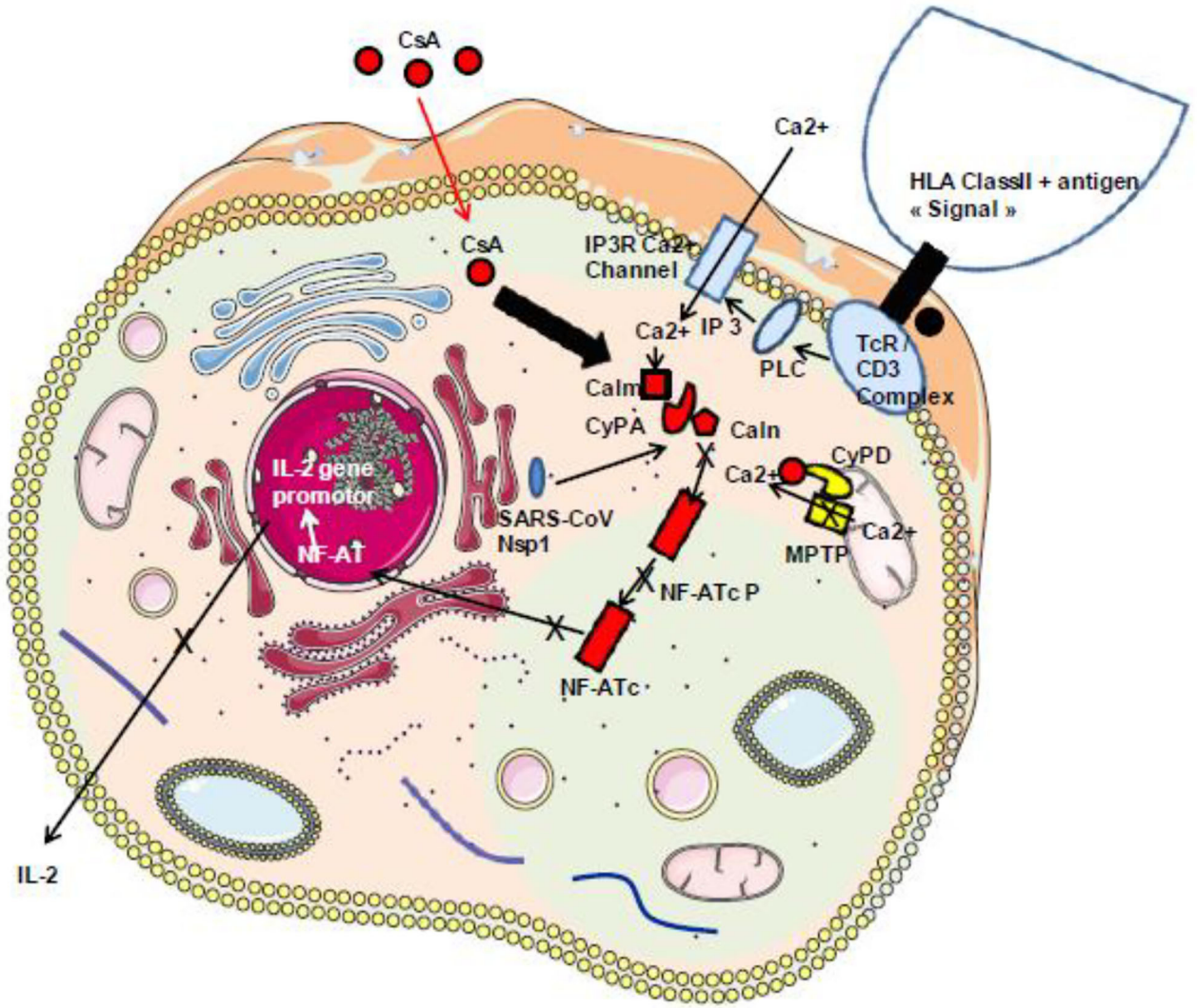

FIGURE 5 | Schematic representation of the classical TcR/CD3-induced activation of IL-2 production. During infection with SARS-CoV-2, the virally induced cell dysregulation leads to the aberrant opening of MPTP inducing mitochondrial release of Ca^{2+} that triggers an abnormal Ca^{2+}/calmodulin activation of calcineurin and dephosphorylation of the cytoplasmic nuclear factor of activated T cells (NF-AT) leading to NF-AT nuclear translocation and the synthesis of IL-2 and other inflammatory cytokines. Under CsA treatment, the CsA/CyPA complex specifically binds to calcineurin and inhibits its phosphatase function. Consequently, the NF-AT remain under its inactive cytoplasmic phosphorylated form. Moreover, by interacting with CyPD, CsA prevents the opening of MPTP and the release of Ca^{2+} that usually lead to cell death. In addition, through binding to CyPA, CsA is expected to upregulate interferon that blocks virus replication. HLA class II, human leukocyte antigen class II; TcR–CD3 complex, T-cell receptor–CD3 complex; PLC, phospholipase C; IP3, inositol 1,4,5-triphosphate; Calm, calmodulin; Caln, calcineurin; NF-ATcP, nuclear factor of activated T-cell cytoplasmic phosphorylated form; NF-ATc, NF-AT cytoplasmic dephosphorylated; PKC, protein kinase C; CsA, cyclosporin A.

with the NS4A protein of the Japanese encephalitis virus (162), suggesting that CyP isoforms are essential to the replication complex of flaviviruses.

Regarding coronaviruses, it was reported that CsA inhibits the human coronaviruses HCoV-NL63, HCoV-229, and SARS-CoV-1, as well as animal coronaviruses such as feline CoV and porcine CoV, suggesting that CyPs are required for the successful replication of most coronaviruses (147). Once inside the cell, the genomic RNA (positive) from each coronavirus is released from the viral particle present in late endosomes. Covered with a cap allowing its anchorage to the ribosome level, this genomic RNA serves as a template for the translation of two large open reading frames (ORF1a and ORF1b). This yields to the synthesis of the polyprotein 1a (pp1a), and following a ribosomal frameshift, it leads to the extended pp1ab polyprotein. After proteolysis, several non-structural proteins (Nsp) are produced including a RNA-dependent RNA polymerase which interacts with other Nsp compounds to form, together with the host

protein including CyP proteins, the endoplasmic-reticulum-derived double-membrane-associated replication transcription complex required for the synthesis of all viral molecules which enter in the composition of *de novo* viral particles (163–165). The antiviral properties of CsA against HCoV-229E and SARS-CoV-1 were confirmed in an independent *in vitro* work which concluded that CsA strongly affects the replication of coronaviruses HCoV-229E and SARS-CoV-1 rendering RNA and protein synthesis almost undetectable (19). It was also reported that CyPA interacts with the SARS-CoV-1 nucleocapsid (N) protein (166, 167). A genome-wide SARS-CoV-1 screening of viral proteins interacting with cellular compounds (human cDNA libraries) performed using the yeast two-hybrid strategy revealed that the Nsp1 protein of SARS-CoV-1 binds FKBPs (147). It was also reported that FK506 inhibits the replication of HCoV-NL63, HCoV-229, and SARS-CoV-1 and that inhibition of HCoV-NL63 replication by FK506 occurs through inhibition of the FKBP1A/B, suggesting that both FKBP and CyP families of PPIases are involved in the replication of coronaviruses (24). It is worth noting that both siRNA-mediated CyPA depletion and shRNA-mediated CyPA depletion so far failed to trigger the reduction of SARS-CoV-1 replication, suggesting either that SARS-CoV-1 transcription mainly involves FKBPs and/or CyP other than CyPA or that the residual CyPA present in cells after treatment was sufficient to achieve the building of the replication complex (19, 168). CsA was also reported to inhibit the replication of MERS-CoV, a result which was more drastic when CsA was combined with interferon (IFN)-α (169). It was reported that CsA upregulates the interferon regulatory factor 1 (IRF1) signaling pathway and that inhibition of IRF1 allows viral replication despite the presence of CsA. The SARS-CoV-1 virulence factor Nsp1 antagonizes the IFN immune response (170, 171).

During the replication cycle of SARS-CoV-2, the RNA-dependent RNA polymerase (RdRp) required for the replication of the virus is active within a complex composed by several non-structural proteins of the virus such as Nsp12, Nsp8, and Nsp7 as well as cellular proteins likely including members of the CyP protein family. Within this replicative machinery (that is a target for the FDA-approved triphosphate metabolite remdesivir), the active site cleft of Nsp12 (RdRp) binds to the first turn of gRNA template, while Nsp8 is involved in the formation of sliding poles regulating the processivity of the RdRp (16, 17). The Nsp12 needs to associate with Nsp8 and Nsp7 to activate its capability to replicate long RNA. The Nsp13 helicase is also present in the SARS-CoV-2 replication complex and facilitates the RdRp function (172). Recently, the antiviral activity of CsA was evaluated *in vitro* on Vero E6 cells infected by SARS-CoV-2 and treated 1 h postinfection with serial drug dilutions, and it was reported an anti-SARS-CoV-2 at 50% effective concentration (EC_{50}) of 3.5 μM to be compared with 1.5 μM for chloroquine and 5.2 μM for lopinavir (21). Interestingly, the non-immunosuppressive CsA derivative alisporivir (Debio-025), previously reported to inhibit the *in vitro* replication of the human coronavirus HCoV-NL63 (173), was assayed for SARS-CoV-2 inhibition on Vero E6 cells infected for 3 h at a MOI of 0.05 and was found to reduce SARS-CoV-2 production in a dose-dependent manner, with an EC_{50} of 0.46 μM (22). These results suggest that CsA inhibits the viral replicative machinery likely through interaction with a member of the CyP family. Although CyPA depletion so far failed to trigger the reduction of SARS-CoV-1 replication (see above), a function for CyPA in SARS-CoV-2 replication cannot be excluded. It was also previously reported that the transmembrane glycoprotein CD147 (also known as extracellular matrix metalloproteinase inducer EMMPRIN) is facilitating viral replication by interacting with the N protein of SARS-CoV-1 through CyPA (146). CD147 was also reported to bind extracellular CyPB and to stimulate T lymphocytes (174). In COVID-19 patients, the anti-CD147 antibody meplazumab was claimed to improve the recovery of patients, suggesting a role for the CyPA/CD147 complex in SARS-CoV-2 replication similar to that previously described for SARS-CoV-1 (175). Finally, in their very elegant work, Gordon and colleagues set up a SARS-CoV-2 protein interactome map which identified 332 high-confidence protein interactions between SARS-CoV-2 proteins and human cellular compounds. This study revealed that the Nsp2 protein of SARS-CoV-2 interacts with FKBP15 and that the ORF8 of SARS-CoV-2 interacts with FKBP7 and FKBP10 (176). Altogether, these results suggest that CsA acts at different levels in infected cells to prevent the SARS-CoV-2 replication cycle (**Figure 6**).

CsA AND CYCLOPHILIN IN THE RENIN–ANGIOTENSIN SYSTEM PATHWAY: IMPLICATION FOR COVID-19

More than two decade ago, it was shown that the formation of abdominal aortic aneurysm in the rat model of elastase infusion was attenuated by CsA treatment (177). CyPA is known to promote atherosclerosis through stimulation of low-density lipoprotein uptake, decrease of endothelial nitric oxide synthase (eNOS) expression, increase of vascular cell adhesion molecule 1 (VCAM-1), and induction of tumor necrosis factor alpha (TNFα) (178). It was reported that deletion of CyPA in mice prevents the formation of abdominal aortic aneurysm in response to infusion of angiotensin II (Ang II) (179).

Although CyPA is an intracellular molecule, it can be secreted from macrophages in response to inflammatory stimuli acting as a chemoattractant of monocytes (63), and it is also secreted by endothelial cells and vascular smooth muscle (VSM) cells and stimulates proinflammatory signals thereby contributing to cardiovascular diseases (180, 181). Extracellular CyPA triggers IκBα phosphorylation that activates the nuclear translocation of NF-κB into the cell nucleus stimulating the transcription of VCAM-1 and E-selectin (66). Indeed, CypA secretion is regulated by Rho-kinase and behaves as a secreted oxidative stress molecule contributing to the pathogenesis of arteriosclerosis, hypertension, and heart failure, and inhibition of Rho-kinase by fasudil reduces the Ang II-induced aortic aneurysm formation (182, 183). Reactive oxygen species (ROS) were found to contribute to the pathogenesis of arteriosclerosis through induction of extracellular signal-regulated kinases ERK1/2 and p38 MAP kinase signaling which stimulated VSM cell growth (184–186). ROS-induced VSM cell growth

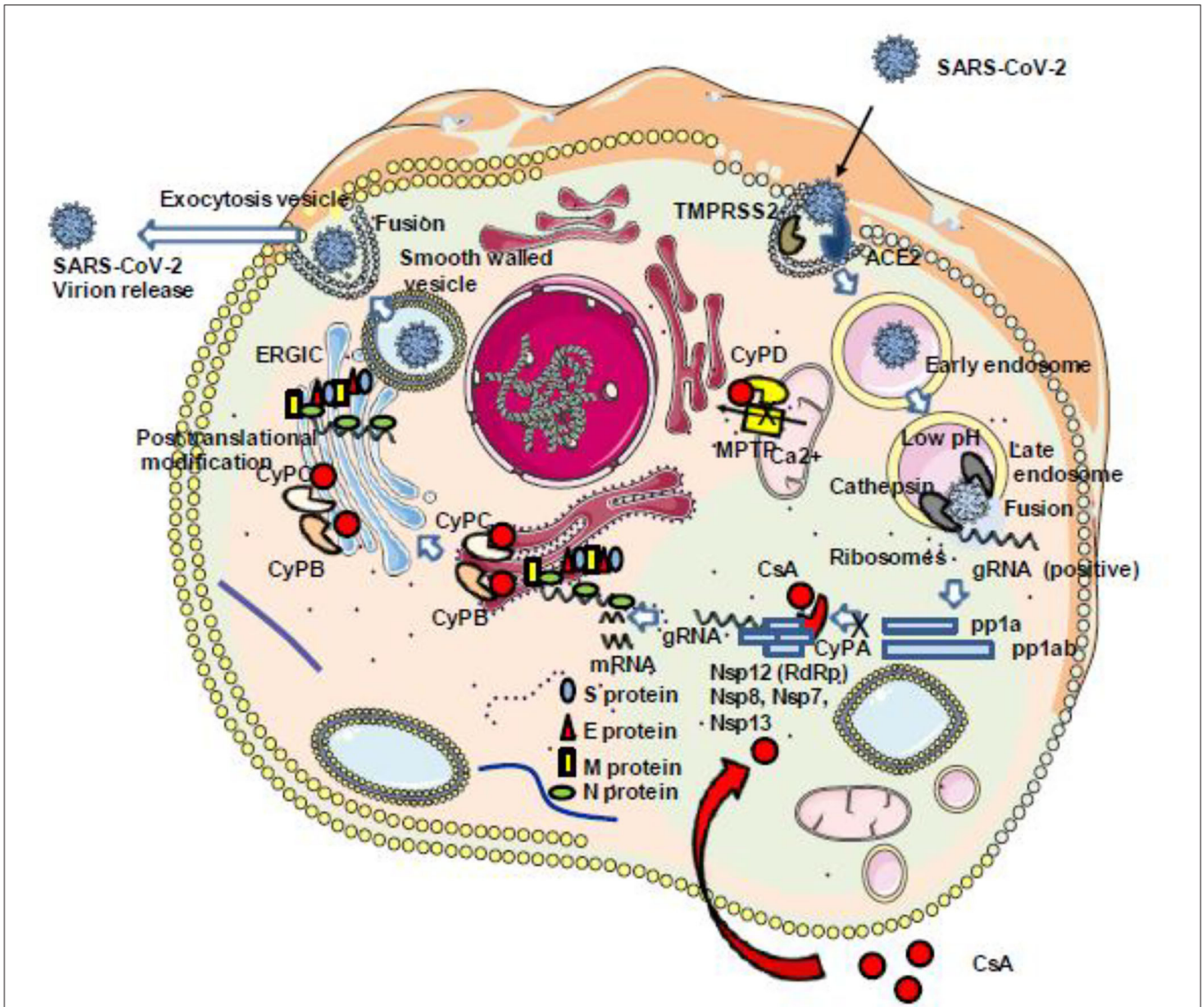

FIGURE 6 | Schematic representation of the antiviral properties of CsA. Once the SARS-CoV-2 genome starts to be transcribed into pp1a and pp1ab, the RNA-dependent RNA polymerase (Nsp12) should interact with several other viral (Nsp8, Nsp7, Nsp13) and cellular (CypA) proteins to construct a replication complex. This complex is required for the viral replication cycle to be completed with the synthesis of the structural proteins S, E, M, and N. This step can be inhibited through the interaction between CsA and CypA (see text for details regarding the different steps of the SARS-CoV-2 cycle which can be inhibited by CsA). ACE2, angiotensin-converting enzyme 2; CsA, cyclosporin A; CyPA, CyPB, CyPC, and CyP, cyclophilins A, B, C, and D; gRNA, genomic RNA; Nsps, non-structural proteins; ERGIC, endoplasmic reticulum–Golgi intermediate compartment.

and proinflammatory signal have been implicated in the revascularization of obstructive coronary artery disease and the pathogenesis of neointima following vascular injury (187). Serum levels of CyPA were found elevated in coronary artery disease (188–190). CypA secreted from blood vessels and heart cells regulates signal pathways and causes a decline of diastolic and systolic function leading to proliferation of cardiac fibroblasts, the occurrence of cardiac hypertrophy, and remodeling (191).

Taniyama and colleagues reported that Ang II activates p38 MAPK inducing an Akt signaling pathway that results in VSM cell activation and suggested that the ROS-sensitive 3-phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates Akt and that a parallel pathway that requires NADPH oxidase (NOX)-dependent production of ROS (including superoxide anions O_2^-, hydrogen peroxide H_2O_2, and hydroxyl radical OH) triggers p38 MAPK activation that in turn activates Akt (186). CyPA was also found to be involved in the translocation of NOX enzymes and the two molecules synergize to increase ROS production (192). Finally, it was also reported that Ang II triggers the release of CyPA and the activation of

metalloproteinase 2 (MMP-2) in VSM cells derived from human abdominal aortic aneurysm (62). Ang II type 1 receptor (AT1R) blockers have been shown to prevent cardiovascular diseases (193). During treatment with simvastatin (a member of the statin family which inhibits the hydroxymethylglutaryl CoA reductase), patients with abdominal aortic aneurysm were found to have reduced CypA mRNA expression as well as reduced CyPA intracellular protein levels (194). Interestingly, in a mice model, deletion of the CyPA gene prevented the formation of abdominal aortic aneurysm usually observed in response to infusion of Ang II (179).

In SARS-CoV-2-infected individuals, the host angiotensin-converting enzyme A (ACE2) monocarboxypeptidase serves as a cell-surface receptor for the virus which interacts with ACE2 by the receptor-binding domain present in its spike (S) protein [reviewed in (195)]. We have recently found evidence that SARS-CoV-2-infected cells have a downregulation of ACE2 mRNA expression and a reduced cell surface expression of ACE2 and that COVID-19 patients have decreased soluble ACE2 and increased levels of Ang II in their plasma (196). Besides the vasoconstrictor and thrombotic effects of Ang II, the dysregulation of the renin–angiotensin pathway with the massive Ang II accumulation is likely to promote the production of proinflammatory cytokine *via* AT1R interaction, by activating metalloprotease 17 (ADAM17) which can process the membrane-anchored TNFα to a soluble TNFα which acts as an activator of NF-KB and IL-6Rα to a soluble form (sIL-6Rα) which can form a complex with IL-6 and activates a STAT3 signaling pathway (197, 198). Since Ang II triggers the release of extracellular CyPA through regulation of Rho-kinase and that extracellular CyPA behaves as a secreted oxidative stress molecule triggering the activation of the NF-κB that stimulates the transcription of VCAM-1 and E-selectin and the overexpression of TNFα the inhibition of CyPA with CsA in COVID-19 patients could reduce atherosclerosis, hypertension, and heart failure. Interestingly, treatment of COVID-19 patients with a recombinant soluble human ACE2 (hrsACE2 from Apeiron Biologics, Vienna, Austria) which can interfere with virus binding but also with Ang II reduced SARS-CoV-2 load and induced a massive decrease of Ang II levels, IL-6, and TNF in patients and showed a strong benefit for the outcome of the patients (199) (**Figure 7**).

CONCLUSION

The emergence of the COVID-19 pandemic about 1 year ago has stressed healthcare systems worldwide, and besides improving the care of patients as knowledge of the disease improves, there was a global race to identify as fast as possible effective drugs to treat SARS-CoV-2-infected patients while waiting to be able to protect individuals with an effective vaccine (200). Since no antiviral was specifically developed against this new coronavirus, the number of clinical trials of molecules expected to interfere with the viral replication cycle or to modulate the immune response has been greater than ever. In this emergency context, the fastest strategy that has been followed by the majority of healthcare teams has been the repositioning of molecules already approved by the US Food and Drugs Administration. Among other molecules, there is ample evidence that CsA may represent a molecule to be tested further in its repurposing therapeutic strategy to treat patients with severe forms of COVID-19. This molecule is widely available, FDA-approved, and affordable. It prevents proinflammatory processes, blocks SARS-CoV-2 replication, and interferes with angiotensin II harmful effects. Recently, Guisado-Vasco et al. (201) reported on the clinical characteristics and outcomes of 607 patients with severe forms of COVID-19 receiving antiviral, antimalarials, glucocorticoids, or immunomodulation with tocilizumab or CsA. From this retrospective observational study (COQUIMA cohort), the authors conclude that among the prescribed therapies, only CsA was associated with a significant (four-fold) decrease in mortality. Moreover, this study adds clear information on the dosing (cumulative dose at least 300 mg) and duration (max 3 weeks) of CsA repurposing in COVID-19.

Therapeutic doses of CsA are usually in the range of 10–20 mg/kg daily when given orally. A wide variability in CsA pharmacokinetics has been observed after the oral or intravenous administration of this drug to patients and varies with respect to the organ grafted, age of the patient, and patient health status. CsA is absorbed in the gastrointestinal tract and almost completely metabolized in both the liver and small intestine by cytochrome P450 family 3 (CYP3A). CsA is also given as intravenous infusion using 2.5–5 mg/kg daily. CsA bioavailability in patients range from 5 to 90%. CsA has the advantage of the intravenous application route which may be crucial for the treatment of critically ill patients with severe forms of COVID-19. However, it is important to emphasize that the serum levels of CsA in conventional treatment fold above the *in vitro* drug concentration required for the inhibition of SARS-CoV-2 replication. The CsA concentration required to inhibit virus replication exceeds the serum concentration of the drug that is usually well below 200 ng/ml (202). A major challenge is to obtain appropriate concentrations of CsA in infected tissues, which will likely require three- to six-fold higher doses than those usually given to patients, which will strongly increase the risks for toxic effects (100). Under these conditions, it is not possible to conclude that the lower COVID-19 mortality reported under CsA treatment is due to an antiviral effect; it could as well result from an anti-inflammatory effect and/or prevention of the deleterious action of Ang II.

Given the variety of side effects of CsA, a careful evaluation of cost/benefit should be done before considering this molecule as a first-line therapy in COVID-19. Nephrotoxicity is the most common adverse effect of CsA treatment and is frequently associated with arterial hypertension (203–205). CsA nephrotoxic effect is dose and duration dependent (206). Vascular effects in the kidney lead to reduced glomerular filtration and impaired sodium excretion. Changes in blood pressure can develop within a few weeks of treatment and sometimes are severe and associated with intracranial hemorrhage, left ventricular hypertrophy, microangiopathic hemolysis, and organ damage (207, 208). This could be a problem as many patients with mild or severe forms of

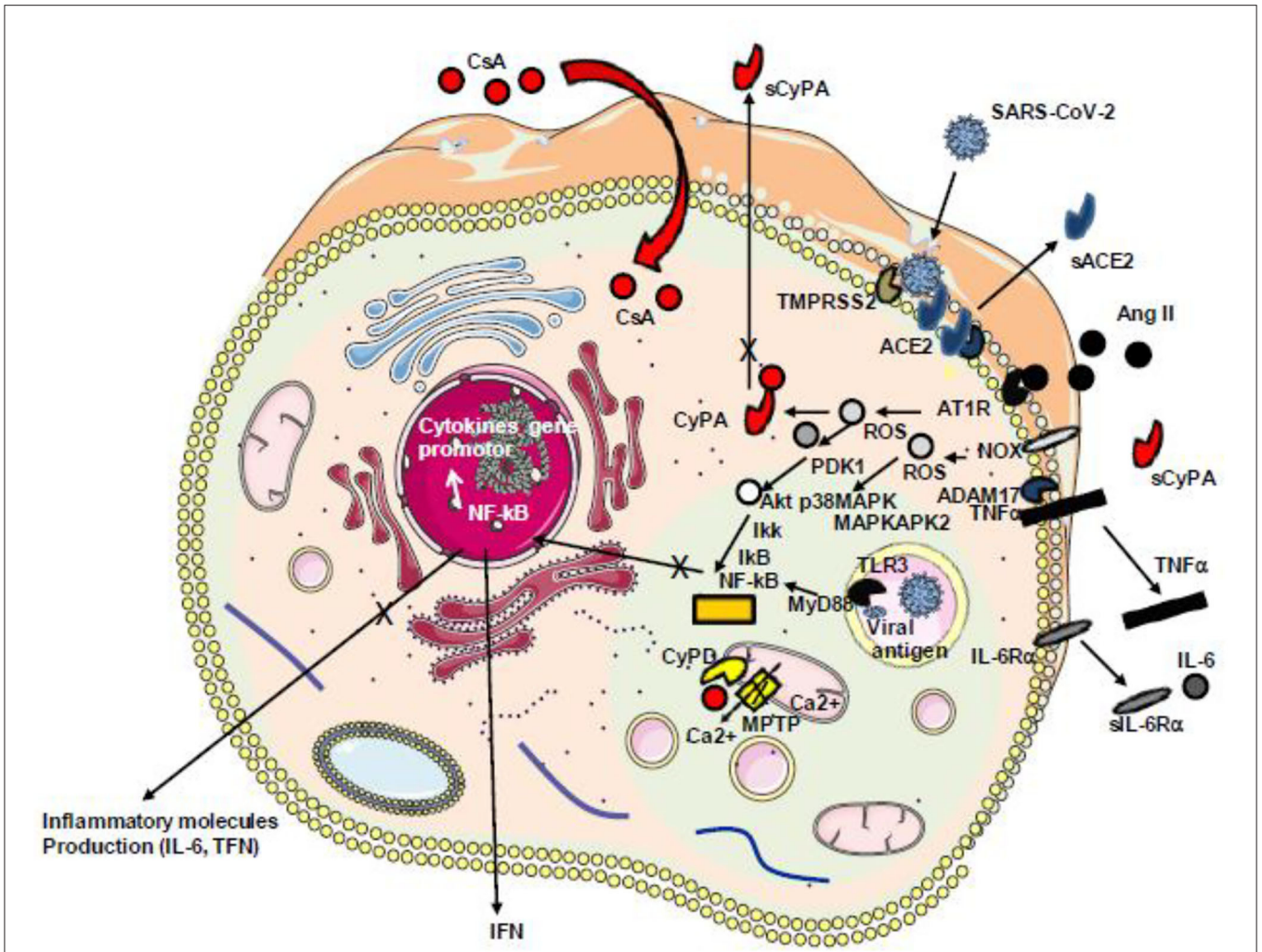

FIGURE 7 | Schematic representation of Ang II/AT1R-induced inflammatory pathway with cytokine release. During infection with SARS-CoV-2, the virus binds ACE2 reducing the ACE2 transcription and inhibiting the capacity of ACE2 to mediate the cleavage of angiotensin II (Ang II) into angiotensin 1–7. The accumulation of Ang II triggers signals through its receptor AT1R inducing ROS production. ROS triggers the secretion of CyPA that acts as a stress factor activating the ERK1/2 kinase and overproduction of ROS through a positive feedback loop. ROS-sensitive 3-phosphoinositide-dependent protein kinase (PDK1) activation that contributes to phosphorylation and activation of Akt. A parallel pathway involves the NOX-dependent generation of ROS that activates the p38 MAP kinase (p38MAPK) which recruits MAPKAPK2 leading to AkT phosphorylation on a second amino acid position leading to full activation of the p38 MAPK–Akt complex, the activation of IKKαβ inducing the release of IkB from the IκB–NF-κB complexes, nuclear translocation of NF-κB, and the production of cytokines including TNF-α and soluble IL-6 receptor (sIL-6R) *via* disintegrin and metalloprotease 17 (ADAM17) followed by the activation of the IL-6 amplifier (IL-6 AMP) which, by feedback regulation, activates both the NF-κB and STAT3 transcription factors and the production of IL-6. SARS-CoV-2 itself activates NF-κB *via* the TLR3 receptor. Ang II, angiotensin II; AT1R, angiotensin II type 1 receptor; ROS, reactive oxygen species; NOX, NADPH oxidase; IKK, IkB kinase; CyPA, cyclophilin A; TLR3, Toll-like receptor 3; NF-κB, nuclear factor κB.

COVID-19 have high blood pressure. In addition, several animal studies have highlighted a vasoconstrictor effect of CsA (209–211). Hypertension and nephrotoxicity must be monitored carefully in patients under CsA therapy. Yet, CsA was reported to protect against Ang II-induced organ damage in transgenic rats harboring human renin and angiotensinogen genes by inhibiting perivascular monocyte/macrophage infiltration and IL-6 and iNOS expression (212). Moreover, many drugs including amphotericin B, aminoglycoside antibiotics, and co-trimoxazole are at risk to potentiate the nephrotoxicity of CsA (202). Indeed, there is a long list of drugs that were proven or suspected to clinically interact with CsA (213) such as anticonvulsants (carbamazepine, phenobarbital, phenytoin, primidone) that reduce CsA blood concentration, antidepressants (fluvoxamine, nefazodone), antimicrobial and antifungal drugs (ketoconazole, fluconazole, itraconazole, metronidazole, fluoroquinolones, macrolides, clarithromycin, erythromycin), antiviral drugs (ritonavir, saquinavir), cardiovascular drugs (amiodarone, calcium channel blockers, amlodipine, nicardipine, verapamil, carvedilol), and hypoglycemic drugs (glibenclamide, glipizide) among others. This list also includes chloroquine and glucocorticoids,

which are sometimes used in COVID-19 therapy. The adverse effects of CsA treatment include nephrotoxicity (risk increased by ACE inhibitors among many other drugs), hypertension, hyperkalemia (risk increased by potassium salts), hyperlipidemia, hypomagnesemia, neurotoxicity (risk increased by imipenem), hepatotoxicity (risk increased by androgens), posttransplant diabetes, gingival hyperplasia (risk increased by nifedipine), and hirsutism. Moreover, CsA was reported as able to augment Ang II-stimulated rise in intracellular free calcium in vascular smooth muscle cells (214) and to increase ADAM17 activity up to three-fold, likely leading to an ACE2 shedding increase detrimental to COVID-19 patients (215, 216).

The data in the literature are clear regarding the effects of CsA on *in vitro* SARS-CoV-2 replication, but these are not the only possible beneficial effects one would expect from CsA experimental use in the treatment of COVID-19 since it can modulate both proinflammatory responses and the RAS pathway. Moreover, as summarized in **Table 3**, several preliminary CsA clinical trials performed on COVID-19 patients are encouraging and suggest that this strategy should be pursued further. In this review, we describe at least three possible mechanisms for which it can be postulated that they are likely to produce a favorable effect on the outcome of COVID-19 patients: (i) an anti-inflammatory effect reducing the production of proinflammatory cytokines, (ii) an antiviral effect preventing the formation of the viral RNA synthesis complex, and (iii) an effect on tissue damage and thrombosis by acting against the deleterious action of angiotensin II. It is also possible that CsA contributes to decrease the lactate/pyruvate ratio in cells by activating the NHE-3 Na^+/H^+ exchanger, thereby counteracting the hypoxic damage induced by SARS-CoV-2 infection (215, 217). Even if CsA has many effects that are likely to improve the outcome of patients infected with SARS-CoV-2, one can of course wonder about the consequence of using a therapeutic drug that exhibits immunosuppressive effects in severe forms of COVID-19 because this could reduce the innate and adaptive immune responses of the patients against the virus (146, 218–220). However, there is an increasing panel of available cyclophilin inhibitors such as alisporivir/Debio-025 (Novartis), Debio-064 (Novartis), SDZ NIM811 (Sandoz, Novartis), SCY-635 (Scynexis Inc., Jersey City, NJ, USA), STG-175 (S&T Global, Woburn, MA, USA), CRV431 (Hepion Pharmaceuticals, Edison, NJ, USA) or CPI-431-32 (Ciclofilin Pharmaceuticals Inc., San Diego, CA, USA), and it is still possible to replace CsA by one of these compounds or compare these molecules in clinical trials. Finally, as recently highlighted by Schuurmans and Hage (221), it will be very important to decide when CsA should be administered to SARS-CoV-2-infected patients and what should be the effective cumulative dose based on oral or intravenous CsA administration, to obtain the most beneficial effects. Originally used as salvage therapy in refractory COVID-19 cases, CsA could soon be seen as a first-line therapy in COVID-19.

AUTHOR CONTRIBUTIONS

CAD, CM, M-DP-M, and DR contributed to the concept of the study. CM designed the tables. CAD designed the figures and wrote the paper. CD provided the histological data. DR obtained the funding for this study. All authors reviewed and approved the final version of the manuscript.

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The Differences and Changes of Semi-Quantitative and Quantitative CT Features of Coronavirus Disease 2019 Pneumonia in Patients With or Without Smoking History

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Objective: To assess CT features of COVID-19 patients with different smoking status using quantitative and semi-quantitative technologies and to investigate changes of CT features in different disease states between the two groups.

Methods: 30 COVID-19 patients with current smoking status (29 men, 1 woman) admitted in our database were enrolled as smoking group and 56 COVID-19 patients without smoking history (24 men, 32 women) admitted during the same period were enrolled as a control group. Twenty-seven smoking cases and 55 control cases reached recovery standard and were discharged. Initial and follow-up CT during hospitalization and follow-up CT after discharge were acquired. Thirty quantitative features, including the ratio of infection volume and visual-assessed interstitial changes score including total score, score of ground glass opacity, consolidation, septal thickening, reticulation and honeycombing sign, were analyzed.

Results: Initial CT images of the smoking group showed higher scores of septal thickening [4.5 (0–5) vs. 0 (0–4), $p = 0.001$] and reticulation [0 (0–5.25) vs 0 (0–0), $p = 0.001$] as well as higher total score [7 (5–12.25) vs. 6 (5–7), $p = 0.008$] with statistical significance than in the control group. The score of reticulation was higher in the smoking group than in the control group when discharged [0.89 (0–0) vs. 0.09 (0–0), $p = 0.02$]. The score of septal thickening tended to be higher in the smoking group than the control group [4 (0–4) vs. 0 (0–4), $p = 0.007$] after being discharged. Quantitative CT features including infection ratio of whole lung and left lung as well as infection ratio within HU (−750, −300) and within HU (−300, 49) were higher in the control group of initial CT with statistical differences. The infection ratio of whole lung and left lung, infection ratio within HU (−750), and within HU (−750, −300) were higher in the control group with statistical differences when discharged. This trend turned adverse after discharge and the values of quantitative features were generally higher in the smoking group than in the control group without statistical differences.

Conclusions: Patients with a history of smoking presented more severe interstitial manifestations and more residual lesion after being discharged. More support should be given for COVID-19 patients with a smoking history during hospitalization and after discharge.

Keywords: AI, COVID-19, cigarette smoke, CT images, quantitative CT technique, interstitial lung changes

INTRODUCTION

Coronavirus Disease 2019 (COVID-19) has spread across the world and the number of confirmed cases is continually rising (1, 2). As of November 24, 2020, 58,712,326 confirmed cases and 1,388,528 death cases from COVID-19 involving 219 countries, areas, or territories had been reported (3). Through thorough research, the epidemiology, clinical symptoms, pathological characteristics, and biological features have been well-established. The development of a vaccine targeting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has also made substantial progress. However, how to prevent COVID-19 patients from lethal medical events, e.g., acute respiratory distress syndrome (ARDS), and to provide medical support for recovered COVID-19 patients after being discharged remain tricky.

Therefore, investigating the risk factors for predicting the outcome of patients with COVID-19 during hospitalization and after discharge is clinically urgent. Previous studies stated that patients of an elderly age and with other disease conditions had worse outcomes (4, 5). A previous study conducted by Hu et.al demonstrated COVID-19 patients with pre-existing chronic obstructive pulmonary disease (COPD) had a higher risk of all-cause mortality (6). Smoking is also reported to be high risk factor for COVID-19 patients (7). Smoking can cause lung injuries, leading to emphysema and fibrosis (8, 9), and is related to higher expression of angiotensin converting enzyme 2 (ACE2), which is the receptor for SARS-CoV-2. So it might also be an independent factor for COVID-19 infection and might worsen the disease prognosis (10). A meta-analysis conducted by Zhao and colleagues discovered that active smoking increases the risk of developing severe COVID-19 by around 2-fold (11). Hence, carefully evaluating the high-risk patients with smoking may facilitate a better treatment scenario.

Computed tomography (CT) has proven to be an important tool in diagnosing and evaluating the response of COVID-19 in clinical practice (12–14). Our previous research (15–17) also discovered that chest CT can be used as a potential tool to diagnose and evaluate the severity of COVID-19. The CT imaging features of COVID-19 patients had been well-described, e.g., bilateral and peripheral distributed ground-glass opacities. Studies also discovered that CT can evaluate the severity and extent of fibrosing interstitial pneumonia (18, 19). However, whether COVID-19 patients with or without smoking history have specific radiographic characteristics is not clear.

With the state-of-the-art data analysis strategy, artificial intelligence (AI) technologies have achieved remarkable success in medical imaging analysis. Numerous studies have shown great potential in automated quantification of lung abnormalities and severity prediction applying AI-based technologies (20–22).

Thus, the aim of this study is to assess CT features of COVID-19 patients with different smoking status using AI-based quantitative and visual scoring methods and to investigate changes of CT features in different disease states between the two groups.

MATERIALS AND METHODS

This retrospective study was approved by the Medical Ethical Committee (Approved Number.2020002), which waived the requirement for patients' informed consent.

