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Endocrinology and COVID-19: A cross-disciplinary topic, volume II

Edited by
Jeff M. P. Holly and Gesthimani Mintziori

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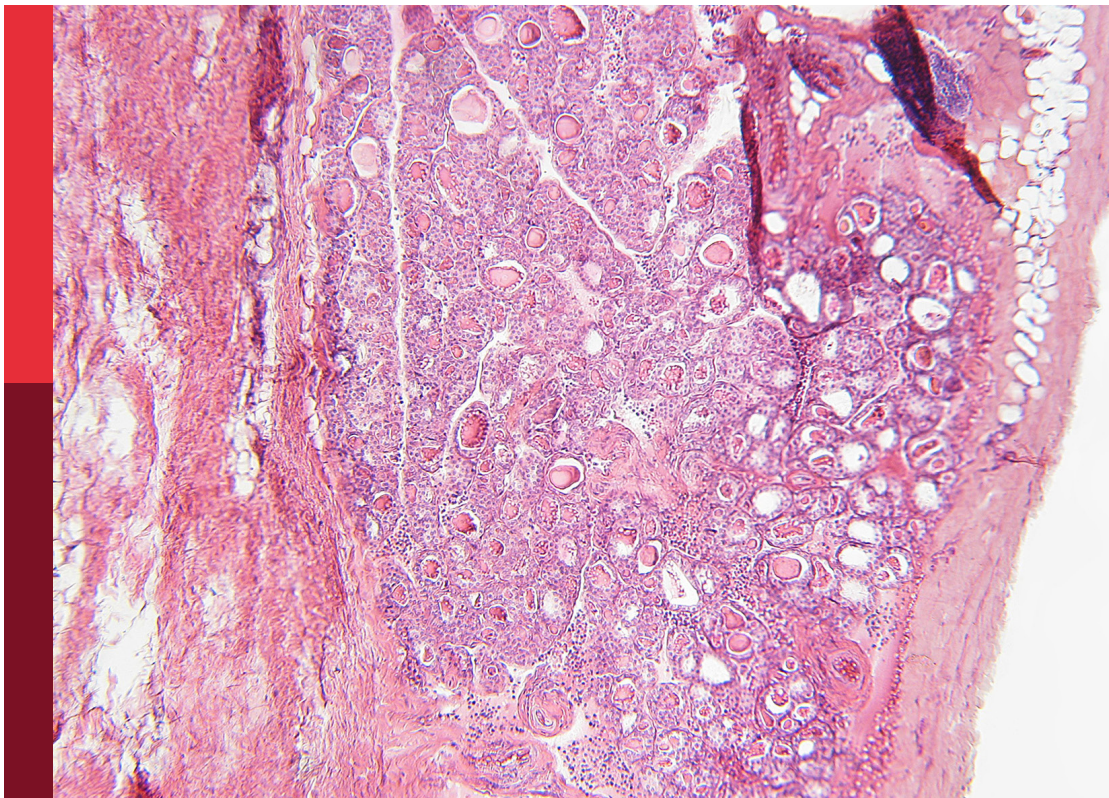

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Endocrinology and COVID-19: A cross-disciplinary topic, volume II

Topic editors

Jeff M. P. Holly — University of Bristol, United Kingdom

Gesthimani Mintziori — Aristotle University of Thessaloniki, Greece

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Table of contents

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Controlled Ovarian Hyperstimulation Protocol in Infertile Patients During the COVID-19 Pandemic

Fei Li[1,2], HuiXia Zhang[1], WeiYi Shi[1], YiFang Wu[1], Ye Tian[1], YiHong Guo[1], HaiXia Jin[1] and Gang Li[1]**

[1] Center for Reproductive Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Center for Reproductive Medicine, The First People's Hospital of Shangqiu, Shangqiu, China

Objectives: To explore the appropriate controlled ovarian hyperstimulation (COH) protocols in infertility patients who received the *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatments during the COVID-19 pandemic.

Materials and Methods: This retrospective cohort study evaluated the efficiency of the early follicular-phase long-acting GnRH-agonist long (EFLL) protocol (a new protocol developed by Chinese clinicians), prolonged pituitary down-regulation of EFLL protocol (Pro-EFLL), and the GnRH-ant protocol for couples meeting the study criteria between February 2020 and June 2020 who were treated by the First Affiliated Hospital of Zhengzhou University during the COVID-19 pandemic, and compared the pregnancy rates and miscarriage rates per fresh transfer cycle, number of retrieved oocytes, endometrial thickness on the day of hCG injection and the number of fertilized oocytes, mature oocytes, fertilized oocytes, and transferable embryos among the three protocols.

Results: We found that the prolonged pituitary down-regulation during the COVID-19 pandemic by utilizing a full-dose of GnRH-a administrated in infertility patients were no differences in clinical outcomes than other protocols, The prolonged pituitary down-regulation protocol and EFLL protocol were associated with a higher Endometrial thickness on the day of hCG injection (12.67 ± 2.21 vs. 12.09 ± 2.35 vs. 10.79 ± 2.38, $P < 0.001$), retrieved oocytes (14.49 ± 6.30 vs. 15.02 ± 7.93 vs. 10.06 ± 7.63, $P < 0.001$), mature oocytes (11.60 ± 5.71 vs. 11.96 ± 6.00 vs. 7.63 ± 6.50, $P < 0.001$), fertilized oocytes (9.14 ± 5.43 vs. 8.44 ± 5.34 vs. 5.42 ± 5.20, $P < 0.001$), and transferable embryos (4.87 ± 2.96 vs. 6.47 ± 5.12 vs. 3.00 ± 3.28 vs. $P < 0.001$) in the GnRH-antagonist protocol.

Conclusion: We recommend that patients start Gn injections 33–42 days after a pituitary downregulated full dose (3.75 mg) of gonadotropin-releasing hormone agonist during the COVID-19 pandemic, even a delay of 2–4 weeks does not affect the implantation rate. The study can provide a more detailed estimate and clinical management strategies for infertile couples during the COVID-19 pandemic.

Keywords: COVID-19 pandemic, controlled ovarian hyperstimulation, pituitary down-regulation, infertility, IVF/ICSI

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Jeff M. P. Holly,
University of Bristol, United Kingdom

Reviewed by:
Kajal Khodamoradi,
University of Miami, United States
Qing Xue,
Peking University First Hospital, China

****Correspondence:***
HaiXia Jin
jhxgl@126.com
Gang Li
lgvigor@126.com

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INTRODUCTION

The coronavirus disease-19 (COVID-19) pandemic started in late December 2019 in Wuhan, Hubei Province, China, and has since spread rapidly around the globe, with many countries being severely affected (Lai et al., 2020; Vermeulen et al., 2020). The disease as an acute respiratory infectious disease has been managed according to A class infectious diseases as stipulated in the Law of China. The Chinese government began enforcing social distancing, including restrictions on gatherings, public transportation and school closures limitations, including reproductive medicine procedures (Xia et al., 2020). During the special period of the epidemic, the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) have come together to jointly affirm the importance of continued reproductive research during the COVID-19 pandemic (Gianaroli et al., 2020; Veiga et al., 2020), committed to continuous monitoring of the effects of COVID-19 on reproduction, collecting data on infertility patients during the pandemic, and helping the majority of patients who seek treatment to ultimately become parents. However, there is no uniform standard on how to deal with infertile people and how to arrange medical treatment during this difficult time (Lupia et al., 2020; Pasquale et al., 2020; Veiga et al., 2020).

In the special period of the COVID-19 pandemic, it is necessary to develop or refine robust controlled ovarian hyperstimulation (COH) protocols to minimize exposure risks, to reduce the rate of cycle cancelations and to alleviate the financial and emotional burden of interrupting treatment for infertile couples due to the epidemic. To meet current needs, one full-dose depot of long-acting gonadotropin-releasing hormone agonist (GnRH-a) per COH cycle would be more suitable and convenient for women than short-acting GnRH-a injections or the GnRH antagonist protocol during the COVID-19 pandemic (Ben-Kimhy et al., 2020; Li F. et al., 2020), because there are fewer incidences of potential exposure. The early follicular-phase long-acting GnRH-agonist long (EFLL) protocol (a new protocol developed by Chinese clinicians) applies a pituitary downregulated full dose (3.75 mg) of dipherelin on days 2–4 of menstruation, and Gn starts 30–42 days later along with confirmation of the pituitary downregulation (Ying et al., 2019; Li F. et al., 2020). A series of studies has suggested its advantages in improving endometrial receptivity, embryo implantation and clinical pregnancy rates (Ren et al., 2014; Schisterman et al., 2020). It is worth emphasizing that the EFLL protocol was initially applied in a Chinese *in vitro* fertilization (IVF) center in 2016, and it has become the mainstream protocol in most reproductive medicine centers now in China (Li F. et al., 2020).

However, due to the interruption of medical treatment by COVID-19, many patients are affected by unexpected clinic closures (Sadeghi, 2020), and Gonadotrophins (Gns) would start > 42 days later along with confirmation of prolonged pituitary downregulation (pro-EFLL). Do these changes affect the outcome of assisted pregnancies in these infertile couples? Which controlled ovarian stimulation management strategies are most appropriate during the COVID-19 epidemic? In view of this, the aim of our study was to evaluate the appropriate COH protocol for infertility patients who received IVF/ICSI treatments during the COVID-19 pandemic. We gathered data comparing the clinical efficacy of the EFLL protocol, prolonged pituitary downregulation of the EFLL protocol and GnRH antagonist protocol before COH through collecting pregnancy rates and miscarriage rates per fresh transfer cycle, which are vital indicators for infertile couples (Vaegter et al., 2017; Veiga et al., 2020). To our knowledge, this is the first study to evaluate prolonged pituitary downregulation in infertile patients during the COVID-19 pandemic to provide a more detailed estimate and clinical management strategies for infertile couples.

MATERIALS AND METHODS

This retrospective cohort study evaluated the efficiency of the EFLL protocol, the pro-EFLL protocol, and the GnRH-ant protocol for couples meeting the study criteria between February 2020 and June 2020 who were treated by the First Affiliated Hospital of Zhengzhou University during the COVID-19 pandemic. We screened eligible subjects and removed 433 patients who had abandoned treatment because of the COVID-19 pandemic, other ovarian hyperstimulation protocols and women who received transplantation genetic screening or transplantation genetic diagnosis or did not have complete laboratory data (e.g., Baseline data, Endocrine data, and Embryo data). Finally, the study analyzed clinical data from 199 cycles with IVF/ICSI in our reproductive medical center. The experimental materials in this study did not include identifiable part icipants data for the purpose of safeguarding patient privacy. This study was approved by the Ethics Committee of Reproductive Medicine Center, the First Affiliated Hospital of Zhengzhou University, China. Informed consent was waived, with approval from the ethics committee. A flow chart and the data processing procedure are listed in **Figure 1**.

Early Follicular Phase Long-Acting GnRH Agonist Long Protocol

For patients undergoing the standard EFLL protocol, we administered 3.75 mg long-acting GnRH agonist (Diphereline, Ipsen, France) on days 2–3 of menstruation. Patients were monitored by sex hormones level and ultrasound measurements. The following criteria were used for down-regulation standard: No functional cysts and follicle sizes larger than 3–5 mm by ultrasound; LH < 5 IU/L, FSH < 5 IU/L, and P < 1 ng/mL. The initial dose of gonadotropin was administered on the basis of the woman's age, AFC, BMI, and ovarian response to stimulation. The trigger was administered with 2000 IU u-HCG (Livzon Pharmaceuticals) in combination with 250 μg r-human chorionic gonadotropin (hCG) (Merck Serono) when most dominant follicles are mature, the Oocytes were then retrieved 36–37 h after the trigger (**Figure 2**).

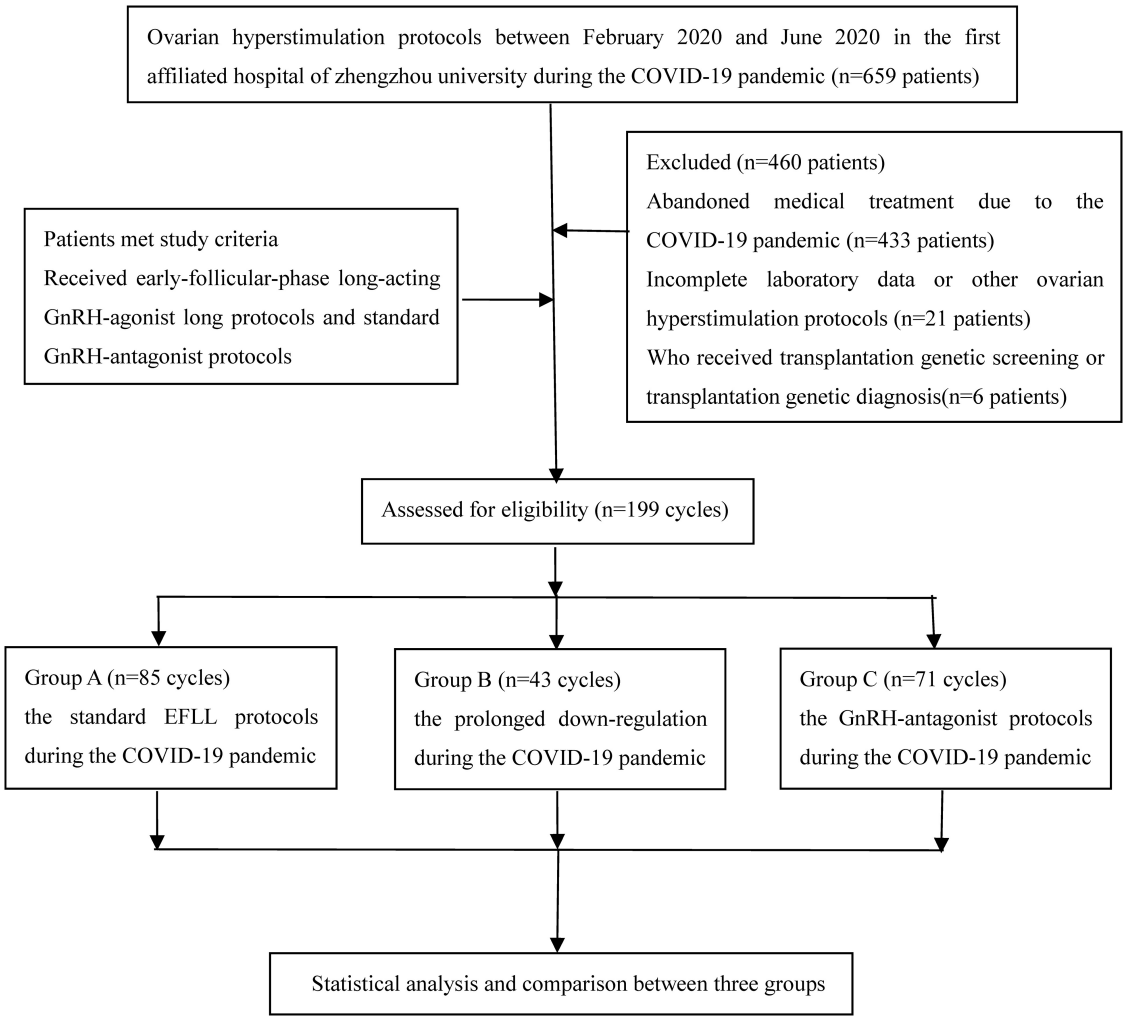

FIGURE 1 | The flow chart.

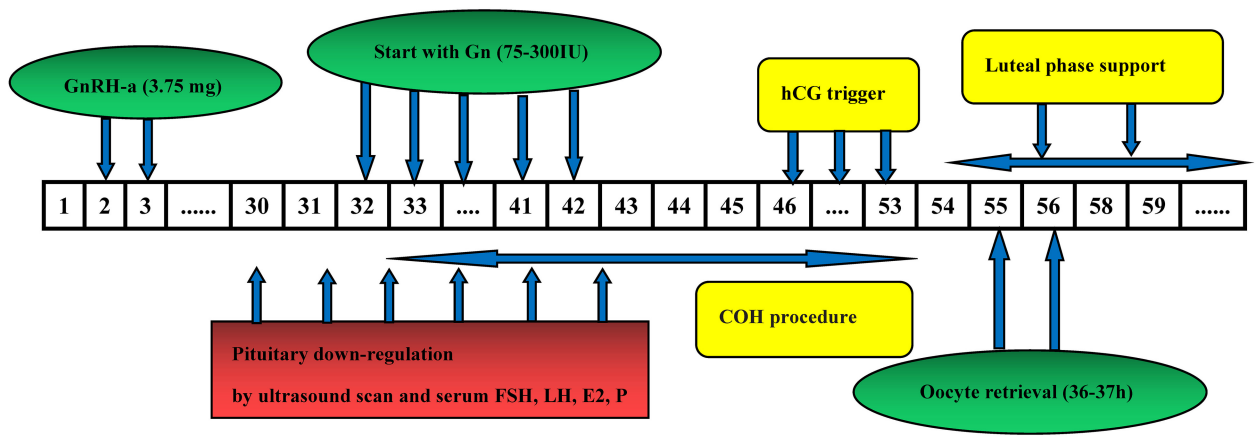

FIGURE 2 | The flow chart of Early follicular phase long-acting GnRH agonist long protocol.

Prolonged Pituitary Down-Regulation of EFLL Protocol

The pro-EFLL protocol is analogous to the standard EFLL protocol as well, on days 2–3 of menstruation, we also administered 3.75 mg long-acting GnRH agonist (Diphereline, Ipsen, France). And all infertile patients were monitored by sex hormones level and ultrasound measurements, however, Gns would start >42 days later along with confirmation of prolonged pituitary down-regulation due to the COVID-19 interrupt medical treatment. The following process is the same as standard EFLL protocol (**Figure 3**).

Gonadotropin-Releasing Hormone GnRH Antagonist Protocol

For the GnRH-ant protocol, COH was started with 72.5–300 IU gonadotropin (Puregon, Organon, Netherlands) on day 2–3 of the menstrual cycle. The initial dose of gonadotropin was administered on the basis of the woman's age, AFC, BMI, and ovarian response to stimulation. A daily dose of 0.25–0.75 mg GnRH antagonist (Cetrotide, Pierre Fabre, Aquitaine Pharm International) was initiated on the sixth day of rFSH stimulation or when the lead follicle reached a mean diameter of 12–14 mm, and the gonadotropin was continued until the day of the trigger administration (5000 IU u-HCG, Livzon Pharmaceuticals or 250 μg r-hCG, Merck Serono in combination with 2000 IU u-HCG). The Oocytes were then retrieved 35–37 h after the trigger (Li F. et al., 2020; **Figure 4**).

Follow-Up Procedure

We performed the follow-up through outpatient visits. The follow-up time began from their first clinical encounter and continued until the pregnancy outcome and miscarriage outcome occurred or the last date of this study, whichever occurred first.

Statistical Analysis

All Data was analyzed using the software R (version 3.6.1) and the Statistical Package for Social Sciences (Version 22.0). The primary outcomes in this retrospective comparative study were pregnancy rates and miscarriage rates per fresh transfer cycle. The secondary outcomes included endometrial thickness, retrieved oocytes, mature oocytes, fertilized oocytes, and transferable embryos. Continuous variables were compared using one-way analysis of variance (ANOVA). Categorical variables were compared using the chi-square test or Fisher's exact test. $P < 0.05$ was considered statistical significance.

RESULTS

Patients who met the study criteria between February 2020 and June 2020 and were treated at the First Affiliated Hospital of Zhengzhou University during the COVID-19 pandemic included 85 patients given the EFLL protocol, 43 patients given the pro-EFLL protocol, and 71 patients given the GnRH antagonist protocol. In the pro-EFLL protocol, the continuous pituitary downregulation time was between 43 and 63 days, 27 patients had a continuous pituitary downregulation time of 43–56 days, and the remaining patients it was between 57 and 63 days. There were no significant differences in the basic characteristics (Age, BMI, FSH, LH, E2, P PRL, AMH, AFC, TSH, FT3, FT4, Blood glucose) among the three groups. The EFLL protocol was associated with a shorter duration of pituitary downregulation (35.73 ± 2.87 vs. 56.56 ± 11.00, $P < 0.001$) and lower FSH levels on the Gn commencing day (3.40 ± 1.87 vs. 4.72 ± 1.90, $P < 0.001$) than the Pro-EFLL protocol. However, there were no significant differences in the pregnancy or miscarriage rates between the two groups (**Table 1**).

Comparison of stimulation variables among the three groups revealed that the EFLL protocol was associated with a lower FSH level (3.40 ± 1.87 vs. 4.72 ± 1.90, $P < 0.001$) than the Pro-EFLL protocol on the Gn commencing day. We found that the EFLL protocol and the Pro-EFLL protocol were associated with a greater endometrial thickness (12.09 ± 2.35 vs. 12.67 ± 2.21 vs. 10.79 ± 2.38, $P < 0.001$), longer duration of Gn use (13.79 ± 1.96 vs. 13.02 ± 2.68 vs. 10.58 ± 2.51, $P < 0.001$), and a lower LH value (0.82 ± 0.81 vs. 1.08 ± 0.93 vs. 5.83 ± 0.99, $P < 0.001$) than the GnRH-ant protocol on the day of the hCG injection (**Table 2**).

We found that the GnRH-ant protocol was associated with a lower number of retrieved oocytes (10.06 ± 7.63 vs. 15.02 ± 7.93 vs. 14.49 ± 6.30, $P < 0.001$), mature oocytes (7.63 ± 6.50 vs. 11.96 ± 6.00 vs. 11.60 ± 5.71, $P < 0.001$), fertilized oocytes (5.42 ± 5.20 vs. 8.44 ± 5.34 vs. 9.14 ± 5.43, $P < 0.001$), and transferable embryos (3.00 ± 3.28 vs. 4.87 ± 2.96 vs. 6.47 ± 5.12, $P < 0.001$) than the EFLL protocol and the Pro-EFLL protocol; however, no statistically significant differences were seen for pregnancy rates (49.4 (42/85) vs. 34.9 (15/43) vs. 39.4 (28/71), $P < 0.001$) or miscarriage rates (11.9 (5/42) vs. 20.0 (3/15/34.9 (15/43) vs. 28.4 (28/71) per transfer cycle (**Table 3**). A comparison of treatment results among the three groups is shown in **Figures 5**, **6**.

DISCUSSION

Our findings indicate that prolonged pituitary downregulation during the COVID-19 pandemic by utilizing a full dose of GnRH-a administered to infertile patients was not associated with differences in pregnancy outcomes, such as pregnancy rates and miscarriage rates per fresh transfer cycle, among the three protocols. In addition, we also found that prolonged downregulation protocols and EFLL protocols can acquire more mature oocytes and transplantable embryos than GnRH-ant protocols. Furthermore, we found that these two protocols were associated with a greater endometrial thickness, longer duration of Gn use, and lower LH value than GnRH-ant protocols on the day of hCG injection. These strategies warrant further investigation. Considering that the ASRM and the ESHRE have no uniform standards on how to treat infertile people and how to arrange medical treatment during these difficult times (Esposito et al., 2020; Simopoulou et al., 2020; Veiga et al., 2020), to meet the current needs, our research results can provide a more detailed view of clinical management strategies for infertile couples during the COVID-19 pandemic.

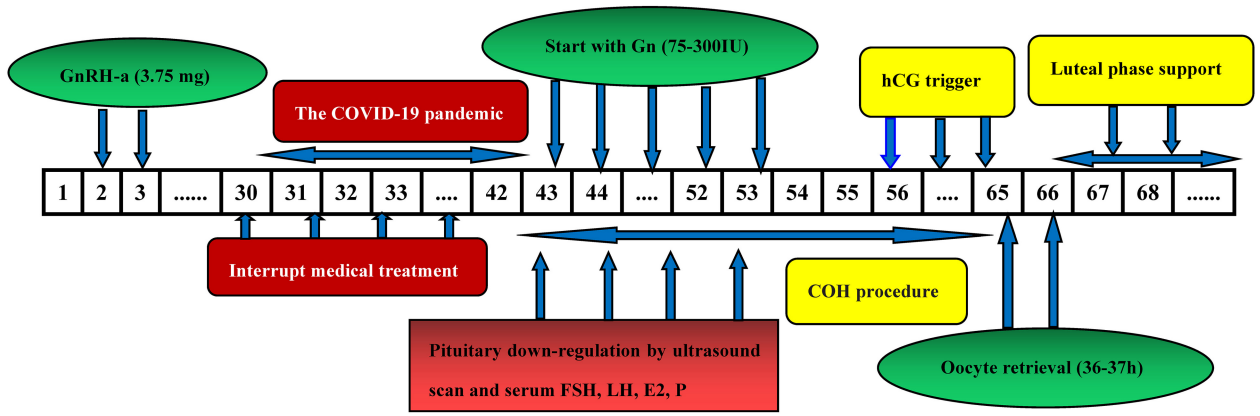

FIGURE 3 | The flow chart of prolonged pituitary down-regulation of EFLL protocol.

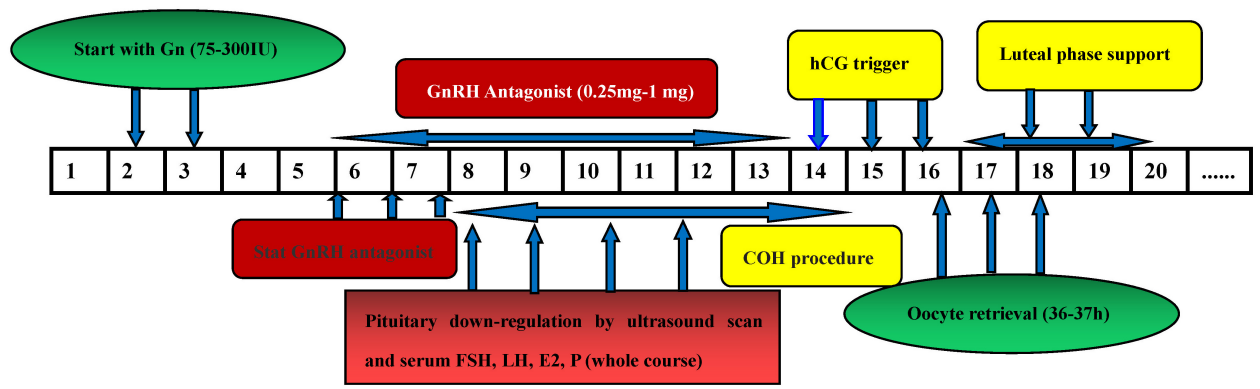

FIGURE 4 | The flow chart of GnRH antagonist protocol. **Figures 5**, **6** comparison of treatment results among the three groups.