Patients

We retrospectively searched the medical records of laboratory-confirmed COVID-19 patients with current smoking status from the Radiology Quality Control Center, Hunan, China, from January 24 to February 18, 2020. Patients who were current smokers or who quit smoking after SARS -Cov-2 infection were classed as having current smoking status. Current smoker was defined as someone who has smoked 100 cigarettes in his or her lifetime and who currently smokes cigarettes (23).The cigarette smoking intensity was quantified in pack-years (number of packs smoked per day multiplied by the number of years smoked) (24). Laboratory-confirmed non-smoking COVID-19 patients admitted during the same period were enrolled as a control group. Non-smokers were defined as patients who had never smoked, or who had smoked <100 cigarettes in his or her lifetime (23). Multiple CT images and clinical characteristics of all included patients were collected and analyzed. The diagnosis of COVID-19 was determined according to the following three methods: (1) isolation of COVID-19, (2) at least two positive results with real-time reverse-transcription polymerase chain reaction (RT-PCR) assay for COVID-19, or (3) a genetic sequence that matches COVID-19. The inclusion criteria of the smoking group was as follows: (1) patients with current smoking status or who quit smoking after infection, and (2) patients with multiple CT scans. The exclusion criteria were as follows: (1) patients with pulmonary lobectomy history; (2) patients with underlying pulmonary disease conditions such as COPD, (3) poor image quality, or (4) patients who quit smoking before SARS-Cov-2 infection. Finally, a total of 30 cases with current smoking history and 56 control cases were enrolled. Follow-up CT images during hospitalization for all patients were collected. The interval of follow-up CTs during hospitalization ranged from 2 to 7 days. All cases were treated strictly and followed the therapeutic principles

based on the guidelines of COVID-19 (Trial Version 8) proposed by the China National Health Commission (25). The basic treatment included symptomatic treatment, recombinant human interferon α2b (aerosol inhalation), and antiviral treatment, such as lopinavir or ritonavir tablets (500 mg twice daily, orally) which were given to all confirmed cases. Corticosteroid treatment and antibiotic treatment were used where appropriate. Invasive mechanical ventilation treatment and extracorporeal membrane oxygenation (ECMO) was used for emergency cases (including severe and fetal cases). Patients who reached recovery standard according to COVID-19 guidelines (trial version 8) were discharged. Discharge criteria was as follows: (1) body temperature returned to normal for more than 3 days; (2) respiratory symptoms significantly relieved; (3) abnormal imaging findings substantially resolved; and (4) viral clearance, e.g., negative nucleic acid test for two consecutive respiratory pathogens (sampling interval $\geq$1 day). Recovered patients underwent another follow-up CT scan within 30 days after leaving hospital. The flowchart was shown in **Figure 1**.

Imaging Analysis

Two thoracic radiologists (with 10 years of experience), who were blinded to smoking history and clinical data, reviewed initial and follow-up CT images independently and resolved discrepancies by consensus. All images were viewed on both lung (width, 1500 HU; level, −700 HU) and mediastinal (width, 350 HU; level, 40 HU) settings. The appearance of emphysema and change pattern of CT imaging were recorded. The CT image features at three time points were calculated and compared using both deep learning-based quantitative method and visual- based semi-quantitative method: initial CT upon admission, follow-up CT when discharged, and follow-up CT after being discharged.

Semi-Quantitative Assessment

A semi-quantitative assessment system was introduced to assess smoking-related interstitial lung changes (26, 27). Visual evaluation included a score of severity and a score of extent. The severity assessment was based on appreciation of five parenchymal abnormalities assumed to reflect increasing severity of lung involvement: ground-glass appearance (score 1), consolidation (score 2), septal thickening (score 3), reticulation (score 4), and honeycombing (score 5). The severity score thus ranged from 0 (no abnormality) to 15 (all abnormalities present). The extent score was obtained by counting the number of bronchopulmonary segments in which any of the previous abnormalities are observed: 1 to 3 segments involved implied a score of 1, 4 to 9 segments implied a score of 2, and more than 9 segments implied a score of 3. The extent score thus ranged from 0 (no abnormality in any segment) to 15 (all 5 abnormalities in more than 9 segments). Finally, severity and extent of disease scores were added to obtain a total score (range: 0–30).

Quantitative Assessment

The uAI software (uAI, Shanghai United Imaging Intelligence Co., Ltd.) was applied for quantitative CT feature assessment. This deep learning-based software could accurately segment the lung as well as the infection regions from chest CT images (28). This tool is based on deep learning, where a VB-net (22) is adopted to fulfill accurate segmentation of lung as well as infection regions from chest CT images. According to segmentation results, quantitative features, which are potentially related to COVID-19, are calculated. Specifically, the lung is segmented and divided into five lung lobes, i.e., superior/middle/inferior lobes of the right lung and superior/inferior lobes of the left lung, and 18 lung segments, with 10 segments in the right lung (denoted as RS1 – 10) and 8 segments in the left lung (denoted as LS1 – 8). In order to minimize the individual bias, we only included calibration features, including the ratio of infection volume to the whole lung, right/left lung, and each lobe/segment and within different HU ranges. Finally, 30 quantitative features were included in this study (**Supplementary Table 1**).

The change pattern of follow-up CT images were also investigated. We defined three imaging changes, namely no change, progress change, and improvement change, which we proposed in our previous study (16). No change referred to no obvious changes presented in chest CT. Progress change referred to the presence of new lesions or the presence of extent involvement area during the treatment. Improvement change referred to the continually absorbed abnormities.

Statistical Analysis

Continuous variables with normal distribution were presented as mean ± standard deviation and compared by *Mann-Whitney U*-test. Continuous variables with non-normal distribution were presented as median (range) and compared by *Mann-Whitney U*-test. Categoric variables were presented as numbers (percentages) and were compared by *Fisher exact* test between smoking and non-smoking groups. The correlations between semi-quantitative results, quantitative results, and smoking intensity were analyzed using the Spearman analysis. Two-sided $p < 0.05$ was considered statistically significant. All statistical analyses were performed with SPSS software (version 19.0, IBM).

RESULTS

Among the 86 included patients, 30 (29 men, 1 women) were in the smoking group, and 56 (24 men, 32 women) were in the non-smoking group. The distribution of age and sex was shown in **Table 1**. There was no statistical difference regarding age between two groups. However, there were fewer females in the smoking group ($p = 0.001$). Clinical type of COVID-19 at baseline and the change patterns of follow-up CT images showed no statistical difference among the two groups (**Table 1**).

Evaluation of Initial CT

There were 14 cases (40%) in the smoking group where emphysema evidence was found on CT images, while only 3 cases (5%) showed emphysema in the control group ($p = 0.001$). Concerning semi-quantitative assessment for interstitial lung changes (**Table 2**), the scores of septal thickening [4.5 (0–5) vs. 0 (0–4)] and reticulation [0 (0–5.25) vs. 0 (0–0)] were significantly higher in the smoking group than in the control group ($p <$ 0.05). The score of consolidation, however, was lower in the

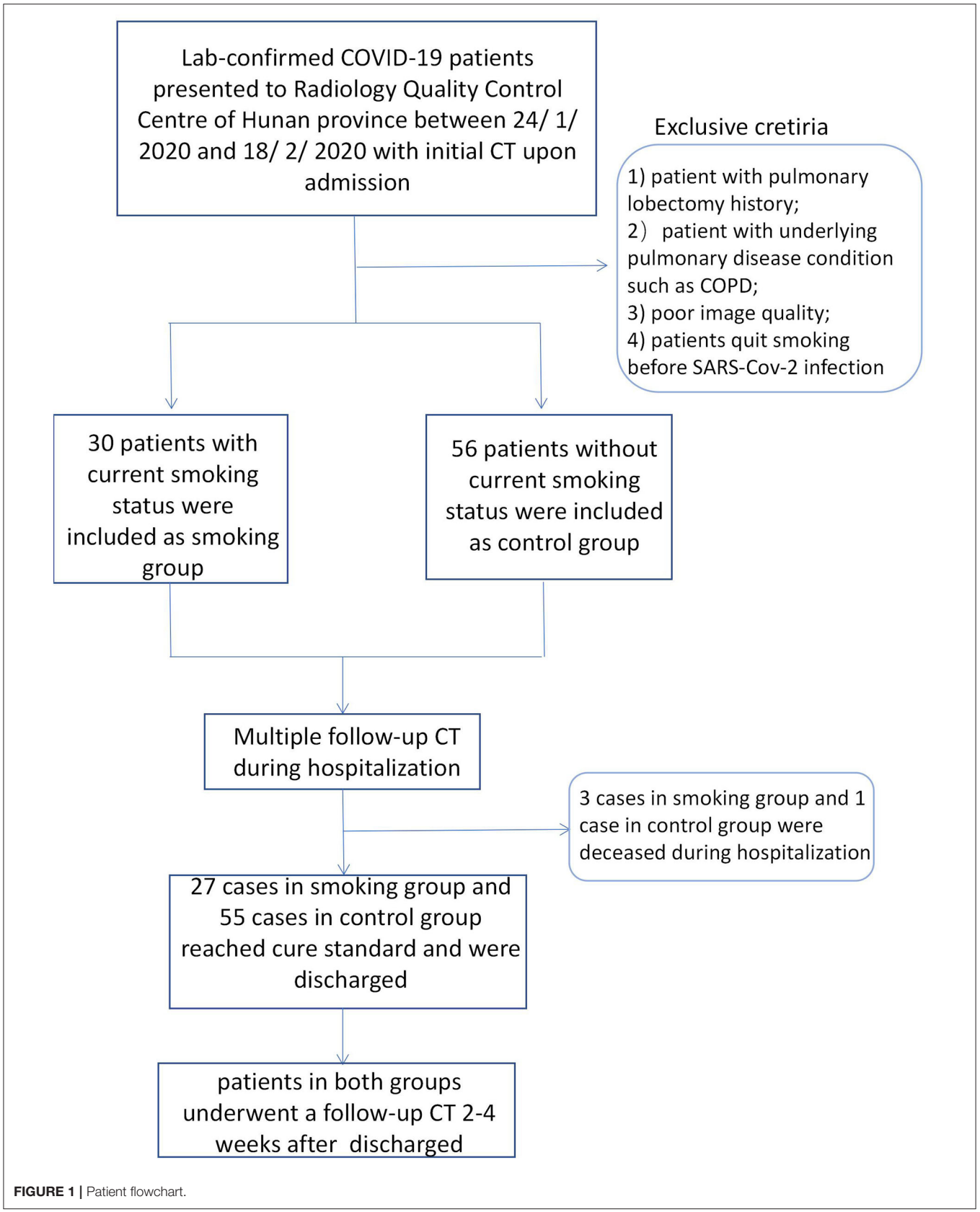

FIGURE 1 | Patient flowchart.

TABLE 1 | Demographic and clinical features.

Basic characteristics	Smoking group ($n = 30$)	Control group ($n = 56$)	*P*-value
Sex			**0.001**
Male	29 (97)	24 (43)	
Female	1 (3)	32 (57)	
Age (years)	50.83 ± 16.05	46.14 ± 13.34	0.152
Clinical type at baseline			0.075
Mild	2 (7)	4 (7)	
Common	22 (73)	49 (87)	
Severe	5 (17)	3 (5)	
Fatal	1 (3)	0	
Imaging features changes			0.056
Improvement change	12 (40)	37 (66)	
Progress change	3(10)	0	
Progressing and then improving change	11 (37)	15 (27)	
No change	4 (13)	4 (7)	
Presence of emphysema	12 (40)	3 (5)	**0.01**
Deceased cases	3 (10)	1 (1)	0.12

The bold values stand for $p < 0.05$.

TABLE 2 | CT features of initial CT.

CT features	Smoking group ($n = 30$)	Control group ($n = 56$)	*P*-value
Quantitative CT features			
Infection ratio in the whole lung (%)	1.2 (0.1–5.5)	4.2 (1.50–7.35)	**0.031**
Infection ratio in inferior lobe of left lung (%)	0.3 (0–4.9)	6.1 (0.52–14.35)	**0.003**
Infection ratio in S6 of left lung (%)	0.05 (0–1.8)	1.8 (0–13.9)	**0.017**
Infection ratio in S7+8 of left lung (%)	0 (0–0.95)	0.45 (0–2.85)	**0.042**
Infection ratio in S9 of left lung (%)	0.05 (0–2.3)	6.6 (0.3–20.1)	**0.001**
Infection ratio in S10 of left lung (%)	0.05 (0–5.05)	3.5 (0.17–17.4)	**0.009**
Infection ratio within HU (−750, −300) (%)	0.75 (0.1–4.5)	2.9 (1.0–6.1)	**0.032**
Infection ratio within HU (−300, 49) (%)	0.1 (0–0.95)	0.85 (0.2–2.2)	**0.012**
Interstitial changes score			
GGO	3 (2–4)	3 (2–3)	0.711
Consolidation	3 (0–3)	3 (0–4)	**0.032**
Septal thickening	4.5 (0–5)	0 (0–4)	**0.001**
Reticulation	0 (0–5.25)	0 (0–0)	**0.001**
Honeycombing sign	0 (0–0)	0 (0–0)	0.173
Total score	7 (5–12.25)	6 (5–7)	**0.008**

The bold values stand for $p < 0.05$.

smoking group than in the control group [3 (0–3) vs. 3 (0–4), $p < 0.05$]. The total interstitial change scores were higher in the smoking group with statistical differences [7 (5–12.25) vs. 6 (5–7), $p < 0.05$]. Results with statistical significance of quantitative assessment of chest CT imaging upon admission were shown in **Table 2**. The infection ratio of whole lung and left lung were higher in the control group than in the smoking group ($p < 0.05$). To be more specific, the infection ratio in inferior lobe, LS6, LS7+8, LS9, and LS10 of left lung were higher in the control group with statistical differences. Infection ratio within HU (−750, −300) [0.75 (0.1–4.5) vs. 2.9 (1.0–6.1), $p < 0.05$] and within HU (−300, 49) [0.1 (0–0.95) vs. 0.85 (0.2–2.2), $p < 0.05$] were also higher in the control group than in the smoking group with statistical differences.

Evaluation of Follow-Up CT When Discharged

There were three patients in the smoking group and one patient in the control group who unfortunately passed away during hospitalization. There were no statistical differences regarding deceased cases between two groups. The remaining 82 cases reached recovery standard and underwent follow-up CT scan when discharged. There were no statistical differences of hospitalization time between the smoking group and control group (19.37 ± 8.49 days vs. 18.47 ± 9.56 days, $p = 0.68$).

The total interstitial change scores when discharged showed no statistical insignificance between two groups (**Table 3**). The score of reticulation was significantly higher in the smoking group than in the control group [0.89 (0–0) vs. 0.09 (0–0), $p = 0.02$]. Results with statistical significance of quantitative CT features when discharged were shown in **Table 3**. The infection ratio of whole lung, the infection ratio of left lung, the infection ratio in inferior lobe, S6, S7+8, S9, and S10 of left lung, and infection ratio within HU (–,−750) as well as infection ratio within HU (−750, −300) were higher in the control group with statistical differences ($p < 0.05$).

Evaluation of Follow-Up CT 2-4 Weeks After Discharged

All recovered patients underwent CT scans 2–4 weeks after being discharged. The interval time of follow-up CT after being discharged is 28.8 ± 0.94 days for the smoking group and 27 ± 4.02 days for the control group. No statistical differences were discovered between two groups concerning follow-up time interval. Regarding the semi-quantitative features, only the score of septal thickening was shown to be higher in the smoking group

TABLE 3 | CT features of follow-up CT when discharged.

CT features	Smoking group ($n = 27$)	Control group ($n = 55$)	*P*-value
Time from admission to discharged (days)	19.37 ± 8.49	18.47 ± 9.56	0.68
Quantitative CT features			
Infection ratio in the whole lung (%)	0 (0–1.7)	1.1 (0.2–3.8)	**0.016**
Infection ratio in the left lung (%)	0.1 (0–1.1)	0.6 (0.1–3.1)	**0.019**
Infection ratio in S6 of left lung (%)	0 (0–0.9)	0.7 (0–6.8)	**0.034**
Infection ratio in S7+8 of left lung (%)	0 (0–0.3)	0.2 (0–1.4)	**0.024**
Infection ratio in S9 of left lung (%)	0 (0–0.2)	0.7 (0–4.8)	**0.004**
Infection ratio in S10 of left lung (%)	0 (0–0.6)	0.6 (0.1–5.6)	**0.004**
Infection ratio within HU (–,−750) (%)	0 (0–0.2)	0.1 (0–0.4)	**0.029**
Infection ratio within HU (−750, −300) (%)	0 (0–1.2)	0.9 (0.1–2.8)	**0.010**
Interstitial changes score	3 (2, 3)	3 (2, 3)	0.364
GGO			
Consolidation	0 (0–3)	0 (0–3)	0.398
Septal thickening	4 (0–5)	4 (0–4)	0.409
Reticulation	0.89 (0–0)	0.09 (0–0)	**0.02**
Honeycombing sign	0.2 (0–0)	0 (0–0)	0.154
Total score	7 (4–11)	7 (5–10)	0.85

The bold values stand for $p < 0.05$.

than the control group ($p = 0.007$) 2–4 weeks after discharge (**Table 4**). There were plenty of quantitative CT features that were higher in the control group than in the smoking group at initial CT and when discharged. Interestingly, this trend turned adverse on follow-up CT 2–4 weeks after being discharged. The values of quantitative features were generally higher in the smoking group than in the control group, without statistical differences (**Supplementary Table 1**).

Correlations Between Semi-Quantitative Results, Quantitative Results, and Cigarette Smoking Intensity

We investigated the relationships between interstitial changes score, quantitative CT features, and cigarette smoking intensity upon admission, when discharged, and 2–4 weeks after discharge. No significant correlations were found in terms of semi-quantitative or quantitative CT features (all $P > 0.05$) measured at all timepoints (**Supplementary Table 4**).

TABLE 4 | CT features of follow-up CT after discharged 2–4 weeks.

Interstitial changes score	Smoking group ($n = 27$)	Control group ($n = 55$)	*P*-value
Interval time of follow-up CT after discharged	28.8 ± 0.94	27 ± 4.02	0.097
GGO	2 (0–3)	0 (0–2)	0.443
Consolidation	0 (0–0)	0 (0–0)	0.528
Septal thickening	4 (0–4)	0 (0–4)	**0.007**
Reticulation	0 (0–0)	0 (0–0)	1
Honeycombing sign	0 (0–0)	0 (0–0)	1
Total score	4 (0–7)	0 (0–6)	0.069

The bold value stand for $p < 0.05$.

Reproducibility

We reanalyzed CT features of both smoking group and control group cases upon admission for intra- and interobserver reproducibility of semi-quantitative assessment.

Reproducibility of semi-quantitative assessment was excellent within observers (intraclass correlation coefficient, 0.988; 95% confidence interval: 0.982, 0.992) and moderate between observers (intraclass correlation coefficient, 0.977; 95% confidence interval: 0.966, 0.984). No intra- or interobserver bias was noted.

DISCUSSION

We comprehensively evaluated and analyzed the radiographic characteristics of 86 patients confirmed as having COVID-19 with or without current smoking status. The study demonstrated that patients with current smoking status presented more severe interstitial manifestations on CT images and may retain more residual lesion after being discharged (**Figures 2**, **3**).

Typical CT features of COVID-19 were well-established by former studies (12, 30), which were consistent with our study. Both the smoking group and control group presented GGO or mixed GGO and consolidation with bilateral, peripheral, and predominately lower lung involvement. The post-mortem biopsy in COVID-19 patients reported pulmonary edema and hyaline membrane formation in both lungs, which might be the underlying pathological driver of GGO sign (31). However, several differences were found between patients confirmed as having COVID-19 pneumonia with or without current smoking status. Quantitative calculation of infection ratios of lung segments and infection ratios of regions within different HU ranges suggested that the control group presented a larger infection ratio than the smoking group and tended to present more GGO and consolidation involvement. However, visual assessment of interstitial changes showed that the smoking group presented more interstitial changes than the control group such as septal thickening and reticulation in the early stage of infection. These interesting phenomena suggest that patients without smoking history in our cohort presented larger lung

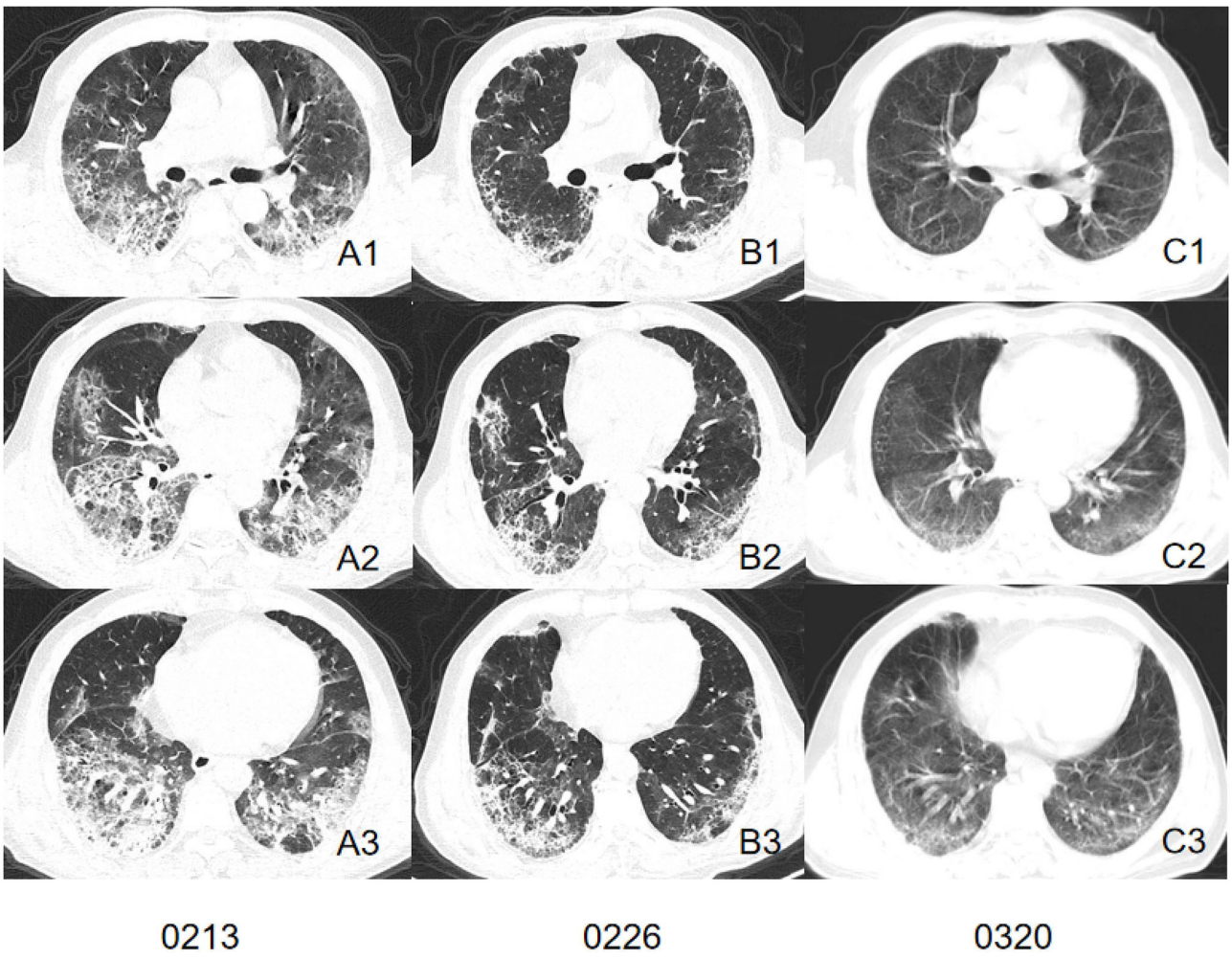

FIGURE 2 | CT images of a patient in smoking group. Initial CT imaging **(A1–A3)** obtained on Feb 13 showed multiple GGO, mixed GGO, and consolidation with bilateral, peripheral, and predominately lower lung involvement. Interlobular septal thickening and reticulation were visible. Follow-up CT when discharged **(B1–B3)** showed absorbed lesion. Interstitial changes remained visible. A follow-up CT conducted 23 days after discharged **(C1–C3)** showed remain residual lesion with subpleural bands involved peripheral area of both lung.

involvement on chest CT images while the smoking group presented more severe interstitial changes, which might have contributed to more residual lesions. The exposure to smoke has been shown to modulate immune and adaptive immune responses when compared with those who had never smoked (32, 33). There was one female (38-year-old) in the control group who died during hospitalization due to a sudden virus-activated "cytokine-storm syndrome." A previous study indicated that lack of exposure to smoke might partly contribute to a stronger immune response to SARS-Cov-2 infection and to the "cytokine-storm syndrome." In this regard, we may assume that the immune system of a current smoker is more tolerant and less reactive compared to patients who have never smoked, which could explain the larger lung involvement presented in the control group. Interestingly, we discovered that GGOs of the smoking group tended to present an uneasily differentiated margin while the control group presented a more defined margin of GGOs. This might suggest the potential of further progress is expected. In contrast, a well-defined margin indicates that the lung manifestations were more restricted (34).

With cigarette exposure, smoking-related lung disease has already occurred before infection, including emphysema and fibrosis. In our study, initial CT scans consisted of former studies with more appearances of emphysema and interstitial changes. Furthermore, an animal study discovered that cigarette smoke disrupted lung endothelial barrier integrity and increased susceptibility to acute lung injury (35). From our results, we discovered that the initial response to SARS-Cov-2 infection for smoking group tended to present more as interstitial changes than the control group since the interstitial scores were higher for smoking patients. Studies have shown that the location and expression of ACE2 was dramatically affected by smoking status. A study conducted by Liu et al. discovered that smoking dramatically upregulates ACE2 expression in the secretory

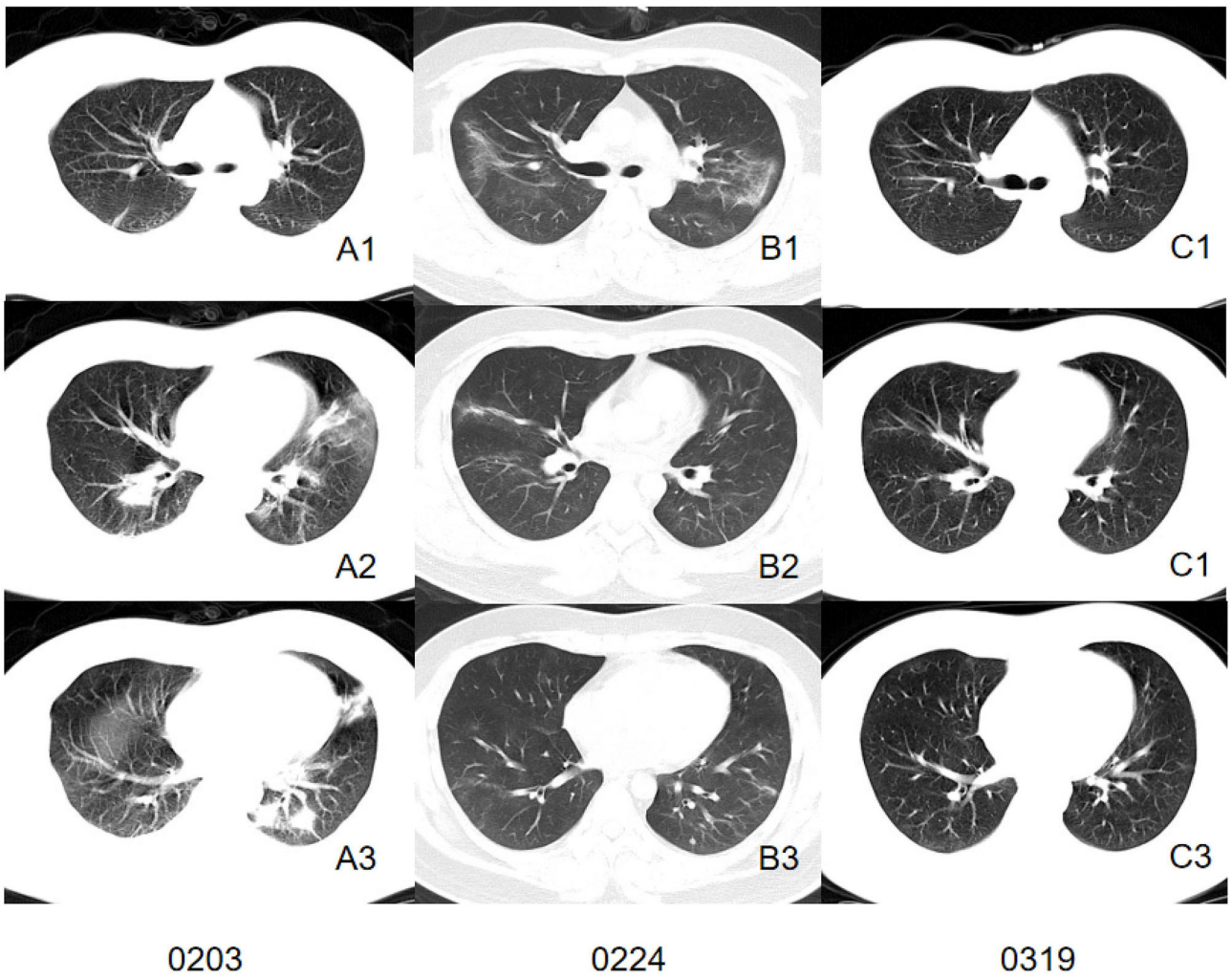

FIGURE 3 | CT images of a patient in control group. Initial CT imaging **(A1–A3)** obtained on Feb 3 showed multiple mixed GGO and consolidation with bilateral, peripheral, and predominately lower lung involvement. Interlobular septal thickening and reticulation were not found. Follow-up CT when discharged **(B1–B3)** showed absorbed lesion. A follow-up CT conducted 24 days after discharged **(C1–C3)** showed completely absorbed lesion.

club cells of the bronchial epithelium, and exacerbates several pathological changes including oxidative stress, hypoxia, and inflammation (36). This might explain the three deceased cases in the smoking group who presented progressing CT change pattern and persistent hypoxemia.

As more recovered COVID-19 patients try to re-embrace their normal life, there is an urgent need to consider the long-term care needs of those affected by COVID-19. At the time of writing, the long-term effects on recovering patients remain unknown. Previous studies reported that the sequelae of patients infected with severe acute respiratory syndrome coronavirus (SARS) and middle eastern Respiratory syndrome coronavirus (MERS) infection were associated with persistent abnormal radiographic change, substantial impairment of exercise and functional capacity, and reduced quality of life (37). Our study discovered that patients without a smoking history tended to have a better response to treatment. This was proven by the assessment of imaging change patterns concerning follow-up CT during hospitalization, which indicated that the involvement of lesions was often shown to be continuously absorbing in the control group, while the majority of cases in the smoking group showed progressive lesion involvement or progressing before absorbing. Also, the follow-up CT after discharge indicated that patients with a smoking history were more likely to have persisting abnormal radiographic changes for a longer time. Therefore, for patients with a current smoking history, more attention should be paid during treatment to prevent disease progression. Also, more frequent follow-up and rehabilitation medical care should be focused on those patients to improve their life-quality after recovery from COVID-19.

Our study had several limitations. Firstly, the population of the smoking group was relatively small due to limited cases presented to our center. Therefore, the effects of smoking on COVID-19 patients might be quite variable in the cohort. The specific impact of different smoking status on COVID-19 disease is controversial. Hypotheses support both a potentially

hazardous impact and a potentially protective effect (29). Our findings demonstrate that patients with a smoking history tend to have more interstitial lung change responses to SARS-Cov-2 infection and poor response to treatment during the course of the disease. Our conclusions need further investigation with a larger study population to be confirmed. We are unable to study differences between subgroups of different time periods of smoking history due to limited smoking cases. It might also explain that our analysis concerning correlations between semi-quantitative results, quantitative results, and smoking intensity showed no statistical difference. Since the number of patients with opposite outcomes is limited (with three deceased cases in the smoking group and one deceased case in the control group), the correlation analysis between CT features and different clinical outcomes is quite difficult to discuss in this cohort. Nevertheless, we are conducting a long-term follow-up study of recovered COVID-19 patients and we shall further investigate the correlations between quantifications of CT features and recovering progress. Secondly, the follow-up time after discharge was relatively short. A long-term follow-up is required. Lastly, more clinical information, such as lung function, should be included in future follow-up research.

In conclusion, patients with smoking history in our study tend to have more interstitial lung change responses to SARS-Cov-2 infection and poor response to treatment during the course of the disease. Follow-up CT images indicated that patients with a smoking history may retain more residual lesions. More support should be given for COVID-19 patients with smoking status during hospitalization and after being discharged.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethical Committee of Second Xiangya Hospital (Approved Number. 2020002). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JL, XX, and SW designed the research and revised the paper. XX, WZ, and ZZ performed the research and acquired the data. All authors drafted and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.663514/full#supplementary-material

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REVIEW
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Potential Effects of Coronaviruses on the Liver: An Update

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The coronaviruses that cause notable diseases, namely, severe acute respiratory syndrome (SARS), middle east respiratory syndrome (MERS) and coronavirus disease 2019 (COVID-19), exhibit remarkable similarities in genomic components and pathogenetic mechanisms. Although coronaviruses have widely been studied as respiratory tract pathogens, their effects on the hepatobiliary system have seldom been reported. Overall, the manifestations of liver injury caused by coronaviruses typically involve decreased albumin and elevated aminotransferase and bilirubin levels. Several pathophysiological hypotheses have been proposed, including direct damage, immune-mediated injury, ischemia and hypoxia, thrombosis and drug hepatotoxicity. The interaction between pre-existing liver disease and coronavirus infection has been illustrated, whereby coronaviruses influence the occurrence, severity, prognosis and treatment of liver diseases. Drugs and vaccines used for treating and preventing coronavirus infection also have hepatotoxicity. Currently, the establishment of optimized therapy for coronavirus infection and liver disease comorbidity is of significance, warranting further safety tests, animal trials and clinical trials.

Keywords: coronavirus, COVID-19, liver injury, liver diseases, drug hepatotoxicity

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INTRODUCTION

Coronavirus (CoV) is a family of viruses that display crown-like structures under electron microscopy, with an outer envelope and positive-stranded RNA as the genomic material (1). These viruses are found widely in many species, including humans, mice, pigs and other animals (2, 3). To date, 7 types of coronaviruses have been shown to cause disease in humans, of which 4 species (alpha CoVs: HCoV-NL63, HCoV-229E; beta CoVs: HCoV-OC43, HCoV-HKU1) can cause self-limiting respiratory symptoms in immunocompromised people, infants and older individuals (4). Another three species (SARS-CoV, MERS-CoV, and SARS-CoV-2) are highly pathogenic to humans, causing respiratory diseases, and the infection may lead to acute respiratory distress syndrome (ARDS), multiple organ failure (MOF) and even death in severe cases (5, 6). As coronaviruses have been widely studied as human respiratory pathogens, their involvement in the hepatobiliary systems needs further investigation.

The occurrence of recent coronavirus outbreaks has revealed that these viruses can mutate to become pathogenic in both humans and animals (7). As virus variations are inevitable and a part of the evolutionary process, outbreaks of coronaviruses will continue to emerge (8). SARS-CoV was the first causative agent of human pathogenic coronavirus outbreak globally, occurring in Guangdong Province of China in 2002–2003 (9), and it can cause severe respiratory syndrome with mortality rate of 9% (7). During this outbreak, ∼8,098 human cases of SARS were reported, and 774

of these patients died of the infection (10). The next coronavirus outbreak that followed the SARS-CoV outbreak was the MERS-CoV outbreak (11). Occurring in 2012, this outbreak involved severe infections in the respiratory tract of infected individuals in Saudi Arabia and other Middle East countries (12). The initial mortality rate of MERS-CoV was ∼50%, but the outbreak was over by 2013, with only a few sporadic cases since (13). Based on the latest update from the WHO, the total number of reported cases of MERS-CoV worldwide was 2,519,866 of the patients died, resulting in a mortality rate of 34.4% (14). The most recent coronavirus outbreak occurred in Wuhan, China, which was also known as the 2019-nCoV outbreak; the virus was recently renamed SARS-CoV-2, and the disease is referred to as COVID-19 (15). The first case of SARS-CoV-2 infection was reported in Wuhan, China, on 31 December 2019 with symptoms of atypical pneumonia (16). This case was later proven to be caused by a novel coronavirus, SARS-CoV-2. According to the WHO, as of 10 AM CET 2nd July 2021, 187,882,032 cases of COVID-19 have been reported, with 4,046,592 deaths, worldwide (17). There were 34,766,404 confirmed cases of SARS-CoV-2 infections in the USA, including 623,039 deaths. In terms of death related to COVID-19, after the USA, the greatest number of deaths due to COVID-19 has been reported in Brazil (534,233), followed by India (408,764).

Despite remarkably high genetic similarity between SARS-CoV and SARS-CoV-2 with regard to gene sequence, the speed at which SARS-CoV-2 spreads is much faster than that of SARS-CoV (18). This may be explained by differences in the structure of spike proteins (S proteins) among coronaviruses (19). The S protein is a 150-kDa protein that is highly N-glycosylated and plays roles in interaction with the endoplasmic reticulum (ER) and receptor attachment (20, 21). Usually, the S protein is cleaved into two functional domains (S1 and S2) by a host protease (furin-like protease) (22, 23). The presence of this special protease cleavage site activates S protein the priming and might improve the efficiency of SARS-CoV-2 transmission (24). The S protein also serves as a ligand on the coronavirus surface, which binds to angiotensin-converting enzyme 2 (ACE-2) (25). SARS-CoV and SARS-CoV-2 use the ACE2 receptor of the host cell, whereas MERS-CoV binds to dipeptidyl-peptidase 4 (DPP4) (26–29). After attaching to the cell membrane, the viral genome enters the cytoplasm and is translated to produce new virions, which can further lead to infection and respiratory disease (30, 31). This mechanism has become the most likely reason for multiple organ dysfunction in patients with coronavirus infection (31, 32).

Abbreviations: SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ACE2, angiotensin-converting enzyme 2; ARDS, acute respiratory distress syndrome; MOF, multiple organ failure; S proteins, spike proteins; ER, endoplasmic reticulum; Dpp-4, dipeptidyl-peptidase 4; ICU, intensive care unit; CCU, critical care unit; ALT, glutamic-pyruvic transaminase; AST, glutamic-oxalacetic transaminease; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; Ang II, angiotensin II; hDpp-4, human DPP-4; hrsACE2, human recombinant soluble ACE2; TNF, tumor necrosis factor; IL-6, interleukin-6; SIRS, systemic inflammatory reaction syndrome; HIRI, Hepatic ischemia–reperfusion injury (HIRI); ROC, reactive oxygen species (ROS); ALD, alcoholic liver disease; NAFLD, non-alcoholic fatty liver disease; ASCO, American Society of Clinical Oncology; ESMO, the European Society for Medical Oncology; ILCA, the International Liver Cancer Association; EASL, the European Association for the Study of the Liver; ASSLD, the American Association for the Study of Liver Diseases; IFNs, Interferons.

Liver Injury in Patients With Coronavirus Infection

Manifestations of Coronavirus-Related Liver Injury

Coronavirus infections are distinguished by continuous fever, cough, fatigue, dyspnea, arthralgias and decreased white blood cells in the serum (13, 33, 34). The severity of coronavirus infection is evaluated by the degree of respiratory symptoms and intensive care unit (ICU) admission (35, 36). It is notable that coronaviruses can influence not only the respiratory system but also the digestive, cardiac and endocrine systems (37, 38). Indeed, one study found that diarrhea occurred in 3.8% of COVID-19 patients and that 43.4% of patients had different degrees of liver function abnormality (39). Moreover, the incidence of liver injury in severe COVID-19 cases (74.4%) was higher than that of patients with mild disease (43.0%) (40). In cases of death due to COVID-19, the incidence of liver injury is 58% (40). According to the autopsy report of SARS patients, many virus particles were observed in the lung and the parenchymal areas and vascular endothelium of other organs, such as the liver (41, 42). The genome of SARS-CoV was also detected in liver tissue by RT-PCR (43). Among the three notable coronaviruses, acute liver injury has been mostly reported in MERS-CoV infection (44), and according to a study from Saad et al., 31.4% of patients have liver dysfunction during MERS-CoV infection (45). The common manifestations of liver injury caused by infections of the three coronaviruses are summarized in **Table 1**.