TABLE 1 | Comparison of baseline parameters between the three groups.

Protocols	EFLL group (*n* = 85)	Pro-EFLL group (*n* = 43)	GnRH-ant group (*n* = 71)	*P*-value
Duration of pituitary down-regulation (days)	35.73 ± 2.87	56.56 ± 11.00[a]	/	<0.001
Age (years)	30.69 ± 4.38	31.26 ± 4.67	32.41 ± 5.97	0.108
BMI (kg/m2)	22.30 ± 2.97	23.08 ± 2.77	23.08 ± 3.57	0.229
Basal FSH (IU/L)	6.46 ± 1.60	6.13 ± 1.44	6.52 ± 1.65	0.427
Basal LH (IU/L)	5.38 ± 3.17	6.66 ± 5.16	5.17 ± 2.37[b]	0.049
Basal E2 (ng/L)	53.73 ± 121.79	56.39 ± 84.14	47.99 ± 43.07	0.878
Basal P (μg/L)	0.37 ± 0.49	0.52 ± 0.78	0.51 ± 0.32	0.147
PRL (ng/mL)	26.99 ± 42.82	18.37 ± 8.64	22.14 ± 38.07	0.421
AMH (ng/mL)	3.80 ± 3.05	3.84 ± 2.77	4.62 ± 4.02	0.269
AFC (numbers)	15.19 ± 6.55	15.35 ± 6.17	14.97 ± 7.58	0.958
TSH (mIU/mL)	2.19 ± 1.11	2.60 ± 1.31	2.35 ± 1.08	0.166
FT3 (pmol/L)	5.13 ± 0.85	5.30 ± 0.57	5.23 ± 0.56	0.398
FT4 (pmol/L)	11.45 ± 1.91	11.49 ± 1.49	11.75 ± 2.13	0.589
Blood glucose (mmol/L)	4.99 ± 0.42	5.03 ± 0.58	4.98 ± 0.48	0.864

Data are shown as means ± standard deviation.
BMI, body mass index; FSH, follicular-stimulating hormone; LH, luteinizing hormone; E2, estradiol; P, progesterone; AMH, anti-Müllerian hormone; AFC, Antral Follicle Countin; TSH, thyroid stimulating hormone.
[a]P < 0.05, vs. early follicular phase long-acting GnRH agonist long protocol (Group A). [b]P < 0.05, vs. prolonged GnRH-a down-regulation in fertility patients during the COVID-19 pandemic (Group B).

TABLE 2 | Comparison of stimulation variables between the three groups.

Protocols	EFLL group (*n* = 85)	Pro-EFLL group (*n* = 43)	GnRH-ant group (*n* = 71)	*P*-value
On the Gn commencing day				
FSH level (IU/L)	3.40 ± 1.87	4.72 ± 1.90[a]		<0.001
LH level (IU/L)	0.63 ± 0.40	0.64 ± 1.20		0.974
E2 level (IU/L)	14.74 ± 45.41	7.82 ± 6.02		0.323
P level (IU/L)	0.22 ± 0.12	0.19 ± 0.19		0.453
On the day of hCG				
Endometrial thickness (cm)	12.09 ± 2.35	12.67 ± 2.21	10.79 ± 2.38[ab]	<0.001
LH value (IU/L)	0.82 ± 0.81	1.08 ± 0.93	5.83 ± 0.99[ab]	<0.001
E2 value (IU/L)	3836.43 ± 2226.29	3276.20 ± 1980.63	3102.54 ± 2477.48	0.115
P-value (IU/L)	0.93 ± 0.51	0.97 ± 0.69	0.76 ± 0.57	0.102
Total dosage of Gn used (IU)	2569.12 ± 957.23	2485.47 ± 943.98	2536.44 ± 853.19	0.888
Duration of Gn used (days)	13.79 ± 1.96	13.02 ± 2.68	10.58 ± 2.51[ab]	<0.001

Data are shown as means ± standard deviation or frequencies.
[a]P < 0.05, vs. early follicular phase long-acting GnRH agonist long protocol (Group A); [b]P < 0.05, vs. prolonged GnRH-a down-regulation in fertility patients during the COVID-19 pandemic (Group B).

TABLE 3 | Comparison of clinical outcomes between the three groups.

Protocols	EFLL group (*n* = 85)	Pro-EFLL group (*n* = 43)	GnRH-ant group (*n* = 71)	*P*-value
No. of oocytes	15.02 ± 7.93	14.49 ± 6.30	10.06 ± 7.63[ab]	<0.001
No. of mature oocytes	11.96 ± 6.00	11.60 ± 5.71	7.63 ± 6.50[ab]	<0.001
Oocyte maturation rates	0.82 ± 0.17	0.81 ± 0.18	0.76 ± 0.23	0.142
No. of fertilized oocytes	8.44 ± 5.34	9.14 ± 5.43	5.42 ± 5.20[ab]	<0.001
Fertilization rates	0.56 ± 0.23	0.63 ± 0.22	0.53 ± 0.27	0.120
No. Of transferable embryos	4.87 ± 2.96	6.47 ± 5.12[a]	3.00 ± 3.28[ab]	<0.001
Pregnancy rates per transfer (%)	49.4 (42/85)	34.9 (15/43)	39.4 (28/71)	0.229
Miscarriage rates (%)	11.9 (5/42)	20.0 (3/15)	25.0 (7/28)	0.358

Data are shown as frequencies (percentages).
[a]P < 0.05, vs. early follicular phase long-acting GnRH agonist long protocol (Group A); [b]P < 0.05, vs. prolonged GnRH-a down-regulation in fertility patients during the COVID-19 pandemic (Group B).

In the special period of the epidemic, it is necessary to develop or refine robust COH protocols to minimize possible exposure. Our study showed that one full-dose depot of long-acting GnRH-a per COH cycle would be more suitable and convenient for infertile couples than GnRH antagonist and short-acting GnRH-a injections during the COVID-19 pandemic because there are fewer possible incidences of potential exposure (Ren et al., 2014; Li F. et al., 2020). Some studies previously observed that a pituitary downregulated full-dose may require a higher dose of gonadotropins for ovarian stimulation (Pan et al., 2019; Xu et al., 2020); however, our study showed that the total dosage of Gns used in our study was not statistically different among the treatment groups, which means a full dose of gonadotropins or prolonged pituitary downregulation would not drastically increase the economic burden for Infertile couples.

We recommend that patients start Gn injections 33–42 days after a pituitary downregulated full dose (3.75 mg) of gonadotropin-releasing hormone agonist during the COVID-19 pandemic. Even a delay of 2–4 weeks does not affect the implantation rate. This would significantly reduce the rate of cycle cancelations and greatly alleviate the financial and emotional burden of interrupting treatment for infertile couples due to the epidemic. Our study can provide a more detailed view of the clinical management strategies for infertile couples during the COVID-19 pandemic; however, these strategies warrant large-scale, prospective and multicenter clinical trials for confirmation in the future.

Our study showed that although the Pro-EFLL protocol was associated with a greater endometrial thickness than GnRH-ant protocols on the day of the hCG injection, the pregnancy rates were not significantly different among the groups. Similar findings were also reported in the study of Wang et al. (2017), Huang et al. (2018), and Liu Y. et al. (2019), and their conclusions are consistent with our findings. However, some studies in the past have shown that endometrial thickness is closely related to pregnancy rates (Haas et al., 2019; Liu W. et al., 2019). Gallos et al. (2018) analyzed 25,767 IVF cycles from the CARE Fertility Group in the United Kingdom and found that when the endometrial thickness was less than 5 mm, the live birth rate was 15.6%, and when the endometrial thickness of 10 mm, the live birth rate was gradually increased to 33.1%. It seems that the thicker the endometrium, the higher the pregnancy rates. We analyzed the reasons of these studies are inconsistent with our results may be caused by the following factors. First,

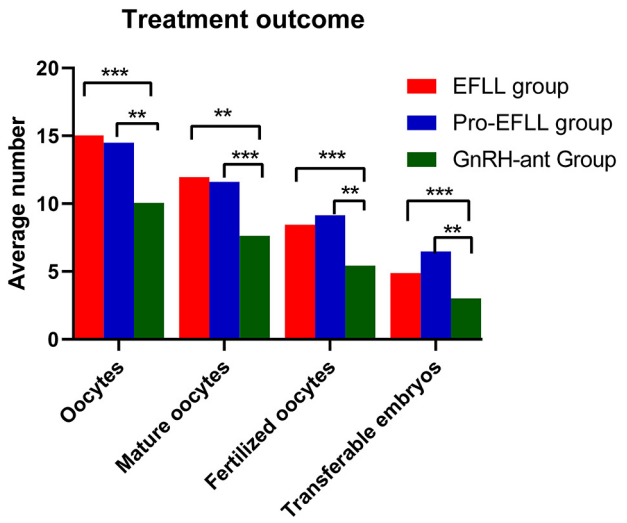

FIGURE 5 | Comparison of oocytes retrieved, mature oocytes, fertilized oocytes, and transferable embryos among EFLL, Pro-EFLL, and GnRH-ant protocol. Values are presented as mean ± standard deviation. **Represents significant difference at $P < 0.01$, ***represents significant difference at $P < 0.001$.

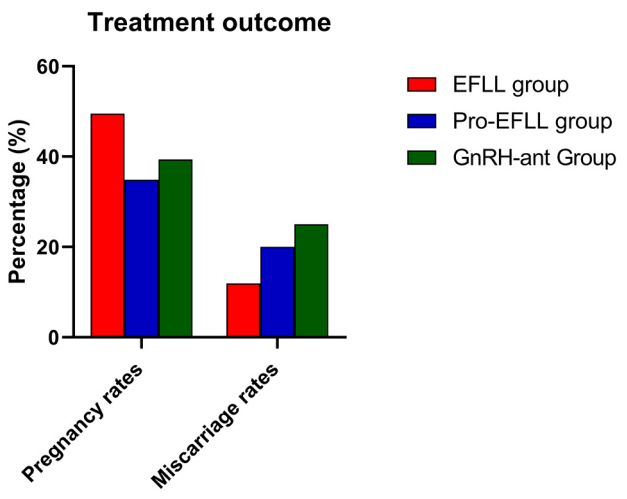

FIGURE 6 | Comparison of pregnancy rates and miscarriage rates among EFLL, Pro-EFLL, and GnRH-ant protocol. Values are presented as frequencies.

endometrial thickness is not the only factor that affects the pregnancy rate since it is influenced by many factors, such as age, endometrial receptivity, and embryo quality (Zhao et al., 2014; Bu and Sun, 2015). Second, these studies did not take into account the effect of basal FSH, AMH, AFC, TSH, FT3, blood glucose or the E2 value on the day of the hCG injection per fresh transfer cycle when adjusting for covariates compared with our work, and previous studies have reported that these variables are related to pregnancy rates and miscarriage rates per fresh transfer cycle (Vaegter et al., 2017; Song et al., 2020). Third, the research populations are different, and there are physiological differences between ethnic Chinese and ethnic Europeans, the physiological difference between the two ethnic groups are reflected in many aspects, such as body mass index difference, altered ovarian morphology and functional changes, Genes associated with reproduction and fertility changes, which might cause different pregnancy outcomes (Mura et al., 1991; Lachance and Tishkoff, 2013; Dumesic et al., 2015).

Furthermore, the EFLL protocol can acquire more mature oocytes and transplantable embryos than the GnRH-ant protocol; however, no statistically significant effects were seen for pregnancy and miscarriage rates per fresh transfer cycle. Some reports suggest that it seemed more strongly impaired to endometrial receptivity by GnRH-a than GnRH-ant treatments, a study revealed that the gene expression profiles of endometrial cells following GnRH-ant treatment are more similar to those during natural cycles using microarray data (Chen et al., 2019), this finding may explain the phenomenon overall. However, some reports on endometrial receptivity have been inconsistent for the GnRH-ant and GnRH-a protocols, and studies have suggested that a full-dose dipherelin injection can be used to achieve long-term suppression of the GnRH agonist, and it can increase endometrial receptivity in patients, although the exact mechanism remains unclear (Lambalk et al., 2017; Wu et al., 2019), so further analysis is required in the future.