The latest studies on SARS-CoV-2 have indicated that the incidence of liver injury in patients with COVID-19 ranges from 14.8 to 53%, manifesting as abnormal glutamic-pyruvic transaminase (ALT), glutamic-oxalacetic transaminase (AST) and bilirubin levels (33, 53, 56). Moreover, compared with mild COVID-19 cases, severe cases show higher levels of plasma ALT and AST (57). The risk of being transferred to the ICU and critical care unit (CCU) is statistically correlated with elevated AST and bilirubin levels, and mortality correlates positively with elevated AST levels (58). Injury to bile duct cells and abnormal gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) levels have also been found in COVID-19 patients (57, 59, 60). This is a transient reaction, and therefore, the ALT levels of most patients usually return to normal after recovery (61). Patients with persistent high ALT level were in severe condition or has basic liver diseases, being found to have higher rates of 30-day mortality and longer hospitalization (62). Albumin is decreased in severe cases (∼26.3–30.9 g/L) and correlates with disease severity and mortality (36, 57, 63). Low levels of prealbumin in severe SARS-CoV-2 patients have also been reported, suggesting that hepatic synthesis is suppressed in these patients (53). Similarly, liver injury in SARS and MERS patients is characterized by mild increases in ALT, AST and bilirubin at the early stage of the disease (35, 44, 50, 51, 64–67). Moreover, great

TABLE 1 | The manifestations of coronavirus-induced liver injury.

	SARS-CoV-2	SARS-CoV	MERS-CoV
Pathological changes	Mild lobular infiltration by small lymphocytes, centrilobular sinusoidal dilation and patchy necrosis	Accumulation of cells in mitosis, ballooning of hepatocytes and mild lobular lymphocytic infiltration.	Moderate portal tract infection, lobular lymphocytic inflammation and hydropic degeneration of hepatic parenchymal cell
	ref: Tian et al. (46)	ref: Chau et al. (47)	ref: Ng et al. (48)
ALT	Elevated (affected proportion: 13.3–28.0%)	Elevated (affected proportion: 52.5–87.0%)	Elevated (affected proportion: 11.0–56.3%)
	ref: Guan et al. (33), Chen et al. (49)	ref: Jiang et al. (50), Liu et al. (51)	ref: Arabi et al. (52)
AST	Elevated (affected proportion: 22.0–58.0%)	Elevated (affected proportion: 37.1–86.9%)	Elevated (affected proportion: 15.0–86.8%)
	ref: Guan et al. (33), Chen et al. (49)	ref: Jiang et al. (50), Liu et al. (51)	ref: Arabi et al. (52)
TB	Elevated (affected proportion: 10.5–18.0%)	Elevated (affected proportion: 30.0%)	Not available
	ref: Guan et al. (33), Chen et al. (49)	ref: Jiang et al. (50)	
Albumin	Decreased (affected proportion: 36.8%)	Decreased (affected proportion: 40.4–72.0%)	Not available
	ref: Zhang et al. (53)	ref: Jiang et al. (50)	
Comorbidity with liver disease	The proportion of severe cases in patients with HBV comorbidity is higher than that of patients without HBV infection. (32.9 vs. 15.3%)	Chronic hepatitis B was not associated with worse clinical outcomes.	Not available
	ref: Wu. et al. (54)	ref: Huang et al. (55)	

ALT, glutamic-pyruvic transaminase; AST, glutamic-oxalacetic transaminease; TB, total bilirubin.

elevation of liver enzymes is an independent factor correlating with a poor prognosis of patients with SARS (68). Although age and pre-existing diseases have been proven to have a significant negative influence on the prognosis of SARS patients, patient age was not significantly different between those with high or low peak ALT levels (69, 70). In a cohort of severe MERS patients, 50% exhibited elevated aminotransferase levels during their time in the ICU (52, 71, 72). Saad et al. illustrated that decreased albumin level is a predictive factor of the severity of MERS (73).

Regarding pathological changes, liver biopsies of SARS patients revealed dramatic increases in eosinophilic bodies and balloon-like hepatocytes, indicating that coronavirus might cause necrosis of hepatocytes (47). Some studies showed that protein 7a, a special protein of SARS-CoV, can also induce necrosis of cell lines belonging to various organs (74, 75). Mild microvascular steatosis and moderate lobular and portal inflammation have been found in the livers of patients with SARS-CoV-2 infection (46). Similar to the observations for SARS and COVID-19 patients, the pathological changes in MERS patients include moderate portal tract infection, lobular lymphocytic inflammation, and hydropic degeneration of hepatic parenchymal cells (48, 76). The definitive mechanism by which liver injury develops in patients with coronavirus infection is unclear, and several pathophysiological theories may explain this phenomenon (**Figure 1**, **Table 2**).

Pathogenic Mechanisms of Coronavirus-Related Liver Injury

ACE2/DPP4-Mediated Damage to Hepatocytes

RAS proteins are encoded by Ras sarcoma oncogenes and belong to a group of small GDP/GTP-binding guanine triphosphatases, which play an essential role in cellular biological behaviors such as proliferation, migration, adhesion, and differentiation (83). Abnormal signaling of RAS occurs in numerous human diseases (84). ACE2 plays an important role in the RAS signaling pathway by upregulating angiotensin II (Ang II), which promotes atherosclerosis, inflammation, and migration of endothelial cells (85). ACE2 is widely present in humans, including in alveolar epithelium, intestinal epithelium and arterial smooth muscle cells (86). Furthermore, it has been confirmed that ACE2 receptors are over-expressed in gastrointestinal epithelium enabling viruses to invade bile duct cells and suppress liver function (40, 86). Herath et al. reported both liver tissue and bile duct epithelium express ACE2 (87). However, the ACE2 expression level in bile duct epithelium was significantly higher than that in liver tissue (88). As bile duct cells play essential roles in hepatic regeneration and the immune activities, upregulation of ACE2 expression in hepatocytes can lead to compensatory proliferation originating from bile duct cells, resulting in liver injury (88, 89). Although SARS-CoV and SARS-CoV-2 can cause liver function abnormality through binding to the ACE2 receptors of bile duct cells, viral inclusions were not observed in the liver biopsies of COVID-19 patients (46). These results indicate that liver injury in patients with coronavirus infection may be the result of bile duct epithelium damage rather than hepatocyte changes (90). Numerous literature have reported liver cirrhosis can also dramatically upregulate ACE2 expression in hepatocytes (91–93). In the normal human liver, ACE2 stains weakly and is limited to the bile duct cells, vascular endothelial cells, and perivenular hepatocytes (86, 87). In the cirrhotic liver, ACE2 staining can be observed in the majority of hepatocytes in the cirrhotic nodules, bile duct and vascular endothelium (88). High expression of

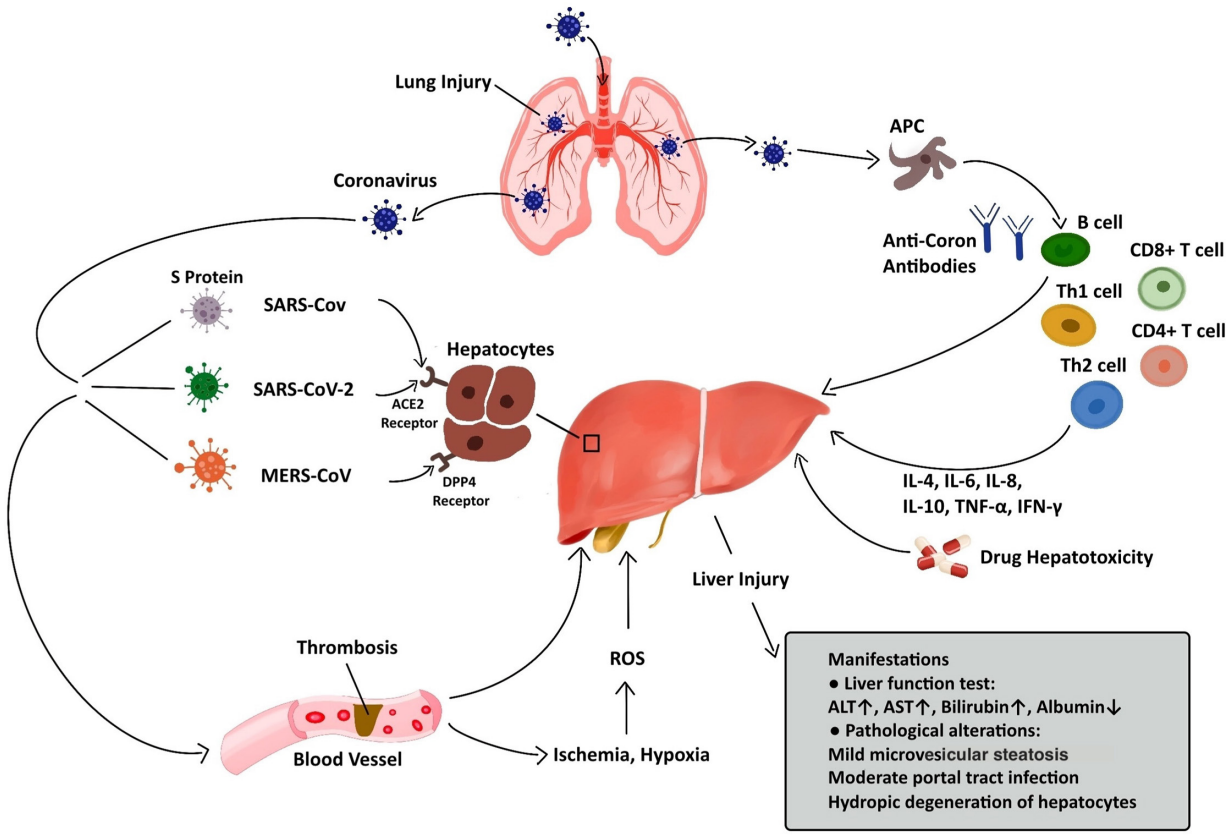

FIGURE 1 | Liver injury caused by coronavirus infection. After entry into the human body through the respiratory tract, coronaviruses can lead to liver injury via several approaches, including ACE2/DPP4-mediated hepatocyte injury, immune-mediated liver injury, ischemia and hypoxia, thrombosis, and drug hepatotoxicity. The manifestations of liver injury involve abnormalities in liver function test and pathological examination.

ACE2 helps more coronaviruses invade hepatocytes and leads to greater virulence of coronaviruses in the liver (87, 94, 95). Thus, patients with both liver cirrhosis and coronavirus infection may have greater extents of liver dysfunction and even higher risks of liver failure compared with normal people (96, 97).

Dipeptidyl peptidase 4 (DPP-4) cleaves a large number of chemokine and peptide hormones involved in the regulation of the immune system (98). DPP-4 is upregulated in the liver, indicating that the liver might be a target organ of MERS-CoV (99, 100). A scientific team built a transgenic murine model which expressed codon-optimized human DPP-4 (hDPP-4) and observed that MERS-CoV can invade into the hepatocytes through DPP-4 and cause hepatocytes injury (101). The hDPP-4 transgenic mouse exhibited mild hepatic injury on the 5th day after MERS-CoV infection, and the pathological manifestations were scattered necrosis of hepatocytes in sinuses and infiltration of numerous macrophages and Kupffer cells (102, 103). On the 9th day, although hepatocytes necrosis was less, fatty changes in hepatocytes were also found (104).

Immune-Mediated Injury

When coronaviruses invade the human body, they activate the immune system, triggering a series of immune activities to eliminate the virus (105–107). The liver plays an essential role in immune activities and contains numerous immune cells that participate in the immune response (108–110). The hepatic acute-phase response (involving cytokines released from immune cells) is a defense reaction to fight against the pathogen and protect vital liver functions (111, 112). T cells play important roles in the anti-coronavirus immune responses, and the balance between the anti-coronavirus response and immune tolerance is maintained by the differentiation of CD4+ and CD8+ T cells (113). During the process of SARS-CoV-2 infection, 80% of immune cells that infiltrate into the liver are CD8+ T cell, and these cells could survive in the inflamed tissue (114). The decrease in the infiltration of CD4+ T cell can lead to depressed B cell activation, along with reduced level of SARS-CoV-2-specific neutralizing antibody and pro-inflammatory cytokine (such as IL-1, IL-6, and TNF-α), so as to affect the clearance of SARS-CoV-2 from the liver (115). Compared with SARS-CoV-2, CD4+ T cell is more susceptible than CD8+ cell during the processes of MERS-CoV and SARS-CoV infections. Liver cells in patients with severe coronavirus infection show various inflammatory changes, such as swelling and steatosis in hepatocytes, proliferation in liver sinus cells, hyperplasia in Kupffer cells and infiltration in immune cells (40, 46, 116).

TABLE 2 | The mechanisms of coronavirus-induced liver injury.

Pathogenic mechanism	Coronavirus type	References	Highlights
ACE2/DPP4-mediated direct injury of hepatocytes	SARS-CoV	Li et al. (27)	ACE2 was shown to be the functional receptor of SARS-CoV. ACE2 will likely contribute to the development of antivirals and vaccines.
	SARS-CoV-2	Bourgonje et al. (28)	ACE2 has been established as the functional host receptor for SARS-CoV-2. ACE2 expression and activity are related to COVID-19 severity. ACE2 inhibitor is a selection of potential treatment modalities for COVID-19.
	MERS-CoV	Wang et al. (29)	The receptor-binding subdomain is critical for viral binding to DPP4 and entry into the target cell.
Immune-mediated injury	SARS-CoV	Duan et al. (68)	IL-1, IL-6, and IL-10 in the serum of SARS patients with abnormal liver function were higher than those in patients with normal liver function.
	MERS-CoV	Mahallawi et al. (77)	IFN-γ, TNF-α, IL-15, and IL-17 were significantly increased in those infected by MERS-CoV.
	SARS-CoV-2	Huang et al. (38)	TNF-α, IFN-γ, IL-6, IL-8, IL-4, and IL-10 were dramatically elevated in COVID-19 patients.
Thrombosis	SARS-CoV, SARS-CoV-2, and MERS-CoV	Giannis et al. (78)	There was a great proportion of patients with hypercoagulable states after coronavirus infection.
	SARS-CoV-2	Llitjos et al. (79)	Elevated D-dimer level and thrombocytopenia were observed in some COVID-19 patients.
Drug hepatotoxicity	SARS-CoV, SARS-CoV-2, MERS-CoV	Sheahan et al. (80)	The anti-corona acitivities of remdesivir has been reported. Redesivir can cause elevation in aminotransferases.
	SARS-CoV	Cao et al. (81)	Lopinavir/Ritonavir can cause elevation in serum amylase and liver enzymes.
	SARS-CoV-2	Fan et al. (39)	A significantly higher proportion of patients with abnormal liver function had received lopinavir/ritonavir after admission.
	SARS-CoV-2	Xu et al. (82)	Tocilizumabcan cause mild elevation in serum aminotransferase, jaundice and occasional reactivation of hepatitis B.

ACE2, angiotensin-converting enzyme 2; DPP-4, dipeptidyl-peptidase 4.

Cytokines can also induce ischemia and hypoxia, which lead to hepatocyte injury and necrosis (117).

Abnormal serum levels of cytokines and chemokines (such as tumor necrosis factor (TNF), interleukin-6 (IL-6), and IL-18) have been detected at the early stage of coronavirus infection (111, 118). Duan et al. found that the concentrations of IL-1, IL-6, and IL-10 in the blood of SARS patients with hepatic dysfunction were higher than those in patients with normal hepatic function, demonstrating the relevance between hepatic injury and the cytokine storms caused by SARS (68). The levels of IL-2-receptor and IL-6 in the serum of patients with SARS-CoV-2 infection were also be found to be elevated and relate to the disease severity (119). Moreover, cytokines secreted by Th1 and Th2 cells (involving TNF-α, IFN-γ, IL-6, IL-8, IL-4, and IL-10), are dramatically elevated in patients with SARS-CoV-2 infection [38]. During the acute phase of MERS-CoV infection, the levels of IFN-γ, TNF-α, IL-15, and IL-17 in the serum of patients were dramatically elevated (77). These results suggest that the systemic inflammatory reaction syndrome (SIRS) and cytokine storms caused by coronavirus infection may be critical mechanisms of liver injury (68, 120, 121). Nonetheless, there is a lack of research on the relationship between pro-inflammatory cytokine activity and liver injury.

Ischemia and Hypoxia

Patients with SARS-CoV-2 infection exhibit different extents of hypoxemia, with more than 40% of patients receiving oxygen treatment (117). Hypoxic liver injury can be marked by increased transaminases in the serum due to dysregulation of the oxygen supply (122). Complications of COVID-19, including ARDS, SIRS and MOF, can lead to hypoxemia, ischemia and shock (123, 124), and microthrombi can disrupt perfusion within the liver. Hepatic sinus endothelial cells also play roles in the occurrence of this phenomenon, as they can respond to inflammatory signals (such as endotoxins with endothelium dysfunction, characterized by reduced vasodilatory responds to acetylcholine and reduced nitric oxide synthase phosphorylation). Hepatic ischemia–reperfusion injury (HIRI) is another familiar pathological process, whose mechanism is closely correlated with reactive oxygen species (ROS), neutrophils, Kupffer cells, and overloaded calcium. HIRI can lead to inflammation and cell injury by activating Kupffer cells, neutrophils, and platelets. Under

the circumstance of ischemia and hypoxia, the cell survival signaling pathway in hepatocytes can be inhibited by glycogen consumption and adenosine triphosphate depletion, resulting in necrosis of these cells (125). Moreover, for patients who develop ARDS, hypoxia can cause oxidative stress responses that facilitate a persistent elevation of ROS (126). ROS and their peroxidized forms can arouse regulation of redox reactions and promote the secretion of pro-inflammatory substances to cause hepatic injury (127, 128). These pathophysiological changes may accentuate liver ischemia and hypoxia, influencing the secretion of hepatotoxic substances and so as to affect hepatic function (125, 129).

Thrombosis

SARS-CoV, MERS-CoV, and SARS-CoV-2 have been reported to lead to hypercoagulable states in patients, thus increasing the chance of thrombosis (78). Previous studies on COVID-19 have shown that 36.2% of patients developed thrombocytopenia, 46.4% of patients had increased D-dimer levels during infection, and the rates were higher in severe than in mild cases (79). It has recently been reported that microvascular thrombosis can lead to end-stage organ injury and can potentially influence hepatic function (130, 131). In the past, elevated levels of serum ALP was considered as a prognostic factor for ischemic stroke and a risk factor for hemorrhagic transformation (132). COVID-19 patients who experienced thrombotic events had dramatically high levels of ALP, though ALP levels were normal or only mildly increased in patients without thrombotic events (133). Recent data suggest that COVID-19 patients have a greater chance of developing disseminated intravascular coagulation (134, 135). Elevated D-dimer levels, the level of degradation products of fibrin, and prolonged prothrombin time have also been shown to be correlated with worse prognosis of patients with SARS-CoV-2 (136). The results of autopsies from Wuhan have revealed lymphocytes and monocytes infiltration in the portal area, with thrombosis and congestion in the sinuses (116). The liver was found to have hepatocyte degeneration along with lobular necrosis and neutrophil infiltration (46, 116). These findings suggest that hypercoagulable states in patients with COVID-19 are a potential reason for liver injury.

Drug Hepatotoxicity

Drug hepatotoxicity is the third leading cause of liver injury after viral hepatitis and alcoholic/non-alcoholic fatty liver disease (137). Based on many clinical studies and animal experiments, several types of drugs have been proven to cause liver injury, including antibiotics, anti-tumor drugs, saikosaponins, anti-tuberculosis drugs, and anti-malarial drugs (138–140). Until now, there are no effective therapeutic treatments for patients with SARS (141, 142). Drugs that were mostly chosen for SARS patients were ribavirin and corticosteroids (143). Ribavirin was used because it had a broad spectrum of activity against RNA viruses, and steroids were chosen because of their anti-inflammatory functions (144, 145). Nevertheless, ribavirin is correlated with obvious hepatotoxicity, including hemolysis, resulting from discontinuation of its use (143). Most patients with SARS-CoV-2 infection have fever and take antipyretic drugs that contain acetaminophen (38, 146). Acetaminophen is known to lead to liver injury, and acetaminophen overdose can induce serious liver injury or even liver failure (147). COVID-19 patients have been treated with lopinavir, abidor, ritonavir, and other antiviral drugs (146). Furthermore, some scientists proposed that HIV protease inhibitors might efficiently inhibit the replication of SARS-CoV-2 (148, 149). However, Shen et al. proved the chance of having liver injury was increased in patients who received both hormone therapy and HIV protease inhibitors (150, 151). Intravenous methylprednisolone was also reported to correlate with acute liver injury, but evidence on the correlation between oral methylprednisolone and liver injury is insufficient (152). We have summarized the effects of several anti-corona drugs on liver function in **Table 3**. Some clinical trials on anti-SARS-CoV-2 drugs are still ongoing (**Table 4**).

EFFECTS OF CORONAVIRUS INFECTION ON PRE-EXISTING LIVER DISEASE

Chronic liver disease is one of the biggest disease burdens, accounting for about 1 million deaths per year worldwide (156–158). As a result, the influence of coronaviruses on various pre-existing liver diseases needs to be further explored; evidence of active viral replication and persistent liver injury after coronavirus infection also calls for further investigation (159). For patients with pre-existing liver diseases, the addition of coronavirus-directed or immune-response-directed liver injury may lead to further hepatic dysfunction, especially for patients with advanced liver diseases. As an example, experience obtained from the SARS pandemic in 2003 showed that comorbidity with hepatitis B can cause more severe liver injury (160). However, if the liver injury caused by COVID-19 is immune-response-directed, the immunocompromised condition of cirrhosis patients and cancer patients may be more beneficial than detrimental (161). Moreover, patients with liver cirrhosis or liver cancer are usually in an immunocompromised state and may be more susceptible to SARS-CoV-2 infection (162, 163). Clinical practice guidance regarding liver disease has been given to healthcare professionals by relevant societies worldwide, including the American Society of Clinical Oncology (ASCO), the European Society for Medical Oncology (ESMO), the International Liver Cancer Association (ILCA), the European Association for the Study of the Liver (EASL), and the American Association for the Study of Liver Diseases (AASLD) (164–169). Here, we summarize the effect of coronavirus infections on the occurrence, development and treatment of four types of liver diseases: viral hepatitis, liver cirrhosis, hepatocellular carcinoma and liver transplantation.

Effect of Coronaviruses on HBV and HCV Hepatitis

HBV and HCV are chronic infections that occur frequently worldwide, with 2 billion people infected and 350 million having chronic infection (170, 171). One study indicated that 3.6 and 0.6% of patients with COVID-19 had a history of hepatitis B and hepatitis C, respectively (172). In a study about hepatic

TABLE 3 | Drug-induced abnormal liver function in patients with coronaviruses infection.

Drugs for coronaviruses infection	Number of cases	Proportion of liver injury	ALT	AST	ALP	Total bilirubin	γ-glutamyltransferase	References
Remdesivir	387	34.0% (130/387)	20.4% (79/387)	20.4% (79/387)	Not available	1% (4/387)	Not available	(153)
Lopinavir/ritonavir	148	37.2% (55/148)	18.2% (27/148)	21.6% (32/148)	4.1% (6/148)	6.1% (9/148)	17.6% (26/148)	(39)
Interferon	31	38.7% (12/31)	38.7% (12/31)	29.0% (9/31)	32.3% (10/31)	16.1% (5/31)	Not available	(154)
Baricitinib	12	58.3% (7/12)	58.3% (7/12)	50.0% (6/12)	Not available	Not available	Not available	(155)
Tocilizumab	20	5.0% (1/20)	5.0% (1/20)	5.0% (1/20)	Not available	Not available	Not available	(82)

ALT, glutamic-pyruvic transaminase; AST, glutamic-oxalacetic transaminease; ALP, alkaline phosphatase.

TABLE 4 | The efficacy and current status of COVID drugs.

Drugs	Mechanisms	Efficacy	Clinical trials
Remdesivir	Inhibiting viral replication by interfering RNA polymerases	To be tested in clinical trials	NCT04292899 NCT04257656 NCT04292730
Lopinavir/Ritonavir	Inhibiting viral replication by interfering protease	Inconsistent results in completed clinical trials	NCT04343768 ChiCTR2000029308
Interferons	Directly inhibit viral replication and transmission and support immune responses to clear viruses.	To be tested in clinical trials	NCT04389645 NCT04276688
Baricitinib	JAK inhibitors	Proven efficacy (baricitinib plus remdesivir)	NCT04401579
Tocilizumab	Humanized mAb targeting IL-6	Do not improve survival	NCT04372186
rhACE2	Completely bind to viral S-protein	To be tested in clinical trials	Not available

biochemical parameters in 324 cases in Shanghai, the percentage of COVID-19 patients with HBsAg positivity was 6.5% (173). Thus, the influence of coronavirus infection on the course of HBV and HCV has attracted widespread attention (174, 175). SARS patients with HBV and/or HCV infection had a higher risk to get liver injury and severe hepatitis because hepatitis virus replication was promoted during SARS-CoV coinfection (55, 67). However, considering coinfection of SARS-CoV, no significant differences in various adverse clinical outcomes between chronic hepatitis B patients and HBsAg-negative patients were detected (176). SARS patients with acute hepatitis and/or decompensated liver cirrhosis have a greater chance to be dead (47). A research team reported that 23/1099 SARS-CoV-2 patients in Wuhan were coinfected by HBV, representing 2.4% of mild cases and 0.6% of severe cases (174). COVID-19 patients also had a higher mortality rate than that of HBV-negative patients (32.9 vs. 15.3%) (54). Liu et al. found that the median time of virus clearance (21 days, 95% CI: 19–29) in COVID-19 patients with HBV infection was longer than that in patients without HBV infection (14 days, 95%, CI: 13–21) (177). These results indicate that coronavirus infection and viral hepatitis interact; thus, exploring the underlying mechanism will be meaningful for optimizing treatment guidance for COVID-19.

The presence of coronavirus infection and complications should be factors considered when doctors develop tailored treatment plans for patients with HBV and/or HCV infection (178). According to the AASLD guidance, we should initiate anti-HBV/HCV therapy in patients under three states: 1) newly diagnosed cases of HBV/HCV; 2) patients without SARS-CoV-2 infections; 3) if resources (involving drug treatments, personnel for approval of therapy, blood testing, follow-up facilities through telemedicine or face-to-face) have not been deployed for SARS-CoV-2 infection (168). HBV reactivations after using tocilizumab or prednisone have been reported in patients with HBV infection; therefore, these two drugs should not be used to avoid HBV reactivation (179). Additionally, according to guidance from AASLD, long-term HBV therapy can be employed for patients with newly diagnosed HBV hepatitis and continued if the patients receive the therapy plan, regardless of whether the patients are infected by SARS-CoV-2 (168). Therefore, therapy guidance for COVID-19 patients with advanced liver disease needs to be established to minimize the risk of liver injury or even liver failure, as both the advantages and disadvantages of an intervention are vital during the treatment of COVID-19.

For hepatitis B patients who are undergoing antiviral treatment and high-dose hormone therapy, discontinuation of anti-HBV therapy might cause reactivation and replication of HBV during SARS-CoV-2 infection (180). Indeed, studies have pointed out that treating HBV/HCV patients with lopinavir and ritonavir can increase the incidence of liver injury (181–183). A clinical study showed that long-term application of ribavirin can lead to serious drug hepatotoxicity in HCV patients, which may be due to metabolic reactions in the body (184). In addition, patients with HBsAg positivity and hepatitis B core antibody positivity treated with corticosteroids showed a higher risk of HBV reactivation, and the incidence of HBV reactivation

correlates with the dosage of corticosteroid treatment (177, 180). Therefore, the clinical status of chronic HBV infection should be systematically evaluated in the setting of corticosteroid use, and nucleotide analog treatment should be taken into consideration to reduce the risk of HBV reactivation or hepatitis flare.

Effect of Coronaviruses on Liver Cirrhosis

It is known that liver cirrhosis is one of the leading causes of death and illness globally; thus, exploring how coronavirus infection influences the course of liver cirrhosis is of great importance (162, 185). For patients with liver cirrhosis and coronavirus infection, the severity of COVID-19 and the incidence of severe complications increase, resulting in a higher liver-related mortality rate compared to patients with COVID-19 alone (49). A clinical study demonstrated that SARS-CoV-2 infection can lead to rapid deterioration in patients with relatively stable liver cirrhosis: 25 COVID-19 patients with Child-Pugh A presented rapid deterioration in hepatic function, and the Child-Pugh scores of over 30% of them increased to B or C after COVID-19 diagnosis (96). The last hospital admission or follow-up visit before COVID-19 diagnosis provides evidence for the importance of SARS-CoV-2 infection in deteriorating hepatic function, which can usually be seen in patients with liver cirrhosis of any etiology (186). However, more evidence is needed to completely clarify the effect of elevated ALT on the disease course of patients with liver cirrhosis and COVID-19 and to further explain the pathogenic mechanism by which coronavirus induces hepatocyte injury (57). The potential cytopathic effect has been demonstrated, as numerous ACE2 receptors might help SARS-CoV-2 enter host liver cells (32). Alternatively, the liver might be indirectly involved in acute inflammatory activity after SARS-CoV-2 infection, as it becomes infiltrated with a large number of macrophages, potential cytokine producers (107).

To date, drugs that have been widely used for COVID-19 treatment included chloroquine, lopinavir/ritonavir, ribavirin, favipiravir, remdesivir, and tocilizumab et al. (146, 187). As the majority of these drugs are metabolized in the liver, abnormal hepatic function might increase the risk of drug hepatotoxicity in COVID-19 patients (188). It is worth noting patients with pre-existing liver diseases, especially liver cirrhosis with Child-Pugh B/C, have a greater chance of experiencing adverse reactions to the above drugs (189). As a result, close and frequent monitoring of hepatic bio-parameters in patients can help in the notification of liver injury and reduce the risk of adverse effects and optimize drug dosages (137). It is recognized that endoscopic variceal screening in healthy individuals should be restricted to people with high risk for variceal bleeding, as well as those with histories of variceal bleeding or portal hypertension (190); otherwise, non-invasive examinations for the diagnosis should be performed (191). To decrease the risk of spreading infection, endoscopy examination in COVID-19 patients need to be restricted to critical situations such as gastrointestinal bleeding.

Effect of Coronaviruses on Liver Cancers

Patients with liver cancers also have a high risk of coronavirus infection, especially if they receive chemotherapy or immunotherapy in the hospital (192). The incidence of COVID-19 in cancer patients at a hospital in Wuhan was 0.79% (12/1,524), higher than that of the whole community during the same period (193). Owing to the serous spread of the COVID-19 pandemic, <50% of them were being continuously treated for their cancer (194). Furthermore, cancer patients have poorer prognosis than patients with COVID-19 alone, with the mortality rate ranging from 5 to 20% (195).

Patients with liver cancer should accept special treatments and take interventions to prevent severe complications of COVID-19 (196). In patient-saturated hospitals, the shortage of clinical and medical resources has largely impeded normal radiological examination, pathological diagnosis and anticancer treatment for patients with liver cancer (197). EASL, ESMO, and ILCA have provided specific guidance for the surveillance, examination and treatment of liver cancer patients with SARS-CoV-2 infection (165, 167, 169, 198). According to the Society of Surgical Oncology, all patients with aggressive liver, pancreatic or gall bladder cancers should undergo surgery (199). For patients who need surgery as well as systemic chemotherapy, neoadjuvant chemotherapy should be considered to delay the surgery.

Screening for esophageal varices and liver cancers is now delayed for all but high-risk patients (186). AASLD guidance suggests that it is appropriate to delay liver cancer surveillance for 2 months after evaluation of the advantages and disadvantages of initiating liver cancer surveillance in COVID-19 patients. Some retrospective studies have indicated that semiannual surveillance can increase the possibility of early detection and improve patients' survival compared with annual surveillance (200). Therefore, delaying screening for over 1 year may lead to progression of liver cancer, resulting in the miss of the best time for operating and even liver failure or death. Delaying HCC surveillance over short periods of time is likely acceptable as the annual HCC incidence is 2–3%, meaning that 98% of people will not develop HCC in a surveillance interval (201). These changes in treatments have potentially increased the risk for variceal bleeding and distant metastasis of liver cancer. Additionally, living donor liver transplantation and locoregional therapy for liver cancers have been delayed in many institutions, possibly increasing both the progression and mortality of liver cancer (168). Selective strategies included using serum biomarkers, increasing outpatient interventions (such as albumin infusions), and integrated telehealth are being strongly recommended by many institutions (202).

Effect of Coronaviruses on Liver Transplantation

After liver transplantation, patients have a greater chance to be infected and/or get severe course of COVID-19 because of their immunosuppressed state (203). These patients are being treated with immunosuppressive drugs and are considered to have greater chances of contracting SARS-CoV-2 infection, resulting in serious complications (1.4% death, 5.0% admitted to the ICU and 15.7% severe disease) (204). Gwilym et al. performed a study involving 151 liver transplant recipients, and reported that previous liver transplantation does not correlate independently

with the mortality of COVID-19 patients (205). In contrast, age and clinical comorbidities were independently correlated with COVID-19-related death in other studies (121, 206). In living donor liver transplantation, ACE2 is a substitute marker for liver regeneration and is upregulated in liver tissue and serum (207). Therefore, during the early postsurgical stage, both liver transplant recipients and donors are more likely to develop SARS-CoV-2 infection because of their elevated ACE2 expression (208). Undiscovered SARS-CoV-2 infection of recipients can increase the risk of developing serious immunosuppression and postsurgical infection, which might cause multiple system organ injury or failure (186). Additionally, a donor with undiscovered SARS-CoV-2 infection may transfer the virus to recipients.

It is reported that using immunosuppressive medicines can modulate the inflammatory activity against SARS-CoV-2 infection (206), and the potential adverse effects need to be considered for liver transplant recipients as well (209). The application of early treatment may also serve as an essential step for the prevention of severe pneumonia in liver transplant recipients (210–212). It is recommended that patients with pre-existing liver disease rapidly receive antiviral treatment (207). According to EASL-ESCMID, special drugs recommended for the treatment of COVID-19 after liver transplantation include remdesivir, chloroquine/hydroxychloroquine with or without azithromycin, lopinavir/ritonavir, tocilizumab et al. (213, 214). Strict screening criteria for organ recipients and donors with coronavirus infection needs to be set to avoid further transmission (215).

Effect of Coronaviruses on Alcoholic Liver Disease and Non-alcoholic Fatty Liver Disease

Patients with alcohol use disorder (AUD) or alcohol liver disease (ALD) are special components of the population with liver diseases (216). The COVID-19 pandemic has resulted in a social environment that leads people to drink at home. Selling of alcohol has increased by 55% in the week ending March 21 compared with the same time last year (217). A Chinese initial report has showed an over 2-fold increase in harmful drinking during the COVID-19 pandemic (218). Same effect was also seen in the USA, in which AUD and ALD is responsible for the highest hospitalization-cost among all chronic liver diseases. ALD patients usually have underlying medical conditions which can lead to higher risks of severe SARS-CoV-2 infection, including obesity with metabolic syndromes, chronic kidney diseases, and corticosteroid treatment for alcoholic hepatitis (219). Actually, patients with severe alcoholic hepatitis should not be treated with standard corticosteroid, especially in localities that are mostly affected by COVID-19 pandemic.

NAFLD is a chronic dysmetabolic disease which has become the most common liver disease in the world, with a prevalence rate of 30% in the western world (220). In addition, NAFLD is not isolated, it is often related to a series of risk factors, metabolic syndromes, and other diseases. The risk of severe SARS-CoV-2 infection also increases by the comorbidity of NAFLD (221). According to a report on 202 COVID-19 patients and their NAFLD status, COVID-19 progression was associated with male sex, age >60, higher BMI, and NAFLD (222). This study also indicated that NAFLD is an independent risk factor for COVID-19 progression (OR 6.4; 95% CI 1.5–31.2). NAFLD is also related to higher risk of abnormal hepatic function and longer clearance time of viruses. In another research, the moderate or high Fibrosis 4 (FIB-4) score can significantly and independently increase the risk of severe COVID-19 progression (223). Therefore, patients with NAFLD show a distinct risk as their metabolic dysfunction and underlying hepatic disorder.

EFFECTS OF ANTI-CORONAVIRUS TREATMENTS ON THE LIVER

Remdesivir

Remdesivir is an antiviral drug which is undergoing clinical trials for treating SARS-CoV-2 infection (224, 225). It was first used for treating Ebola virus infection with clinical experiments still on (226, 227). Results from ongoing experiments *in vitro* and *in vivo* demonstrated the activity of remdesivir against Paramyxoviruses, Filoviruses, and Coronaviruses (80). One study reported adverse events in three patients after using remdesivir, including nausea, vomiting, gastroparesis, and rectal bleeding (153). They also presented increased ALT and AST levels at 1–5 days after receiving the drug (228). However, it remains unclear whether this biochemical change was due to remdesivir or the virus because a large percentage of severely COVID-19 cases develop hepatic dysfunction. At present, there are insufficient data to give a definite adverse effect profile for remdesivir. Conclusive evidence of its effectiveness and adverse effects and calls for further clinical trials (229).

Lopinavir/Ritonavir

Lopinavir and ritonavir, inhibitors of the HIV protease, are two HIV-1 drugs approved by the FDA (230, 231). Recently studies found that the antiprotease activity of these two drugs seem to be effective to against the SARS-CoV-2 (232). The adverse effects observed in ICU patients involved pancreatitis, hepatitis, liver decompensation, prolonged PR intervals and congenital QTc prolongation (233). Previous studies found that the serum amylase and hepatic enzymes were elevated in SARS patients using lopinavir/ritonavir (81). A recent study indicated that CYP3A4 metabolic pathways played essential roles in ritonavir-mediated hepatotoxicity (234). CYP3A participates in the generation of electrophilic content and oxygen free radicals, which covalently bind to macromolecular substances within hepatocytes, causing membrane lipid peroxidation and destruction of membrane integrity (210). Lopainavir/Ritonavir can also act on Ca^{2+}-ATPase on the cell membrane, disrupt the balance between internal and external Ca^{2+} concentrations and dysregulate the biofunction of key organelles (including mitochondria and ER), resulting in injury or even necrosis of hepatocytes (233). Furthermore, overdose of lopinavir/ritonavir can stimulate ER stress pathways in the liver, inducing liver necrosis and inhibiting hepatocyte proliferation (233), and it also initiates inflammatory responses and worsens liver injury by aggravating oxidative stress (235). Several clinical experiments

proved that the combined use of lopinavir/ritonavir with other drugs is effective in patients with COVID-19 (183, 232, 236). However, a study reported that the usage of lopinavir/ritonavir combined with arbidol cannot efficiently promote clearance of SARS-CoV-2 in patients (237). Administration of lopinavir and ritonavir seems to only be beneficial for patients who are at the early-stage of SARS-CoV and MERS-CoV infection (182, 238). Fan et al. found that a high percentage of patients exhibited abnormal levels of hepatic enzymes (57.8%) after receiving lopinavir/ritonavir compared with patients with normal liver function (31.3%) (39).

Interferons

Interferon (IFN) is a type of endogenous signaling molecules secreted by host cells during the immune response to pathogen (239). Increased IFN levels activate the immune system to clear pathogens and suppress pathogen replication (106). There are two subclasses of IFNs which participate in the immune responses: IFN-a and IFN-b. IFN-a initiates effective host-mediated immune activity, which has shown value in the treatment of viral infections (including HBV and HCV) and cancers (240). IFN-b was originally used to treat the autoimmune multiple sclerosis (241). Non-specific immune-mediated reactions may be promising for other viral diseases, including SARS-CoV-2 (107, 111). Nevertheless, patients receiving IFN can also generate neutralizing antibody which decreases the efficiency of viral elimination (242). The adverse effects included leukopenia, lymphopenia, autoimmune hepatitis, and thyroid disease (154, 243).