It is worth emphasizing that if patients require more COH cycles to achieve better cumulative live birth rates, our research shows that GnRH-a protocols is significantly superior to the GnRH-ant protocol. An animal model study suggested that follicles and embryos quality was significantly enhanced by increasing concentrations of GnRH-a and its receptor (Liu M. et al., 2018). One study showed that the optimal serum LH concentration on the commencing day of ovarian stimulation after downregulation with GnRH-a was 0.1 IU/L∼1 IU/L, and when serum LH levels are less than 0.1 IU/L, exogenous LH is required to increase the number of follicles and embryos. When the serum LH is greater than 1 IU/L, it has been proven to have adverse effect on embryo quality. Our study showed that serum LH levels on the commencing day of ovarian stimulation after downregulation were 0.63 ± 0.40 IU/L (EFLL Group) and 0.64 ± 1.20 IU/L (prolonged Group), which are similar to those in the study of Li G. et al. (2020). In view of these findings, we think that it is better to give patients a pituitary downregulated full dose (3.75 mg) of dipherelin on days 2–4 of menstruation during the COVID-19 pandemic.

The clinical value of this study is that it is the first to observe the effect of prolonged GnRH agonist downregulation on pregnancy and miscarriage rates per fresh transfer cycle in Chinese patients with infertility during the COVID-19 pandemic. The findings of this study should be helpful for developing clinical management strategies for infertile couples. However, there are some limitations in the present study (Jansen et al., 2020; Niehus et al., 2020): (1) Retrospective cohort studies are always associated with selection bias issues, with selection bias being introduced, one might expect the result to be skewed in some ways. (2) In this study, our research subjects were all Chinese patients with infertility being given IVF/ICSI treatments during the COVID-19 pandemic.

Therefore, there is a certain deficiency in the universality and generalizability of the research. (3) Because we excluded women who received transplantation genetic screening or other ovarian hyperstimulation protocols, the findings of this study cannot be used for these groups of people.

In conclusion, in the special period of the COVID-19 pandemic, prolonged pituitary downregulation by utilizing a full dose of GnRH-a administered to infertility patients showed no differences in clinical outcomes, such as pregnancy or miscarriage rates per fresh transfer cycle, among the different protocols. The prolonged downregulation protocol and the EFLL protocol can acquire more mature oocytes and transplantable embryos than the GnRH-ant protocol. We recommend that patients start Gn injections 33–42 days after a pituitary downregulated full dose (3.75 mg) of gonadotropin-releasing hormone agonist during the COVID-19 pandemic. Even a delay of 2–4 weeks does not affect the implantation rate. Our study can provide a more detailed estimate and clinical management strategies for infertile couples during the COVID-19 pandemic; however, these strategies warrant large-scale, prospective and multicenter clinical trials for confirmation in the future.

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 was followed the Declaration of Helsinki Guideline and approved by the Ethics Committee of Reproductive Medicine Center, the First Affiliated Hospital of Zhengzhou University, China. Ethical code: ZZDX-2020-T260-16.

AUTHOR CONTRIBUTIONS

GL, HJ, and FL conceived of and designed the experiments. GL, YT, HZ, and WS selected and supervised suitable patients. GL, YW, and FL obtained basic clinical data including age, body mass index, FSH, LH, estradiol, progesterone, and AMH levels, total dosage of gonadotropin used, duration of gonadotropin use, oocyte number, and live birth rate per transfer. GL provided overall supervision. GL, HZ, and FL drafted the manuscript. All authors reviewed this manuscript.

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REVIEW
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Regulation of Angiotensin-Converting Enzyme 2: A Potential Target to Prevent COVID-19?

Yue Hu [*†], *Lihuan Liu* [†] *and Xifeng Lu*

Department of Physiology, Basic Medical College, Shenzhen University, Shenzhen, China

The renin–angiotensin system (RAS) is crucially involved in the physiology and pathology of all organs in mammals. Angiotensin-converting enzyme 2 (ACE2), which is a homolog of ACE, acts as a negative regulator in the homeostasis of RAS. ACE2 has been proven to be the receptor of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which caused the coronavirus disease 2019 (COVID-19) pandemic. As SARS-CoV-2 enters the host cells through binding of viral spike protein with ACE2 in humans, the distribution and expression level of ACE2 may be critical for SARS-CoV-2 infection. Growing evidence shows the implication of ACE2 in pathological progression in tissue injury and several chronic conditions such as hypertension, diabetes, and cardiovascular disease; this suggests that ACE2 is essential in the progression and clinical prognosis of COVID-19 as well. Therefore, we summarized the expression and activity of ACE2 under various conditions and regulators. We further discussed its potential implication in susceptibility to COVID-19 and its potential for being a therapeutic target in COVID-19.

Keywords: SARS-CoV-2, COVID-19, angiotensin-converting enzyme 2, renin–angiotensin system, therapeutic target

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Bo Chen, Sichuan University, China

****Correspondence:***
Yue Hu
huyue886420@126.com

†These authors share first authorship

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an acute respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1). It was first reported in December 2019 in Wuhan, Hubei Province of China; as of March 24, 2021, it has resulted in more than 100 million infections and 3.48 million deaths worldwide. SARS-CoV-2 is predominantly transmitted through direct contact or respiratory droplets in a proximity- and time-dependent manner, and it often requires close contact of within 6 feet over a period of 15 min or longer (2). Common COVID-19 symptoms include fever, dry cough, fatigue, and dyspnea, whereas severe symptoms are accompanied by systemic infection and pneumonia because the lungs are the primary target of the disease (3). The disease also causes damages to other organs such as the heart, liver, and kidneys as well as organ systems such as the intestinal, circulatory, and immune systems. Infected patients experience different symptoms to different degrees, ranging from asymptomatic, mild respiratory infections to severe acute respiratory syndrome, which results in organ failure, shock, acute respiratory distress symptoms, heart failure, arrhythmias, renal failure, and eventually death (4).

Angiotensin-converting enzyme 2 (ACE2) has been reported to be a potent negative regulator of the renin–angiotensin system (RAS), which encodes protein as a functional receptor for the spike glycoprotein of the human coronaviruses SARS, SARS-CoV-2, and HCoV-NL63, and it is a key pathogenic factor for coronavirus infection in host cells (5, 6). In the RAS, ACE2 degrades angiotensin (Ang) II and converts it into Ang-(1-7), which is a vasodilative, antiproliferative, and antiapoptotic agent (7). Thus, the balance between Ang I/Ang II and Ang-(1-7)/Ang-(1-9) in the RAS is disrupted by the combination of coronaviral spike glycoprotein and ACE2 (8), which changes the permeability of cell membranes and leads to organ damage. In particular, SARS-CoV-2 infects type II alveolar epithelial cells through ACE2, thus inducing lung injury (9). The virus then continues to invade the cells of other organs such as the heart, kidney, liver, and intestine by binding the ACE2 receptor through blood circulation; this triggers an excessive immune response, which produces numerous inflammatory cytokines and an imbalance in T-helper-1 and T-helper-2 cells, thus causing a cytokine storm and ultimately leading to multiple organ dysfunction syndrome (10).

As mentioned above, the entry of SARS-CoV-2 into host cells is facilitated through the binding of the viral spike protein with the extracellular domains of the transmembrane ACE2 proteins, resulting in the downregulation of surface ACE2 expression (11). Clinical research has indicated a direct link between the downregulation of tissue ACE2 and the imbalance of the RAS in patients with COVID-19, which promotes the development of multiorgan injuries caused by SARS-CoV-2 infection (12). Because of the crucial role of ACE2 in SARS-CoV-2 infection, potential therapeutic strategies include the prevention of the binding of human ACE2 and the receptor-binding domain of the viral spike protein or the direct and indirect regulation of ACE2 expression, small molecule inhibitors, drugs, ACE2 antibodies, or single-chain antibody fragments against ACE2, which may influence its activity. In addition, a novel strategy for the rapid detection of SARS-CoV-2 has been reported (13), which is based on the function of ACE2 as a receptor of the spike protein and detects samples in a lateral flow immunoassay without DNA extraction and quantitative reverse transcriptase-polymerase chain reaction.

Therefore, understanding the expression and activity of ACE2 regulation in various conditions may help predict SARS-CoV-2 infection in patients with COVID-19 under different conditions and clinical prognosis. In this review, we summarize the regulation of ACE2 expression and activity under various conditions and regulators and discuss its role as a potential therapeutic target in COVID-19.

RAS AND ACE

The RAS is a major regulator of blood pressure and fluid homeostasis (**Figure 1**). It interacts with the kallikrein–kinin system and plays a key role in the cardiovascular system (14). Renin cleaves angiotensinogen to form Ang I, which is then cleaved by ACE to generate Ang II. Ang II can bind to Ang II type 1 and 2 receptors (AT1/2R) and induce vessel contraction and increase blood pressure, thus acting as an effector of the RAS (15, 16). A homolog of ACE, ACE2, was discovered approximately two decades ago (17). This homolog is a single transmembrane protein with 805 amino acids, containing a HEXXH zinc-binding domain that is homologous to the enzyme activity site of ACE, sharing 42% similarities with ACE at the amino acid level (18). However, ACE2 is insensitive to classic ACE inhibitors (ACE-Is) such as captopril, and it plays a role completely opposite to that of ACE (19). ACE2 can recognize Ang I and cleave it into Ang-(1-9), which is quickly converted to Ang-(1-7) by ACE (19, 20). Moreover, ACE2 isolated from the human heart only hydrolyzes Ang II but not Ang I. These findings suggest that Ang II is the preferred substrate of ACE2 (21). Thus, ACE2 can act as an antagonist of ACE functions by degrading Ang II and its consequent vasoconstrictive effects. Furthermore, Ang-(1-7) has been identified as a ligand for Mas receptor, which is a seven-transmembrane G-protein-coupled receptor. Upon ligand binding, Mas receptor induces intracellular signaling cascades, including the activation of protein kinase B and induction of nitric oxide production, exerting vasoprotective functions in contrast to the hypertensive and proliferative functions of Ang II–AT1R/AT2R axis (22). Moreover, ACE2 can act on not only the RAS but also several peptides from other systems, such as neurotensin (1–14), apelin13, dynorphin (1–14), and some of the kinin metabolites (23, 24).

Unlike the ubiquitous expression of ACE, ACE2 is predominantly expressed in the heart, kidneys, and testes. However, ACE2 expression can also be observed in the gastrointestinal tract, lungs, and liver but to a lesser extent. In general, genetic deletions of ACE2 impair cardiovascular functions, but the exact impacts are various. Crackower et al. (25) found that ACE2 deficiency severely reduces cardiac contractility but it does not affect blood pressure, whereas Gurley et al. (26) reported that ACE2 knockout (KO) mice showed no changes in the cardiac structure or function; however, they showed slight increases in blood pressure. Moreover, ACE2 KO mice were shown to be more susceptible to Ang II-induced hypertension (26). In addition to its effects on the cardiovascular system, ACE2 deficiency influences the functions of the liver, whereas its expression is relatively abundant. In addition, ACE2 KO mice have been reported to show abnormal lipid accumulation in the liver and impaired glucose metabolism (27). Furthermore, ACE2 plays a protective role against liver damage; the loss of ACE2 function in the liver may accelerate the development of liver injury such as liver fibrosis (28). In the lungs, ACE2 is primarily expressed in epithelial cells. Deleting ACE2 does not impair lung functions but augments lung injury induced by cigarette smoke exposure, which is likely to be due to the increased activation of matrix metalloproteinases.

ACE2 plays a crucial role in avian influenza H5N1 and H7N9 virus infections as well as in SARS-CoV and SARS-CoV-2 infections (**Figure 2A**). Furthermore, ACE2 deficiency

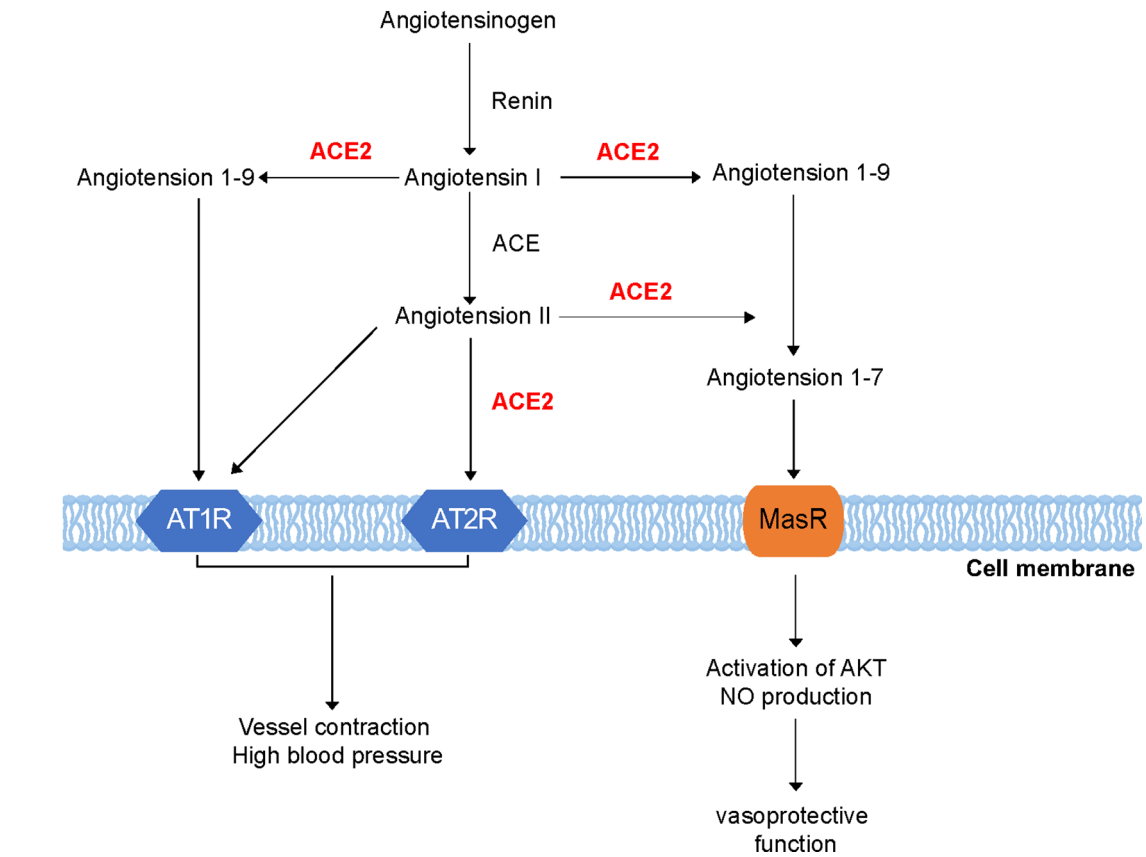

FIGURE 1 | Renin–angiotensin system. Renin cleaves angiotensinogen to form Ang I, which is then cleaved by angiotensin-converting enzyme (ACE) to generate Ang II. Ang II can bind to Ang II type 1 and 2 receptors (AT1/2R). ACE2 is a single transmembrane protein, which can recognize Ang I and cleave it into Ang 1-9, which is quickly converted to Ang 1-7 by ACE. Moreover, Ang-(1-7) has been identified as a ligand for Mas receptor, which is a seven-transmembrane G-protein coupled receptor. Mas receptor may induce intracellular signaling cascades such as protein kinase B and induction of nitric oxide (NO) production.

increases the severity of H7N9-virus-induced lung injury, which may be attenuated by blocking AT1R, suggesting that enhanced Ang II/ATR1 signaling is a major cause of lung injury during infection with the avian influenza virus. Notably, although ACE2 receptor mediates virus infection, both SARS-CoV and H7N9 virus downregulate its expression in the lungs shortly after viral infection; however, the expression of ACE remains unaffected. Thus, it is not surprising that serum Ang II levels were increased in patients with H5N1 and H7N9 infection. Moreover, the administration of human recombinant ACE2 protein reduces lung injury induced by the avian influenza H5N1 virus (29, 30).

SOLUBLE ACE2 AND RECOMBINANT ACE2

In addition to membrane-bound ACE2, a soluble form of ACE2 (sACE2) also exists in circulation. ACE2 may be constitutively cleaved to release two distinct major soluble forms. The deglycosylated molecular masses of the larger and smaller soluble forms are approximately 80 and 70 kDa, respectively (31). A 70-kDa ACE2 was purified from astrocyte cell culture, which converted Ang II into Ang-(1-7), suggesting that this protein is a secreted form of the enzyme (32). A 60-kDa sACE2 was also purified from the mesangial cells from mice, which generated Ang-(1-7) from Ang II that prevented exposures to high levels of this vasoconstrictive peptide and exert a protective effect in renal hemodynamics (33). Because sACE2 contains a completely catalytic domain, it may still be capable of cleaving Ang II in circulation. Circulating sACE2 activity levels have been associated with chronic systolic heart failure in humans, and after intensive medical therapy, increases in baseline serum sACE2 levels have predicted a significant reduction in the risk of death after cardiac transplantation (34).

sACE2 is enzymatically active and partially inhibits virus entry into the target cells of human airway epithelia. Mutant and chimeric ACE2 proteins showed that a point mutation in the ACE2 ectodomain, L584A, markedly attenuated shedding. The

resultant ACE2-L584A mutant trafficked to the cell membrane and facilitated SARS-CoV entry into target cells, suggesting that the ACE2 ectodomain regulates its release and that residue L584 might be part of a putative sheddase "recognition motif". Both wild-type ACE2 and the ACE2-L584A mutant supported productive infection with the SARS-CoV, indicating that sACE2 generation is not required for the protein to function as a coronavirus receptor (35). Soluble recombinant ACE2 (rACE2) has been reported to prevent the rapid hypertension elicited by Ang II by reducing its level and increasing Ang-(1-7) level in plasma, and during Ang II infusion, rACE2 degraded Ang II and thus normalized blood pressure (36). Thus, rACE2 may provide a novel therapeutic target and be used as a potential antihypertensive drug. Notably, ADAM17, which is a disintegrin and metalloprotease 17, cleaves ACE2 between Arg (708) and Ser (709), forming a 20-amino acid transmembrane peptide (37). The binding of Ang II with AT1R can promote sACE2 formation by increasing ADAM17 activity (38), which generates sACE2, reducing surface ACE2 expression (**Figure 2B**). We suggest that rACE2 can be important in SARS-CoV-2 treatment.

Overall, on the basis of the known molecular functions of the RAS, ACE2 and related genes play central roles in the RAS activity, associated pathologies, and virus infection, including the global pandemic, COVID-19. Thus, it is extremely necessary and useful to summarize the regulators of ACE2 for further research.

TRANSCRIPTIONAL AND TRANSLATIONAL REGULATION OF ACE2

Transcription Factor Regulation

Similar to other components of the RAS, ACE2 expression is also well regulated to maintain appropriate RAS activities. Ang II can suppress ACE2 expression *via* AT1R in neuronal cells, forming a negative feedback loop. Ang-(1-7) can antagonize the Ang II-induced downregulation of ACE2 expression, although it does not influence ACE2 expression in neuronal cells. It has been revealed that the regulation of ACE2 by Ang II/AT1R requires sequences to lie between 481 and 516 base pairs upstream of the transcription initiation site of human ACE2; although the exact transcription factor (TF) mediating Ang II/AT1R-regulated ACE2 expression is yet to be identified, it is evident that the ATTTGGA motif, which is a binding sequence for TF Ikaros, is indispensable for this regulation (39). There are also three binding sites that are highly conserved in mammals for hepatic nuclear factor 1 (HNF1), which is a TF that regulates various hepatic genes and plays an important role in homeobox gene family expression of the liver and kidney. Knocking out HNF1 in mice induces death around weaning after a progressive wasting syndrome with a markedly enlarged liver (40). Both HNF1α and HNF1β can increase ACE2 expression in insulin-producing cells, including pancreatic β cells and insulinoma cells. Notably, HNF1α increases ACE2 gene expression in primary cells from pancreatic islets through evolutionarily conserved motifs in the proximal promoter region (41). In addition, HNF4α—a master regulatory protein in the liver and an important TF in angiotensinogen gene regulation—targets ACE2 and influences its mRNA expression (42).

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric TF comprising a regulatory α subunit (HIF-1α) and a constitutive β-subunit (HIF-1β) (43). Notably, HIF-1α indirectly regulates ACE2 through the downregulation of ACE2 expression for the accumulation of Ang II catalyzed by ACE. The expression levels of ACE2 mRNA increase during the early stages of hypoxia and decrease to near baseline levels at the later stages after HIF-1α accumulation in pulmonary artery smooth muscle cells. Therefore, direct regulation of ACE and bidirectional regulation of ACE2 by HIF-1α during hypoxia could play a protective role during the development of hypoxic pulmonary hypertension (44).

Epigenetic Regulation

Except for the TF, epigenetic regulation could also occur in ACE2 mRNA expression, such as the regulation of histone acetylase/deacetylase and microRNAs. Silent information regulator T1 is a histone deacetylase and a transcriptional mediator, which exerts protective effects *via* the deacetylation of its target proteins such as proteins involved in cellular stress resistance and genomic stability. Silent information regulator T1 binds to the ACE2 promoter; this binding may increase after treatment with the antimicrobial peptide mimic 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) in Huh7 cells, which increases ACE2 mRNA expression levels under the hypoxic conditions induced. In contrast, the inhibition of Silent information regulator T1 activity eliminates the 5-aminoimidazole-4-carboxamide ribonucleotide-induced increase in ACE2 expression (45). The ACE2 mRNA expression level increases by increasing the occupancy of histone H3 acetylation, which binds on ACE2 promoter region in rabbit models of high-cholesterol diet-induced atherosclerosis treated with atorvastatin. Additionally, the epigenetic regulation of ACE2 may be another realistic way to treat atherosclerosis and cardiovascular disorders (46).

MicroRNAs are endogenous, small (19–25 nucleotides), and non-coding RNAs, which can target specific genes and function as the negative regulators of gene expression by inhibiting the translation of or promoting the degradation of targeted mRNAs. The role of microRNAs in regulating the standard and novel cardiac RAS during aerobic exercise training in rats with left ventricular hypertrophy has indicated that exercise can increase miRNA-27a and miRNA-27b targeting ACE and miR-143 targeting ACE2, inducing higher mRNA expression and protein levels of ACE2, followed by an increase in Ang-(1-7) and AT2R (47) levels. Additionally, in patients with uremia, circulating miR-421 shows enhanced higher expression than that in healthy individuals; it targets leucocytic ACE2 and decreases its transcripts. The association between miR-421 and ACE2 may contribute to lower expression of the enzyme in the leukocytes of chronic kidney diseases, further supporting the development of atherosclerotic events (48), which suggests that miRNA can also be a target for atherosclerosis. In cardiovascular disease, miR-421 could be a potential regulator of ACE2 involved in the

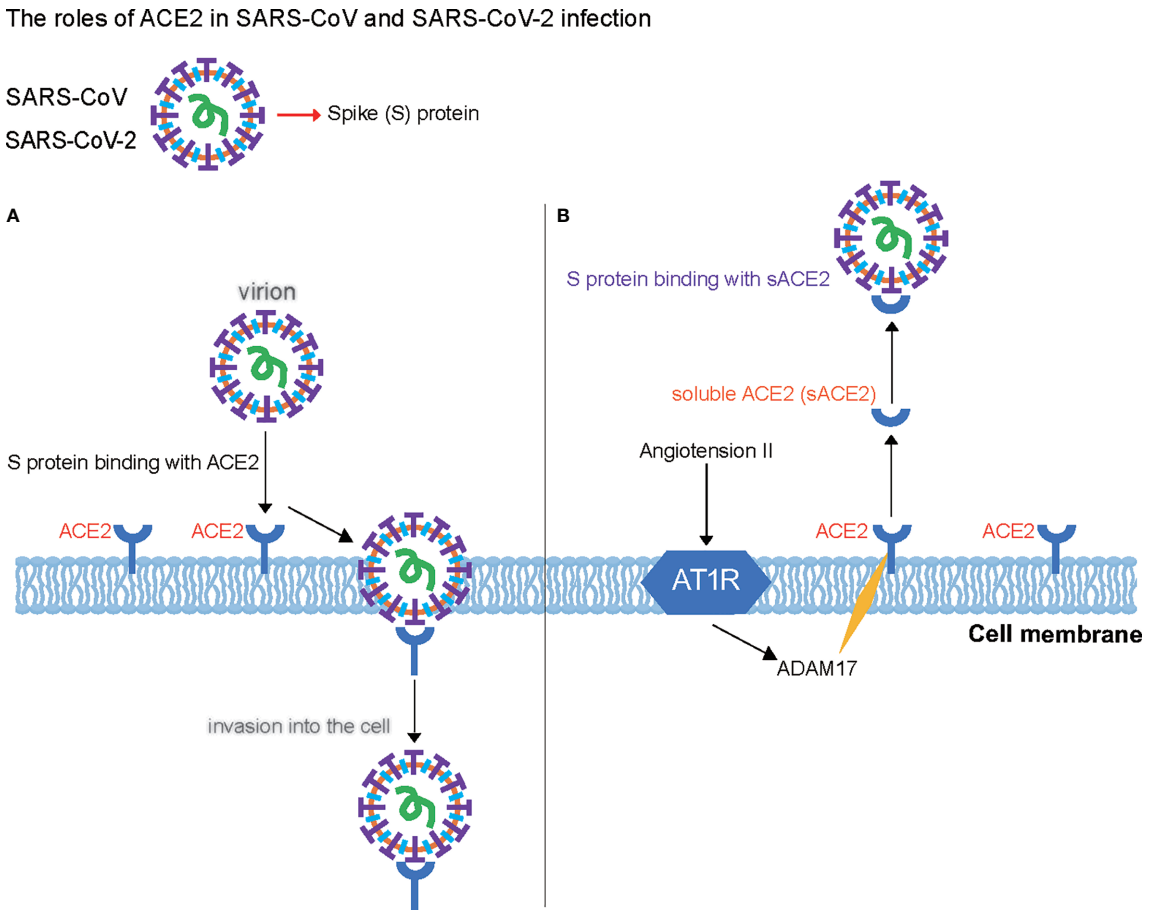

FIGURE 2 | Roles of angiotensin-converting enzyme 2 (ACE2) in severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2 infection. **(A)** ACE2 acts as the key receptor in avian influenza H5N1 and H7N9 virus infections as well as in SARS-CoV and SARS-CoV-2 infections. **(B)** Roles of a soluble form of angiotensin-converting enzyme 2 (sACE2) and A disintegrin and metalloprotease 17 (ADAM17) in cells infected with SARS-CoV and SARS-CoV-2. ADAM17 cleaves ACE2 and forms a 20-amino acid transmembrane peptide. The binding of Ang II with AT1R promotes sACE2 formation by increasing ADAM17 activity, which generates sACE2 and binds to the spike protein of SARS-CoV-2 as the virus receptor.

development of thrombosis and downregulation of its protein level in both primary cardiac myofibroblasts and transformed cells. Notably, miR-421 levels show significant interpatient variabilities, which are consistent with the alteration in ACE2 expression (49).