Baricitinib

Baricitinib is a JAK-STAT inhibitor for the treatment of rheumatoid arthritis patients who should not take more than one TNF antagonist (244, 245). Barcitinib was proven to affect the hyperinflammatory status which happened during SARS-CoV-2 infection and might avoid endocytosis and viral infection by depressing AAK1 activity (246, 247). Scientists should pay attention to the increasing number of reports on infections and thrombosis after using JAK inhibitors for the treatment of COVID-19 (248, 249). We should also evaluate adverse hepatic effects, particularly liver injury, cholestasis and hepatitis, which unexpectedly developed in a non-negligible number of cases (155). To our knowledge, this is the first strong evidence for a potential correlation between baricitinib and drug-induced liver injury, which is a rare and unpredictable adverse effect requiring case-by-case evaluation to exclude other possible reasons for the injury, including the application of drugs with recognized effects of drug-induced liver injury (250, 251).

Tocilizumab

Tocilizumab is a monoclonal antibody against IL-6 receptors, which is originally used for treating rheumatoid arthritis (252, 253). As study from a medical institution in Wuhan reported that 20 severe COVID-19 cases all exhibited rapid decrease in fever after adding tocilizumab to lopinavir, methylprednisolone, and oxygen therapy, increased the oxygenation efficacy to 5% and the hospital discharge rate to 95% (254). Further studies are undergoing for evaluating the efficacy of combining tocilizumab with other antivirals drugs (82, 255). Tocilizumab can lead to moderate elevation in serum aminotransferase, which is usually short-lived and asymptomatic, but it is also correlated with jaundice and occasional reactivation of HBV (256, 257). Tocilizumab need to be withheld when the serum neutrophil is lower than 1,000 cells/mm^2, platelet is lower than 100,000 cells/mm^2, and/or hepatic enzymes are higher than three times of the upper normal limit (258).

EFFECTS OF CORONAVIRUS VACCINES ON THE LIVER

Vaccines against SARS-CoV-2 will be vital for avoiding the spread of the virus and alleviating social panic, but multiple aspects should be considered to prevent an activated innate inflammatory response, increased incidence of autoimmune diseases, and vaccine-induced liver injury (259). In general, the development of vaccinations is costly, and it usually takes long time to finish strict animal and clinical trials before approval for public applications (260). However, under the situation of the COVID-19 outbreak, the medical community is facing tremendous pressure to rapidly develop effective vaccines (261). In past pandemics such as those involving Ebola, H1N1, SARS, and MERS, vaccine development was unable to be completed owing to the end of the pandemic and the reallocation of scientific funds (262–265). Since July 2nd, 2020, there has been 158 vaccine candidates for COVID-19, 135 of which are in the preclinical or the developing stage. Until now, mRNA-1273 (266), Ad5-nCoV (267), INO-4800 (268), LV-SMENP-DC (269), Pathogen-specific aAPC (270), and ChAdOx1 (271) have entered the phase II/III clinical trials (**Table 5**).

Under a pandemic situation, vaccines with the greatest potential to treat COVID-19 are protein sub-unit vaccines, viral vectored vaccines, and RNA- or DNA-based vaccines (272, 273). Plasmid DNA and mRNA vaccines have attracted scientists' attention and effort as they might be applicated to prophylaxis and therapy for personalized treatment and social health solutions (274). These two vaccines can be rapidly and directly produced from the sequence of the targeted protein by general manufacturing methods, either human or virus in origin (275). For vaccinations, constructing a genetic sequence for the antigen rather than deactivating the pathogen or constructing a recombined protein is simpler and quicker and reduces the potential risks of working with live pathogens (276).

These vaccines do not require culture in the lab; they reduce the risk of exposure to live viruses and encode targeted antigens without generating other toxins, but this does not mean that they do not have risks (277, 278). The pitfall of possibly effective adjuvant inflammation is the potential hepatotoxicity of RNA- or DNA-based vaccines (276). As mentioned above, antivirals and anticancer drugs that contain engineered nucleoside analogs can be toxic (138–140), and such toxicity cannot be predicted by preclinical trials and safety tests due to species difference between human and animal (260). The clinical adverse effects include myopathy, acute pancreatitis,

TABLE 5 | The efficacy and current status of COVID vaccines.

Vaccine	Platform	Efficacy	Safety	Stage of development
BNT162b2 BioNTech/Fosun Pharma/Pfizer	3 LNP-formulation encapsulated mRNA	95%	Contraindicated if there is a history of severe or immediate allergy to any component of the vaccine	FDA EUA
mRNA-1273 Moderna/NIAID	Prefusion stabilized S protein mRNA encapsulated in LNP	94.1%	Contraindicated if there is a history of severe or immediate allergy to any component of the vaccine	FDA EUA
AZD1222 ChAdOx1nCoV-19/University of Oxford/AstraZeneca	Chimpanzee adenovirus vector displaying Spike protein on its surface	70.4%	Cases of transverse myelitis have been reported	Phase 3 clinical trial ISRCTN89951424 NCT04516746 NCT04540393 CTRI/2020/08/027170
Ad5-nCoV CanSino Biological Inc	Adenovirus serotype 5 expressing Spike protein	96%	Defective vector replication	Phase 3 clinical trial NCT04526990 NCT04540419
Ad26 CoV S1 Janssen Pharmaceutical	Adenovirus serotype 26 expressing Spike protein	72%	Low Seroprevalence of antibodies	Phase 3 clinical trial NCT04505722 NCT04614948
NCX-CoV2373 Novavax	Full length recombinant SARS-CoV-2 glycoprotein nanoparticles adjuvanted with Matrix M	89.3%	Adjuvant of M-matrix may be allergenic	Phase 3 clinical trial NCT04533399
CoronaVac Sinovac	Formalin inactivating whole virus particles + alum adjuvant	50.4%	Inactivated SARS CoV-2 with alum hydeoxide adjuvant	Phase 3 clinical trial NCT04456595 NCT04582344 NCT04617483
BBIBP-CorV Sinopharm Wuhan Institute of Biological Products/Beijing Institute of Biological Products	Inactivated SARS-CoV-2	100%	Inactivated whole virion SARS-CoV-2	Phase 3 clinical trial NCT04612972

lipodystrophy, hepatic steatosis, and neural injury (273, 274). Vaccine hepatotoxicity was found in preclinical studies with a potential mRNA target obtained from lipid nanoparticles for Crigler-Najjar syndrome, being chosen because only a small dose of protein is required (279). The expression of the mRNA is believed to play a potential role in hepatotoxicity, and repeat dosages were applied (280). In a clinical trial for the mRNA rabies vaccine, self-limited adverse effects reflected by innate immune activities were discovered, even though the authors stated that the vaccine was generally safe (278). However, adverse events to this extent are not observed when using DNA vaccines (272, 275). The double-stranded structure of the DNA plasmid is regarded as a substance that stimulates the immune system through non-TLR pathways (274). Indeed, plasmid DNA also acts on the TBK1-Sting pathways (275), leading to the secretion of IFN-1, which serves as an adjuvant for the initiation of inflammatory responses against antigens (281). It should also be noted that for monoclonal antibodies, repeat administration of mRNA would likely be required, which may increase the efficacy as well as the risk of toxicity (276). Thus, finding the balance of inflammation and deleterious toxicity by controlling adjuvant activities of mRNA remains a work in progress.

Chronic liver disease (CLD) is a contraindication to multiple COVID-19 vaccines, such as: Pfizer Biontech vaccine (Bnt 162B2) (282), Moderna vaccine (mRNA-1273) (283), Chadox1 nCoV-19 vaccine (AZD1222) (284), etc. As a result, there is little data on COVID-19 vaccination in patients with CLD. Given the reduced immunogenicity of non-coronavirus vaccines in patients with CLD, it is unclear whether the COVID-19 vaccine will produce an adequate and durable immune response to the virus as in healthy people (285). The role of increased liver disease severity in determining the immune response to COVID-19 vaccine is unclear. Although no significant hepatotoxicity was reported in the trials conducted, the number of registered patients with liver disease was too small to draw definitive conclusions about the safety of the vaccine in this population. Many clinical trials in patients with liver disease are currently under way worldwide. In view of CLD patients at higher risk of COVID-19-related death, EASL and AASLD recommended that priority for COVID-19 vaccination should be given to patients with advanced liver disease and those who have undergone liver transplantation for more than 3 months (286, 287). Patients with chronic liver disease who are taking antivirals or immunosuppressive drugs should not stop taking drugs before and after vaccination.

DISCUSSION

The recent COVID-19 pandemic has become a threat to global health, and the virus is still evolving. Lessons from previous outbreaks of coronaviruses and influenza epidemics suggest that viral infections can lead to severe respiratory syndromes and corresponding complications (such as abnormal liver function, cardiac insufficiency and renal failure) as a result of the combination of systemic and partial inflammatory responses. As the most important metabolic organ in the human body, the liver is dramatically affected by coronavirus infection. On the other hand, pre-existing liver diseases also influence the severity and motility of patients with coronavirus infection.

SARS-CoV, MERS-CoV, and SARS-CoV-2 are three coronaviruses with remarkable genetic similarity and can all cause acute respiratory inflammation in humans. Compared with MERS-CoV, SARS-CoV, and SARS-CoV-2 share greater similarity, as both attach to host cells using the ACE2 receptor; in contrast, MERS-CoV binds to DPP4 of the host cells. Nonetheless, the manifestations of liver injury are rather the same among them, characterized by decreased albumin and elevation in ALT, AST, liver enzymes and bilirubin. Increases in GGT and ALP are also observed in COVID-19 patients, suggesting injury of bile duct cells in the liver. Thus, liver injury might also be the result of bile duct cell injury, as liver biopsies obtained from a few COVID-19 patients did not show viral inclusions but rather microvesicular steatosis. The pathological changes that occur are also similar among the three coronaviruses, commonly manifesting as microvascular steatosis and moderate lobular and portal inflammation. Based on previous studies, the frequency and extent of liver injury in severe cases with coronavirus infection were remarkably higher than those in mild cases. As a result, we conclude that these three coronaviruses can cause liver injury that results in similar manifestations and that the degree of liver injury correlates positively with the severity of infection.

Although numerous clinical studies have indicated a strong correlation between liver injury and coronaviruses, the mechanism by which coronaviruses damage hepatocytes and affect hepatic function is still unclear. Several pathophysiological theories have been proposed. First, ACE2-mediated hepatocyte damage is known to be the most direct effect of SARS-CoV-2 infection on the liver. Upregulation of ACE2 in hepatocytes facilitates the invasion of SARS-CoV-2 and causes greater virus virulence in the liver. Considering the important role of ACE2 in coronavirus infection, hrsACE2 may become a promising therapy for patients with SARS-CoV or SARS-CoV-2 infection. Second, immune activity is largely enhanced during coronavirus infection. Once infected with coronaviruses, a large number of cytokines (IL-6, IL8, IFN-γ, and TNF-α, etc.) are secreted by immune cells and released into the blood, inducing inflammation in various tissues or even ARDS, SIRS and MOF. This suggests that immunotherapy is essential for patients with coronavirus infection, and accordingly, interferon-α and corticosteroids are widely used owing to their anti-inflammatory function. However, as immune dysfunction leads to serious consequences, close monitoring of serum cytokines is necessary during immunotherapy. Third, hypoxia can cause persistent elevation in reactive oxygen species, which can promote the secretion of various pro-inflammatory substances that induce liver injury. Therefore, monitoring hypercoagulable states in patients, including thrombocytopenia and increased D-dimer and ALP levels, will be meaningful for preventing thrombosis and further ischemia and hypoxia. To summarize, all of these factors can affect hepatic function and cause liver damage during the course of coronavirus infection. As liver injury is due to multiple factors, more attention should be paid to the pathogenic mechanisms involved, not only in laboratory experiments but also in clinical monitoring and follow-up visits.

The acquisition and clearance of coronavirus infection is largely dependent on the health condition of the patient as well as and any pre-existing diseases. Hepatitis B and C, liver cirrhosis, liver cancer, and immunosuppressive drugs after liver transplantation generally lead to an immunocompromised state. As the numbers of infected individuals and clinical studies of SARS-CoV-2 are much greater than those of SARS-CoV or MERS-CoV, the correlation between pre-existing liver disease and COVID-19 is clearer and more convincing than that for SARS or MERS. Due to delayed SARS-CoV-2 clearance in those with HBV infection, the severity and mortality rate is higher in patients with HBV infection than in those with HBV negativity. For those who have already developed liver cirrhosis, the Child-Pugh scores are likely to increase because of liver injury caused by COVID-19. Moreover, complications of COVID-19 occur earlier and to a larger extent in patients with systemic immunocompromised status. COVID-19 also largely influences the treatment of liver diseases. For hepatitis B/C patients undergoing anti-HBV treatment, discontinuation of high-dose corticosteroid therapy might cause reactivation of HBV during SARS-CoV-2 infection. Furthermore, lopinavir and ritonavir have been proven to increase the chance of developing liver injury in patients with HBV or HCV infection. To prevent the risk of virus transmission, certain examinations such as endoscopy and vascular radiography are being restricted to only severe emergencies (for example, internal bleeding). Although many widely recognized institutions (including ASCO, ESMO, ILCA, EASL, and AASLD) have provided guidance for liver disease treatment in the presence of SARS-CoV-2 infection, more optimized treatments need to be explored to accomplish the lowest risk of disease deterioration and complications.

At present, drugs that are used for treating coronavirus infection include remdesivir, lopinavir/ritonavir, interferon-a, baricitinib, tocilizumab, ACE2 inhibitors, and hrsACE2. Unfortunately, no drug has been proven to be absolute effective for coronavirus therapy. In fact, there are still no excellent drugs for therapy of SARS and MERS, which emerged many years ago. The difficulty in finding optimized drugs for coronavirus infection is mainly due to severe adverse effects. Remdesivir, lopinavir, and ritonavir have all been reported to increase the probability of liver injury, and the extent of liver injury is closely related to the dose of these drugs. IFNs have the potential to initiate a non-specific immune response, causing hepatocyte damage and autoimmune hepatitis and increasing the risk of developing severe complications such as ARDS and SIRS. As

a JAK inhibitor, baricitinib can increase the risk of thrombosis and further lead to liver injury. Tocilizumab can also reactivate HBV in SARS-CoV-2 coinfection, which will delay the recovery of both viral hepatitis and COVID-19. ACE inhibitors and hrsACE2 might impede invasion of coronaviruses and also attachment to host cells in various tissues (such as the lung, liver, gastrointestinal tract and kidney), preventing organ damage. Overall, vaccines against coronaviruses will be vital in preventing their outbreaks, but multiple factors need to be considered to avoid an activated innate inflammatory response, an increased incidence of autoimmune diseases, and vaccine-induced liver injury. Although vaccines with the greatest potential are RNA- or DNA-based vaccines, their positive and adverse effects are seldomly detected due to species differences between humans and lab animals. Therefore, the development of vaccinations usually takes a long time to carry out strict animal and clinical trials before being approved for public applications, which is a challenging task for both society and scientists.

Here, we discussed the potential effects of three coronaviruses and their related treatments on the liver, yet there remains a huge lack of clinical and laboratory experiments to provide strong evidence. The reasons can be summarized as follows:

1) The clinical data of patients have not been fully explored. As the main manifestations of coronavirus infections are respiratory inflammation and damage, most studies have focused on impacts on the lung. However, coronavirus infections can also cause severe complications in other organs, such as the kidney and liver. Therefore, close monitoring of hepatic biochemical parameters is essential to reduce the deterioration of liver disease and death from liver failure. In general, other known factors with hepatotoxicity must be excluded when evaluating the significance of a factor on liver injury.
2) Some studies are waiting for follow-up data for COVID-19 patients, as well as data from COVID-19 patients with long course of disease. Exploration on the relationship between coronavirus infections and chronic liver diseases, such as liver cirrhosis, liver cancers and liver transplantation, is of great significance, and long-term data collection is needed. A systematic record of clinical information and liver diseases, including biochemical indicators, virus-related indicators, clinical symptoms, immunity states and psychological states of each stage of the disease. We should also utilize information on family history to deeply illustrate the relationship between pre-existing liver disease and COVID-19-induced liver injury.
3) Genetic regulatory mechanisms of coronavirus infections warrant further exploration. For example, the level of ACE2 expression in hepatocytes can largely decide the extent of direct liver injury, but upregulation of ACE2 expression does not occur in all patients. Therefore, genetic variations and transcriptome data obtained from second-generation sequencing can provide more ideas regarding the mechanism of COVID-19 susceptibility and its complications.
4) Suitable animal models are urgently needed. For studying the relationship between pre-existing liver diseases and COVID-19-induced liver injury, it is better to establish an animal model that has both liver diseases and COVID-19. The safety and potential adverse events of drugs and vaccines that might occur in COVID-19 patients should be evaluated in animal experiments.

CONCLUSION

Our understanding of coronaviruses, their diagnosis, treatment, and prevention is rapidly evolving. As the pandemic spreads and new evidence is published, it is important to study the effect of coronaviruses on the liver and identify the risk factors for hepatic complications in patients with coronavirus infection. There is an urgent need to develop a clinical guidance for liver diseases patients with coronavirus infection. A complete record of patients with coronaviruses infection with systematic recording of clinical information and liver diseases will be useful to the identification of hepatic complications, the development of hepatic complications risk models, and the prediction of response to treatment.

AUTHOR CONTRIBUTIONS

XW and JL conducted literature retrieval and data collection. XW summed up the information and wrote the first draft. LY and ZL took part in revising the article critically and made substantial contributions to the conception and design. All authors approval for the version for publication and agree to be accountable for all aspects of the work.

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Clinical Characteristics of Cancer Patients With COVID-19: A Retrospective Multicentric Study in 19 Hospitals Within Hubei, China

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Objective: This study aimed to determine the association between prognosis of COVID-19 patients with and without cancer. Moreover, we compared the prognosis of cancer patients subjected to anti-tumor therapy with those who have not undergone anti-tumor therapy in the past 6 months.

Methods and Results: A total of 7,926 adult patients with COVID-19 were retrospectively enrolled in Hubei Province,China between December 31, 2019 and February 20, 2020. Two hundred and seventy seven cancer patients (cancer group, median age 64 [IQR 56–70] years; 50.90% male) and 7,649 non-cancer patients were identified (non-cancer group, median age 55 [IQR 42–64] years; 48.19% male). The mortality rate was lower in the non-cancer group compared to the cancer group (4.50 vs. 9.03%; $P < 0.001$). The duration between onset and admission shorter in the cancer group (Days, 9 [IQR 5–18]) compared to the non-cancer group (Days, 10; [IQR 6–19]; $P = 0.036$). ICU occupancy was higher in the cancer group (n[%], 30[10.83%]) than in the non-cancer group (n[%], 314[4.11%]). In reviewing the anti-tumor therapy, data from 277 selected cancer patients were obtained out of which 74 patients had undergone anti-tumor therapy (mean age 65 [IQR 51–67] years; 45.95% male), 203 had not undergone anti-tumor therapy (non-anti-tumor therapy group, mean age 63 [IQR 53–75] years; 49.75% male) in the past 6 months. The mortality rate for the anti-tumor therapy group and the non-anti-tumor therapy group was similar (9.46 vs. 8.87%; $P = 0.879$).

Conclusion: The mortality rate was higher in COVID-19 patients with cancer compared to those without cancer. Moreover, anti-tumor therapy in the past 6 months did not worsen the prognosis of cancer patients with COVID-19.

Keywords: COVID-19, SARS-CoV-2, pneumonia, cancer, antitumor therapy

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INTRODUCTION

COVID-19 is currently a global pandemic. About 122,000 patients have been infected with COVID-19 in China with 4.63% death rate based on the data from the Chinese Center for Disease Control. There have been 207.17 million confirmed cases of COVID-19, including 4.36 million deaths on Aug 16th, 2021, reported to WHO (1).

Previous studies focused on the general epidemiologic survey, clinical presentation, or prognosis of mild and severe pneumonia cases (2–7). However, limited studies exist on the epidemiology and prognosis in tumor patients infected with SARS-CoV-2 (8–11).

Herein, this study reports general epidemiologic survey, clinical presentation, and prognosis of a subgroup based on a multicentric observational outcome study from a large cohort of COVID-19 patients in Hubei province. A higher mortality rate was reported to be associated with cancer patients infected with SARS-CoV2. Of note, recent anti-tumor therapy does not jeopardize the prognosis of cancer patients with COVID-19. We hope that the findings from this study can provide insights to others (12) who are similarly confronted with the COVID-19 challenges that arise from cancer research.

METHODS

Study Procedure and Patient Cohorts

This observational multicenter cohort study was performed in 19 tertiary hospitals in China. The admission of patients in the 19 hospitals were performed uniformly according to an executive order issued by the Chinese government (13). This study was approved by the Central Ethics Committees (Clinical Ethical Approval No. 2020010) and ratified by the Institutional Ethics Committees in each hospital without any alterations. Informed consent from patient was waived by the ethics committees from each hospital due to the emerging pandemics. COVID-19 diagnosis was determined through clinical manifestations, chest CT, or real-time RT-PCR according to WHO interim guidance and the New Coronavirus Pneumonia Prevention and Control Program (5th edition) published by the National Health Commission of China (14, 15). The inclusion criteria were patients diagnosed with COVID-19. The following exclusion criteria were used to determine the patient cohort: Age less than 18, incomplete medical records, without exact outcome (discharge or death). pregnancy, acute lethal organ injury (e.g., acute myocardial infarction, acute pulmonary embolism, or acute stroke), decompensated or end stage of chronic organ dysfunction (e.g., decompensated cirrhosis, decompensated chronic renal insufficiency, or severe congestive heart failure), acquired immune deficiency syndrome (AIDS), severe trauma (e.g., parenchymatous organ rupture, bleeding, fracture). Based on these criteria, a total of 7,926 COVID-19 patients admitted from December 31, 2019 to February 20, 2020 in 19 designated hospitals in Hubei Province, China were initially evaluated for inclusion. Exactly 277 patients having cancer or have a history of cancer were selected to represent the cancer group with COVID-19. The other 7,649 patients were enrolled in the non-cancer group.

Chest computerized tomography (CT) or throat-swab specimens were obtained from all patients upon admission. A team of physicians evaluated the severity of COVID-19. The demographics, clinical characteristics, medical history, laboratory tests, radiological reports, therapeutic intervention, and outcome data were obtained from the electronic medical records of the patients. The final date of follow up for determining the outcome was April 16th, 2020.

Data Collection

The clinical end point was defined by death or recovery at the time of discharge from the hospital. Following data were collected including patient demographic information (age, gender), medical histories and underlying diseases [e.g., hypertension, diabetes mellitus, chronic obstructive pulmonary disease (COPD), chronic liver disease, chronic kidney disease], tumor type and location, prior treatment history (chemotherapy, radiation therapy, targeted therapy, surgery), physical examination findings and clinical manifestation (fever, cough, fatigue, dyspnea, and comorbidities), laboratory data [e.g., complete blood count, C-reactive protein (CRP), procalcitonin (PCT), hepatorenal function test, serum cardiac enzyme concentration], radiologic report data [unilateral or bilateral infiltrates was classified according to computed tomography (CT) scan of the chest], invasive or non-invasive therapeutic interventions [e.g., antibiotics, antivirals, Chinese patent medicine, vasoactive drugs, hormone therapy drugs, invasive or non-invasive ventilation, renal replacement therapy, extracorporeal membrane oxygenation (ECMO) therapy] and clinical outcomes [sepsis and septic shock, acute respiratory distress syndrome (ARDS), acute kidney injury (AKI), acute liver injury (ALI), acute myocardial injury (AMI), disseminated intravascular coagulation (DIC), ICU stay, clinical end point]. All identity information of patients was removed and recoded before data analysis. The database did not contain any patient identity or confidential information. Data were independently reviewed and confirmed by two experienced physicians to guarantee accuracy. When there was disagreement between the two examiners, a third physician was called in to give an opinion. Criteria definition is shown in **Supplementary Table 4**.

Statistics

Continuous variables were presented as the mean±SD for normally distributed continuous variables while median and interquartile range (IQR) for non-normally distributed continuous variables. For all categorical variables, binary dummy variables and percentage (%) were introduced to represent the categorical values. Continuous variables were studied using the *T*-Test or Mann-Whitney *U*-test. Associations between categorical dependent variables and independent categorical variables were evaluated using Pearson's chi squared test or Yates's correction for continuity analysis. A difference was considered significant if the two-side *p*-value was less than 0.05. Statistical analyses were carried out using SPSS Statistics v23.0 (IBM, Armonk, NY, USA) and the statistical analysis

Abbreviations: AIDS, acquired immune deficiency syndrome; AKI, acute kidney injury; ALI, acute liver injury; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMI, acute myocardial injury; ARDS, acute respiratory distress syndrome; AST, aspartate aminotransferase; CI, confidence interval; COPD, chronic obstructive pulmonary disease; CoV-2, coronavirus 2; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; CT, computed tomography; DIC, disseminated intravascular coagulation; ECMO, extracorporeal membrane oxygenation; IQR, interquartile range; PCT, procalcitonin; SARS, severe acute respiratory syndrome; TBIL, total bilirubin; ULN, upper limit of normal.

TABLE 1 | Clinical characteristics and Comorbidities of patients with COVID-19 in Non-cancer group and cancer group on admission.

Parameters	Total population	Non-cancer group	Cancer group	*P*
	***N* = 7,926**	***N* = 7,649**	***N* = 277**	
	Median (range) or n (%)	**Median (range) or n (%)**	**Median (range) or n (%)**	
Clinical characteristics on admission				
Symptom onset to admission, median (IQR), day	10 (6–19)	10 (6–19)	9 (5–18)	0.036*
Age, median (IQR), y	55 (43–64)	55 (42–64)	64 (56–70)	<0.001**
Male gender, n (%)	3,827 (48.28%)	3,686 (48.19%)	141 (50.90%)	0.375
Fever, n (%)	5,757 (72.63%)	5,539 (72.41%)	218 (78.70%)	0.021*
Cough, n (%)	5,038 (63.56%)	4,878 (63.77%)	160 (57.76%)	0.041*
Fatigue, n (%)	2,568 (32.40%)	2,486 (32.50%)	82 (29.60%)	0.311
Dyspnea, n (%)	1,297 (16.36%)	1,254 (16.39%)	43 (15.52%)	0.700
Comorbidities on admission				
COPD (%)	61 (0.76%)	60 (0.78%)	1 (0.36%)	0.658
Diabetes Mellitus (%)	867 (10.94%)	840 (10.98%)	27 (9.75%)	0.271
Hypertension (%)	1,964 (24.78%)	1,880 (24.58%)	84 (30.32%)	0.030*
Chronic liver disease, n (%)	142 (1.79%)	133 (1.74%)	9 (3.25%)	0.103
Chronic renal diseases, n (%)	15 (1.94%)	148 (1.93%)	6 (2.17%)	0.784

*P-values were generated by the comparison between Non-cancer group and Cancer group, *P < 0.05, **P < 0.01.*

package R-3.6.3 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

The Association Between Prognosis of COVID-19 Patients With and Without Cancer

To determine the prognosis difference between COVID-19 patients with and without cancer, the study cohort included 7,926 COVID-19 patients admitted to 19 hospitals in Hubei, China. Of the 7,926 patients, 277 participants with cancer were defined as cancer group [median age 64 (IQR 56–70) years; 50.90% male] and the leaving 7,649 were defined as the non-cancer group [median age 55 (IQR 42–64) years; 48.19% male]. Patient characteristics and comorbidities at admission are shown in **Table 1**.

The cancer group was characterized by older age, lower prevalence of cough and higher prevalence of fever at presentation compared to the non-cancer group. Besides, the duration between onset and admission was shorter in the cancer group than in the non-cancer group [days, 9 (IQR 5–18) vs. 10(IQR 6–19); $p = 0.036$] (**Table 1**). The thoracic CT findings revealed higher prevalence of pulmonary infection lesions in cancer group compared to the non-cancer group (**Supplementary Table 1**). Laboratory examination on admission indicated increased liver enzymes (AST increase 22.02 vs. 14.77%, $p < 0.001$), kidney function abnormalities (CREA increase 10.83 vs. 5.43%, $p < 0.001$) and increased C-reactive protein (38.27 vs. 29.45%, $p = 0.002$) and procalcitonin level (27.80 vs. 16.16% $p < 0.001$) in the cancer group compared to the non-cancer group (**Supplementary Table 1**).

Regarding the inpatients medical treatment, compared to the non-cancer group, we found that patients in the cancer group suffered more from sepsis and septic shock (7.22 vs. 1.78%; $p < 0.001$), ARDS (18.05 vs. 10.16%; $p < 0.001$), AKI (7.22 vs. 2.01%; $p < 0.001$), ALI (13.36 vs. 6.86%; $p < 0.001$), AMI (12.64 vs. 4.93%, $p < 0.001$), DIC (2.53 vs. 0.43%, $p < 0.001$; **Table 2**). Moreover, the cancer group experienced more antiviral drugs (74.73 vs. 67.67%; $p = 0.013$), antibiotics (71.84 vs. 59.17%; $p < 0.001$), vasoactive drugs (15.52 vs. 4.52%; $p < 0.001$), hormone therapy drugs (35.02 vs. 25.70%, $p < 0.001$), ICU treatment (10.83 vs. 4.11%, $p < 0.001$), non-invasive ventilation (9.75 vs. 5.87%, $p = 0.008$) and invasive ventilation (6.14 vs. 2.26%, $p < 0.001$) compared to the non-cancer group (**Table 2**). Clinical end point showed a higher mortality rate in the cancer group than the non-cancer group (9.03 vs. 4.50%, $p < 0.001$; **Table 2**).

Prognosis of Cancer Patients Subjected and Not Subjected to Anti-Tumor Therapy in the Past 6 Months

A total of 277 COVID-19 cancer patients were enrolled in this subgroup study cohort. The cancer types of patients and num. of patients underwent anti-tumor treatment are displayed in **Supplementary Table 3**. We analyzed cancers of different sites, anti-tumor treatment in the past 6 months did not increase the mortality rate **Supplementary Table 3**. Of note, 74 patients underwent anti-tumor therapy in the past 6 months and were defined as anti-tumor therapy group [median age 65 (IQR 51–67) years; 45.95% male] whereas, the other 203 patients were classified as the non-anti-tumor therapy group [median age 63 (IQR 53–75) years; 49.75% male]. Patient characteristics at admission are highlighted in **Table 3**. Comparison of the general data (age, sex, onset to admission time, medical histories and underlying diseases, physical exam

TABLE 2 | Treatment and Outcome of patients with COVID-19 in Non-cancer group and cancer group.

Parameters	Total population	Non-cancer group	Cancer group	*P*
	***N* = 7,926**	***N* = 7,649**	***N* = 277**	
	Median (range) or n (%)	**Median (range) or n (%)**	**Median (range) or n (%)**	
Treatment				
Antiviral drug, n (%)	5,383 (67.92%)	5,176 (67.67%)	207 (74.73%)	0.013*
Antibiotics, n (%)	4,725 (59.61%)	4,526 (59.17%)	199 (71.84%)	<0.001**
Traditional Chinese medicine, n (%)	6,268 (79.08%)	6,050 (79.10%)	218 (78.70%)	0.874
Antidiabetic drug, n (%)	1,028 (12.97%)	970 (12.68%)	58 (20.94%)	<0.001**
Vasoactive drug, n (%)	389 (4.91%)	346 (4.52%)	43 (15.52%)	<0.001**
Systemic corticosteroids, n (%)	2,063 (26.03%)	1,966 (25.70%)	97 (35.02%)	<0.001**
Immunoglobin, n (%)	1,554 (19.61%)	1,478 (19.32%)	76 (27.44%)	<0.001**
Nasal cannula oxygen inhalationd, n (%)	5,127 (64.69%)	4,903 (64.10%)	224 (80.87%)	<0.001**
Invasive ventilation, n (%)	190 (2.40%)	173 (2.26%)	17 (6.14%)	<0.001**
Noninvasive ventilation, n (%)	476 (6.01%)	449 (5.87%)	27 (9.75%)	0.008**
Renal replacement therapy, n (%)	76 (0.96%)	73 (0.95%)	3 (1.08%)	0.922
Extracorporeal membraneoxygenation, n (%)	22 (0.28%)	21 (0.27%)	1 (0.36%)	0.755
Outcome				
Sepsis and Septic shock, n (%)	156 (1.97%)	136 (1.78%)	20 (7.22%)	<0.001**
ARDS, n (%)	827 (10.43%)	777 (10.16%)	50 (18.05%)	<0.001**
Acute kidney injury, n (%)	174 (2.20%)	154 (2.01%)	20 (7.22%)	<0.001**
Acute liver injury, n (%)	562 (7.09%)	525 (6.86%)	37 (13.36%)	<0.001**
Acute myocardial injury, n (%)	412 (5.20%)	377 (4.93%)	35 (12.64%)	<0.001**
DIC, n (%)	40 (0.50%)	33 (0.43%)	7 (2.53%)	<0.001**
ICU stay, n (%)	344 (4.34%)	314 (4.11%)	30 (10.83%)	<0.001**
Mortality, n (%)	369 (4.66%)	344 (4.50%)	25 (9.03%)	<0.001**

*P-values were generated by the comparison between Non-cancer group and Cancer group, *P < 0.05, **P < 0.01.*

findings and clinical manifestation, radiologic report data) showed no statistical difference between the non-antitumor therapy group and antitumor therapy ($p > 0.05$; **Table 3** and **Supplementary Table 2**).

Regarding treatment, patients in the anti-tumor therapy group experienced a higher accuracy rate of ALI (21.62 vs. 10.34%; $p = 0.015$) compared to the non-anti-tumor therapy group. The accuracy rate of sepsis and septic shock, ARDS, AKI, AMI, and DIC were similar between the two groups. Treatments (antibiotics, antiviral drugs, Chinese patent medicine, vasoactive drugs, hormone therapy drugs, ICU treatment, non-invasive ventilation, invasive ventilation) had no statistical difference between the two groups (**Table 4**). Clinical end point showed no statistical difference in the mortality rate for the two groups (anti-tumor therapy group, 9.46% vs. non-anti-tumor therapy group, 8.87%, $p = 0.879$; **Table 4**).

DISCUSSION

In the present study, we reported a considerably high prevalence of COVID in tumor patients (277/7, 926, 3.49%). We speculate the reason is two-fold. First, tumor patients were highly exposed to the risk of virus infection, because they require more hospitalization or outpatient visits. Also, the immune homeostasis of the tumor patients was blunted (16, 17), especially those receiving chemotherapy, immunosuppressive therapy or molecular-targeted drug therapy, even those newly diagnosed patients have not been subjected to treatment or patients with best supportive care, their immune status were compromised (18).

Findings from this study indicated that cancer patients are characterized by older age, but lower prevalence of cough and higher prevalence of fever at onset stage. Of note, the duration between onset and admission was shorter in the cancer group than in the non-cancer group. This may be attributed to the blunted immune status in cancer patients. Previous studies have demonstrated that cancer patients have a higher risk of severe events (19). This study reported that the physiological severity of illness such as sepsis and septic shock, ARDS, AKI, ALI, AMI, and DIC occurred frequently in cancer patients. Besides, they also experienced more antiviral drugs, antibiotics, vasoactive drugs, hormone therapy drugs, ICU treatment, non-invasive ventilation and invasive ventilation. This indicates the health care providers more resources such as medications, medical supplies, modern equipments, employees were needed to allocate to the population. Even though, higher mortality occurred in the patients of cancer group. These findings remind us, to decrease the incidence of virus infection, phone communication or network consulting service must be considered by both physicians and patients as a way to improve care and follow-up and to reduce unnecessary

TABLE 3 | Clinical characteristics and Comorbidities of cancer patients with COVID-19 in Non-antitumor group and Antitumor group on admission.

Parameters	Total population	Non- antitumor group	Antitumor group	*P*
	***N* = 277**	***N* = 203**	***N* = 74**	
	Median (range) or n (%)	**Median (range) or n (%)**	**Median (range) or n (%)**	
Clinical characteristics on admission				
Symptom onset to admission, median (IQR), day	9 (5–18)	9 (5–20)	9 (6–18)	0.990
Age, median (IQR), y	64 (56–70)	63 (53–75)	65 (51–67)	0.788
Male gender, n (%)	141 (50.9%)	101 (49.75%)	40 (45.95%)	0.526
Fever, n (%)	218 (78.70%)	159 (78.33%)	59 (79.73%)	0.801
Cough, n (%)	160 (57.76%)	118 (58.13%)	42 (56.76%)	0.838
Fatigue, n (%)	82 (29.60%)	66 (32.51%)	16 (21.62%)	0.079
Dyspnea, n (%)	43 (15.52%)	30 (14.78%)	13 (17.57%)	0.571
Comorbidities on admission				
COPD (%)	1 (0.36%)	1 (0.49%)	0(0%)	0.598
Diabetes Mellitus (%)	27 (9.75%)	21 (10.34%)	6 (8.11%)	0.578
Hypertension (%)	84 (30.32%)	58 (28.57%)	26 (35.14%)	0.293
Chronic liver disease, n (%)	9 (3.25%)	7 (3.45%)	2 (2.7%)	0.941
Chronic renal diseases, n (%)	6 (2.17%)	5 (2.46%)	1 (1.35%)	0.924

P-values were generated by the comparison between Non-antitumor group and Anti-tumor group.

visits to hospitals during the outbreak (20–22). Urgent and semi-urgent patients who require hospital treatment or check-up should do more protections to prevent nosocomial infection.

Several oncology societies have developed guidelines on cancer care during COVID-19 pandemic. However, there are some questions that remain open, including the risk of impairing the outcome when treatment stopped, continued, or modified for the patient's well-being and the "distraction effect" of the pandemic, which is represented by the risk of shifting total attention away from standard clinical care to COVID-19 only (23). ESMO, NICE and French guidelines suggested to use a tiered approach to categorize patients into different priority levels to receive active cancer therapy (24). This study reports that patients who underwent anti-tumor therapy in the past 6 month exhibited a higher accuracy rate of ALI. In the previse study, *Yekedüz E* reported chemotherapy increased the risk of death from COVID-19 in cancer patients, but there was no safety concern for immunotherapy, targeted therapies, surgery and radiotherapy (25). *Song K* demonstrated a possible association between recent receipt of oncologic treatment and a higher risk of death among patients with carcinoma who are hospitalized with COVID-19 (26). However, the findings of the current study do not support the previous research (19, 25). One unanticipated result was that the risk accuracy rate for severe events such as sepsis and septic shock, ARDS, AKI, AMI, and DIC were similar in the anti-tumor group compared with patients not subjected to anti-tumor therapy in the past 6 months. Interestingly, treatments of both populations experienced also had no statistical difference. Anti-tumor therapy (chemotherapy, radiotherapy, targeted therapy) in the past 6 months did not affect the mortality of cancer patients.