Regulation by Other Factors

Several factors regulate ACE2 mRNA expression levels during disease progression. For example, transforming growth factor-β, which is pivotal in diabetic nephropathy, has a negative feedback effect on the mRNA expression and protein level of ACE2 and its cell membrane-binding receptor Mas. Thus, it can decrease ACE2, Mas, and Ang-(1-7) conversion from Ang II in high glucose-cultured NRK-52E cells, suggesting a possible treatment for diabetic renal fibrosis (50). Additionally, nuclear factor erythroid 2-related factor 2 functions as a master regulator of redox balance in cellular cytoprotective responses. The loss of this factor upregulates the expression of renal proximal tubule cellular ACE2 and its Mas receptor, followed by an increase in urinary Ang-(1-7) levels and downregulation of the expression of angiotensinogen, ACE, and profibrotic genes in Akita mice (51).

Curcumin, a yellow pigment extracted from the rhizomes of *Curcuma longa*, which exhibits pharmacologic properties such as anti-inflammation and fibrosis properties, increases ACE2 protein levels and enhances its expression in the intermyocardium relative to animals with Ang II infusion (52). Rosiglitazone, which is a peroxisome proliferator-activated receptor gamma ligand that acts as an insulin sensitizer and exerts cardiovascular action, increases ACE2 and Ang-(1-7) protein levels and decreases the Ang II level to lower blood pressure in a peroxisome proliferator-activated receptor gamma-dependent manner in male Wistar rat models (53).

Regulation by Chinese Medicine

As mentioned above, ACE2 is an important enzyme that attaches to the cellular membranes in the lungs, arteries, heart, kidney,

and intestines and functions in the pathophysiology of lung and cardiovascular diseases. Some drugs regulate ACE2 protein levels during disease processes. For example, Yinhenhao decoction is a traditional Chinese medicine that has antifibrotic effects that reduce hepatic fibrogenesis in the bile duct ligation rat liver model. This is achieved by decreasing the standard RAS pathway components and transforming growth factor-β1 down expression, which elevates the ACE2 protein level to recover and rebuild self-regulation of the RAS (54). In addition, Sini decoction, which is used widely for treating clinical diseases, alleviates *Escherichia coli*-induced acute lung injury in mice by markedly enhancing ACE2 protein levels to activate the ACE2–Ang-(1-7)–Mas pathway and equilibrate the ACE–Ang II–AT1R and ACE2–Ang-(1-7)–Mas axis (55). Diminazene aceturate, which is the most widely used therapeutic agent for trypanosomiasis and has been shown to prevent pulmonary hypertension, increases the protein level of ACE2 to prevent the progression of asthma by altering the levels of AKT, p38, NF-κB, and other inflammatory markers in male Wistar rats (56). Some other regulators such as RAS regulators and drugs influence ACE2 functions at transcriptional and translational levels and its activities.

On the basis of these ACE2 regulators described above, we summarized the available data, including the data regarding their effects on ACE2 through mRNA expression, protein level alteration, and activities in **Table 1**.

PHARMACOLOGICAL REGULATION OF ACE2

Considering that the primary symptoms of COVID-19 reported to date include hypertension, atherosclerosis, diarrhea, glaucoma, anosmia, ageusia, skin lesions (dermatitis), autoimmune inflammation of the central nervous system, and damage to organs such as the lungs, heart, kidneys, and testicles, all these diffuse COVID-19 disorders are likely associated with an overreaction of the RAS in patients with COVID-19 (57). Current pharmacotherapies aim to inhibit multiple levels of the RAS through distinct modes of action. Because ACE2 is the key negative modulator of the RAS, its gene transcription and translation level and the catalytic activities are modified owing to the intricate nature of the RAS. The presence of several RAS modulators functioning at different levels in this system results in various effects on ACE2. In **Table 2**, we summarize the major studies, including the effects of various RAS modulators (as drugs) on ACE2 in mRNA expression, protein levels, and activities. The effects elicited by these different drugs on ACE2 depend on several factors, including the various systems studied, disease progression stage, and drug usage.

Although ACE2 is not the direct cellular target of these therapies, ACE2 gene transcription, translation, and catalytic activity are also modified owing to the intricate nature of the RAS. For example, angiotensin-receptor blockers (ARBs) and

TABLE 1 | Transcriptional and translational regulators of angiotensin-converting enzyme 2.

Class	Gene name	Condition	System	Impact on ACE2
Transcription factor	Hepatic nuclear factor 1 (HNF1), comprising HNF1α and HNF1β	Healthy	*In vivo* in insulin-producing cells, including pancreatic β cells and insulinoma cells	Increase ACE2 mRNA expression level (40, 41)
	HNF4α	Healthy	*In vivo* in the liver of mice	Target ACE2 and affect its mRNA expression level (42)
	Hypoxia-inducible factor 1 (HIF-1), comprising HIF-1α and HIF-1β	Hypoxic pulmonary hypertension	*In vivo* in pulmonary artery smooth muscle cells	Target ACE2 and indirectly decrease ACE2 mRNA expression level for the accumulation of Ang II catalyzed by ACE (43, 44)
Epigenetic regulation	Histone deacetylase: silent information regulator T1	Hypoxic	*In vivo* in Huh7 cells	Bound to the ACE2 promoter and increase ACE2 mRNA expression level (45)
	Histone H3 acetylation	Atherosclerosis	In the heart of rabbits with high-cholesterol diet-induced atherosclerosis treated with atorvastatin	Bound to the ACE2 promoter and increase ACE2 mRNA expression level (46)
	MicroRNAs: miR-143	Left ventricular hypertrophy	In aerobic exercise training rats	Increase ACE2 mRNA expression and protein level (47)
	MicroRNAs: miR-421	Uremic	Human	Target leucocytic ACE2 and decrease its transcripts (48)
		Thrombosis	In both primary cardiac myofibroblasts and transformed cells	Decrease ACE2 protein level (49)
Other factors	Transforming growth factor-β	High-glucose-cultured	In NRK-52E cells	Decrease ACE2 mRNA and protein level (50)
	Nuclear factor erythroid 2–related factor 2 (Nrf2)	Nrf2 knockout	In Akita mice renal proximal tubule cells	Increase ACE2 expression (51)
	Curcumin	Myocardial fibrosis	In male Sprague-Dawley rats	Increase ACE2 protein level (52)
	Rosiglitazone: a peroxisome proliferator-activated receptor gamma ligand	Hypertension	*In vivo* in male Wistar rats	Increase ACE2 protein level (53)
Chinese medicine	Yinhenhao decoction	Hepatic fibrogenesis	*In vivo* in the bile duct ligation rat liver model	Increase ACE2 protein level (54)
	Sini decoction	Acute lung injury	*In vivo* in rats	Increase ACE2 protein level (55)
	Diminazene aceturate	Asthma	*In vivo* in male Wistar rats	Increase ACE2 protein level (56)

TABLE 2 | Studies investigating the impacts of several cardiopulmonary diseases and renin–angiotensin system modulators on angiotensin-converting enzyme 2 expression and activity.

Drug class	Drugs	Condition	System	Impact on ACE2
ACE-I	Lisinopril	Healthy	*In vivo* in rat renal cells	Decrease in ACE2 mRNA expression (combination with low-sodium diet) (58)
			In vivo in rat cardiac (LV) cells	Increase in ACE2 mRNA expression (59)
		HTN	*In vivo* in transgenic Ren2 rats cardiac and renal cells	Increase in ACE2 mRNA expression and activity (60)
	Enalapril	MI	*In vivo* in rat cardiac (LV) and plasma cells	Increase in ACE2 mRNA expression and activity 8 weeks post-MI (61)
	Captopril	ALI	*In vivo* in rat pulmonary tissue and *in vitro* in rat pulmonary microvascular endothelial cells	Increase in ACE2 protein level (62)
	Any ACE inhibitors	Likely HTN	*In vivo* in human intestinal cells	Increase in intestinal ACE2 mRNA expression (63)
ARB	Losartan	Healthy	*In vivo* in rat cardiac (LV) cells	Increase in ACE2 mRNA expression and activity (64)
			In vivo in rat cardiac (LV)/renal cells	Potentiation of renal upregulation of ACE2 mRNA expression (65)
		ARDS	*In vivo* in rat BALF	Increase in ACE2 activity (66)
	Olmesartan	HTN	*In vivo* in rat aorta/carotid artery cells	Increase in ACE2 mRNA expression and activity (67)
	Irbesartan	Healthy	*In vivo* in rat aorta cells	Significantly increase mRNA expression and protein level (68)
	Telmisartan	HTN	*In vivo* in rat cardiac (aorta) cells	Decrease in ACE2 activity (69)
		Healthy	*In vivo* in rat renal cells	Increase in ACE2 mRNA expression and protein level (70)
	Eprosartan	HF	*In vivo* in rat cardiac cells	Increase in ACE2 activity (71)
MRB	Eplerenone	Healthy	*In vivo* in rat peritoneal macrophages/cardiac cells	Increase in ACE2 mRNA expression and activity (72)
	Spironolactone	HF	*In vivo* in human monocyte-derived macrophages	Increase in ACE2 mRNA expression and activity (72)
RI	Aliskiren	DN	*In vivo* in rat renal cells	Decrease in ACE2 activity (73)

ACE-I, angiotensin-converting enzyme inhibitor; ALI, acute lung injury; ARB, angiotensin-receptor blocker; DN, diabetic nephropathy; HF, heart failure; HTN, hypertension; LRTI, lower respiratory tract infection; LV, left ventricle; MI, myocardial infarction; MRB, mineralocorticoid receptor blocker; RI, renin inhibitor.

ACE-Is mostly increased ACE2 mRNA expression and protein level in the heart, kidneys, and thoracic aorta, but the activity varies across experimental models and tissues for ACE-Is (**Table 2**). Lisinopril treatment in healthy rats fed with low-sodium diet decreases the ACE2 mRNA levels in renal cells (58), whereas it increases ACE2 mRNA expression in cardiac cells (59). In addition, lisinopril used in transgenic Ren2 rats in hypertension increases ACE2 mRNA level and activity in the heart and kidneys (60). Moreover, in other ACE-I treatments, ACE2 mRNA expression and activity are both increased by enalapril in myocardial infarction rat cardiac and plasma cells (61). ACE2 protein levels are increased by captopril in rat pulmonary tissue and pulmonary microvascular endothelial cells *in vitro* under conditions of acute lung injury (62). Moreover, ACE2 mRNA expression levels were increased in human intestinal cells by any ACE-Is under hypertension condition (63). These findings may be attributed to the tissue-specific regulation of ACE2 as higher ACE2 protein levels were reported in the heart, but ACE2 activity was higher in the kidneys of Sprague–Dawley rats, adding to the complexity of the tissue in the RAS (74). Therefore, the mechanisms behind the augmentation of ACE2 mRNA levels by ACE-Is and ARBs require further characterization. Moreover, mineralocorticoid receptor blockers such as spironolactone, which prevents increases in both ACE and AT1R mRNA levels and the associated increase in AT1R density from aldosterone signaling in cardiomyocytes (75, 76), increase ACE2 mRNA expression and activity in monocyte-derived macrophages from patients with chronic heart failure (72). Furthermore, eplerenone increases ACE2 mRNA expression and activity in healthy rat peritoneal macrophages or cardiac cells (72). The renin inhibitor, aliskiren, decreases the ACE2 activity in diabetic nephropathy renal cells (**Table 2**) (73).

Clinical researchers have reported that patients with COVID-19 with these comorbidities of cardiovascular disease, diabetes, and chronic hypertension have been treated with RAS modulators such as ACE-Is, ARBs, mineralocorticoid receptor blockers, and renin inhibitors (77). ACE-Is and ARBs are commonly used therapeutic drugs for hypertension. Animal experiments have revealed that these drugs decrease the systolic pressure in healthy rats and upregulate ACE2 levels (78). However, some researchers still doubt the safety and effect of using these drugs on patients with COVID-19. As ACE2 expression is suppressed in hypertension and may be further deprived by the SARS-CoV-2 upon infection, the application of ARBs may protect against pulmonary injury under careful blood pressure management.

CONCLUSIONS

Since the discovery of ACE2, progress has been made in elucidating its biochemical actions and fundamental role in cardiovascular diseases and as a receptor for SARS-CoV-2 attachment. ACE2 functions a negative regulator of the RAS by metabolizing Ang II into the beneficial peptide Ang-(1-7); this important biochemical and physiological property is being harnessed as a potential therapeutic target in patients with cardiovascular diseases. The activation of the RAS axis due to the binding of SARS-CoV-2 to ACE2, which leads to the direct

loss of ACE2 and indirect loss of ACE2 *via* proteolytic processing and shedding, partly drives the systemic manifestations of COVID-19.

To date, there is no effective drug for the treatment of COVID-19. Although different types of vaccines have been approved to be used worldwide, the number of vaccines is limited and the protection efficiency varies across countries and regions. Therefore, the severity of the COVID-19 pandemic remains intact and the number of infected people keeps increasing, especially in India. Furthermore, at least three types of SARS-CoV-2 mutants have been identified: B.1.1.7, which was first found in the United Kingdom (79); E484K, which was first found in South Africa (80); and the Indian mutant (the delta variant), which has higher transmission efficiency and stronger pathogenicity than other variants of the virus (81). Therefore, effective drugs are urgently required for the treatment of COVID-19, especially for patients with comorbidities such as hypertension, cardiovascular disease, and lung injury. Of note, several drugs are being developed to treat COVID-19, and some of them are in phase 3 of the clinical trial. For example, colchicine (phase 3) is investigated to determine whether short-term treatment with colchicine reduces the rate of death and lung complications associated with COVID-19. Convalescent plasma has been also used in an efficacious therapy to prevent the progression from mild to severe/critical COVID-19.

Although the putative effects of ACE2 downregulation on the cardiovascular system in the course of the COVID-19 pandemic requires more intensive studies, patients with COVID-19 with these comorbidities of cardiovascular disease have already been treated with RAS and ACE2 regulators. Recent research supports continued use of drugs such ARBs or ACE-Is for patients who have been already using these medications before SARS-CoV-2 infection (82). Thus, we speculate that ACE2-based regulation strategies may become one of the most promising approaches for future therapies and improve disease prognosis in COVID-19. This review will serve as a point of reference for the use of these related drugs.

AUTHOR CONTRIBUTIONS

YH and XL designed the content. YH and LL wrote the main manuscript. LL performed the data collection. YH, LL, and XL edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Immuno-Endocrinology of COVID-19: The Key Role of Sex Hormones

Flavia Tramontana[1], *Sofia Battisti*[2,3,4], *Nicola Napoli*[1] *and Rocky Strollo*[5*]

[1] Department of Medicine, Unit of Endocrinology & Diabetes, Università Campus Bio-Medico di Roma, Rome, Italy, [2] Radiology Department, Azienda Unità Sanitaria Locale (AUSL) Romagna M. Bufalini Hospital, Cesena, Italy, [3] Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Meldola, Italy, [4] Dipartimento di Medicina Specialistica, Diagnostica e Sperimentale (DIMES), Alma Mater Studiorum-Universita di Bologna, Bologna, Italy, [5] Department of Science and Technology for Humans and the Environment, Università Campus Bio-Medico di Roma, Rome, Italy

Epidemiological evidence shows clear gender disparities in the Coronavirus 2019 Disease (COVID-19) severity and fatality. This may reflect the contribution of gender-related factors, such as sex hormones, to COVID-19 pathogenesis. However, the mechanism linking gender disparities to COVID-19 severity is still poorly understood. In this review, we will pinpoint several elements involved in COVID-19 pathogenesis that are regulated by the two main sex hormones, estrogen and androgen. These include tissue specific gene regulation of SARS-CoV2 entry factors, innate and adaptive immune responses to infection, immunometabolism, and susceptibility to tissue injury by cytopathic effect or hyper-inflammatory response. We will discuss the mechanistic link between sex hormone regulation of COVID-19 pathogenetic factors and disease severity. Finally, we will summarize current evidence from clinical studies and trials targeting sex hormones and their signalling in COVID-19. A better understanding of the role of sex hormones in COVID-19 may identify targets for therapeutic intervention and allow optimization of treatment outcomes towards gender-based personalised medicine.

Keywords: COVID-19, sex hormones, immuno-endocrinology, immune response, estrogen

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***Correspondence:**
Rocky Strollo
r.strollo@unicampus.it

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) caused by the novel coronavirus SARS-CoV-2 represents a global health threat which has caused globally almost five million deaths by October 2021 (https://covid19.who.int/). Epidemiological evidence shows clear gender disparities in COVID-19 severity and fatality, placing gender as a main factor associated with a more severe disease, along with older age and cardiometabolic comorbidities. Although there is no significant sex difference in the proportion of individuals infected with SARS-CoV-2, males face double the risk of developing critical or fatal disease compared with females (1, 2). The sex gap is closed in prepubescent individuals, where both sexes are relatively protected from COVID-19 complications compared to adults (3, 4). This may reflect a possible contribution of gender-related factors, such as sex hormones, to COVID-19 pathogenesis (5, 6). However, out of 45 COVID-19 randomized controlled trials published by December 2020, only eight reported sex-disaggregated results or subgroup analyses (7).

In this review, we will pinpoint several elements involved in COVID-19 pathogenesis that are, at least partially, regulated by the two main sex steroids, estrogen and androgen. These include tissue specific gene regulation of SARS-CoV-2 entry factors, innate and adaptive immune responses to infection, immunometabolism, and susceptibility to tissue injury by cytopathic effect or hyper-inflammatory response. We will discuss the mechanistic link between sex-hormone regulation of COVID-19 pathogenetic factors and disease severity. Finally, we will summarize current evidence from clinical studies and trials targeting sex steroids and their signalling in COVID-19.

SEX HORMONES CONTROL VIRUS-HOST INTERACTION

SARS-CoV-2 is a single-stranded RNA-enveloped virus which uses the angiotensin-converting enzyme 2 (ACE2) as main access door to host cells. Entrance is facilitated by a host type 2 transmembrane serine protease, TMPRSS2, that is responsible for priming of the viral S glycoprotein. Increased tissue (co-) expression of ACE2 and TMPRSS2 at the virus entry sites may enhance infection, while downregulation may prevent SARS-CoV-2 binding to target cells. Both elements are under genetic control of sex steroids. ACE2 belongs to a subgroup of genes escaping X-chromosome inactivation with higher expression in men in several tissues (8), including a slight tendency for male-biased expression in the lung. The predominant male-biased expression of ACE2 is in line with the demonstrated higher ACE2 activity in males partially driven by sex steroids (9). Similarly, plasma ACE2 concentration has been found to be higher in men than in women possibly reflecting the expression at the tissue level (9). Sex steroids acts on the modulation of ACE2 expression in a tissue specific manner. According to studies in mice, estrogen receptor (ER) *alpha* activation by estradiol downregulates kidney ACE2 whereas ovariectomy, which is a state of estrogen deprivation, increased ACE2 activity and its expression in kidney and adipose tissue (10). Estrogen may also downregulate ACE2 in differentiated airway epithelial cells (11), the main SARS-CoV-2 entry site. It has been shown that ACE2 expression in primary isolated human airway smooth muscle (ASM) cells was lower in women compared to men, and significantly upregulated by testosterone (12). Furthermore, consistent with the age-dependent decline in circulating sex steroids, males experience lower ACE2 level than females in the late stage life (13).

Taken together, data suggest that the two main sex steroids produce opposite effects on ACE2 regulation. While estrogen tend to favour downregulation of the SARS-CoV-2 main receptor in several tissues, testosterone may enhance its expression.

TMPRSS2 is an androgen responsive gene (14). Its expression in human lung epithelial cells is upregulated by androgen while downregulated by androgen deprivation (15). Exogenous treatment with androgen was shown to be associated with an increased expression of TMPRSS2 in human type 2 pneumocytes (15). Therefore, part of the sex-based disparities in COVID-19 severity may be explained by high androgen in males that contribute to disease severity by promoting viral replication (16). In a study on 118 patients with primary prostate cancer, where *TMPRSS2* gene is a therapeutic target, androgen deprivation therapy was associated with a lower risk of SARS-CoV-2 infection (odds ratio 4.05; 95% CI 1.55–10.59). While these data needs validation in larger cohorts, they provide support to the association between androgen control of TMPRSS2 expression and risk of COVID-19. More recently, Samuel et al. performed a high-throughput screen with a library of 1443 FDA-approved drugs and a subsequent *in silico* screen of more than 9 million drug-like compounds to detect drugs effective in reducing ACE2 protein levels in cardiac cells and lung organoids. The authors found that the most effective drugs were linked to androgen receptor signalling inhibition (17). Inhibitors of 5-alpha reductase, which dampen androgen signalling, were able to downregulate both ACE2 and TMPRSS2 in lung epithelial cells and cardiac cells, leading to a lower SARS-CoV-2 infectivity in lung organoids (17).

Therefore, the increased (co-)expression of ACE2 and TMPRSS2 in SARS-CoV-2 target tissues may explain the higher occurrence of COVID-19 complications in males. However, whether sex hormones-dependent modulation of ACE2 or TMPRSS2 in the lung or other SARS-CoV-2 target tissues correlates with COVID-19 susceptibility or severity needs to be further elucidated.

SEX HORMONES CONTROL ANTI-VIRAL IMMUNE RESPONSE

Gender is a key host factor influencing immune response, leading to differences in severity, prevalence, and pathogenesis of infection, with males generally more susceptible than females (18).

According to experimental evidence from the severe acute respiratory syndrome (SARS) caused by the SARS-CoV, another *beta*-coronavirus closely related to SARS-CoV-2, estrogen status is key in determining disease severity through modulation of the immune response. Ovariectomy of SARS-CoV infected female mice or treatment with an ER antagonist increased mortality compared to treatment with tamoxifen (a selective ER modulator) (19). The protective role of ER signalling has been linked to the induction of the "anti-viral status" mediated by type I interferon (IFN-I), a first line cytokine involved in host defence (4). In the SARS-CoV model, reduced survival was due to a robust viral replication and delayed IFN-I signaling which promoted accumulation of pathogenic inflammatory monocyte-macrophages, resulting in elevated lung pro-inflammatory cytokines and dysfunctional virus-specific T-cell responses (20). Thus, ER signalling may prime the IFN-I response and prevent viral replication. In contrast, a delayed IFN-I activation would generate a response-lag unable to compensate robust viral replication, thus leading to uncontrolled hyperinflammation. Data in humans provide

support to this hypothesis. It has been shown that long-term treatment of post-menopause women with estradiol enhanced IFN-I response *via* the *toll-like* receptor (TLR) 7 pathway (21). TLR7 represents a sentinel receptor of the innate immune response to viral RNA from SARS-CoV-2 and other coronaviruses. Recognition of viral RNA by TLR7 expressed by dendritic cells triggers signalling cascades that result in the production of large amount of IFN-I. TLR7 is also sex biased as it is encoded by X chromosome and escapes X inactivation in B cells through epigenetic modifications (22). Thus, the increased expression of TLR7 in females compared to male can potentiate priming of IFN response by ER signalling, providing prompt antiviral defence and subsequent antibody production. Accordingly, it has been recently showed that loss of function in TLR7 gene resulted in a severe disease in young male patients after being infected with SARS-CoV-2 (23).

Estrogen can also modulate adaptive responses displaying a diphasic effect ranging from immunosuppressive at high concentration to immunostimulatory at lower concentration (19). For example, lymphocyte activation (e.g., proliferation and IFN-γ production) classically follows a diphasic dose-response to estrogen concentrations—low dose stimulation and high dose inhibition. Thus, in older women, the residual low levels of estrogen may up-regulate T cell IFN-γ, inducing effector T helper 1 proliferation and antibody production, all factors that sustain anti-viral immune responses. This may partially counterbalance the age dependent decline in adaptive immune responses (24).

Androgens present different effects on both innate and adaptive responses, which are often opposite to estrogens, and may explain the overall increased susceptibility to viral infections in males compared to females. First, testosterone is immune suppressive on dendritic cells (a main source of IFN-I) and reduces cytokine production by such cells, which is consistent with the reduced IFN-I response to TLR7 stimulation in males compared to females. Second, androgens inhibit T-helper 1 differentiation, thus potentially delaying the mounting of specific antiviral responses. Finally, testosterone directly enhances production of the immunosuppressive cytokine IL-10 by $CD4^+$ T-cells, leading again to suppressed IFN-I response as well as impaired survival and differentiation of B cells (14). In a recent report of 136 SARS-CoV-2 PCR-positive patients, low testosterone and high estradiol were associated with disease severity in COVID-19 patients. Furthermore, both male and female COVID-19 patients presented elevated estradiol levels which positively correlated with plasma IFN-γ levels (25). However, it should be noted that men with acute or subacute illness are known to develop a transient functional secondary hypogonadism. Therefore, testosterone assessment at hospital admission may not reflect the real androgen status.

Therefore, the androgen and estrogen status can significantly affect immune response to viral infection. While estrogen promote the "anti-viral state" induced by IFN-I (innate immunity) and the development of anti-SARS-CoV-2 specific responses (adaptive immunity), androgen may delay the mounting of prompt and effective anti-viral response.