A note of caution is due here since a substantial proportion of patients who have longer disease courses may be clinically cured, or those new discovered patients incidentally during COVID-19 therapy may be mixed in the group not subjected to anti-tumor therapy. Even though, it is particularly encouraging to find that the patients who underwent antitumor treatments (chemotherapy, radiotherapy, targeted therapy) in the past 6-month still showed no worse outcome compared with the other group. The results of our study are similar to the results of the study conducted by *Jee J* et al. In their study, patients treated with cytotoxic chemotherapy did not have an increased risk of worse COVID-19 course (27). Interestingly, *K Yang* reported receiving chemotherapy within 4 weeks before symptom onset, and male sex were risk factors for death during admission to hospital (8). However, the effect of treatment on its postponement of cancer patients cannot be ignored (28, 29), the diagnostic or treatments' delays would result in life-years lost. We proposed that streamlined efficient anti-tumor treatment should not be affected or cancelled. Originally prescribed antitumor regimens are recommended when sufficient resources and standard precautions can be ensured.

However,there are still some limitations in our study and we have a lot of works to do in the future. First, patients enrolled were infected with early variants of the virus during the time frame of the first outbreak. The effect to cancer patients might change accompanied by viral mutations. Subsequent variants which might potentially result to a slightly different patient presentation. Second, our main aim was to check whether anti-tumor therapy in the past 6 months could worsen the prognosis of cancer patients with COVID-19. Treatment and outcome of different types of cancer with COVID-19 in non-antitumor group and antitumor group were illustrated. However, the older age for the cancer group might be biased by the types of cancer detected, especially as older patients might have a greater proportion of comorbidities. Limitations in sample size have hampered a

TABLE 4 | Treatment and Outcome of patients with COVID-19 in Non-antitumor group and Antitumor group.

Parameters	Total population	Non- antitumor group	Antitumor group	*P*
	N = 277	*N* = 203	*N* = 74	
	Median (range) or n (%)	Median (range) or n (%)	Median (range) or n (%)	
Treatment				
Antiviral drug, n (%)	207 (74.73%)	147 (72.41%)	60 (81.08%)	0.142
Antibiotics, n (%)	199 (71.84%)	144 (70.94%)	55 (74.32%)	0.579
Traditional Chinese medicine, n (%)	218 (78.70%)	162 (79.80%)	56 (75.68%)	0.458
Antidiabetic drug, n (%)	58 (20.94%)	42 (20.69%)	16 (21.62%)	0.866
Vasoactive drug, n (%)	43 (15.52%)	31 (15.27%)	12 (16.22%)	0.848
Systemic corticosteroids, n (%)	97 (35.02%)	66 (32.51%)	31 (41.89%)	0.148
Immunoglobin, n (%)	76 (27.44%)	54 (26.60%)	22 (29.73%)	0.606
Nasal cannula oxygen inhalationd, n (%)	224 (80.87%)	166 (81.77%)	58 (78.38%)	0.525
Invasive ventilation, n (%)	17 (6.14%)	12 (5.91%)	5 (6.76%)	0.981
Noninvasive ventilation, n (%)	27 (9.75%)	19 (9.36%)	8 (10.81%)	0.719
Renal replacement therapy, n (%)	3 (1.08%)	2 (0.99%)	1 (1.35%)	0.692
Extracorporeal membrane oxygenation, n (%)	1 (0.36%)	1 (0.49%)	0 (0%)	0.598
Outcome				
Sepsis and Septic shock, n (%)	20 (7.22%)	13 (6.40%)	7 (9.46%)	0.385
ARDS, n (%)	50 (18.05%)	33 (16.26%)	17 (22.97%)	0.198
Acute kidney injury, n (%)	20 (7.22%)	14 (6.90%)	6 (8.11%)	0.730
Acute liver injury, n (%)	37 (13.36%)	21 (10.34%)	16 (21.62%)	0.015*
Acute myocardial injury, n (%)	35 (12.64%)	26 (12.81%)	9 (12.16%)	0.886
DIC, n (%)	7 (2.53%)	5 (2.46%)	2 (2.70%)	0.748
ICU stay, n (%)	30 (10.83%)	22 (10.84%)	8 (10.81%)	0.995
Mortality, n (%)	25 (9.03%)	18 (8.87%)	7 (9.46%)	0.879

*P-values were generated by the comparison between Non-antitumor group and Anti-tumor group, *P < 0.05.*

more stratified analysis to reduce the effect of selection bias. In addition, we only conducted analysis and research in cohorts in Hubei, China. For patients in other regions, the conclusions we observed may be subject to geographic influences, including local medical conditions, economic levels, and government policies.

Although the current study enrolled limited patients, the findings confirmed the vulnerability of cancer patients with the SARS-CoV-2 (30). Moreover, we suggest that tumor patients should be considered as a special population because they are more susceptible to infection with SARS-CoV-2 due to immune change and social medical need, Antitumor treatments do not expose tumor patients to more risk of severe complications or higher mortality when they have SARS-CoV-2 infection. The antitumor treatments for tumor patients during pandemic should be recommended cautiously.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors. Meanwhile, the proposal with detailed aims, statistical plan, and other information/materials may be required and investigated by the 19 hospitals to guarantee the rationality of requirement and the security of the data.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Central Ethics Committees and Institutional Ethics Committees in Zhongnan Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HW and YY: conception and design and acquisition of data (acquired and managed patients, provided facilities, etc.). DG and HW: development of methodology. DG, HW, and QZ: analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis), writing, review, and revision of the manuscript. DG and YY: administrative, technical, and material support (i.e., reporting or organizing data, constructing databases). YY: study supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.614057/full#supplementary-material

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BRIEF RESEARCH REPORT
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Vaccine Hesitancy Is a Barrier to Achieving Equitable Herd Immunity Among Racial Minorities

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Introduction: Racial minority groups have been disproportionately affected by the 2019 novel coronavirus disease (COVID-19). Vaccine hesitancy may be a major barrier to achieving equitable herd immunity and must be addressed to reduce the excess morbidity and mortality of COVID-19 in disproportionately affected communities. This study aimed to determine if COVID-19 vaccine hesitancy, and its factors vaccine complacency and confidence, are more prominent among disproportionately affected racial minority groups.

Methods: We collected data from participants aged 18 years or older from the four most populous U.S. states, including New York, California, Florida, and Texas, and Canada. Data were collected using a web-based survey platform. Data are available at http://www.covid19-database.com.

Results: Data from 4,434 participants were included [mean (SD) age = 48.7 (17.2) and 50.4% women]. Vaccine hesitancy was higher in Black, Indigenous (Native American and Indigenous People of Canada, including First Nations, Inuit and Métis), and Latinx compared to White participants, while no difference was found between East Asian and White participants. The group differences in vaccine hesitancy for Indigenous and Black compared to White participants remained after controlling for sociodemographic factors. Determinants of vaccine complacency were equivalent between disproportionately affected racial groups and white participants. Vaccine confidence (i.e., trust in vaccine benefit) was generally lower in all racial groups compared to White participants. Differences in vaccine mistrust comparing Black and East Asian to White participants remained after controlling for sociodemographic factors.

Discussion: Disproportionately affected racial minorities may have higher vaccine hesitancy and lower confidence in COVID-19 vaccines. Public health and other relevant government services should address vaccine hesitancy among racial minorities using a culturally sensitive, community-centered approach to attain equitable herd immunity.

Keywords: COVID-19, racial minorities, herd immunity, vaccine hesitancy, vaccine acceptance, 3C model

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INTRODUCTION

The 2019 novel coronavirus disease (COVID-19) has disproportionately affected racial minorities in the United States (U.S.) and Canada, resulting in higher rates of infection, hospitalization and death (1–5). Black, Indigenous, and Latinx (i.e., people of Latin American cultural or ethnic identity) populations have had ≥2.6 times higher case rates (2) and ≥3.3 times higher mortality rates than non-Latinx White individuals (6). To address these health disparities, the National Academies of Sciences, Engineering, and Medicine (NASEM) and the World Health Organization (WHO) have recommended prioritization of racial minorities who are socioeconomically and epidemiologically disadvantaged for COVID-19 vaccines (2, 7). To accomplish this in the U.S., indices are available, including the Social Vulnerability Index (8) and the Area Deprivation Index (9), to guide the equitable distribution of vaccines based on regional socioeconomic status.

Despite efforts to promote equitable distribution of vaccines, vaccine hesitancy is a likely barrier to achieving herd immunity and reducing the excess morbidity and mortality attributable to COVID-19. Some evidence suggests that Indigenous (Native American and Indigenous People of Canada, including First Nations, Inuit and Métis), Black, and Latinx individuals have higher rates of vaccine hesitancy; however, this research lacks information on the key underlying drivers (10–13). An effective framework for equitable vaccine allocation to disproportionately affected racial minorities must address vaccine hesitancy in these groups to ensure equitable herd immunity.

According to the WHO Strategic Advisory Group of Experts (SAGE), vaccine hesitancy emerges when individuals (1) *lack confidence* in the safety and effectiveness of the vaccine and the system recommending and providing it; (2) are *complacent*, in that they do not believe the vaccine-preventable disease is serious and vaccination is not necessarily required to prevent infection; and (3) perceive that access to the vaccine is *inconvenient*, uncomfortable or unaffordable (14). The present paper focuses on vaccine hesitancy and the determinants of vaccine complacency and confidence. Governments are tasked with the responsibility of ensuring vaccines are convenient [i.e., easily accessible, affordable, and delivered in a comfortable and culturally sensitive manner (14)].

In a large sample of people in the U.S. and Canada, this study aimed to determine whether there are differences in vaccine hesitancy, complacency, and confidence across the following racial/ethnic groups: Indigenous, Black, Latinx, East Asian and White. We hypothesized disproportionately affected racial minority groups would have higher COVID-19 vaccine hesitancy attributable to differences in the determinants of lower vaccine confidence compared to East Asian and White participants.

METHODS

We collected data from 4,434 participants aged 18 years or older from the four most populous U.S. states, including New York (n = 1001), California (n = 1001), Florida (n = 501), and Texas (n = 503) and from English-speaking Canada (n = 1936). Data are available at http://www.covid19-database.com. Data were collected at three time points, in May and July 2020 using a web-based survey. The survey was developed, pre-tested, and collected using a web-based platform *Dynata*, a global market research company. We placed a quota restriction on age to ensure that data from a representative sample of participants from the U.S. and Canada were collected. We aimed to include approximately an equal number of respondents from the following age ranges: 18–24, 25–34, 35–44, 45–54, 55–64, and 65+ years of age from each region. Responses were collected between May to August 2020. The study was approved by our institution's Research Ethics Board (REB). All participants provided informed consent prior to starting the survey.

Participants provided sociodemographic information and completed a battery of assessments to assess vaccine hesitancy and the determinants of vaccine complacency and confidence in relation to COVID-19. Participants were asked to select the racial or ethnic group that best describes them. Participants that selected the following categories were included for analysis: "Indigenous" (Native American, American Indian, First Nations, Inuit and Métis), "Black," "Latinx" (Hispanic), "East Asian" (Chinese, Japanese, or Korean), and "White." These categories were chosen based on the NIH guidelines for racial and ethnic categories and by Statistics Canada (15, 16). Not all racial groups were included in the analysis, including Arab/West Asian, Filipino, Southeast Asian, South Asian, and "Other groups." Our study was a direct follow-up investigation of an editorial published in *JAMA* that discussed the prioritization of COVID-19 vaccinations in disproportionately affected racial minorities (1). As a result, we focused our investigation on these disproportionately affected groups, which included Indigenous, Black, and Latinx individuals. Participants' degree of vaccine hesitancy was assessed using a single-item that asked how likely they are to get vaccinated if a vaccine for COVID-19 becomes available. The answer option ranged from "1, Definitely" to "6, Definitely Not," with a higher score representing greater hesitancy. Assessments of the determinants of vaccine complacency and confidence are presented in **Table 1**.

One-way analysis of variance (ANOVA) or chi-square (χ^2) statistics were performed to examine the differences in sociodemographic, vaccine hesitancy, complacency, and confidence variables between racial groups. Participants who identified as being White were used as a reference group in all pairwise comparisons. Bonferroni correction for multiple comparisons was applied and a threshold of $p < 0.002$ (i.e., 0.05/29 comparisons) was used to establish significance. For exploratory purposes, the analyses were repeated for Canada and the United States separately. Multivariate analyses of variance (MANOVA) were subsequently performed to examine the differences in vaccine hesitancy, vaccine complacency and confidence between the racial groups, controlling for sociodemographic variables found to be significantly associated with vaccine hesitancy, including age, education, religion, region of residence, healthcare worker status, income, employment status, and political affiliation. Bonferroni correction for multiple comparisons was applied and a threshold of $p < 0.003$ (i.e., 0.05/16 comparisons) was used to establish significance.

TABLE 1 | Differences in sociodemographic and vaccine hesitancy, complacency, and confidence determinants between racial groups.

	Indigenous (*N* = 48)	Black (*N* = 219)	Latinx (*N* = 338)	East Asian (*N* = 529)	White (*N* = 3,300)	
	Mean (SD) or *N* (%)					*t* (df) and *p*-value
Vaccine hesitancy score	3.1 (1.8)	3.5 (1.8)	2.6 (1.7)	2.4 (1.5)	2.2 (1.5)	$F_{(4,4429)} = 41.22$, $p < 0.001^{*1,2,3}$
Sociodemographic determinants						
Age	43.4 (17.3)	40.3 (17.3)	40.4 (17.0)	39.8 (14.2)	51.6 (16.7)	$F_{(4,4429)} = 100.48$, $p < 0.001^{*5,6,7,8}$
Gender (man/woman[a])	24 (51.1%) /23 (48.9%)	90 (41.3%) /128 (58.7%)	147 (43.6%) /190 (56.4%)	240 (45.5%) /287 (54.5%)	1,681 (51.1%) /1,606 (48.9%)	$\chi^2(4) = 17.43$, $p = 0.002$
Education (years)	15.0 (4.0)	14.0 (4.0)	14.0 (4.0)	16.3 (3.5)	15.0 (4.0)	$F_{(4,4428)} = 21.27$, $p < 0.001^{*4,6,7}$
Religion (yes/no[a])	22 (52.4%) /20 (47.6%)	160 (76.2%) /50 (23.8%)	250 (77.9%) /71 (22.1%)	215 (42.7%) /289 (57.3%)	2,133 (66.9%) /1,055 (33.1%)	$\chi^2(4) = 153.90$, $p < 0.001^{*3,8}$
Region						
New York/California[a]	12 (25.0%)	105 (47.9%)	158 (46.7%)	228 (43.1%)	1,336 (40.5%)	–
Canada	31 (86.1%)	48 (21.9%)	26 (7.7%)	259 (49.0%)	1,316 (67.0%)	$\chi^2(4) = 119.51$, $p < 0.001^{*6,7}$
Florida/Texas	5 (13.9%)	66 (30.1%)	154 (45.6%)	42 (7.9%)	648 (33.0%)	$\chi^2(4) = 77.22$, $p < 0.001^{*3,8}$
Population density						
1,000 or less[a]	4 (8.3%)	6 (2.8%)	6 (1.8%)	5 (1.1%)	103 (3.2%)	–
1,000–29,999	7 (14.6%)	16 (7.5%)	27 (8.0%)	20 (4.2%)	396 (12.3%)	$\chi^2(4) = 2.30$, $p = 0.681$
30,000–99,999	13 (27.1%)	36 (16.9%)	54 (16.0%)	68 (14.4%)	515 (16.0%)	$\chi^2(4) = 7.13$, $p = 0.129$
100,000 or more	21 (43.8%)	133 (62.4%)	207 (61.2%)	378 (80.3%)	1,961 (60.8%)	$\chi^2(4) = 19.74$, $p = 0.001^{*4}$
Household income						
<$20,000[a]	3 (6.5%)	39 (18.7%)	48 (15.0%)	24 (4.8%)	178 (5.7%)	–
$20,000–$59,999	19 (41.3%)	77 (37.0%)	116 (36.1%)	116 (23.4%)	770 (24.7%)	$\chi^2(4) = 22.69$, $p < 0.001^{*2,3}$
$60,000–$99,999	12 (26.0%)	53 (25.4%)	90 (28.0%)	159 (32.1%)	896 (28.8%)	$\chi^2(4) = 62.00$, $p < 0.001^{*6,7}$
$100,000–$139,999	9 (19.6%)	20 (9.6%)	34 (10.6%)	94 (18.9%)	597 (19.2%)	$\chi^2(4) = 93.81$, $p < 0.001^{*6,7}$
$140,000 or more	3 (6.5%)	19 (9.1%)	33 (10.3%)	103 (20.8%)	675 (21.7%)	$\chi^2(4) = 114.77$, $p < 0.001^{*6,7}$
Employment status						
Unemployed	8 (16.7%)	41 (18.7%)	52 (15.4%)	66 (12.9%)	358 (10.8%)	$\chi^2(4) = 14.51$, $p = 0.006^{*2}$
Employed[a]	30 (62.5%)	112 (51.1%)	180 (53.3%)	352 (68.8%)	1,762 (53.4%)	–
Student	1 (2.1%)	27 (12.3%)	46 (13.6%)	51 (9.9%)	94 (2.8%)	$\chi^2(4) = 102.93$, $p < 0.001^{*2,3,4}$
Retired	8 (16.7%)	30 (13.7%)	42 (12.4%)	43 (8.4%)	921 (27.9%)	$\chi^2(4) = 112.40$, $p < 0.001^{*6,7,8}$
Healthcare worker (yes/no[a])	5 (10.4%) /43 (89.6%)	50 (22.8%) /169 (77.2%)	56 (16.6%) /282 (83.4%)	91 (17.2%) /438 (82.8%)	379 (11.5%) /2,921 (88.5%)	$\chi^2(4) = 37.47$, $p < 0.001^{*4,6}$

(Continued)

TABLE 1 | Continued

	Indigenous (*N* = 48)	Black (*N* = 219)	Latinx (*N* = 338)	East Asian (*N* = 529)	White (*N* = 3,300)	
	Mean (SD) or *N* (%)					*t* (df) and *p*-value
Political spectrum						
Communism left wing or socialism	1 (2.1%)	10 (4.6%)	20 (5.9%)	18 (3.4%)	203 (6.2%)	$\chi^2(4) = 14.68$, $p = 0.005^{*8}$
Liberal	14 (29.2%)	73 (33.3%)	103 (30.5%)	165 (31.2%)	945 (28.6%)	$\chi^2(4)=3.71$, p=0.447
Center[a]	16 (33.3%)	92 (42.0%)	142 (42.0%)	217 (41.0%)	1,067 (32.3%)	–
Conservative	15 (31.3%)	33 (15.1%)	70 (20.7%)	120 (22.7%)	1,025 (31.1%)	$\chi^2(4) = 55.36$, $p < 0.001^{*6,7,8}$
Fascism right wing or authoritarianism	2 (4.2%)	11 (5.0%)	3 (0.9%)	9 (1.7%)	60 (1.8%)	$\chi^2(4) = 11.04$, $p = 0.026$
Alcohol use (yes/no[a])	23 (47.9%) /25 (52.1%)	119 (54.3%) /100 (45.7%)	189 (55.9%) /149 (44.1%)	277 (52.4%) /252 (47.6%)	2,267 (68.7%) /1,033 (31.3%)	$\chi^2(4) = 86.09$, $p < 0.001^{*5,6,7,8}$
Cigarette use (yes/no[a])	10 (20.8%) /38 (79.2%)	40 (18.3%) /179 (81.7%)	58 (17.2%) /280 (82.8%)	72 (13.6%) /457 (86.4%)	666 (20.2%) /2,634 (79.8%)	$\chi^2(4) = 13.84$, $p = 0.008^{*8}$
Electronic cigarette use (yes/no[a])	9 (18.8%) /39 (81.3%)	34 (15.5%) /185 (84.5%)	52 (15.4%) /286 (84.6%)	59 (11.2%) /470 (88.8%)	415 (12.6%) /2,885 (87.4%)	$\chi^2(4) = 6.42$, $p = 0.170$
Cannabis use (yes/no[a])	19 (39.6%) /29 (60.4%)	47 (21.5%) /172 (78.5%)	58 (17.2%) /280 (82.8%)	55 (10.4%) /474 (89.6%)	614 (18.6%) /2,686 (81.4%)	$\chi^2(4) = 38.78$, $p < 0.001^{*1,8}$
Complacency determinants						
Perceived susceptibility to infectious disease	3.6 (1.0)	3.5 (1.1)	3.4 (1.0)	3.7 (0.9)	3.4 (1.1)	$F_{(4,4429)} = 11.26$, $p < 0.001^{*4}$
Perceived seriousness of COVID-19	4.1 (1.2)	4.4 (0.9)	4.5 (0.9)	4.4 (0.9)	4.5 (0.9)	$F_{(4,3546)} = 1.42$, $p = 0.225$
Perceived safety of social distancing measures	4.0 (1.0)	3.7 (1.2)	3.5 (1.3)	3.4 (1.1)	3.6 (1.1)	$F_{(4,3546)} = 6.78$, $p < 0.001^{*8}$
Perceived safety of going out in the community	3.1 (1.4)	3.1 (1.2)	3.0 (1.3)	2.9 (1.2)	3.1 (1.3)	$F_{(4,3546)} = 4.83$, $p = 0.001^{*4}$
Perceived likelihood of a second wave of COVID-19	3.9 (1.1)	4.0 (1.1)	4.2 (1.0)	4.1 (0.9)	4.0 (1.0)	$F_{(4,3546)} = 1.50$, $p = 0.199$
Tested positive for COVID-19 (self)						$\chi^2(4) = 5.34$, $p = 0.254$
Tested positive	2 (4.2%)	5 (2.3%)	9 (2.7%)	7 (1.3%)	99 (3.0%)	
Not tested or tested negative[a]	46 (95.8%)	214 (97.7%)	329 (97.3%)	522 (98.7%)	3,201 (97.0%)	
Tested positive for COVID-19 (someone close)						$\chi^2(4) = 40.74$, $p < 0.001^{*8}$
Tested positive	14 (29.2%)	72 (32.9%)	140 (41.4%)	117 (22.1%)	923 (28.0%)	
Not tested or tested negative[a]	34 (70.8%)	147 (67.1%)	198 (58.6%)	412 (77.9%)	2,377 (72.0%)	
COVID-19 health risk factors[b]	1.0 (1.2)	0.6 (1.1)	0.6 (1.2)	0.4 (0.9)	0.8 (1.1)	$F_{(4,4429)} = 16.60$, $p < 0.001^{*8}$

(Continued)

TABLE 1 | Continued

	Indigenous ($N = 48$)	Black ($N = 219$)	Latinx ($N = 338$)	East Asian ($N = 529$)	White ($N = 3,300$)	
	Mean (SD) or *N* (%)					*t* (df) and *p*-value
Confidence determinants						
Mistrust of vaccine benefit	2.9 (1.4)	2.9 (1.4)	2.6 (1.3)	2.6 (1.1)	2.3 (1.2)	$F_{(4,4429)} = 18.10$, $p < 0.001^{*1,2,3,4}$
Worries over unforeseen future effects	4.0 (1.3)	4.1 (1.3)	3.8 (1.3)	3.8 (1.1)	3.7 (1.3)	$F_{(4,4429)} = 9.30$, $p < 0.001^{*2}$
Concerns about commercial profiteering	3.5 (1.5)	3.7 (1.4)	3.4 (1.4)	3.2 (1.3)	2.9 (1.5)	$F_{(4,4429)} = 25.89$, $p < 0.001^{*2,3,4}$
Preference for natural immunity	3.7 (1.3)	3.5 (1.3)	3.4 (1.4)	3.3 (1.2)	3.2 (1.4)	$F_{(4,4429)} = 6.04$, $p < 0.001^{*2,3}$
Positive attitudes toward holistic health approaches	12.7 (5.0)	12.8 (5.4)	12.7 (5.1)	12.7 (4.1)	11.8 (4.2)	$F_{(4,4429)} = 8.11$, $p < 0.001^{*2,3,4}$
Positive attitudes toward complementary and alternative medicine	22.2 (4.3)	22.4 (4.3)	23.0 (4.2)	23.3 (3.9)	23.6 (5.0)	$F_{(4,4429)} = 5.77$, $p < 0.001^{*6}$
Mistrust in Government's management of COVID-19	23.8 (8.7)	26.0 (9.3)	26.1 (8.6)	25.3 (8.1)	26.0 (9.1)	$F_{(4,4429)} = 1.45$, $p = 0.215$

[a] *Reference variable.*

[b] *One point was assigned for each health risk factor (i.e., heart disease, hypertension, lung disease, diabetes, cancer, chronic kidney disease, obesity, and weakened immune system) to derive a total health risk factor score for COVID-19.*

[*] $p < 0.002$ *(0.05/29 comparisons).*

Bonferroni corrected p-value < 0.05 with White as the reference group:

[1] *Indigenous > Whites;* [2] *Black > Whites;* [3] *Latinx > White;* [4] *East Asian > White;* [5] *White > Indigenous;* [6] *White > Black;* [7] *White > Latinx;* [8] *White > East Asian.*

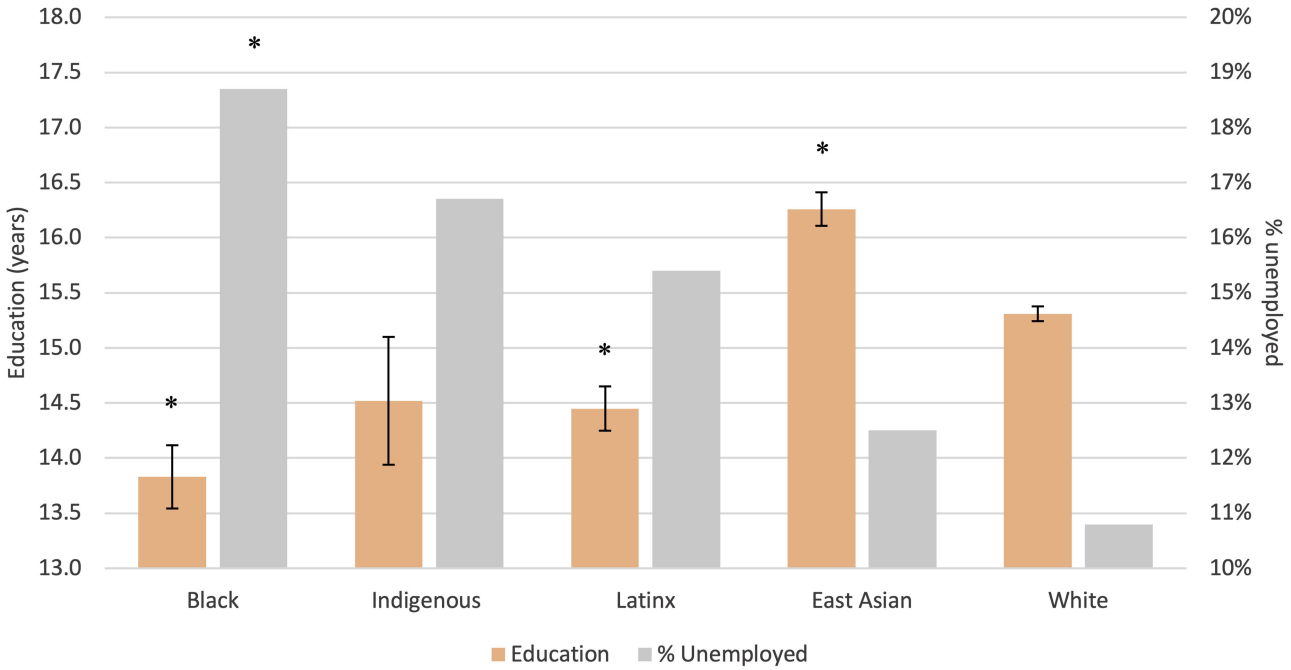

FIGURE 1 | Differences in years of education and % unemployed between racial groups. *Bonferroni corrected p-value < 0.05 with White as the reference group; Error bars represent standard error.

Statistical analyses were performed using IBM SPSS Statistics (version 26, IBM Corp., Armonk, N.Y., USA). The EQUATOR Reporting Guidelines were followed. Additional survey details can be found in **Supplementary Material 1** and the full list of variables and data collected for the survey are available online at http://www.covid19-database.com.

RESULTS

The mean age was 48.7 (SD = 17.2) and 50.4% of the participants were women. The majority of participants were White (74.4%). One percent of the participants were Indigenous, 4.9% Black, 11.9% East Asian, and 7.6% Latinx. Indigenous, Black, and Latinx participants were more socioeconomically disadvantaged than East Asian and White participants. Sociodemographic characteristics of the participants included in the study can be found in **Table 1**; **Figure 1**; **Supplementary Materials 2, 3**.

In the unadjusted analyses, vaccine hesitancy was significantly higher in Black, Indigenous, and Latinx compared to White participants (**Table 1**; **Figure 2**). When controlling for sociodemographic factors, the group difference in vaccine hesitancy remained for Indigenous and Black vs. White participants, but not between Latinx and White participants. Separate unadjusted analyses by country showed higher vaccine hesitancy in Black compared to White participants in both Canada and the U.S., but no significant differences between Latinx and White participants in Canada and between Indigenous and White participants in the U.S. (**Supplementary Materials 4–7**).

In terms of determinants of vaccine complacency, disproportionately affected racial minority groups perceived COVID-19 with the same degree of seriousness as White participants. East Asian participants were more likely than White participants to believe they are susceptible to infectious disease and less likely to perceive the current social distancing and community restrictions to be safe and restrictive enough. East Asian participants also had a fewer number of health risk factors for COVID-19 compared to White participants. The group differences in the determinants of vaccine complacency remained when controlling for sociodemographic differences, with the exception of health risk factors for COVID-19, which became non-significant.

Vaccine confidence was generally lower in all racial minority groups compared to White participants. Attitudes toward vaccinations, including mistrust in vaccine benefit (**Figure 2**), worries over unforeseen future effects of vaccines, concerns about commercial profiteering, and preference for natural immunity were generally higher in disproportionately affected racial minorities compared to White participants. Black, Latinx, and East Asian participants had more positive attitudes toward holistic health approaches compared to White participants, although attitudes toward complementary and alternative medicine were more positive in White compared to Black participants. There were no group differences with respect to trust in Government's management of COVID-19. Group differences in the determinants of vaccine confidence remained when controlling for sociodemographic differences (**Supplementary Material 8**).

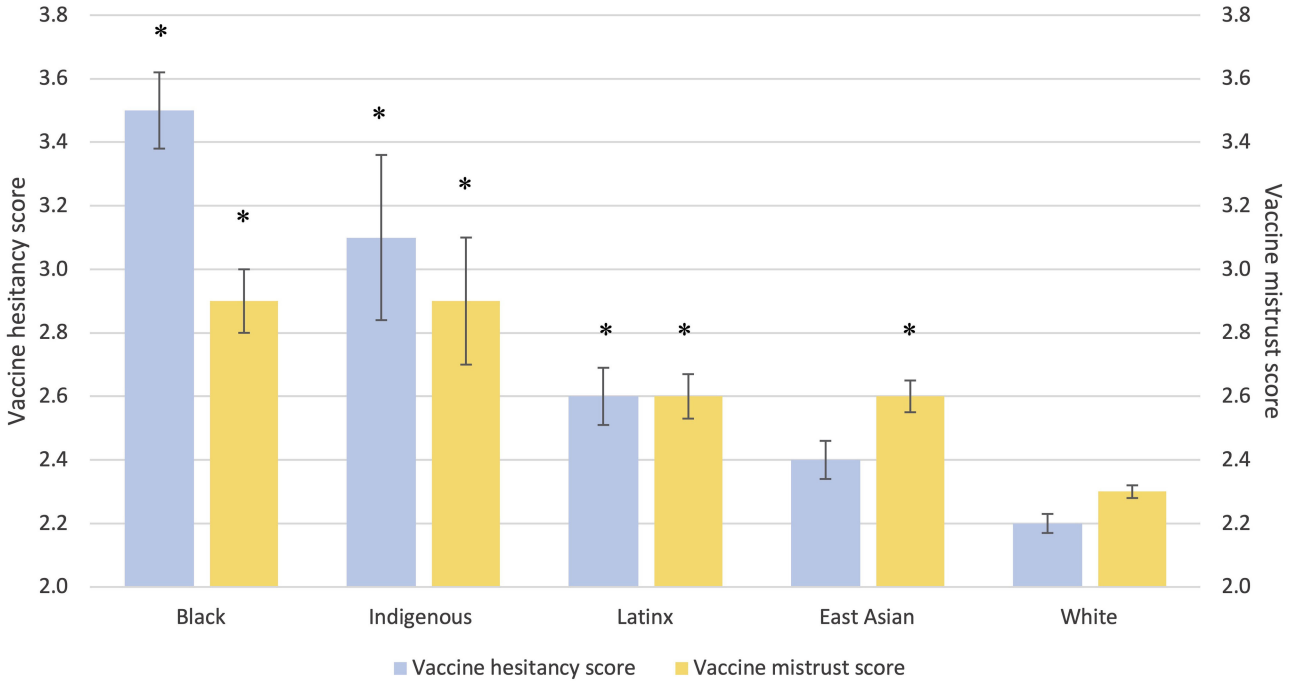

FIGURE 2 | Differences in vaccine hesitancy and vaccine mistrust scores between racial groups. *Bonferroni corrected p-value < 0.05 with White as the reference group; Error bars represent standard error.

DISCUSSION

Addressing vaccine hesitancy prior to the availability of vaccines for COVID-19 is essential to achieve equitable herd immunity among racial minorities who have been disproportionately affected by COVID-19. At the time of this study, only 43.7% of Indigenous, 33.4% of Black, and 56.5% of Latinx are "very probably" to "definitely" likely to get a COVID-19 vaccine, as compared to 59.6% of East Asians and 67.4% of Whites.

Racial minorities had lower vaccine confidence, while no notable group differences were found in vaccine complacency. In other words, all groups viewed COVID-19 with the same degree of seriousness, yet differed in their degree of vaccine confidence. In the current sample, racial minority groups disproportionately affected by COVID-19 were more socioeconomically disadvantaged and more likely to be personally affected by COVID-19. Disproportionately affected groups had lower years of education, higher unemployment, and less income, and were also generally younger, more religious, and less conservative than White participants. Notably, group differences in vaccine hesitancy between Indigenous and Black compared to White participants remained after accounting for these sociodemographic differences. The persistence of group differences after accounting for socioeconomic disparities may reflect the historical and contemporary systemic factors that contribute to mistrust in medical interventions among racial minorities in North America. These include the Tuskegee Experiment where Black American men were deceived subjects of an observation study of untreated syphilis and the Qu'Appelle BCG Vaccine Trial in which First Nations children of the Qu'Appelle reserves in southern Saskatchewan were subjects of a vaccine trial for tuberculosis, while their impoverished living conditions were left unaddressed (17, 18).

There are a few limitations to this study. First, only English-speaking participants who are familiar with using a computer were included. Second, the sample size for Black, Indigenous, and Latinx participants was relatively low compared to East Asian and White participants. As such, some of the null results may be attributed to a lack of statistical power. Third, we recognize that the use of racial and ethnic categorizations as employed in this study are imperfect. Participants had the opportunity to select the category they most identified with, which was felt to be the best means to overcome this limitation where only a single response option was available. Participants were also offered the option of choosing "other" if they did not feel one of the categories represented them. It is possible some participants may not identify with the nomenclature of the racial and ethnic categories and thus were not included in the study. Fourth, the study included a convenience sample and thus may not be representative of the general population. Lastly, the efficacy and the specific risks associated with the COVID-19 vaccines were unknown at the time of the study.

In summary, disproportionately affected racial minority groups may have higher vaccine hesitancy, in particular lower COVID-19 vaccine confidence. If the societal objective is to ensure the equitable attainment of herd immunity among racial minority communities disproportionately affected by COVID-19, in addition to optimizing vaccine accessibility [i.e., ensuring

vaccines are easily accessible and affordable (14)], special efforts ought to be made within these communities to bolster vaccine confidence using a culturally sensitive, community centered approach. Moreover, "in times of famine and pestilence," local and national governments may have the legal responsibility to achieve this aim (19).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are available, without undue reservation, at http://www.covid19-database.com.

ETHICS STATEMENT

All studies involving human participants are reviewed and approved by the Centre for Addiction and Mental Health. Participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PG formulated the research aims, designed the research methodology, provided oversight in executing the study, and wrote the first draft of the manuscript. JK conducted statistical analyses and assisted with writing the manuscript. LQ assisted with designing the study and interpreting the data. SW assisted with designing the study and edited the manuscript. EB assisted with formulating the research aims, designing research methodology, validating research outputs, and editing the manuscript. BA assisted with formulating the research aims, interpreting the data, and edited the manuscript. BP assisted with interpreting the data and edited the manuscript. AG-G provided oversight in executing the study and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.668299/full#supplementary-material

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Tracing Management and Epidemiological Characteristics of COVID-19 Close Contacts in Cities Around Chengdu, China

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Introduction: Close contacts have become a potential threat to the spread of coronavirus disease 2019 (COVID-19). The purpose of this study was to understand the epidemiological characteristics of close contacts of confirmed or suspected cases of COVID-19 in the surrounding cities of Chengdu, China, so as to provide a basis for the management strategy of close contacts.

Methods: Close contacts were determined through epidemiological investigation of indicated cases, and relevant information was entered in the "Close Contact Information Management System." Retrospective data of close contacts from January 22 to May 1, 2020 were collected and organized. Meanwhile, the contact mode, isolation mode, and medical outcome of close contacts were descriptively analyzed.

Results: A total of 986 close contacts were effectively traced, with an average age of (36.69 ± 16.86) years old, who were mainly distributed in cities of eastern Chengdu. The frequency of contact was mainly occasional contact, 80.42% of them were relatives and public transportation personnel. Besides, the time of tracking close contacts and feedback was (10.64 ± 5.52) and (7.19 ± 6.11) days, respectively. A total of seven close contacts were converted to confirmed cases.

Conclusions: Close contacts of COVID-19 have a risk of invisible infection. Early control of close contacts may be helpful to control the epidemic of COVID-19.

Keywords: close contact, COVID-19, epidemiological, epidemic prevention and control, tracking

INTRODUCTION

In December 2019, a series of unexplained pneumonia cases appeared in Wuhan, Hubei, China (1–3), which was subsequently identified by etiological identification as a novel coronavirus, named the coronavirus disease 2019 (COVID-19). Although many details of the emergence of the virus are still unknown, several pieces of evidence have confirmed human-to-human transmission (4–6). Afterwards, the World Health Organization (WHO) announced it as a Public Health Emergency of International Concern (PHEIC) on January 30, 2020 (7). COVID-19 has spread worldwide, which has caused more than 239 million cases and 4.87 million deaths as of October 18, 2021.

To control the further spread of the epidemic, the Wuhan government has implemented a "lockdown" (8). Unfortunately, this period coincides with the traditional mass movement before the Spring Festival, that is, a form of "going home." As a result, more than 5 million people have left Wuhan, which undoubtedly increased the risk of infection in other areas (9). Chengdu, located in the southwest of China, is an important transportation hub in China. The increase in population mobility also increased the import of infectious diseases. Since the first COVID-19 case reported in Chengdu on January 22, there have been 166 cases as of May 1, 2020. Existing data showed that the epidemic of COVID-19 in Chengdu was dominated by imported cases, and most patients were close contacts of confirmed cases, that is, "second-generation cases." How to "contain" the "three links" of infectious diseases and timely and accurate detection and tracking of close contacts are still a major focus and difficulty in epidemic control.

Close contact tracing is an intervention that requires the index case to provide as much information as possible about contacts who have acquired the risk of infection within a given period of time before the test results are available (10). Close contact management has become one of the core strategies to reduce additional transmission (11). Jing et al. (12) have provided important insights into the factors affecting the transmission of COVID-19 primary cases and the susceptibility of their close contacts.

Existing studies have confirmed that effective concentration or home isolation of close contacts could restrain the spread of COVID-19 to a certain extent, which could also create a good living and development environment (11, 13). At the same time, collecting accurate epidemiological data through contact tracing can increase the awareness of the epidemic and draw up effective intervention measures.

Since the outbreak of the COVID-19 epidemic, Chengdu has adopted strict case isolation treatment, close contact tracing, and medical observation measures, which have effectively prevented the spread of the epidemic. From the perspective of close contact tracing, this study aims to understand the epidemiological characteristics and tracing management of close contacts transferred from Chengdu to surrounding cities, and at the same time, scientifically and reasonably determine the quarantine objects, so as to provide a basis for epidemic prevention and control.

MATERIALS AND METHODS

Data Collection

Close contacts were determined following the "Management Plan of Close Contacts of COVID-19 Cases" in the "COVID-19 Prevention and Control Program" of the China Health Commission (14). Possible close contacts were determined through epidemiological investigation of confirmed, asymptomatic, or suspected cases. Moreover, some of the information on close contacts came from the personnel of public security, tourism, and other departments or areas who request assistance in the investigation.

Close contacts refer to people who have not had effective protection from suspected or confirmed cases (within 1 meter) from 2 days before symptoms appear, or 2 days before sampling asymptomatic samples, including people who are living together, studying together, those under diagnosis and treatment, and those sharing transportation, etc. Relevant information was entered into the "Close Contact Information Management System" by health workers. Retrospective data of close contacts from January 22 to May 1, 2020 were used in our analysis.

Close Contact Management Measures

According to the distribution of close contacts in the inner districts and counties of Chengdu, the basic information of close contacts was entered into the system by the prevention and control personnel of the local jurisdiction. Once after identification, the close contacts were subjected to centralized or home isolation for medical observation for 14 days; body temperature and respiratory symptom were monitored approximately every day. In addition, the SARS-CoV-2 nucleic acid test was performed at least twice before the quarantine was ended, with an interval of more than 24 h each time. If there was no abnormality, isolation was terminated.

Research Content

We collected the information of close contacts among people who were isolated due to COVID-19, which included the basic information of close contacts, relationship with original cases, mode of isolation observation, mode of contact, location of contact, and presence of clinical symptoms, etc.

Statistical Analysis

The retrospective data and relevant information of the close contacts were collected through the "Close Contact Information Management System" and the database was established. Data were statistically sorted and analyzed by SPSS version 22.0 software (IBM Corp, NY, USA). The qualitative data were statistically described by frequency, composition ratio or rate, and statistically analyzed by chi-square test. $P < 0.05$ was considered statistically significant. ArcGIS version 10.5 software (Environmental Systems Research Institute, Redlands, CA, USA) was used to describe the spatial distribution of close contacts.

RESULTS

Screening of Close Contacts

According to the epidemiological investigation, 11,079 close contacts were tracked by May 1, 2020. Among them, 8,348 cases were local management close contacts in Chengdu and 1,057 cases were in other provinces. Through further screening of close contacts in cities around Chengdu, 986 cases were finally included in this study (details are shown in **Figure 1**).

Abbreviations: COVID-19, coronavirus disease 2019; WHO, World Health Organization; PHEIC, Public Health Emergency of International Concern.

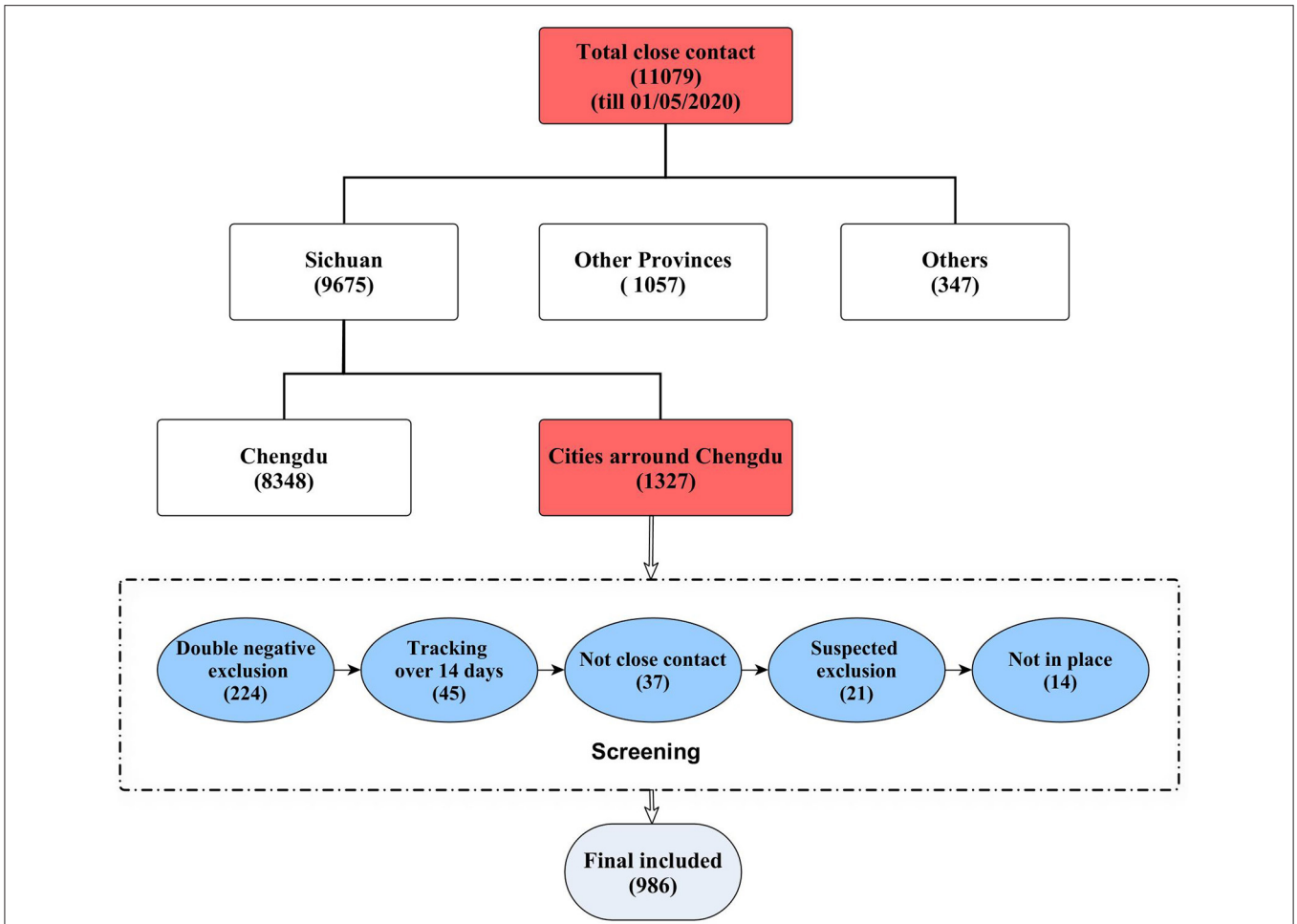

FIGURE 1 | The flow chart of close contact screening.

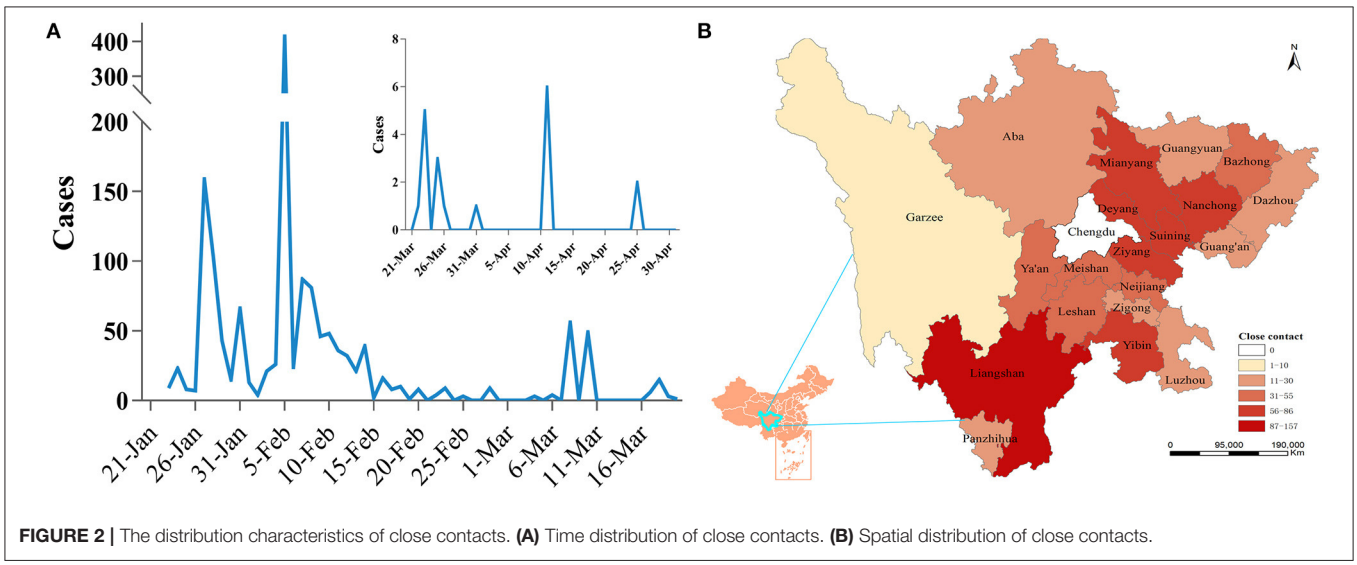

FIGURE 2 | The distribution characteristics of close contacts. **(A)** Time distribution of close contacts. **(B)** Spatial distribution of close contacts.

Distribution Characteristics of Close Contacts

Through the analysis of the discovery time of all close contacts, the distribution presented as three different peaks, which were mainly concentrated from January 26 to February 14 (accounting for 82.85%). After a stable period of nearly 20 days, another surge appeared on March 8 and then stabilized again (**Figure 2A**). A total of 986 close contacts were distributed in 20 cities around Chengdu (ranging from 1 to 157, average: 46.96). Except for the Liangshan Prefecture, the cities with more close contacts were mainly located in the eastern part of Chengdu, accounting for 51.52% (**Figure 2B**).

TABLE 1 | The basic characteristics of close contacts.

Index	Cases (*n*%)	χ^2	*P*-value
Gender			
Male	558 (56.59)	17.53	<0.001
Female	428 (43.41)		
Age			
0–<15	87 (8.82)		
15–<30	256 (25.96)		
30–<45	226 (22.92)	2399.19	<0.001
45–<60	304 (30.83)		
≥60	113 (11.46)		
Relationship with cases			
Relatives	297 (30.12)	692.84	<0.001
Fellow passengers	496 (50.30)		
Colleague	23 (2.33)		
Diagnosis	32 (3.25)		
Others	138 (14.00)		
Personnel classification			
Medical staff	20 (2.03)	1836.58	<0.001
Non-medical staff	966 (97.97)		
Contact frequency			
Occasionally	599 (60.75)	789.33	<0.001
General	207 (20.99)		
Often	180 (18.26)		
Contact location			
Domicile	295 (29.92)	773.85	<0.001
Restaurant	23 (2.33)		
Vehicle	496 (50.30)		
Hospital	94 (9.53)		
Others	78 (7.91)		
Contact mode			
Vehicle	496 (50.30)	627.5	<0.001
Dinner together	211 (21.40)		
Domesticity	136 (13.79)		
Diagnosis and treatment	132 (13.39)		
Others	11 (1.12)		
Isolation mode			
Centralized isolation	712 (72.21)	1865.32	<0.001
Home isolation	236 (23.94)		
Hospital treatment	6 (0.61)		
Other	32 (3.25)		

Basic Characteristics of Close Contacts

Among the close contacts, there were 558 men and 428 women, with a male:female ratio of 1.30:1. The average age of close contacts was (36.69 ± 16.86) years old, which mainly concentrated in the age group of 15–60 years (79.72%); no significant difference was found in the distribution of different ages ($P < 0.001$). The frequency of contact between close contacts and cases was mainly occasional contact (60.75%), and the relationship with cases was mainly relatives (30.12%) and co-passengers (50.30%), and most of them were in the same train compartment (70.35%, data not shown). Contact places were mainly residential and transportation (81.54%), and the method of contact was mainly sharing rides and gatherings (71.70%), while hospital contact accounted for about 9.53% (**Table 1**).

Time Index Analysis

Through the analysis of time indexes of close contacts, it was found that the time of tracking close contacts was (10.68 ± 5.46) days, and the feedback time of other cities after receiving assistance in the investigation was (7.24 ± 6.14) days. The time from case discovery to close contacts release was (4.81 ± 4.14) days, which was longer than the actual isolation time (4.17 ± 4.40; $t = 3.175$, $P = 0.002$; **Table 2**).

Outcome of Close Contacts

Among the 986 close contacts, 18 had symptoms, mainly manifested as upper respiratory symptoms such as cough, runny nose, and sore throat (data not shown). A total of seven close contacts were converted to confirmed cases, with the majority of them frequent contacts (42.86%). Meanwhile, the seven cases were mainly the relatives and co-passengers of indicated cases, and the main contact mode was eating together (42.86%; **Figure 3**).

DISCUSSION

COVID-19 has caused a widespread pandemic, and human-to-human transmission was discovered as early as the beginning of the epidemic (5, 15, 16). With the increase in population mobility, it is undoubtedly possible for the disease to spread further. As for the close contact management policy, the general

TABLE 2 | Close contact discovery and isolation time.

Index	Time (days)	P_{25}	P_{75}
Close contact tracking time	10.64 ± 5.52	6.31	15.00
Feedback time	7.19 ± 6.11	7.00	11.00
Supposed isolation time	4.81 ± 4.14	1.00	7.69
Actual isolation time	4.17 ± 4.40	3.00	5.00

Data were expressed as mean ± S.D, and the 25th and 75th percentiles were described.

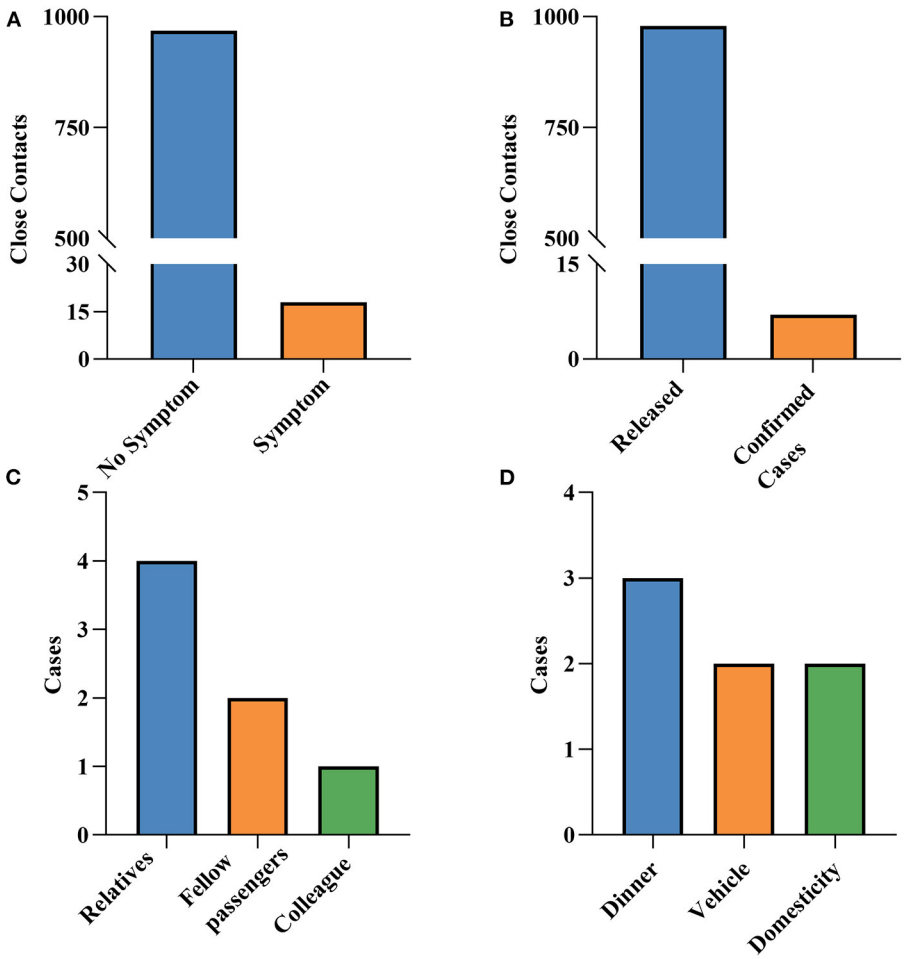

FIGURE 3 | The outcome of close contacts. **(A)** Whether they have symptoms or not. **(B)** Status of close contacts. **(C)** Relationship with indexed cases. **(D)** Contact mode with indexed cases.

policy of China and WHO is more or less the same. However, with the changes of epidemic situation and normalization management, China's management measures for close contacts have been gradually revised and improved. It is mainly reflected in defining the number and times of nucleic acid detection, so as to understand the outcome of close contacts as soon as possible. Through the close contacts tracking of suspected cases of COVID-19 in Chengdu, as of May 1, 2020, 986 close contacts in cities around Chengdu were brought into effective medical observation, and 7 were converted to confirmed cases (attack rate 0.71%), significantly lower than other cities in China (3.7%) (17) and Ireland (7.0%) (18). These results suggested that tracking and management of close contacts could effectively reduce the delay between infection and isolation, thus preventing the further spread of the virus.

During the COVID-19 pandemic, the impact of community environment is enormous, which has proved that early and strict isolation tracking was an effective strategy to limit clusters (19, 20). Close contact tracing mainly includes identification, listing, and tracking, and is an important aspect of epidemic control and often needs the help of all sectors of society (21). Besides, it is also a tedious task that requires a lot of human resources and cannot be fully implement in areas with widespread transmission (22–24). How to accurately identify and track management is still a difficult problem. At present, the most commonly used tracking technologies in the world are software and applications such as the CoV-SCR web-app (25, 26), which provides convenience for secret connection management. But there are still drawbacks. At the outbreak of the epidemic in Chengdu, the Chengdu Center for Disease Control and Prevention urgently developed a "Close Contact Information Management System" to dynamically identify cases and their close contacts. To some extent, this restrained the spread of the epidemic and the occurrence of second-generation cases.

Evidence so far showed that the transmission of COVID-19 occurred in the prodromal stage of mild illness of the infected person, and the interpersonal activities contributed to the spread of infection (8, 27). To curb the spread of the disease, the Chinese government has blocked the source city since January 23, 2020. However, the large-scale population movement during the Spring

Festival may have contributed to the spread of the disease (7, 9). According to the big data of the Sichuan Mobile Network, from January 10 to January 20, as many as 22,000 people entered Chengdu from Wuhan. At the same time, COVID-19 carriers among them may have spread the virus to their contacts through work, travel, and gatherings (28), which undoubtedly increased the difficulty of epidemic prevention and control. The analysis of 986 close contacts found that the main contacts were passengers and relatives (80.42%), while the main modes were transportation and gatherings (71.70%), indicating that the key population to focus on for epidemic prevention and control should be co-passengers and relatives.

Similar to SARS and MERS, hospital transmission was a serious problem of COVID-19, or even worse. A recent retrospective study showed that 1,716 health workers were infected, accounting for 3.84% of the total cases (11). In this survey, medical personnel accounted for 2.03% of close contacts, and 9.13% of people became new close contacts through diagnosis and treatment and contact in the hospital. Nosocomial infections have greatly increased the burden on the health system and hindered early infections from obtaining timely medical support (29). In turn, it also suggested that the prevention and control of nosocomial infection may hinder the spread of the epidemic to a certain extent.

Our observational study has several limitations of importance for its interpretation, which mainly manifested in determining the possibility of recall bias and selection bias. First, tracking contacts through interviews are prone to recall bias, because individuals may not be able to recall events that occurred 14 days ago accurately, resulting in omissions or prolonging the finding time of some close contacts. Thus, close contact tracing systems or software seem to be particularly important (24). Besides, due to the existence of exclusion factors, it is indeed possible to determine the existence of selection bias, which may also have a certain impact on the attack rate. Fortunately, through the control of close contacts, the spread of the epidemic caused by close contacts has not been confirmed, minimizing the possibility of second-generation cases. At the same time, no association was found between the missing close contacts and previous cases. In addition, the data analyzed in this study seem to be out of date at this stage. To avoid this defect, we will analyze the latest data in our future research and compare the data differences between the two stages.

CONCLUSION

Collectively, these findings illustrated that transportation and gatherings were the main ways to cause close contact infection. While focusing on co-passengers and relatives, we should also pay attention to nosocomial infection. Isolating close contacts at home or intensively for 14 days and monitoring their health every day could be part of the active case detection. We believe that if the public is encouraged to maintain their own contact list every day, this will help greatly to reduce the time and effort for contact tracing.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KY: conceptualization, methodology, formal analysis, and writing—original draft. JD: data curation and writing—original draft. LW: data curation and software. SJ: methodology and supervision. RL: software and validation. XT: conceptualization and project administration. ZL: data analysis and manuscript modification. All authors reviewed and agreed upon the final version of the manuscript.

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Serum Lactate Dehydrogenase Level as a Prognostic Factor for COVID-19: A Retrospective Study Based on a Large Sample Size

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Background: In this study, we investigated the relationship between serum lactate dehydrogenase (LDH) level and disease progression and prognosis of patients with COVID-19.

Methods: We retrospectively reviewed the information of 1,751 patients with COVID-19 from Leishenshan Hospital in Wuhan, China. Univariate and multivariate Cox regression analyses as well as Logistics regression analyses, and Kaplan-Meier curves were used to determine the association between LDH levels and the prognosis of COVID-19 patients.

Results: LDH was an independent risk factor for in-hospital death no matter it was taken as classified variable and continuous variable (all $P = 0.001$) but not for severe or critical illness status. The Kaplan-Meier curves for LDH level showed that an elevated level of LDH was associated with in-hospital death.

Conclusions: In patients with COVID-19, the increased LDH level is associated with a higher risk of negative clinical prognosis and higher mortality. This will provide a reference for clinicians and researchers to understand, diagnose, and treat patients with COVID-19. Further prospective studies with larger sample sizes are needed to verify these findings.

Keywords: COVID-19, lactate dehydrogenase (LDH), SARS-CoV-2, prognostic factor, Leishenshan Hospital

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INTRODUCTION

The world is currently experiencing a major coronavirus disease 2019 (COVID-19) pandemic (1–3). Although COVID-19 can cause severe illness, the case fatality rate is relatively low (4). As of 22 April 2020, more than 2,500,000 cases were reported worldwide, with more than 170,000 deaths.

Leshenshan Hospital is hosted by Zhongnan Hospital and is a temporary, specialized 1,600-bed hospital designated for the treatment of patients with COVID-19. From February 8, 2020 to

April 15, 2020, 1,880 patients with confirmed COVID-19 were admitted. Lactate dehydrogenase (LDH) is one of the enzymes of the glycolytic pathway that catalyzes the conversion of pyruvate to lactate with concurrent conversion of reduced nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD) (5). Elevated LDH levels have been shown to be associated with more severe disease and increased mortality in multiple diseases (5–7).

Clinicians and researchers have been making efforts to understand and cure COVID-19; however, knowledge of its pathogenesis is limited (8–10). In our study, we investigated the effect of serum LDH levels on the disease progression and prognosis of patients with COVID-19. There is usually a normal range for the measurement of LDH in clinical application. The study group assignment in this study was generated based on the normal range of LDH level but we also took LDH as a continuous variable when conducting analyses so that we can intuitionally detect the relation between LDH level and the prognosis of COVID-19 patients.

MATERIALS AND METHODS

Study Design and Patients

We conducted a retrospective cohort study of 1,880 patients with laboratory-confirmed SARS-CoV-2 infection, who were admitted to Leishenshan Hospital in Wuhan, China, with COVID-19 between February 9 and March 18, 2020. The medical records of these patients were reviewed by two experienced physicians, and detailed information on patient demographics, clinical features, laboratory test results, computed tomography (CT) images, and treatment were extracted. A total of 129 patients who did not have an LDH test or whose LDH test results were missing were excluded, leaving 1,751 patients for the analysis. Of these patients, 1,653 had an LDH level within the normal range (125–343 U/L), 43 had a low LDH (<125 U/L), and 55 had an elevated LDH (>343 U/L). Considering the clinical implications of the LDH results, patients with a normal or decreased LDH level were assigned to one group and compared with the 55 patients with an elevated LDH level. LDH was also taken as a continuous variable when conducting the analyses for the prognosis of COVID-19 patients. All the laboratory findings were baseline data including LDH level.

Definitions

The primary outcome in this study was the occurrence of death during the period of hospitalization. The illness status was defined according to the seventh edition of the Chinese management guideline for COVID-19 published by the Chinese National Health Commission (11). We acquired records of the illness status on admission and the highest level of illness status of patients during their hospitalization. The latter was also used as an outcome in this study. Mild and common cases were assigned in one group while severe and critical cases were combined into one group when illness status was used as an outcome in analysis. The survival time in this study was defined as the period from the day that patients on admission to the day deaths occurred or follow-up stopped and it was described as "follow-up days." An axillary temperature over 37.3°C was defined as fever. A semi-quantitative score system based on the results of the CT images was generated to evaluate the pulmonary lesions of COVID-19 patients. Each of the image characteristics including ground-glass opacities, reticulation or cords change, consolidation, and pleural effusions were assigned 1 point. Score 1 was the sum of the points mentioned above. Score 2 was assigned based on the area of the lung lobes involved: no involvement (0 points); <25% involvement (1 point); 26–50% (2 points); 51–75% (3 points); >75% (4 points). The total score was equal to the sum of score 1 and score 2.

Ethics Approval and Patient Consent

This study obtained the approval of the Research Ethics Commission of the Zhongnan Hospital of Wuhan University (approval number: 2020074). The requirement for informed consent was waived because the study was retrospective.

Statistical Analysis

All the statistical analyses were performed using SPSS Version 23.0 (IBM Corp, Armonk, NY, USA). Comparisons between the low/normal and elevated LDH level groups for categorical data were performed using the chi-square test or Fisher's exact test if the number of observations was limited. Comparisons of continuous variables were performed using independent group *t*-tests when the data were normally distributed, or Mann-Whitney U test when the data were not normally distributed. Univariate and multivariate Cox regression analyses were conducted for investigating the relation between in-hospital death and LDH level, while Logistics regression analyses were generated for detecting the relation between illness status and LDH level. Factors which were significant associated with primary outcomes in univariate analyses were selected into adjustment when conducting multivariate analyses. For intuitionally detecting the relation between LDH level and the prognosis of COVID-19 patients, LDH level was taken as both classified variable and continuous variable in the regression analyses. Based on the result of regression analyses, Kaplan-Meier survival analyses were used to explore whether LDH levels were associated with prognosis. Curve fitting analysis was performed to assess the relation between CT performances and survival time. Two-sided p-values < 0.05 were regarded as statistically significant.

RESULTS

Demographics, Clinical Information, and Treatment

The mean age of the patients in the elevated LDH group was 63.66 ± 14.49 years, which was higher than that in the normal/decreased LDH group (57.51 ± 14.36, $P = 0.002$; **Table 1**). Severe and critical cases account for a major part of patients with elevated LDH level no matter on

TABLE 1 | Demographic and clinical information for COVID-19 patients in different LDH level.

Covariate	LDH normal or decreased group (n = 1,696)		LDH elevated group (n = 55)		P-value
Age, year, mean ± SD	57.51 ± 14.36		63.66 ± 14.49		0.002
Sex					
Female	890	52.50%	26	47.30%	0.447
Male	806	47.50%	29	52.70%	
Comorbidity	487	60.30%	36	73.50%	0.066
Cardiovascular disease	331	41.00%	22	44.90%	0.587
Pulmonary disease	82	10.60%	5	13.90%	0.533
Nervous system disease	50	6.20%	4	8.20%	0.581
Endocrine disease	129	16.00%	6	12.20%	0.488
Malignancy	56	6.90%	3	6.10%	0.828
Digestive system disease	41	5.10%	4	8.20%	0.347
Illness status of COVID-19 on admission					
Mild	649	38.30%	14	25.50%	<0.001
Common	770	45.40%	14	25.50%	
Severe	260	15.30%	20	36.40%	
Critical	17	1.00%	7	12.70%	
The highest level of illness status at hospitalization					
Mild and common	903	53.40%	5	9.30%	<0.001
Severe	756	44.70%	36	66.70%	
Critical	33	2.00%	13	24.10%	
The highest level of oxygen support					
Low flow oxygen therapy	250	86.20%	6	33.30%	<0.001
High flow oxygen therapy	39	13.40%	7	38.90%	
Tracheal intubation	1	0.30%	4	22.20%	
ECMO	0	0.00%	1	5.60%	
Symptoms when admitted to the hospital					
Fever or myalgia	575	79.00%	35	79.50%	0.929
Respiratory system symptoms	588	80.80%	35	79.50%	0.842
Digestive system symptoms	74	10.20%	6	13.60%	0.444
Nervous system symptoms	24	3.30%	2	4.50%	0.655
CT score 1 in the first time	2.31 ± 0.71		2.58 ± 0.72		0.084
CT score 2 in the first time	2.30 ± 0.78		2.63 ± 0.65		0.053
CT total score in the first time	4.62 ± 1.28		5.21 ± 1.10		0.035
Antiviral therapy	811	99.10%	41	100.00%	0.552
Antibiotic therapy	484	99.40%	34	100.00%	0.646
Anticoagulation treatment	103	6.10%	21	38.20%	<0.001
Use of corticosteroid	90	5.30%	17	30.90%	<0.001
Death	8	0.50%	7	12.70%	<0.001
Follow-up days, mean ± SD	19.26 ± 8.893		22.96 ± 10.38		0.004

admission or in the highest level of illness severity during hospitalization (both $P < 0.001$; **Table 1**). In addition, more patients in the elevated LDH group required critical airway management [tracheal intubation or extracorporeal membrane oxygenation (ECMO)] ($P < 0.001$; **Table 1**), and those in the elevated LDH group had significantly higher in-hospital mortality (12.7%) than those in the normal/decreased LDH group (0.50%, $P < 0.001$; **Table 1**). Patients in the elevated LDH group were also more likely to need anticoagulation treatment and corticosteroids (both $P < 0.001$; **Table 1**).

Laboratory Findings

As shown in **Table 2**, there were significant differences in most laboratory indexes according to the LDH level. The median interleukin-6 in the elevated LDH group was above the normal range and was significantly higher than that in the normal/decreased LDH group, as was D-dimer, indicating that patients with elevated LDH had a more intense inflammatory responses and more of these patients were in a hypercoagulable state. In addition, patients with elevated LDH were more likely to have lymphopenia ($P < 0.001$). However, the prevalence of SARS-CoV-2 immunoglobulin M

TABLE 2 | Outcomes of laboratory tests for COVID-19 patients in different LDH level.

Covariate	LDH normal or decreased group (n = 1,696)	LDH evaluated group (n = 55)	*P*-value	Reference
Interleukin-6, pg/mL	1.50 (1.50–3.74)	17.77 (4.17–49.24)	<0.001	0.00–7.00
Procalcitonin, ng/mL	0.04 (0.03–0.05)	0.11 (0.06–0.21)	<0.001	<0.05
Alanine aminotransferase, U/L	22.00 (15.00–36.00)	44.00 (29.00–80.00)	<0.001	9.00–50.00
Aspartate aminotransferase, U/L	19.00 (16.00–26.00)	42.60 (31.00–86.00)	<0.001	15.00–40.00
Albumin, g/L	37.80 (35.10–40.10)	32.80 (30.30–36.20)	<0.001	40.00–55.00
Creatine kinase, ng/mL	51.00 (36.00–74.00)	91.00 (50.00–172.00)	<0.001	18.00–198.00
Total bilirubin, μmol/L	9.10 (7.00–11.90)	9.50 (6.00–18.00)	0.344	5.00–21.00
Direct bilirubin, μmol/L	3.10 (2.40–4.20)	4.60 (2.60–8.00)	<0.001	0.00–7.00
Indirect bilirubin, μmol/L	5.70 (4.30–7.80)	4.80 (3.40–7.60)	0.085	1.50–1.80
Creatinine, μmol/L	64.10 (54.10–76.00)	67.10 (57.40–90.00)	0.730	64.00–104.00
Ureanitrogen, mmol/L	4.80 (3.90–5.80)	5.90 (4.10–8.19)	0.001	2.80–7.60
INR	0.97 (0.93–1.01)	1.01 (0.97–1.10)	<0.001	0.85–1.15
Prothrombin time, s	11.30 (10.90–11.70)	11.7 (11.3–12.73)	<0.001	9.40–12.50
Thrombin time, s	17.60 (17.00–18.30)	16.95 (16.23–18.00)	0.001	10.30–16.60
Activated partial thromboplastin time, s	27.20 (24.55–30.40)	28.20 (24.98–33.13)	0.095	25.10–36.50
Fibrinogen, g/L	2.92 (2.51–3.67)	4.08 (3.09–4.75)	<0.001	2.38–4.98
D-dimer, mg/L	0.37 (0.21–0.87)	1.37 (0.61–4.07)	<0.001	<0.50
White blood cell count, $\times 10^9$/L	5.68 (4.70–6.8)	6.92 (5.27–9.40)	<0.001	3.5–9.5
Neutrophil count, $\times 10^9$/L	3.25 (2.53–4.23)	4.53 (3.35–7.89)	<0.001	1.8–6.3
Lymphocyte count, $\times 10^9$/L	1.62 (1.27–1.99)	0.96 (0.59–1.35)	<0.001	1.1–3.2
Monocyte count, $\times 10^9$/L	0.50 (0.40–0.63)	0.57 (0.41–0.74)	0.108	0.1–0.6
Red blood cell count, $\times 10^9$/L	4.12 (3.77–4.49)	3.90 (3.52–4.40)	0.035	4.3–5.8
Hemoglobin, g/L	126.00 (115.00–137.00)	122.00 (107.00–134.00)	0.125	130–175
Hematocrit, %	38.00 (34.90–40.90)	36.90 (32.20–40.20)	0.041	40.00–50.00
Platelet count, $\times 10^9$/L	229.00 (188.00–277.00)	203.00 (141.00–280.00)	0.020	125.00–350.00
IgM (+) of SARS-CoV-2	199 (35.20%)	9 (40.90%)	0.584	(–)
IgG (+) of SARS-CoV-2	504 (29.70%)	19 (34.50%)	0.316	(–)

TABLE 3 | Univariate and multivariate Cox regression analysis for the survival of patients in different LDH level.

Group		Cox regression analysis			
		HR	95% CI		*P*-value
Univariate analysis	LDH normal or decrease group	ref			
	LDH evaluated group	26.626	9.624	73.661	<0.001
Multivariate analysis*	LDH normal or decrease group	ref			
	LDH evaluated group	4.491	1.218	16.560	0.024

**Adjust for age, the history of cardiovascular disease, WBC, PLT, lymphocyte count, D-Dimer.*

TABLE 4 | Univariate and multivariate cox regression analysis for the survival of patients and logistics regression analysis for the severity of patients when taking LDH as a continuous variable.

Group		Cox regression analysis				Logistics regression analysis			
		HR	95% CI		*P*-value	OR	95% CI		*P*-value
Univariate analysis	LDH level	1.002	1.001	1.002	<0.001	1.012	1.010	1.014	<0.001
Multivariate analysis*	LDH level	1.006	1.002	1.009	0.001	1.003	0.992	1.014	0.577

**Adjust for age, the history of cardiovascular disease, WBC, PLT, lymphocyte count, D-Dimer.*

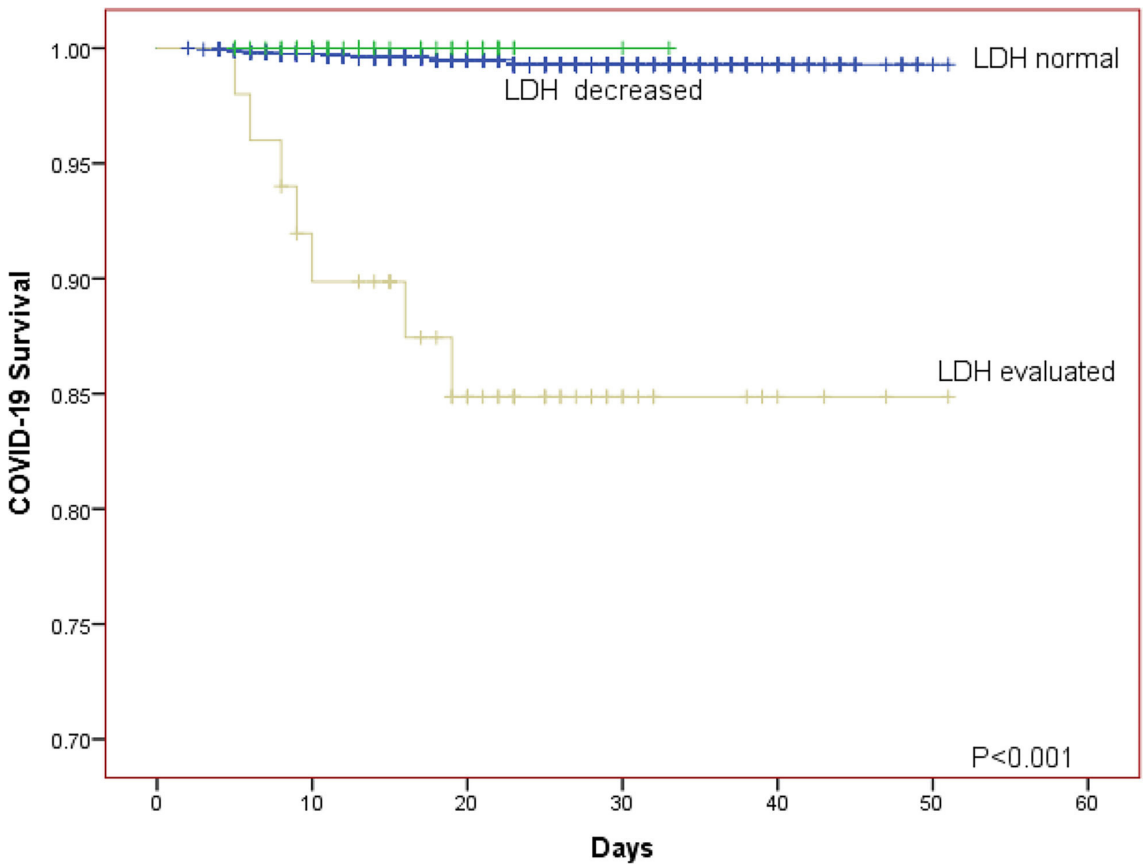

FIGURE 1 | The Kaplan-Meier curves for the survival of COVID-19 patients in different LDH level (normal group, decreased group, evaluated group).

and immunoglobulin G did not differ significantly according to the LDH level.

Analysis for the Relationship Between Prognosis and LDH Level

The univariate Cox regression analysis showed that patients in the elevated LDH group had a higher risk of in-hospital death than those in the normal or decreased LDH group [hazard ratio (HR): 26.626, 95% confidence interval (CI): 9.624–73.661, $P < 0.001$; **Table 3**]. The result of univariate logistics regression analysis presented the same tendency that elevated LDH group suffered higher risk of developing into sever or critical illness status than those in the normal or decreased LDH group [odds ratio (OR): 11.216, 95% CI: 4.447–28.288, $P < 0.001$; **Supplementary Table 1**]. The adjustment factors included in the multivariate Cox regression model were age, history of cardiovascular disease, white blood cell count, platelet count, lymphocyte count, and D-dimer. The results of the multivariate analysis showed that an elevated LDH level was an independent risk factor for in-hospital death ($P = 0.024$; **Table 3**). Elevated LDH level was not related to sever or critical illness status after adjustment ($P = 0.997$; **Supplementary Table 1**).

LDH was taken as a continuous variable in further analysis. The result was similar to the previous analysis which LDH level was divided into groups. LDH was an independent risk factor for in-hospital death (univariate analysis $P < 0.001$, multivariate analysis $P = 0.001$; **Table 4**) but not for severe or critical illness status (univariate analysis P<0.001, multivariate analysis $P = 0.557$; **Table 4**). Each unit increase in LDH level was associated with higher risk of death (univariate analysis: HR = 1.002, 95 CI%: 1.001–1.002; multivariate analysis: HR = 1.006, 95 CI%: 1.002–1.009).

The Kaplan-Meier curves was further generated for descripting the relationship between survival of patients and LDH level. The Kaplan-Meier curves for LDH level showed that patients in the elevated LDH group had worse prognosis than those with normal/decreased LDH regardless of whether the patients were divided into two or three LDH groups (both $P < 0.001$; **Figures 1**, **2**).

Curve Fitting Analysis for the Evaluation of CT Images

Figure 3 shows the result of the curve fitting analysis for CT images and days that the CT scan was done. Score 1 for all patients reached the peak at 20 days (2.5 points, **Figure 3A**), while score 2 for all patients reached the lowest point at 12 days (2.40 points, **Figure 3B**). The total score reached the peak at 19 days (4.90 points, **Figure 3C**). Similarly, score 1 for patients

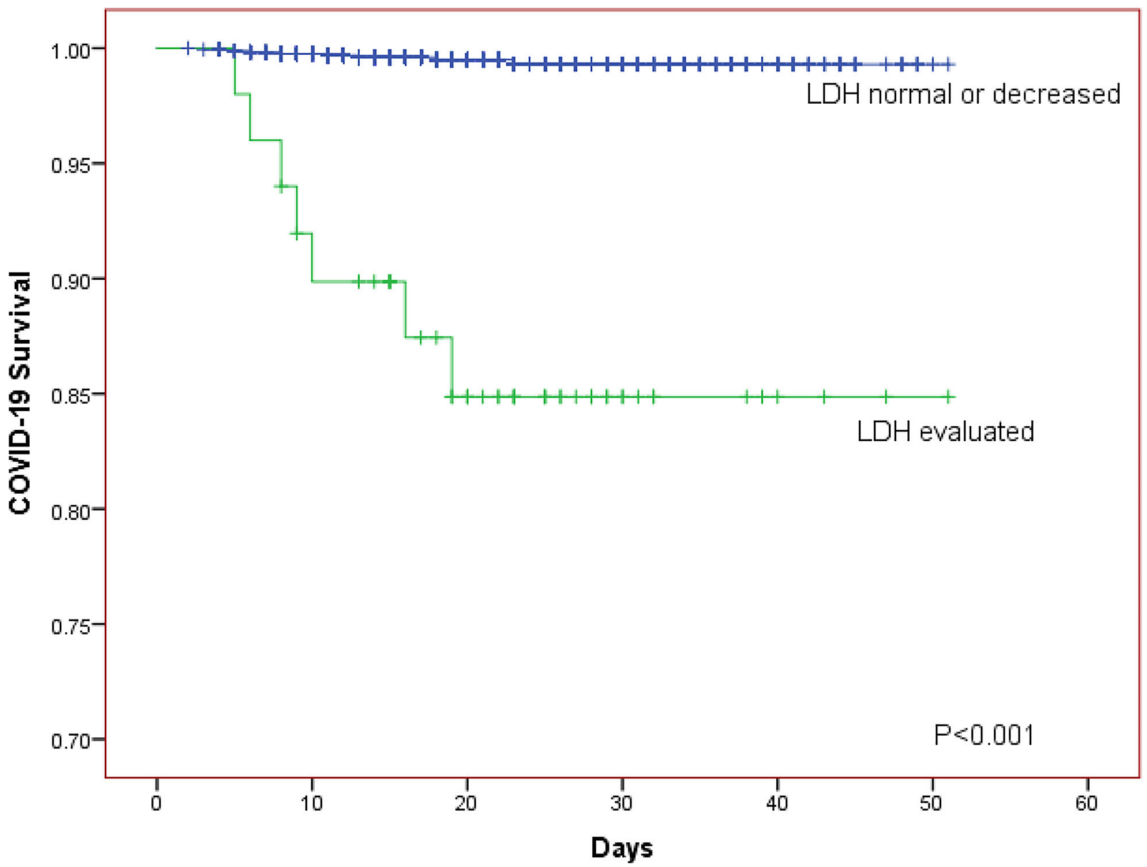

FIGURE 2 | The Kaplan-Meier curves for the survival of COVID-19 patients in different LDH level (normal or decreased group, evaluated group).

with normal/decreased LDH reached the peak of 2.42 at 21 days (**Figure 3D**), score 2 reached the peak of 2.30 at 16 days (**Figure 3E**) and total score reached the peak of 4.70 at 20 days (**Figure 3F**). For patients with evaluated level of LDH, score 1, and total score reached the peak of 2.90, 5.80 on 19, 16 days, respectively (**Figures 3G,I**). However, the tendency of score 2 for patients with elevated LDH level tended to be descending (**Figure 3H**).

DISCUSSION

We investigated the effect of LDH on the clinical course and survival of patients with laboratory-confirmed COVID-19 based on a large sample with 1,880 patients and found that patients with elevated LDH were associated with higher mortality on univariate or multivariate Cox regression analysis no matter LDH was taken as classified variable or continuous variable. The Kaplan-Meier curves for COVID-19 progress also showed the same tendency.

In addition to the reticulocyte count, indirect bilirubin levels, serum haptoglobin, and LDH levels have been used as markers of hemolysis (5). In another study, Tasaka et al. (12) found that measuring LDH levels could help improve the diagnosis of pneumocystis pneumonia. Furthermore, they found that the HIV-positive patients had higher LDH levels than HIV-negative patients. These studies reveal that LDH plays an important role in differentiating disease, including that of the immune system. In our study, although we did not compare the LDH levels in patients with COVID-19 with the LDH levels in patents with other types of pneumonia or the normal population, an elevated LDH level was predictive of higher mortality in patients with COVID-19. Therefore, LDH was shown to be associated with disease diagnosis and prognosis.

COVID-19 patients with higher LDH levels tended to be older, and were more likely to require respiratory support. On the other hand, the patients in the elevated LDH group had similar comorbidities to the other patients. In patients with pneumonia, the presence of comorbidities may adversely affect the clinical course and the outcome (13). In our cohort, the prevalence of pulmonary disease did not differ according to the LDH level; therefore, the comorbidities did not act as confounders of the association between LDH levels and survival in patients with COVID-19. Previous study found that the levels of LDH in severe cases of COVID-19 were significantly higher than both non-severe cases of COVID-19 and healthy control group, while the LDH level of non-severe cases were also higher than healthy group (14). In this study, LDH level was associated with severe or critical illness status in univariate logistics regression analysis, which was in accordance with the result of previous study.

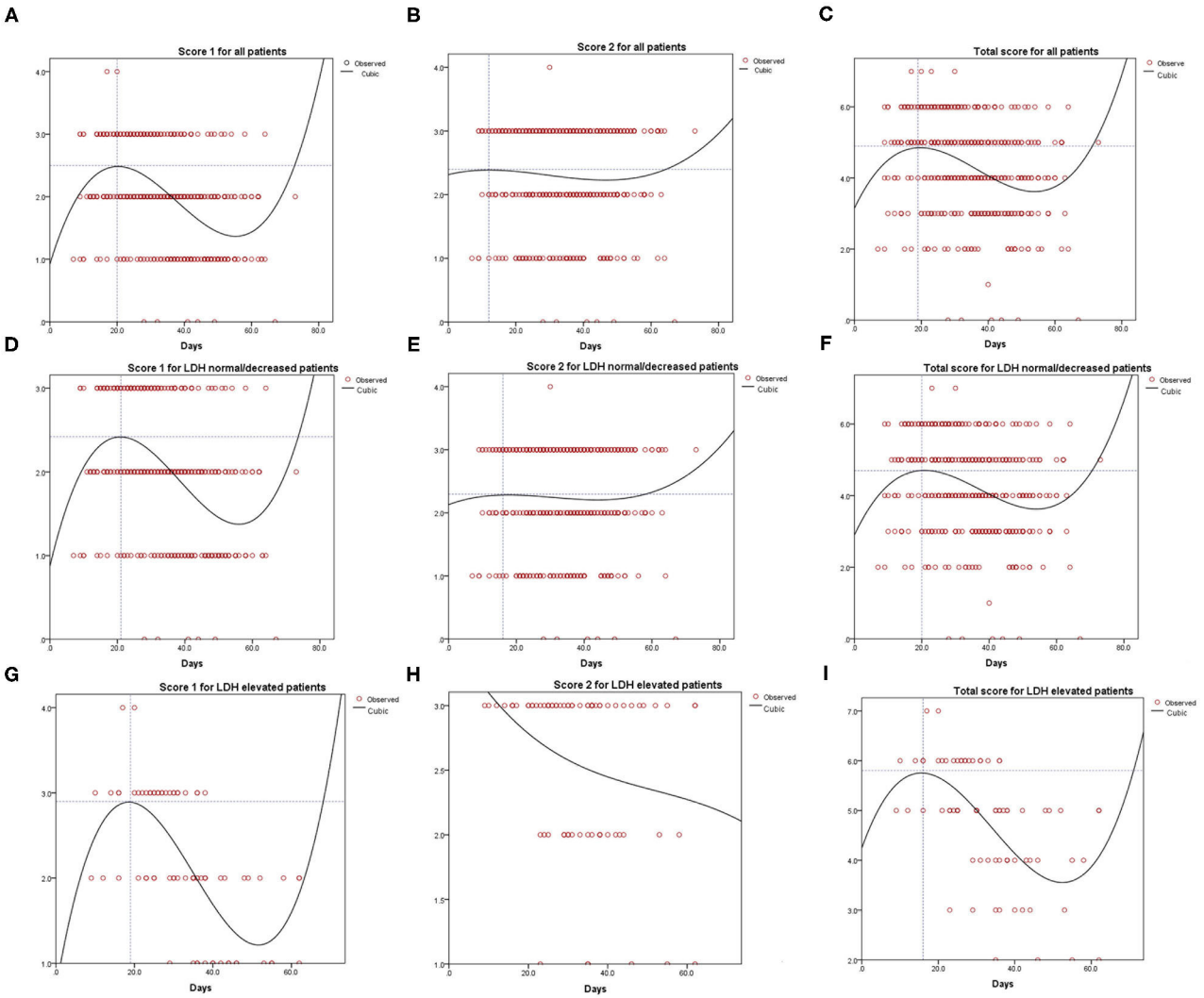

FIGURE 3 | Curve fitting analysis for all the COVID-19 patients **(A–C)**, LDH normal or decreased group **(D–F)** and LDH evaluated group **(G–I)**.

However, significant differences were not found in multivariate regression analysis which contained adjustment of confounding factors including age, the history of cardiovascular disease, WBC, PLT, lymphocyte count, D-Dimer. We hypothesized that elder patients and patients with the history of cardiovascular disease essentially burden higher risk of cardiac muscle or lung interstitial damage.

In the early phase of COVID-19, CT images reveal multifocal peripheral and basal ground-glass opacities, crazy paving patterns, traction bronchiectasis, and air bronchogram signs (15, 16). However, depending on various factors, such as the evolution of the disease course or severity, comorbidity and therapy, CT presentations are dynamic and manifestation patterns often overlap (13, 17–19). With the progression of the clinical course, the CT manifestations include pleural effusion, irregular interlobular, and septal thickening (16). In this study, the CT manifestations were evaluated and presented as score 1 (imaging feature type), score 2 (lesion distribution), and the total score (score 1 plus score 2) by two independent radiologists to record the dynamic changes (20). Fitting curve for imaging manifestations types of lung inflammation and lesion distribution in normal or lower LDH group showed a trend of first rise then descend, however, higher LDH group patients showed a trend of rapidly rising and then rapidly falling (**Figures 3G,I**) or presented as a trend of declining all along (**Figure 3H**). This may be because patients with elevated LDH tended to have severe clinical symptoms of pneumonia and were then transported to the hospital for unified and timely medical treatment.

In addition, the use of antiviral therapy and antibiotic therapy did not differ according to the LDH level among the patients in our study. However, a higher proportion of patients with elevated LDH received anticoagulation treatment and corticosteroid. Drug treatment, especially the use of corticosteroids, may

slow virus clearance due to its immunosuppressive effect (21, 22). This may affect the disease course and biochemical indicators, including LDH; therefore, further research is needed to determine the effects of corticosteroids and anticoagulants on LDH in patients with COVID-19.

Other studies have found that an elevated LDH level is a sensitive biomarker for lymphoproliferative disorders (23, 24). Ghobrial et al. (25) and Boothpur et al. (26) identified that serum LDH was one of the negative prognostic factors for overall survival and recurrence. Many studies have found a significant drop in T lymphocyte subsets and an increase in inflammatory cytokines in patients with COVID-19 (8, 27). Nguyen et al. (24) demonstrated that the SARS-CoV-2 virus could enable cross-protective T-cell based immunity in a comprehensive *in silico* analysis. In our study, patients in the elevated LDH group had a higher white blood cell count but lower lymphocyte count than patients with normal/decreased LDH. Overwhelming inflammation and cytokine-associated lung injury could be important factors in initiating severe events in patients with COVID-19 (28), Therefore, LDH may affect the clinical course of COVID-19 by causing inflammation and lung injury, and influencing T-cell based immunity.

Jiang et al. found that patients with COVID-19 had IgG and IgM antibodies which specifically combine with SARS-CoV-2 proteins, particularly the N protein and S1 protein (29). They also found that S1 specific IgG signal positively correlates with the level of LDH (29). However, whether serum lactate dehydrogenase have any similar physiological function or pathological pathway to affect the clearance of SARS-CoV-2 remains unclear and warrants further study.

This study has some limitations. Heterogeneity is an unavoidable limitation of retrospective studies Data of patients were collected retrospectively, which inevitably led to biases in our study. Another limitation is a lack of research on the mechanism of serum lactate dehydrogenase levels as a common risk factor for COVID-19 progress and prognosis. In addition, the role of drug interference such as glucocorticoids, antiviral and antibacterial treatment cannot be excluded. Further multi-center prospective studies with a larger sample size are needed to verify the findings and to determine the pathogenic mechanism by which LDH exerts an effect on patients with COVID-19.

CONCLUSION

Our study revealed that LDH level is an independent risk factor for the survival of patients with COVID-19 and a high LDH level is a predictor of mortality in patients with COVID-19. However, LDH level seems not to be associated with severe or critical illness status. This study will provide a valuable reference for clinicians and researchers to understand, diagnose, and treat patients with COVID-19, although prospective studies with a larger sample size are needed to verify the findings and to determine the pathogenic mechanism by which LDH exerts an effect on patients with COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

This study obtained the approval of the Research Ethics Commission of the Zhongnan Hospital of Wuhan University (approval number: 2020074). The requirement for informed consent was waived because the study was retrospective.

AUTHOR CONTRIBUTIONS

YH, ZLiu, and JL: conception and design. LG, KLu, and XW: administrative support. KLi and ZX: provision of study materials or patients. QW and JC: collection and assembly of data. YH, CZ, and ZLi: data analysis and interpretation. YH and ZLiu: manuscript writing. MW, WY, and XW: final approval of manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.671667/full#supplementary-material

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Lower Rate of Daily Smokers With Symptomatic COVID-19: A Monocentric Self-Report of Smoking Habit Study

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Background: Identification of prognostic factors in COVID-19 remains a global challenge. The role of smoking is still controversial.

Methods: PCR-positive in- and outpatients with symptomatic COVID-19 from a large French University hospital were systematically interviewed for their smoking status, use of e-cigarette, and nicotinic substitutes. The rates of daily smokers in in- and outpatients were compared using the same smoking habit questionnaire to those in the 2019 French general population, after standardisation for sex and age.

Results: The inpatient group was composed of 340 patients, median age of 66 years: 203 men (59.7%) and 137 women (40.3%), median age of both 66 years, with a rate of 4.1% daily smokers (CI 95% [2.3–6.9]) (5.4% of men and 2.2% of women). The outpatient group was composed of 139 patients, median age of 44 years: 62 men (44.6%, median age of 43 years) and 77 women (55.4%, median age of 44 years). The daily smoker rate was 6.1% (CI 95% [2.7–11.6], 5.1% of men and 6.8% of women). Amongst inpatients, daily smokers represented 2.2 and 3.4% of the 45 dead patients and of the 29 patients transferred to ICU, respectively. The rate of daily smokers was significantly lower in patients with symptomatic COVID-19, as compared to that in the French general population after standardisation by age and

sex, with standardised incidence ratios (SIRs) of 0.24 [0.12–0.48] for outpatients and 0.24 [0.14–0.40] for inpatients.

Conclusions: Daily smoker rate in patients with symptomatic COVID-19 is lower as compared to the French general population

Keywords: tobacco, SARS-CoV-2, cross sectional, COVID-19, smoking-epidemiology

INTRODUCTION

The COVID-19 pandemic continues to affect socially heterogeneous patient cohorts. As such, identifying relevant risk factors could allow national public health authorities to implement more targeted and efficient measures to control its spread. The role of smoking, in particular, has been implicated with a worse prognosis in patients with COVID-19 (1), although this remains controversial (2).

Amongst patients hospitalised by *severe acute respiratory syndrome coronavirus-2* (SARS-CoV-2), the crude prevalence of active smokers ranges from 1.4 to 12.5% in China (1, 3–10) to 1.3–5.1% in the USA (11, 12). These data early in the pandemic suggested that the prevalence of active smokers amongst inpatients and outpatients with COVID-19 was much lower compared to the general population. However, these data did not take into account key confounders such as age and sex. Additionally, these studies included mostly hospitalised patients in whom the reported rate of active smoking may be indirectly related to their likelihood of having respiratory or cardiovascular comorbidities. Such patients are more likely to be queried about their smoking status and receive appropriate counselling. On the contrary, the smoking prevalence may be underreported for patients who present with a non-smoking-related condition. These patients are less likely both to be asked about their smoking status and to have it accurately recorded in their medical records. Hence, we consider that the link between active smoking and the risk of SARS-CoV-2 infection has yet to be accurately determined.

To study this, we conducted an observational study that compares the rates of daily active smokers in two groups of patients with COVID-19: (1) admitted or inpatients and (2) non-admitted or outpatients. All data were collected using a dedicated *smoking habit* questionnaire (13). We also standardised our data by patient age and gender.

MATERIALS AND METHODS

Study Design

This was a cross-sectional study investigating the smoking status of patients with COVID-19 who were managed either as in- or outpatients. The inpatients had developed severe symptomatic disease whereas the outpatients had the mild form. We determined the patients' active smoking status using the same *smoking habit questionnaire* as that used in the recent French National Survey of Tobacco Consumption 2019 (13). This allowed us to standardise comparison between our cohort and the national population after accounting for age and gender.

All patients with a confirmed diagnosis of COVID-19 *via* PCR at the Pitié-Salpêtrière Hospital in Paris were eligible. We recruited them from two sources: inpatients [those hospitalised in the medical wards of medicine (excluding ICU)] and outpatients (those after the medical consultation deemed as being well enough to isolate at home). The patients in ICU were excluded as their clinical status made detailed interviewing unfeasible. All inpatient data were collected from 23 March to 9 April 2020 whereas all outpatient data were collected from 28 February to 30 March 2020. We also followed up with all inpatients a month later to collect relevant outcome data.

As per the recommendation of our Ethics and Research Committee of Sorbonne University (2020-CER-2020-13), informed consent was waived.

Study Endpoints and Definitions

We verified the smoking status of patients by specifically asking whether they were active or former smokers (or had never smoked). For the active smokers, we also asked for further details such as daily or occasional consumption and also the number of cigarettes smoked daily. We used the same definition as that of the Annual Survey of Tobacco Consumption in France (Public Health France Smoking Barometer) (13). Daily smokers were defined as individuals reporting daily consumption of cigarettes or other tobacco products (e.g., cigars, cigarillos, pipe, and shisha). Occasional smokers were those who reported infrequent consumption. Our group of former smokers included anyone who had smoked in the past (occasionally or daily) but had been abstaining before their COVID-19 diagnosis. The term "never smoker" defined patients who had never smoked.

In addition, all patients were asked whether they had used any nicotine replacement therapy (NRT, including e-cigarettes). We asked all former smokers about the duration since they had last smoked and asked active smokers whether they had quit since their diagnosis of COVID-19.

Finally, we extracted the following data from the medical records: admission status (in- or outpatient), age, sex, whether they were healthcare workers, and relevant comorbidities (e.g., diabetes, hypertension, obesity, immune deficiency, and COPD). For the inpatients, we also extracted the following outcomes at one month after their clinical presentation: admission status (with or without ICU stay), discharged without any ICU, or death (in ICU or the ward).

All COVID-19 diagnoses were based on a PCR-positive test from a nasopharyngeal swab.

TABLE 1 | Clinical characteristics and smoking habits of patients with COVID-19.

	Outpatients ($N = 139$)			Inpatients ($N = 340$)			Outpatient/inpatient comparison *p*-value*
	Male ($n = 62$)	Female ($n = 77$)	All	Male ($n = 203$)	Female ($n = 137$)	All	
Median (IQR) age (yr)	43 [32–55]	44 [32–54]	44 [32–55]	66 [55–76]	66 [56–79]	66 [55–77]	<0.001
Coexisting disorder							
High blood pressure	9 (15.3 %)	7 (9.6 %)	16 (12.1 %)	84 (41.4 %)	58 (42.3 %)	142 (41.8 %)	0.004
Diabetes	4 (6.8 %)	3 (4.1 %)	7 (5.3 %)	54 (26.6 %)	41 (29.9 %)	95 (27.9 %)	<0.001
Obesity	4 (6.78 %)	6 (8.2 %)	10 (7.6 %)	28 (14.3 %)	19 (14.1 %)	47 (14.2 %)	0.003
Immune deficiency	4 (6.8 %)	1 (1.4 %)	4 (3 %)	34 (16.7 %)	26 (19 %)	60 (17.6 %)	<0.001
COPD	2 (3.4 %)	0 (0 %)	2 (1.5 %)	17 (8.4 %)	10 (7.3 %)	27 (7.9 %)	0.381
Smoking status							0.38
Active	3 (5.1 %)	5 (6.8 %)	8 (6.1 %)	11 (5.4 %)	3 (2.2 %)	14 (4.1 %)	
Active occasional	3 (5.1 %)	3 (4.1 %)	6 (4.5 %)	4 (2 %)	0 (0 %)	4 (1.2 %)	
Former	21 (35.6 %)	20 (27.4 %)	41 (31.1 %)	76 (37.6 %)	35 (25.7 %)	111 (32.8 %)	
Never smoker	32 (54.2 %)	45 (61.6 %)	77 (58.3 %)	111 (55 %)	98 (72.1 %)	209 (61.8 %)	
Missing data	4 (6.5 %)	3 (3.9 %)	7 (5.0 %)	1 (0.5 %)	1 (0.7 %)	2 (0.6 %)	

**Except for age, p-value corresponds to logistic regression models adjusted on age and sex.*

Smoking Rates in the National Reference Population

The French population was used as a reference to compute the standardised incidence ratio (SIR). The incidence of daily smokers had already been reported by the French National Survey 2019 (Santé Publique France Health Barometer) (13). This is an annual cross-sectional survey performed on a representative sample of French metropolitan area residents (age range of 18–85) based on a two-stage random sample (13). This survey involved 10, 352 residents and the same definitions of daily smokers, occasional smokers, former smokers, and never smokers as detailed above. The age and gender were reported only for 18–75-year-old active daily smokers but not for occasional active smokers, former smokers, or non-smokers. The rate of active daily smokers in the 76–85-year-old group was reported globally and not by gender.

Statistical Analyses

A descriptive analysis was performed within each patient group. The qualitative variables were described as frequencies and percentages whereas the quantitative variables were described as median and interquartile range. We accounted for any differences in age and gender between the patient groups *via* Wilcoxon's rank-sum test and chi-squared test. We accounted for any significant differences in comorbidities and smoking status *via* logistic regression (adjusted by age and gender) instead.

The SIRs were used to compare daily smoker rates between the inpatient and outpatient groups, respectively, with those of the reference population. We also separately estimated the SIR in healthcare workers and non-healthcare workers seen in outpatients (as healthcare workers were overrepresented). To estimate SIR and its 95% confidence interval in each group, we used a Poisson regression model with log link and reference rate as offset. Finally, to compare the SIRs between the two groups, we introduced the group variable in the model.

All patients were included in the main analysis. Those older than 75 were analysed as part of the 65–75-year-old group for standardisation (considering the reference rates of daily smokers were 10.4% in men and 9% in women). In our view, this is a conservative approach as the rate of daily smokers decreases with age (only 4.8% of daily smokers amongst the 76–85-year-old French cohort in 2019). As we were unable to confirm the smoking status in six outpatients and two inpatients, we did not include them in the main analysis. Overall, we performed two sensitivity analyses: (1) after excluding patients older than 75 and (2) considering those patients with missing smoking status as daily smokers.

All analyses were performed at a two-sided α level of 5%, using R software, version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/).

RESULTS

Patient Demographics and Clinical Characteristics

The demographic and clinical characteristics of the two groups are shown in **Table 1**. Overall, we included 340 inpatients and 139 outpatients. The outpatients' cohort was younger than the inpatient cohort (median age of 44 vs. 66, respectively) (**Figure 1**). Their gender distributions were very different too. In the inpatient group, 59.7% were men compared to 40.3% women whereas, in the outpatient group, 44.6% were men compared to 55.4% women.

The inpatient group was composed of 203 men (59.7%, median age of 66) and 137 women (40.3%, median age of 66). Fourteen patients in this group were identified as daily smokers. This equated to a rate of 4.1% [CI 95%: 2.3–6.9] with 5.4% being men and 2.2% being women. Amongst them, four smoked five or fewer cigarettes daily, three smoked six to 10 cigarettes, one

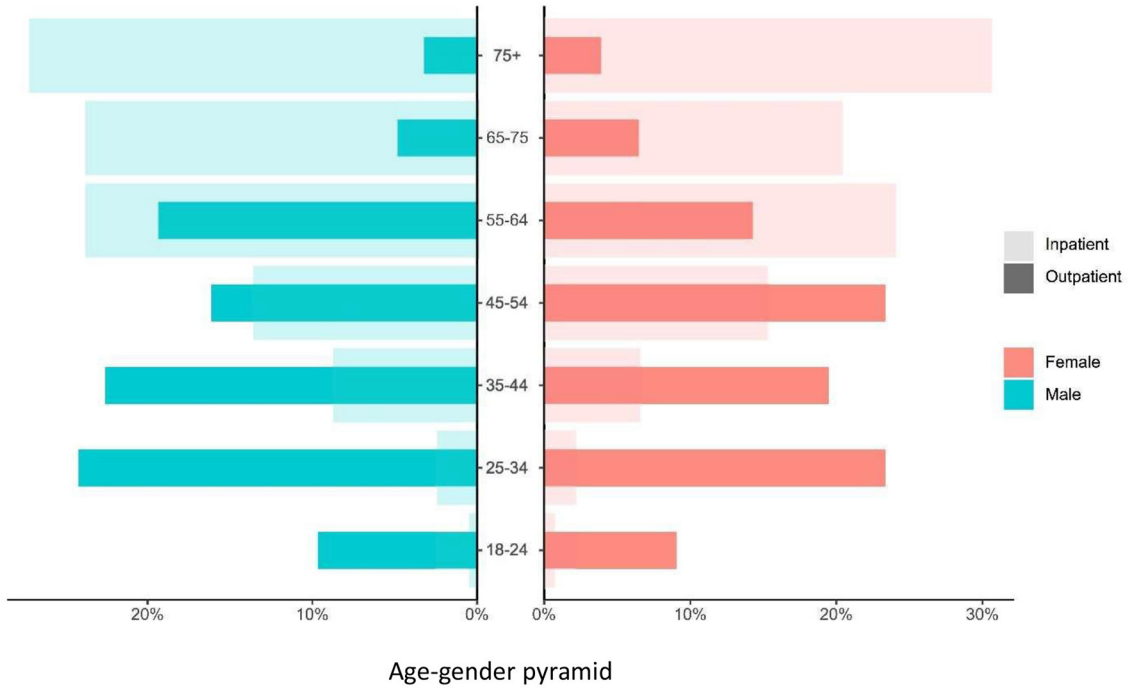

FIGURE 1 | Age and sex distribution in inpatients and outpatients with COVID-19. Dark- and light-shaded histograms represent nonadmitted (outpatients) and admitted (inpatients) patients with confirmed COVID-19 status, respectively. Red represents female patients and blue represents male patients.

smoked 15 cigarettes, and five smoked 20 or more. We did not have the data for one patient in this group.

With regard to the former smokers in the inpatient cohort (n = 111, 32.8%), we had information on the abstinence period for all except six. Five former smokers (4.8%) had been abstinent for 2 months, 2 (1.9%) for 6 months, and 98 (93.3%) for more than a year before being infected with COVID-19. Two former smokers (1.9%) were using nicotine substitutes (one with e-cigarettes and one with patches) at the time of disease onset.

The outpatient group was composed of 62 men (44.6%, median age of 43) and 77 women (55.4%, median age of 44). Sixty-eight (51.5%) in this group were healthcare workers. Smoking status was missing for seven patients. Eight patients were identified as daily smokers. This equated to a rate of 6.1% [CI 95%: 2.7–11.6] (5.1% of men and 6.8% of women). Amongst them, three smoked <5 cigarettes daily, three smoked 6–10, and two smoked 20 cigarettes or more. Since their diagnosis of COVID-19, two stopped smoking completely without NRT.

With regard to the former smokers in the outpatient cohort (n = 41, 31.1%), two (4.9%) had been abstinent for 3 months and 39 (95.1%) for more than a year before being infected with COVID-19. Two (4.9%) were using nicotinic substitutes of which one used e-cigarettes. Finally, amongst the 77 non-smokers, none were using a nicotinic substitute.

Unsurprisingly, the inpatient group was also more multimorbid than the outpatient group. Examples of contributing conditions (after age and gender adjustment) included the following:

- hypertension [OR_{adj} = 2.5 (95% CI; 1.4–4.8), p = 0.004]
- diabetes [OR_{adj} = 5.4 (95% CI; 2.4–13.7) $p < 0.001$]
- obesity [OR_{adj} = 3.7 (95% CI; 1.7–8.9), p = 0.002]
- immune deficiency [OR_{adj} = 12.45 (95% CI; 4.6–44.3), $p < 0.001$].

The odds ratio of COPD was not significantly different; OR_{adj} = 2.0, p = 0.38.

Comparing the Daily Smoker Rate With the French Population

In the main analysis (**Figure 2**), age- and gender-adjusted SIRs of daily smokers were 0.24 [0.12–0.48] and 0.24 [0.14–0.40] for outpatients and inpatients, respectively (**Table 2**). Within the outpatients' group, the SIR was 0.17 [0.05–0.53] for the healthcare workers subgroup and 0.32 [0.13–0.76] for the others. Our sensitivity analyses also yielded similar results (**Table 2**).

Of note, the daily smoker rate within the 76–85-year-old patients was 1.6% (inpatients) and 3.8% (outpatients). This was lower than the 4.8% observed in the corresponding age-specific French population (2019 data).

Outcome of Inpatients With COVID-19

We followed up with all patients in this cohort one-month post-presentation (regardless of active admission status) (**Table 3**). Fifty-four (15.9%) were still on the medical ward whereas 29 (8.5%) had been transferred to ICU. There had been 46 deaths (13.5%) in ICU or the ward. Finally,

211 (62.1%) had been discharged without requiring any ICU stay.

Amongst the 14 daily smokers, all were discharged except for one who was transferred to ICU and one who died. Twenty-three former smokers (20.7%) and 21 non-smokers (10%) died whist 11 former smokers (9.9%) and 17 non-smokers (8.1%) were transferred to ICU. Thus, active smokers represented 2.2 and 3.4% of the 45 deaths and the 29 patients transferred to ICU, respectively.

DISCUSSION

Our monocentric study shows that the rate of daily smokers is significantly lower amongst the patients with symptomatic COVID-19 compared to the French population. This was regardless of the patients' admission status. The SIRs of daily smokers in the outpatients and inpatients groups were identical at 0.24 [0.12–0.48] and 0.24 [0.14–0.40], respectively, which is 76% lower than that of the French population (after adjusting for age and gender).

TABLE 2 | Standardised incidence ratios for daily smokers.

	SIR CI 95%	*p*-value
Main analysis—Inpatients	0.24 [0.14–0.40]	<0.001
Main analysis—Outpatients	0.24 [0.12–0.48]	<0.001
Sensitivity analysis excluding patients older than 75—Inpatients	0.27 [0.15–0.46]	<0.001
Sensitivity analysis excluding patients older than 75—Outpatients	0.18 [0.08–0.40]	<0.001
Sensitivity analysis considering the patients with missing smoking status as daily smokers—Inpatients	0.27 [0.17–0.44]	<0.001
Sensitivity analysis considering the patients with missing smoking status as daily smokers—Outpatients	0.43 [0.26–0.71]	<0.001
Outpatient healthcare workers	0.17 [0.05–0.53]	<0.001
Outpatients without healthcare workers	0.32 [0.13–0.76]	<0.001

Main analysis involved all included patients. Patients older than 75 were analysed in the 65–75 years of age range for standardisation.

However, the SIRs did not differ between outpatients and inpatients, suggesting that the potential role of smoking in modulating COVID-19 is independent of the infection severity. We also did not identify a link between infection severity and the number of cigarettes consumed daily. As per the 2019 national data, the mean number of cigarettes smoked daily was 12.5 (13.5 for men and 11.4 for women) (13). Moreover, we also found that nicotinic substitutes had been rarely used by former smokers and never by non-smokers. These findings were in line with our national data indicating that e-cigarette use was low in France overall (4.4% daily users) and that they were very rarely used by non-smokers (1% of e-cigarette users).

Previous studies have also reported a low rate of active smokers amongst patients with COVID-19. In China, this was 1.4–12.6% (1, 3–10) (compared to 27.3% of all adult smokers nationally) whereas, in the USA, this was 1.3% nationally (CDC data) (compared to 14% of all adult smokers nationally). In New York City, this rose to 5.1% instead (11, 12).

Our study collectively investigated the smoking status of outpatients and inpatients infected with COVID-19. Hence, at the time of the study, it was not possible to accurately assess whether the severity of COVID-19 infection was related to active smoking. Patients with severe COVID-19 are generally more multimorbid and may have been previously advised to quit smoking. In the initial data from China, the smoking status of both inpatients and outpatients was not considered separately (1, 3–10). The Centers for Disease Control and Preventions (CDC, USA) found the incidence of active smokers to be 1.3% for their national cohort of patients with COVID-19. More specifically, this was 1% for outpatients, 2% for patients hospitalised but not in an ICU, and 1% for patients admitted to ICU (14). However, it is important to consider that many patients overall did not have their smoking status even recorded in their medical records. Moreover, all other previous studies (except two) have only reported the crude rates of active smokers and not included a control group or the corresponding national population. In those two studies that did include a reference national population, there was neither any statistical comparison nor adjustment for age or gender distribution (1, 14).

Our findings are confirmed by those of other international cohorts. For example, in one Italian study involving patients with COVID-19 admitted to medical wards only, the proportion of active smokers was significantly lower in the COVID-19

TABLE 3 | Outcomes of patients.

	n	Discharged	Still hospitalised	Transferred to ICU	Died
Daily smokers	14	9 (64.3%)	4 (28.6%)	1 (7.1%)	1 (7.1%)
Occasional	4	4 (100.0%)	0 (0%)	0 (0%)	0 (0%)
Former smokers	111	58 (52.3%)	19 (17.1%)	11 (9.9%)	23 (20.7%)
Nonsmoker	209	141 (67.5%)	30 (14.4%)	17 (8.1%)	21 (10.0%)
Smoking status unknown	2	0 (0%)	1 (50.0%)	0 (0%)	1 (50.0%)
Total	340	211	54	29	46

The following outcomes at 1 month after clinical presentation were identified: discharged without any ICU stay, ongoing hospitalisation in the medical ward without ICU admission, or if they occurred earlier: transferred to ICU and still alive at day 30 and finally death (in ICU or the medical ward).

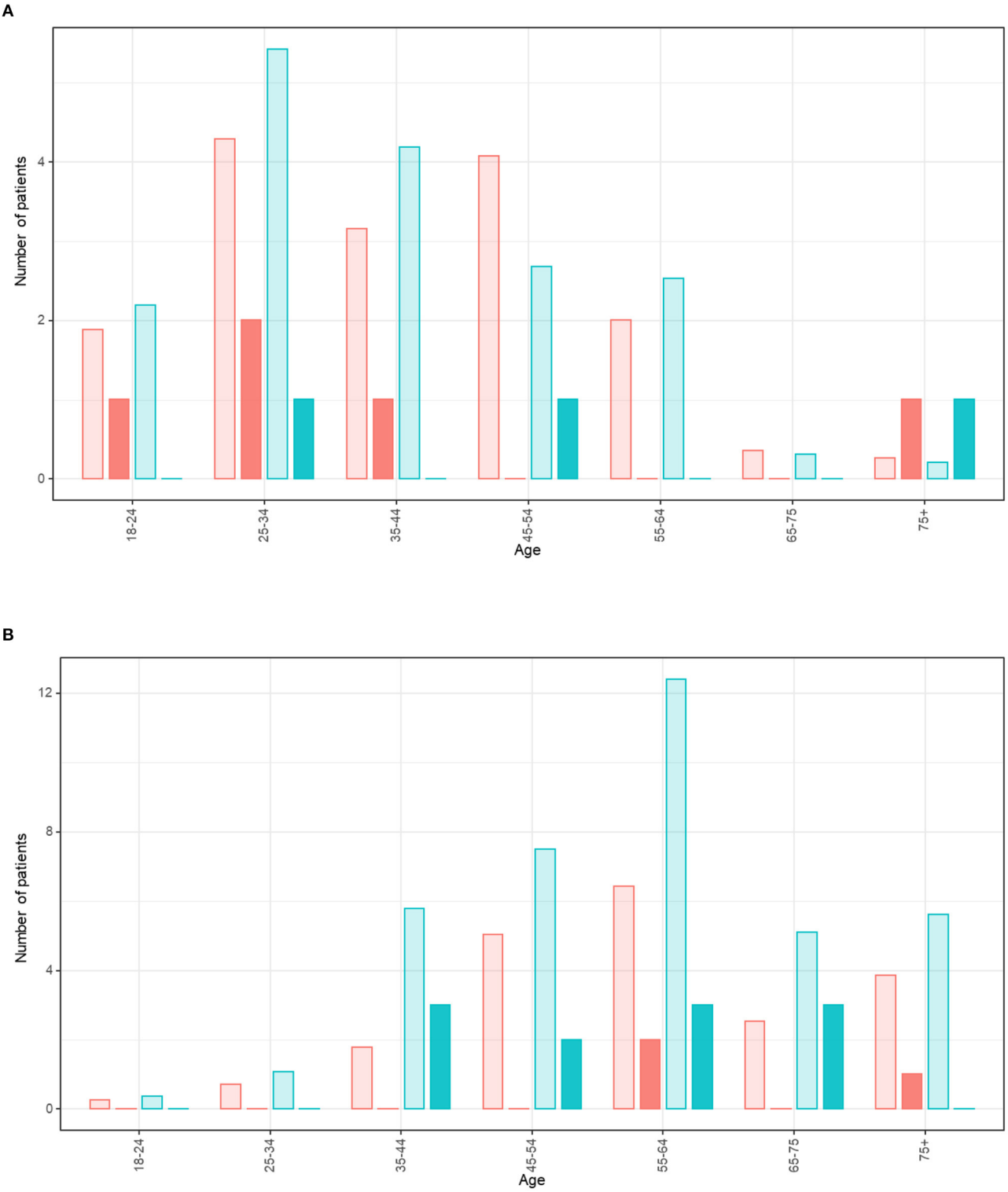

FIGURE 2 | Expected and observed number of cases of daily smokers amongst patients with COVID-19 (categorised by age and gender). **(A)** For outpatients. **(B)** For inpatients. The red bars represent female smokers and blue bars represent male smokers. The bars with lighter shading represent the expected number of daily smokers of each age and gender amongst the patients with COVID-19 in reference to 2019 French general population. The dark bars represent the observed number of daily smokers of each age and gender amongst the patients with COVID-19.

group compared to the non-COVID-19 group (4.1% vs. 16%, $p = 0.00003$). Active smokers were also significantly less likely to be hospitalised for COVID-19 compared with non-smokers after adjusting for age and gender (*OR* 0.14; 95% CI, 0.06–0.31, $p < 0.001$) (15). Moreover, in a prospective cohort study using routinely collected data from 1,205 general practitioners in England with 8.28 million participants aged 20–99 years, the proportion of light, moderate, and heavy smokers was also significantly lower in the 19,486 patients who had COVID-19 compared to the total population (5.66 vs. 13.4%, 0.8 vs. 2.58%, and 0.5 vs. 1.19%, respectively) (16, 17). A cross-sectional study in the UK, analysing the smoking status of 3,802 patients registered with the Royal College of General Practitioners Research and Surveillance Centre primary care sentinel network, also found a lower rate of COVID-19 positivity amongst active smokers (11.4%) compared to non-smokers (17.9%) (18). Similar findings were also identified amongst individuals living in homeless shelters in Chicago (19).

Domestically in France, such findings have also been replicated in other regions. In one study ($n = 661$), the active smokers had a lower risk of confirmed COVID-19 compared to non-smokers (7.2 vs. 28.0%; age-adjusted *OR* = 0.23; 95% CI = 0.09–0.59). This association remained significant after adjustment for occupation too (20). Similarly, during the COVID-19 breakthrough that occurred on the Charles de Gaulle aircraft carrier between 21 January to 13 April 2020, the rate of active smokers was lower amongst the COVID-19 infected crewmembers compared to their non-infected colleagues (45 vs. 58%). As per the univariable analysis, this equated to an odds ratio of 0.59 (95% CI; 0.45–0.78; $p < 0.001$) for active smokers vs. former or non-smokers (21). Another study covering the clinical characteristics and factors associated with hospital admission or death in 43,103 adult outpatients described a lower rate of worsening amongst patients who reported being current smokers. The current tobacco use odds ratio was 0.67 [0.47–0.95] for clinical worsening association (22).

Overall, our study has multiple strengths. In contrast to reported work, our study was specifically designed to assess smoking habits in patients with COVID-19. Early studies discussed above had assessed patient smoking status depending on what was recorded in the medical files (1, 3–10). This aspect is often underreported by most clinicians except those involved in respiratory or cardiovascular medicine. We systematically asked patients about their smoking habits and the use of nicotinic substitutes. Although we conducted this study with a systematic and standardised investigation of smoking habits and the use of nicotinic substitutes, in the French context of care, where the smoking status does not impact the access to the best level of care, we cannot completely exclude that self-report in smoking habits might be underestimated and underreported in a context of emergency crisis, but this is unlikely. Moreover, our rate of missing data, one of the most frequent caveats of studies reported so far, was very low (1.9%). Additionally, to completely rule out the impact of missing data on the conclusion of our study, we did a sensitivity analysis that considered patients with missing smoking status as daily smokers. In this analysis, the SIR remained significantly below, thus demonstrating the robustness of our results. Furthermore, we calculated this using the same definitions as those within the French Annual National Survey of Smoking (Public Health France Barometer) (13). Finally, we investigated apart from the association of daily smoking with COVID-19 separately in outpatients and inpatients, which provides additional relevant information to previous studies.

Our study has also certain limitations. First, this work was performed in early 2020 whereas our data on the national reference population dated from 2019. Whilst the difference between both years is likely minimal, we know from previous data that the rate of daily smokers in France has declined in recent years (from 26.9% in 2017 to 24.0% in 2019). In addition, our work looked into the patient population at one hospital and could not be representative of the general population. Our SIRs were calculated based on the assumption that our cohort who mainly originated from the catchment area around a Parisian hospital had the same smoking habits as the general French population. This is important to consider as the rates of smoking rates are lower in the Paris region (22.1% in 2017) compared to other French regions (26.9% in France overall in 2017) (23).

Furthermore, healthcare workers were overrepresented in our outpatient group due to the wider availability of testing at their workplace. Healthcare workers represent a heterogeneous population with similarly heterogeneous rates of smoking habits in France (24) and elsewhere. In a systematic review and meta-analysis, the prevalence of tobacco use in healthcare workers was 21% (31% in men and 17% in women) (25). Additionally, even when estimating the SIR separately in healthcare and non-healthcare outpatients, we still observed significantly lower daily smokers rates in the outpatients than in the general population. Notably, this difference was not identified within our inpatients' cohort where healthcare workers were not overrepresented. It is thus very unlikely that the very low SIRs that were estimated both for the out- and inpatient groups are the result of the study setting (we observed a 76% decrease in the COVID-19 population as compared to the French population, which is very substantial). Smoking rates may differ across ethnic, social status, and socio-professional categories. However, those information were not available in the French national Baromètre Santé survey, preventing us from standardising on these variables. Finally, due to the lack of separately available age or gender data, we were unable to calculate the adjusted SIRs of other subgroups such as former smokers or non-smokers.

A further issue with our study is that we could not include patients admitted to ICU. Hence, we were not able to conclude whether active smoking was associated with very severe forms of COVID-19. Importantly, in our study, active smokers represented 2.2% of the patients who died and 3.4% of those transferred to ICU, respectively. This compares favourably with the rate of 4.1% active smokers in the inpatient cohort. This was also replicated in a multicentre cohort study of 4,244 ICU patients in France, in which the rate of active smokers rate was very low (4%) amongst ICU patients with COVID-19 (26). In addition, Hippisley-Cox et al. also showed a low rate of smokers amongst patients admitted to ICU compared to the total population (3.65 vs. 13.4%, 0.54 vs. 2.58%, and 0.16 vs. 1.19%, respectively) (16).

A further potential issue with our methodology is that the information gleaned was self-reported by patients. This is important to consider in the light of established literature on social desirability bias, which suggests that patients underestimate their real cigarette consumption (27). Although we used the same methodology as the national smoking survey, we consider that any potential bias would have equally affected our study cohort and the national reference cohort in a similar manner. Moreover, as access to healthcare in France is not based upon any private insurance-based health incentive or otherwise, there was no patient advantage in underreporting their smoking status.

In addition, the smoking status in our study was only assessed in patients with symptomatic COVID-19 whereas a proportion of infected individuals can remain asymptomatic (28). Thus, we cannot conclude whether daily smoking is associated with SARS-CoV-2 infection, or with symptomatic forms of this infection. The recent study by Fontanet et al. (20) that relied on SARS-CoV-2 serologies and thus considered both symptomatic and asymptomatic COVID-19 highlighted a decrease in the risk of COVID-19 of the same order of magnitude and provides an answer to this question.

Finally, although our study provides an important perspective in COVID-19 care, our findings remain observational. All things considered, our data may suggest that the effect of tobacco smoking on COVID-19 could be mediated by nicotine rather than whole tobacco smoke. Nicotine can modulate the angiotensin converting enzyme 2 (ACE2) receptor (29–31), which SARS-CoV-2 uses for cellular entry (32–34). This in turn modulates the nicotinic acetylcholine receptor (35). Hence, we hypothesise that SARS-CoV-2 alters the control of the nicotine receptor through acetylcholine. This would explain why previous studies also identified an association between smoking and COVID-19 severity (1, 3, 6). As hospitals generally impose smoking cessation and nicotine withdrawal at the time of hospitalisation, tobacco (nicotine) cessation could lead to the release of nicotine receptors, whose expression is already upregulated in smokers. This could propagate a "rebound effect" responsible for the worsening of disease observed in hospitalised smokers. However, this hypothesis needs further investigation.

The conclusions of our study should be handled with caution. In the light of the possible increased risk of the severe form of COVID-19 amongst smokers once infected and of the long-term harmful consequences of smoking, which is responsible for a very heavy public health burden with more than 78,000 deaths per year in France, our findings need careful consideration and cannot be translated into a clinical practice despite recent studies supporting our conclusions. We want to reaffirm here the deleterious effects of tobacco.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Sorbonne University Comité Ethique et Recheche (2020—CER-2020-13). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MM, FT, SL, and ZA designed the study, analysed the data, and wrote the manuscript. VP, CM-P, JP, JH, EM, GG, EC, PH, AC, TS, and ZA recruited the patients, analysed and reviewed the data, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Universal Testing Policy for COVID-19 in Pregnancy: A Systematic Review

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Background: The coronavirus disease (COVID-19) has spread at an accelerated rate. WHO reported that in the general population, the majority are either asymptomatic or mildly infected. In view of the high risk of SARS-CoV-2 transmission from a pregnant woman to her newborn, healthcare workers and other patients, it is a raised concern whether universal testing should be implemented in this targeted population. The current guidelines have not recommended a universal testing policy. In certain European countries, however, the policy was implemented by some hospitals in regions with high prevalence of COVID-19 infection.

Aim(s): To assess the justification for universal screening of pregnant women for COVID-19 prior to admission in labor through systematic review of antenatal prevalence of asymptomatic infection, hence risk of inadvertent spread of infection.

Materials and Methods: Three databases confined to PubMed, Ovid and Science Direct were used to search for articles from November 2019 onwards published in the English language. The search was conducted using the keywords "COVID-19" or "coronavirus" or "SARS-CoV-2" and "pregnancy" or "pregnant" or "obstetric" or "labor" and "universal" or "testing" or "prevalence". The review was registered with PROSPERO.

Results: The search result retrieved 34 studies, with the majority consisting of retrospective cohort studies, while other studies such as prospective cohort study, research letters and a case series were also identified. A total of 19,958 pregnant women were universally tested until the date of report. Overall, the prevalence of universal testing among pregnant women presenting to labor and delivery units are higher in Western regions. From the total number of pregnant women 5.3% tested positive and among these, the majority (75.5%) did not manifest any symptoms at the time of testing.

Conclusion: In areas with high prevalence of COVID-19 infection, the implementation of a universal testing policy among pregnant women presenting to labor and admission units may be cost effective in helping to curb disease transmission.

Systematic Trial Registration: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020184248, PROSPERO: CRD42020184248.

Keywords: severe acute respiratory syndrome coronavirus 2, pregnancy, universal testing, prevalence, policy

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INTRODUCTION

Coronavirus disease (COVID-19), a respiratory illness that is caused by a novel coronavirus (SARS-CoV-2) is a global public health crisis and emergency. Since the World Health Organization (WHO) announced it as a pandemic on 11 March 2020, the virus has continued to spread rapidly and tremendously worldwide (1). As of 6 February 2021, the number of individuals infected globally has reached over 105 million of the population, with more than 2 million deaths[1]. Large studies of five vaccine candidates' efficacy and safety results have been publicly reported through press releases and several countries have begun implementing public vaccination, however it is still too early to perceive widespread benefit[2], as viral mutations continue to be reported.

A report by the WHO stated that approximately 80% of the COVID-19 infected population are either asymptomatic or mildly symptomatic (2). Asymptomatic patients are those who have positive test result for SARS-CoV-2 without symptom manifestation. A study by researchers at Johns Hopkins University showed that universal testing increased COVID-19 case detection by more than 200 percent in general as compared to targeted testing, and concluded that more testing resources are needed to curb the infection (3). The study also summarized that unrecognized asymptomatic cases can hinder preventive strategies, as well as increase the risk of the virus spreading (3). As pregnant women are also affected by coronavirus, this disease has drawn attention around the world, whether a universal testing policy should be imposed on all pregnant women who attend labor and admission units. In the United States, the National Institutes of Health categorized the disease severity into: asymptomatic, mild, moderate, severe critical illness (4). Knowledge regarding the capability of the virus to spread from an asymptomatic patient is still limited and poorly understood (5). Liu et al. described two out of 15 cases of pregnant women who were asymptomatic at presentation and underwent testing in view of contact history, in whom pneumonic lesion of COVID-19 was identified upon computed tomography evaluation (6). A study in an affiliated pair of New York City hospitals revealed 14 out of 43 (32.6%) pregnant women who were initially either asymptomatic and presented for obstetrically indicated labor induction, or remained asymptomatic upon presentation, and were subsequently identified to have positive COVID-19 infection upon universal testing at labor unit admission (7). Such asymptomatic pregnant women are at higher risk of infecting their newborns upon birth, healthcare workers and other patients if they are not identified.

According to the WHO, the decision to perform a test should be based on clinical and epidemiological factors that meet the suspected case definition for COVID-19 (8). In order for a test to be used for screening procedures in early disease detection, it should fulfill certain criteria such as validity, reliability, yield, cost, acceptance and follow-up services (9). It is more desirable and cost-effective to conduct universal testing in a population where the prevalence is high (10). This review therefore aims to look at reported prevalence rates of COVID-19 and thus explore the need for a universal testing policy for COVID-19 among pregnant women especially at the time of admission for delivery. It should be borne in mind that inadvertent exposure of healthcare workers to undiagnosed COVID-19 positive patients is an occupational hazard that comes with dire consequences, not only on the health and life of the worker, but also on healthcare services as a result of staff shortage due to quarantine and illness. Infection from an asymptomatic pregnant woman with COVID-19 infection who comes in labor in particular is a hazard to the healthcare worker that we should be seriously concerned about.

METHOD

Study Design

This is a systematic review of literature that was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. The study protocol and review were registered with PROSPERO (CRD42020184248). Two other systematic reviews were registered simultaneously in the same PROSPERO proposal but are dealt with separately.

Literature Search Strategy

A thorough and comprehensive literature search for studies published from November 2019 onwards was conducted and limited to English language publications. Three different electronic databases (PubMed, Ovid and Science Direct) were searched using the keywords "universal testing," "COVID-19" and "pregnancy." The PICOS terms used are as shown in **Table 1**. Additional relevant studies found from the references were also retrieved. The Boolean operator 'AND' was used to combine parts of the subject terms and 'OR' was used to expand the search. Only the latest publication would be chosen when there were similar studies with more than one publication.

Screening of Articles for Eligibility and Quality Assessment

The articles identified from the databases and additional resources were screened for eligibility. First, the title and abstract were screened. Second, eligible studies had to meet all the inclusion criteria developed from the research question using PICOS (Population, Intervention, Comparator, Outcome, Study) design as shown in **Table 2**. Exclusion criteria includes patients known to have previously been tested positive for SARS-CoV-2 infection. Full articles were retrieved and read in the event of any doubt or uncertainty regarding the content relevance during the abstract screening. After a comprehensive list of abstracts was obtained, the articles were retrieved and reviewed in full-text. One researcher screened all studies and the results were

[1]https://www.worldometers.info/coronavirus/ (accessed February 6, 2021).
[2]https://www.who.int/news-room/q-a-detail/coronavirus-disease-(covid-19)-vaccines (accessed February 5, 2021).
Abbreviations: COVID-19, Coronavirus Disease 2019; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; PPE, Personal Protective Equipment; WHO, World Health Organization; CDC, Centers for Disease Control and Prevention; MMAT, Mixed Method Appraisal Tools.

collated and reviewed by the second researcher. In the event of disagreement involving the study selection, a third reviewer would be consulted to reach a consensus.

Data Extraction

The following information was manually extracted from each study: year and country of publication, name of first author, study design, sample size/number of pregnant women who participated, trimester, number of pregnant women with positive or negative COVID-19 infection and number of asymptomatic infected pregnant women. The relevant data extracted was organized into tables using an Excel® spreadsheet. Gray literature was searched for any written policy of universal testing for COVID-19 in pregnancy.

Data Synthesis and Quality Assessment

Information retrieved was analyzed and interpreted. The primary outcomes assessed were the number of population with positive COVID-19 infection through universal testing, the number of asymptomatic pregnant women, and their prevalence. The information was synthesized using a narrative (descriptive) method. The quality of each study was independently evaluated by the first researcher using the Mixed Methods Appraisal Tools (45).

RESULTS

The selection process of articles and inclusion in the systematic review was summarized in **Figure 1** using the PRISMA flow diagram for systematic review. The initial search yielded a total of 356 articles. Other sources such as references from searched articles yielded three additional articles for this review. After removing the duplicates, 185 articles were screened for keywords relevance from the title and abstract. The full-text versions of the publications were reviewed in case of uncertainty. Only those that fulfill the inclusion criteria shown in **Table 1** and English publications were included for eligibility assessment. The full texts of these studies were fully examined. Eventually, only a total of 34 articles were included in this review, consisting mainly of retrospective cohort studies, followed by research letter, prospective cohort studies and case series. The data from these 34 studies was further summarized in **Table 2**. A total of 19,958 pregnant women worldwide were universally tested for COVID-19 infection upon arrival at labor and delivery admission units.

TABLE 1 | PICOS criteria for inclusion and exclusion of studies.

Parameter	Inclusion criteria	Data extraction
Population	Pregnant women presented to labor and delivery admission unit	Location
Intervention	Universal testing on all pregnant women presented to labor and delivery admission unit	Prevalence of positive test for COVID-19
Comparator	None	
Outcome	Pregnant women with positive test for COVID-19	Prevalence of symptomatic and asymptomatic women with COVID-19 positive test
Study	Case reports/observational studies	Type of study design

Risk of Bias

By using the Mixed Methods Appraisal Tools (MMAT) (3), the risk of bias of the studies were summarized in **Table 2**. In general, the individual studies had low to moderate range of risk of bias due to adequate approach to the research question and findings, with presence of coherence among the sources, data collection and analysis. In contrast, research letters and case series had moderate to serious risk of bias due to poor inclusion criteria. However, the clinical cases were presented clearly with clear messages provided.

Main Findings

This systematic review reports the prevalence of universal testing policy worldwide. It is notably found that the policy is adopted mostly in Western countries, as the implementation of the policy is highest in regions such as New York, Italy, Spain and Portugal. About two thirds (13,165/19,958 or 66.0%) of the population tested were from the United States, one of the countries with the highest number of population affected by the disease. From the total number of 19,958 pregnant women tested, an average of 5.3% were found to be infected. The total positive test rate ranged from 0.4 (12) to 27.0% (40). We also found that 1.3% (260/19,958) of the total number of pregnant women presenting to labor and admission units were asymptomatic women who tested positive for COVID-19 infection. Out of the total number of positive tests for COVID-19, the proportion of asymptomatic pregnant women (75.5%) was markedly higher than symptomatic pregnant women (24.5%). Guidelines vary in terms of recommendation for testing for COVID-19 among pregnant women. The ACOG recommends universal testing in areas with high prevalence of the infection (46).The guidelines issued by the Indian Council of Medical Research recommends universal testing of "all pregnant women in/near labor who are hospitalized for delivery" (47). However, other guidelines may not state such a stand clearly (48), and may rely on clinical screening as first line (49).

DISCUSSION

This systematic review reports with great concern that the prevalence of asymptomatic COVID-19 patients is threefold that of symptomatic patients, thus seriously raising the question of universal testing, particularly in pregnancy, where there is prolonged close contact with multiple healthcare workers especially when the patient is in labor. Although prevalence varies across the globe and several vaccines have been successfully tested and now implemented, mutants of SARS-CoV-2 appear every so often, hence other preventive measures such as limiting contact and physical distancing still matters.

It is good to note some move toward advocating universal testing of pregnant women attending labor and delivery units, given the recent spike in the prevalence of asymptomatic

TABLE 2 | Summary of studies reviewing the outcome of universal testing and the prevalence of asymptomatic pregnant women with positive SARS-CoV-2.

No	First Author	Country	Title	Universally tested pregnant women *N*	COVID-19 Infection				Study design	RoB
					Negative *n* (%)	Positive				
						Total *n* (%)	Asymptomatic *n* (%)	Symptomatic *n* (%)		
1	Prabhu et al. (11)	United States (New York)	Pregnancy and postpartum outcomes in a universally tested population for SARS-CoV-2 in New York City: a prospective cohort study	675	605 (89.6)	70 (10.4)	55 (78.6)	15 (21.4)	Prospective cohort	++
2	Fassett et al. (12)	United States (Los Angeles)	Universal SARS-CoV-2 screening in women admitted for delivery in a large managed care organization	3,923	3,906 (99.6)	17 (0.4)	17 (100.0)	0 (0.0)	Retrospective cohort	+
3	Vintzileos et al. (13)	United States (New York)	Screening all pregnant women admitted to labor and delivery for the virus responsible for coronavirus disease 2019	161	129 (81.1)	32 (19.9)	21 (65.6)	11 (34.4)	Retrospective cohort	++
4	Campbell et al. (14)	United States (New York)	Prevalence of SARS-CoV-2 among patients admitted for childbirth in Southern Connecticut	770	740 (96.1)	30 (3.9)	22 (73.3)	8 (26.7)	Retrospective cohort	+
5	LaCourse et al. (15)	United States (Washington)	Low prevalence of SARS-CoV-2 among pregnant and postpartum patients with universal screening in Seattle, Washington	188[a]	182 (97.3)	5 (2.7)	1 (20.0)	4 (80.0)	Retrospective cohort	++
6	Miller et al. (16)	United States (Chicago)	Clinical implications of universal severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) testing in pregnancy	635	612 (96.4)	23 (3.6)	10 (43.5)	13 (56.5)	Research letter	++
7	London et al. (17)	United States (New York)	The relationship between status at presentation and outcomes among pregnant women with COVID-19	75	65 (86.7)	10 (13.3)	10 (100.0)	0 (0.0)	Retrospective cohort	++
8	Goldfarb et al. (18)	United States (Boston)	Universal SARS-CoV-2 testing on admission to the labor and delivery unit: low prevalence among asymptomatic obstetric patients	757	737 (97.4)	20 (2.6)	9 (45.0)	11 (55.0)	Retrospective cohort	+
9	Ochiai et al. (19)	Japan	Universal screening for SARS-CoV-2 in asymptomatic obstetric patients in Tokyo, Japan	52	49 (94.2)	3 (5.8)	3 (100.0)	0 (0.0)	Retrospective cohort	++
10	Bianco et al. (20)	United States (New York)	Testing of patients and support persons for coronavirus disease 2019 (COVID-19) infection before scheduled deliveries	155	131 (84.5)	24 (15.5)	24 (100.0)	0 (0.0)	Retrospective cohort	++
11	Ferrazzi et al. (21)	Italy	SARS-CoV-2 infection testing at delivery: a clinical and epidemiological priority	1,566	1,517 (96.9)	49 (3.1)	27 (55.1)	22 (44.9)	Retrospective cohort	+
12	Herraiz et al. (22)	Spain (Madrid)	Universal screening for SARS-CoV-2 before labor admission during COVID-19 pandemic in Madrid	203[a]	199 (99.0)	2 (1.0)	1 (50.0)	1 (50.0)	Retrospective cohort	++
13	Sutton et al. (23)	United States (New York)	Universal screening for SARS-CoV-2 in women admitted for delivery	215[a]	181 (84.7)	33 (15.3)	29 (87.9)	4 (12.1)	Research letter	+++

(Continued)

TABLE 2 | Continued

No	First Author	Country	Title	Universally tested pregnant women *N*	COVID-19 Infection				Study design	RoB
					Negative *n* (%)	Positive				
						Total *n* (%)	Asymptomatic *n* (%)	Symptomatic *n* (%)		
14	Gagliardi et al. (24)	Italy (Tuscany and Liguria)	Universal severe acute respiratory syndrome coronavirus 2 testing of pregnant women admitted for delivery in 2 Italian regions	533	530 (99.4)	3 (0.6)	2 (66.7)	1 (33.3)	Research letter	++
15	Yassa et al. (25)	Turkey	Outcomes of universal SARS-CoV-2 testing program in pregnant women admitted to hospital and the adjuvant role of lung ultrasound in screening: a prospective cohort study	296	273 (92.2)	23 (7.8)	12 (52.2)	11 (47.8)	Retrospective cohort	++
16	Berkowitz et al. (26)	United States (Ohio)	Implementation of universal testing for SARS-CoV-2 in pregnant women with intended admission for delivery	518[b]	482 (98.1)	10 (1.9)	7 (70.0)	3 (30.0)	Research letter	+++
17	Santos et al. (27)	Portugal	Prevalence of SARS-CoV-2 infection in asymptomatic pregnant women and their partners in a tertiary care hospital in Portugal	428	426 (99.5)	2 (0.5)	2 (100.0)	0 (0.0)	Research letter	+++
18	Abeysuriya et al. (28)	United Kingdom	Universal screening for SARS-CoV-2 in pregnant women at term admitted to an East London maternity unit	180[c]	171 (96.1)	7 (3.9)	6 (85.7)	1 (14.3)	Retrospective cohort	++
19	Buckley et al. (29)	United States (New York)	Universal testing of patients and their support persons for severe acute respiratory syndrome coronavirus 2 when presenting for admission to labor and delivery at Mount Sinai Health System	307	257 (83.7)	50 (16.3)	50 (100.0)	0 (0.0)	Research letter	+++
20	Doria et al. (30)	Portugal	COVID-19 during pregnancy: a case series from an universally tested population from the north of Portugal	103	91 (88.4)	12 (11.6)	11 (91.7)	1 (8.3)	Case series	+++
21	Bender et al. (31)	United States (Pennsylvania)	Universal testing for severe acute respiratory syndrome coronavirus 2 in 2 Philadelphia hospitals: carrier prevalence and symptom development over 2 weeks	318	310 (97.5)	8 (2.5)	8 (100.0)	0 (0.0)	Retrospective cohort	+++
22	Vinuela et al. (32) (2020)	Spain (Madrid)	SARS-CoV-2 screening of asymptomatic women admitted for delivery must be performed with a combination of microbiological techniques: an observational study	100	91 (91.0)	9 (9.0)	9 (100.0)	0 (0.0)	Retrospective cohort	+++
23	Tanacan et al. (33)	Turkey (Ankara)	The rate of SARS-CoV-2 positivity in asymptomatic pregnant women admitted to hospital for delivery: experience of a pandemic center in Turkey	206	203 (98.5)	3 (1.5)	3 (100.0)	0 (0.0)	Prospective cohort	+++

(Continued)

TABLE 2 | Continued

No	First Author	Country	Title	Universally tested pregnant women *N*	COVID-19 Infection				Study design	RoB
					Negative *n* (%)	Positive				
						Total *n* (%)	Asymptomatic *n* (%)	Symptomatic *n* (%)		
24	Saviron-Cornudella et al. (34)	Spain (Madrid)	Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) universal screening in gravids during labor and delivery	266	260 (97.7)	6 (2.3)	4 (66.7)	2 (33.3)	Retrospective cohort	++
25	Reale et al. (35)	United States (Massachusetts)	Patient characteristics associated with SARS-CoV-2 infection in parturients admitted for labor and delivery in Massachusetts during the spring 2020 surge: a prospective cohort study	2,945	2,852 (96.8)	93 (3.2)	80 (86.0)	13 (14.0)	Prospective cohort	+
26	Pineles et al. (36)	United States (Texas)	Racial-ethnic disparities and pregnancy outcomes in SARS-CoV-2 infection in a universally-tested cohort in Houston, Texas	935	858 (91.8)	77 (8.2)	66 (85.7)	11 (14.3)	Retrospective cohort	++
27	Naqvi et al. (37)	United States (Los Angeles)	Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) universal testing experience on a Los Angeles labor and delivery unit	82	81 (98.8)	1 (1.2)	0l (0.0)	1 (100.0)	Prospective cohort	++
28	Mei-Dan et al. (38)	Canada (Toronto)	Questionnaire-based vs universal PCR testing for SARS-CoV-2 in women admitted for delivery	446	442 (99.1)	4 (0.9)	3 (66.7)	1 (33.3)	Prospective cohort	+
29	Maru et al. (39)	United States (New York)	Universal screening for SARS-CoV-2 infection among pregnant women at Elmhurst Hospital Center, Queens, New York	124	78 (62.9)	46 (37.1)	33 (71.7)	13 (28.3)	Retrospective cohort	++
30	Hcini et al. (40)	French Guiana	Maternal, fetal and neonatal outcomes of large series of SARS-CoV-2 positive pregnancies in peripartum period: a single-center prospective comparative study	507	370 (73.0)	137 (27.0)	103 (75.2)	34 (24.8)	Prospective cohort	+
31	Figueiredo et al. (41)	Portugal (Porto)	Systematic screening for SARS-CoV-2 in pregnant women admitted for delivery in a Portuguese maternity	184	173 (99.9)	11 (0.1)	9 (81.8)	2 (18.2)	Prospective cohort	+
32	Waghmare et al. (42)	India (Maharashtra)	Universal screening identifies asymptomatic carriers of SARS-CoV-2 among pregnant women in India	1,140	999 (87.6)	141 (12.4)	284 (73.8)	37 (26.2)	Prospective cohort	++
33	Diaz-Corvillon et al. (43)	Chile	Routine screening for SARS CoV-2 in unselected pregnant women at delivery	583	546 (93.7)	37 (6.3)	16 (43.2)	21 (56.8)	Prospective cohort	+
34	Blitz et al. (44)	United States (New York)	Universal testing for coronavirus 2019 in pregnant women admitted for delivery: prevalence of peripartum infection and rate of asymptomatic carriers at four New York hospitals within an integrated healthcare system	382	318 (83.3)	64 (16.7)	45 (70.3)	19 (29.7)	Retrospective cohort	++
	TOTAL			19,958	18,896 (94.7)	1,062 (5.3)	802 (75.5)	260 (24.5)		

N, Pregnant women who were universally tested; a, Asymptomatic and inconclusive (n = 1); b, Results were not obtained within clinically relevant time frame (n=26); c, Two women (n = 2) excluded as one was previously suspected and other one declined testing; RoB, Risk of bias; +, Low risk of bias; ++, Medium risk of bias; + + +, Serious risk of bias.

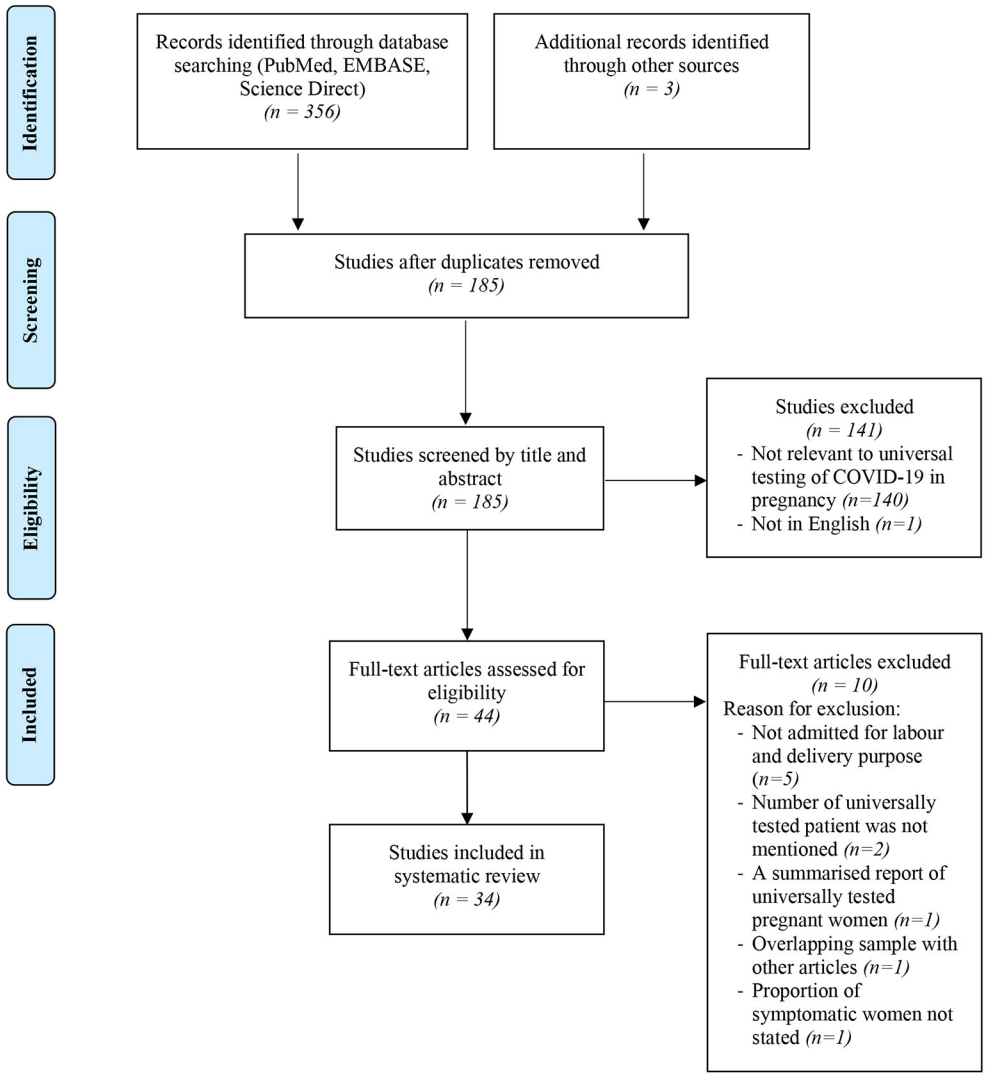

FIGURE 1 | Flowchart showing inclusion in systematic review of studies reporting on prevalence of universal testing policy for COVID-19 in pregnant women.

COVID-19 cases worldwide. Recently, the American College of Obstetricians and Gynecologists published an update on the recommendation to consider universal testing for pregnant women especially in high prevalence areas (46). In a study by Bianco et al., universal screening using the telephone as a screening tool is inadequate as 24 patients who were previously not identified as likely to be COVID-19 positive via such screening, were tested positive from the universal testing (20). Therefore, the findings of this systematic review implies that healthcare workers and other patients are at significant risk of exposure and getting infected with COVID-19, if universal testing of pregnant women is not implemented in high prevalence areas. The alternative measure is universal precaution i.e., wearing of personal protective equipment (PPE) when handling all cases. However, universal usage of PPE will result in wastage of a precious commodity that has been in short supply.

Routine SARS-CoV-2 testing would require the use PPE. On the other hand, in the case of patients with reported symptoms but received negative results, PPE use could be avoided. In general, universal testing may result in an overall increase in terms of PPE usage. Therefore, given the potential increased need for supply, the implementation of universal testing could pose a challenge to the current hospital supply systems. An increased demand for PPE would occur and facilities with limited access to PPE would suffer greatly.

The implementation of universal testing in pregnancy, however, can act as a multipronged approach to reduce the risk of SARS-CoV-2 transmission, particularly in healthcare facilities in regions with high prevalence of the infection. In view of longer exposure between pregnant women and healthcare professionals before, during and after delivery, universal testing in this specific population can assist in infection control operations. It can help protect the safety of newborns, hospital staff, and other patients.

In addition, it also allows priority clinical care to be given to both the infected mother and her baby at the time of birth and during the postpartum period, in terms of appropriate further treatment such as management of delivery, counseling for breastfeeding and newborn skin-to-skin contact. It is important to bear in mind that the COVID-19 prevalence rate is extremely fluid and has a tendency to escalate rapidly[2], hence policies and guidelines should be formulated in a flexible manner so as to be enable prompt response with day to day changes in the situation.

Attempts have also been made to elucidate clinical or simple laboratory predictive risk factors for COVID-19 infection among pregnant women in order to proceed to conduct targeted antigen testing (50–52). This may be more feasible options in the long term, especially from the health economics point of view. It can also be implemented irrespective of the local prevalence of the disease.

The prevalence rate from universal testing appears to mirror the rate within the local general population (37). The current compiled review is useful in planning preventive strategies in the interest of the health of pregnant mothers, their babies, and mitigating the risk of healthcare workers. In regions with low COVID-19 prevalence, the approach may be different. The research question on universal testing needs to be addressed carefully. The incubation period for COVID-19 is reported to be between 5 and 14 days and the duration of immunity is still being studied. For populations with low prevalence, using a screening checklist and restricting diagnostic testing only for those with positive screening may be a more cost-effective option. A cost-effectiveness study on universal testing is in order before universal testing can be recommended as a policy. The issue of timing of testing in relation to pregnancy and labor also needs to be considered and are not easy decision points.

Strength and Limitations

The strength of our study is as a systematic review that looks at universal testing policy for COVID-19 in pregnancy at the point of admission for labor and delivery. It is useful in guiding policy making in relation to preventive measures and testing for the infection. One limitation of this review is the nature of the studies retrieved. Although the majority of studies included are retrospective cohort studies, case reports and research letters that were retrieved are expected to have high risk of bias. Apart from that, we did not report the prevalence of COVID-19 in the general population in individual studies as they were likely to be underreported to various degrees, depending on the extent of mass testing in a particular population. As a result, we were not able to compare the prevalence of infected cases by different regions and countries. It is quite difficult to do this retrospectively for all locations as the local prevalence changes fairly rapidly and the studies were time-sensitive, several of them limiting the study period to 1 or 2 weeks only.

CONCLUSION

This review looks at the outcome of a universal testing policy in terms of prevalence of asymptomatic pregnant women in various populations. Given the high rate of asymptomatic pregnant women in certain regions of the world, universal testing may provide enhanced safety to the public and healthcare workers in these areas, but cost will be increased from various angles. Although the current trend of universal testing predominates only in developed countries, more studies involving developing and less developed countries should be conducted to provide valuable information of the need for such a policy of universal testing for COVID-19 in pregnancy. Universal testing provides benefits in areas with high prevalence of disease, hence testing for the background prevalence in representative samples of pregnant women in various regions should be considered in order to guide policy making. Above all, in areas with high prevalence of COVID-19, the strategy of universal testing of pregnant women before admitting them for delivery is essential and must be implemented rigorously in order to protect the women, their newborns, and in-contact healthcare workers so as to curb the spread of infection in the community.

AUTHOR CONTRIBUTIONS

NAFH led the data synthesis, collection and analysis, while being supervised by ZAM. RS gave an expert clinical advice on methodology and community health, while AHMK and RAR gave expert clinical advice on obstetrics. All authors critically revised the manuscript for important intellectual content and reviewed and approved the final version.

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