SEX HORMONES AND IMMUNOMETABOLISM IN COVID-19

Excess adiposity may provide and sustain a proinflammatory *milieu* that promotes an imbalanced immune response towards hyperinflammation. This may trigger a cytokine storm, leading to impaired T-cell anti-viral specific activity and exacerbate disease severity. Sex steroids have a clear role in shaping fat distribution. Males accumulate more visceral fat than females and age-related decline in sex steroids in humans is linked to greater fat accumulation in central regions. For example, downregulation of estrogen signalling through ER-alpha knockout lead to obesity in both male and female mice (26). Serum estrogen decline after menopause is associated with abdominal fat accumulation, while hormone replacement therapy reduces visceral fat (27), implying a key role of estrogen in regulating fat mass. Similarly, hypogonadism in men is associated with visceral adiposity while increasing testosterone concentration in men induces a reduction in total fat mass (28). However, while estrogens show beneficial effects on body fat regulation also in males, androgens have opposite effects in females. Women with polycystic ovary syndrome exhibit hyperandrogenism concomitant with visceral fat accumulation (29). Moreover, treatment with anabolic steroid having androgenic activity was associated with increased visceral fat accumulation (30). This is consistent with experimental evidence showing that treatment of female mice with testosterone results in greater body weight and fat mass that are sustained throughout adult life (31).

Female type fat distribution is associated with lower systemic inflammation, lower risk of developing cardiometabolic diseases and less severe COVID-19. We have recently shown that abdominal fat distribution characterized by increased visceral (VAT) and lower subcutaneous adipose tissue (SAT) is strongly associated with COVID-19 severity. SAT was higher in females than males, and inversely associated with the need of intensive treatment. Furthermore, each millimetre increase in VAT thickness increased risk of admission to intensive care unit by 16%, independently of body mass index (32). VAT has important immunological functions strongly contributing to the production of proinflammatory molecules such as IL-1β, IL-6, and TNF-α. One-third of the circulating IL-6 is produced by adipocytes and adipose tissue matrix (33). Low adiponectin and leptin resistance states associated with obesity display immune characteristics that partially resemble those seen in COVID-19 (34). In subjects with obesity, T-cell subpopulations (CD3+, CD4+, CD45RO+, CD8+) and their proliferative response to polyclonal mitogens are suppressed (35). These abnormalities are reversed with energy restriction (which decreases leptin) (36). In subjects with obesity, increased leptin levels correlate with circulating TNF-α, which displays a suppressive effect on lymphocytes count (35). This is in line with evidence suggesting that COVID-19 patients have a four-fold increase in leptin levels compared to non-infected controls (37). Adipokine levels are also under the control of sex steroids. Estradiol levels are directly associated with serum leptin while male steroids

decrease leptin gene expression and secretion from human adipocytes (38). Conversely, low adiponectin levels in men *vs*. women appear to be predominantly mediated by male sex steroid hormones (39).

On the other hand, SARS-CoV-2 infection might enhance VAT inflammation. Mesenteric VAT, which surrounds the small intestine, is the first line of defence against pathogens translocated from the intestine to the circulation (40). Over 50% of COVID-19 patients test positive for SARS-CoV-2 RNA in stool, and 10% have gastrointestinal symptoms consistent with a SARS-CoV-2 cytopathic effect on enterocytes (41). According to single-cell RNA-sequencing data, the enterocyte is one of the main cells co-expressing high levels of the SARS-CoV-2 entry factors ACE2 and TMPRSS2 (42), suggesting that the gut may act as potential entry site of SARS-CoV-2. Virus recognition by the gut immune system may trigger an immunoinflammatory response spreading to mesenteric VAT and exacerbating local inflammation.

Therefore, the interaction between sex steroids, immune response and immuno-metabolic factors may generate an immunoendocrine environment that sustains infection and promotes COVID-19 progression at multiple levels.

SEX HORMONES AND SEX INFLUENCE VACCINE RESPONSES

COVID-19 vaccination campaign has started with a total of 6.5 billion vaccine doses that have been administered (https://covid19.who.int/info) and nearly 48% of the world population has received at least one dose of a COVID-19 vaccine (43). First reports on COVID-19 vaccine unfortunately were not powered to provide evidence of safety and efficacy by sex (44) although the point estimates of efficacy for subgroups was also high, consistent with that observed in the overall study population (45). However, evidence on other vaccines have shown differences in response or efficacy according to gender. For instance, the antibody response to seasonal influenza vaccines has been shown to be at least twice as high in females compared to males (18). A more robust protective antibody response that can facilitate vaccine efficacy in women was also observed after vaccination against influenza, hepatitis A and B, rubella, measles, mumps, herpes simplex and dengue viruses (46). This greater response may also explain why women experienced more frequent and severe adverse effects (18) as reported in the first month of the COVID-19 vaccine rollout (https://www.cdc.gov/mmwr). According to EUDRAVigilance report the suspected adverse drugs reaction of COVID-19 vaccines ranged from 59.0% to 72.0% in women and from 26.1% to 39.1% in men (https://www.adrreports.eu/en/). Mechanisms of these discrepancies may be related to differences in both innate and adaptive immunity as women have usually greater T cells activation, proliferation and cytotoxic activity as well as higher immunoglobulin basal levels and B cells number compared to men (47, 48). Moreover, studies in mice have demonstrated that while estrogen promote the development of antibodies testosterone may suppress it (47, 48). Indeed, a lower antibody response was observed to influenza vaccination in men compare to women, particularly in those with higher levels of testosterone at the time of vaccination (49). Despite this, only few studies have so far considered sex as a possible element that may affect COVID-19 vaccine response. Latest reports indicated a higher and similar efficacy in the vaccine arm compared to placebo for both men and women (50, 51). However, a recent meta-analysis including sex-disaggregated data from BNT162b2-BioNTech/Pfizer, mRNA-1273-Moderna, Ad26.COV2.S-Johnson&Johnson/Janssen showed a significantly increased efficacy in men compared to women. Males resulted to have a 33% reduced risk of developing COVID-19 compared to females (52). Data from a report on 248 healthcare workers undergoing the BNT162b2 vaccine showed a tendency for greater antibody response in females compared to males seven days after the second dose, although this difference was not significant (p=0.055) (53).

Current results are still controversial indicating that the efficacy of COVID-19 vaccines has not been adequately addressed in terms of sex and that the influence of sex and sex hormones is still poorly understood. Larger longitudinal studies are needed to clarify whether sex and sex steroids significantly affect the development of effective SARS-CoV-2 vaccine response.

SEX HORMONES INFLUENCE INFLAMMATION AND SUSCEPTIBILITY TO TISSUE INJURY

Although there is no direct evidence available from studies carried out in SARS-CoV-2 infected subjects, literature data support the concept that sex steroids may influence susceptibility or protection to tissue injury of organs targeted by COVID-19 complications. The major morbidity and fatality from COVID-19 is due to acute viral pneumonitis that evolves to acute respiratory distress syndrome (ARDS) (54). This is characterised by hyaline membrane changes, microvessel thrombosis with exudative and proliferative phases of diffuse alveolar damage (55), sometimes superimposed by bacterial pneumonia. In a LPS-induced model of acute lung injury, male mice developed increased airway hyperresponsiveness and inflammation compared with their female counterparts (56). Treatment with testosterone enhanced inflammatory responses in females to a level that was similar to that showed in males. In contrast, gonadectomy reduced airway inflammation in males but not females suggesting that androgens sustain the proinflammatory action of LPS-induced lung insult (56). Ovariectomized females showed an increment in the neutrophil content in bronchoalveolar lavage fluids, myeloperoxidase activity in whole lung, and leak of albumin into the lung compared with intact females (57). However, estrogen replacement was found to be effective in reducing all these lung injury features by suppressing cell adhesion molecules and proinflammatory cytokines. In the carrageenan-induced pleurisy model, which represents a well-known murine model

of inflammation, tissue damage was exacerbated by ER blockage (58). Several mechanisms may help to explain the protective effect of estrogen against acute lung injury and resolution of inflammation, including regulation of apoptosis (59) and nitric oxide production.

COVID-19 can also lead to a number of extrapulmonary manifestations (60). Among those, cardiovascular complications (myocardial dysfunction and arrythmias, acute coronary syndromes, and thrombotic complications) occur in over a third of hospitalised COVID-19 patients and are associated with a significant mortality risk (61). Direct cytopathic myocardial injury, systemic inflammation, virus-mediated endothelial damage, and hypoxia are some of the potential factors involved in these complications. Estrogen offers a vascular protective effect that may partially explain the gender discrepancy in COVID-19 deaths (62). Acute administration of estrogen in male rabbit have been shown to be protective against ischaemia, reducing infarct size by 20% (63, 64). Direct membrane signalling mediated by estrogen lead to vasodilation through nitric oxide release. Similarly, ER-alpha signalling mediated preservation of endothelial cell structure and function by preventing apoptotic pathway activation (65). Estrogen cardioprotective properties suggest that estrogen status may reduce susceptibility to cardiac injury, endotheliitis and subsequent cardiovascular complications associated with COVID-19 (60). However, direct evidence from COVID-19 studies is needed.

WHAT IS THE CLINICAL EVIDENCE FOR ESTROGEN AND ANTI-ANDROGENIC THERAPIES IN COVID-19?

Evidence that pharmacological modulation of estrogen and/or androgen signalling can prevent SARS-CoV-2 infection or disease severity is limited to a few observational studies. A retrospective study involving over 68,000 cases has studied the effect of exogenous estradiol administration on COVID-19 deaths. The authors found that death risk in women over 50 years of age receiving estradiol treatment was significantly reduced compared to those who were untreated (hazard ratio 0.29, 95% CI 0.11 to 0.76) (66). Montopoli

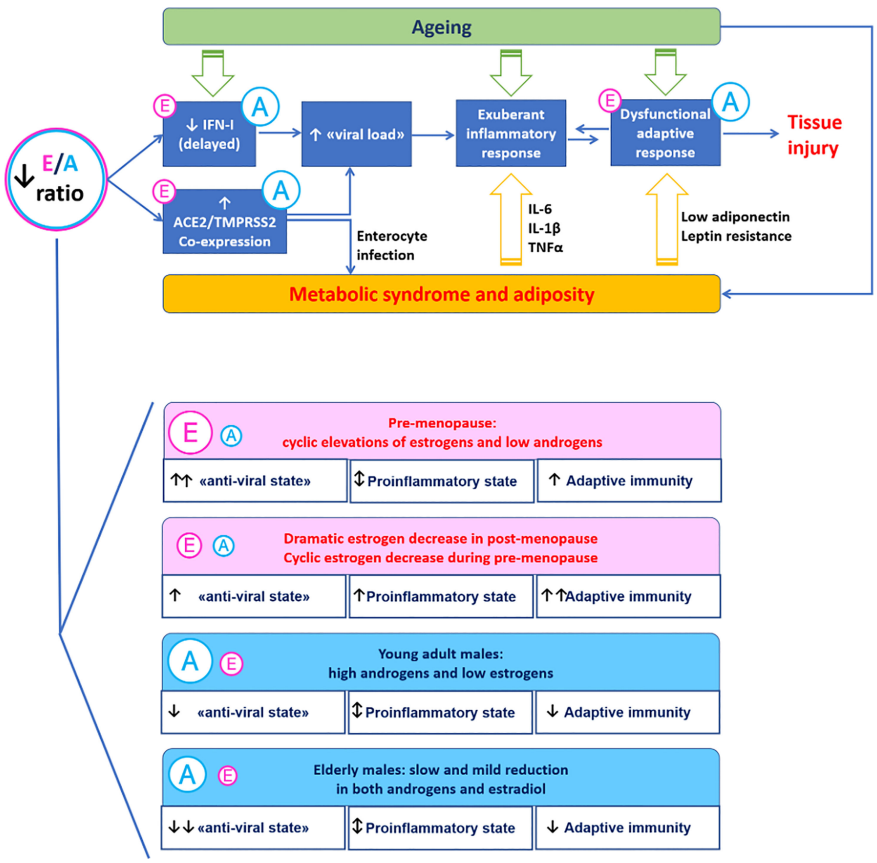

FIGURE 1 | A mechanistic model for the immunoendocrinology of COVID-19. Differences in estrogen to androgen balance due to ageing and gender may modulate SARS-CoV-2 entry factors ACE2 and TMPRSS2 involved in virus-host interaction. Estrogen promote the "anti-viral state" induced by interferon type I (innate immunity) and the development of anti-SARS-CoV-2 specific responses (adaptive immunity). The proinflammatory *milieu* associated with excess visceral adiposity promotes SARS-CoV-2 infection and may be directly involved in the infection trough the enterocyte-adipose tissue axis.

et al. (14) observed that men treated with androgen depravation therapy for prostate cancer were protected against SARS-CoV-2 infection. Prostate cancer patients receiving androgen deprivation therapy had a significantly lower risk of SARS-CoV-2 infection compared with patients who did not receive it (4/5273 *vs.* 114/37161; odds ratio 4.05; 95% CI 1.55-10.59). In another observational study on 100 patients with androgenic alopecia and laboratory confirmed SARS-CoV-2 infection, treatment with dulasteride, which prevent testosterone conversion to dihydrotestosterone by inhibiting the 5-*alpha* reductase, was associated with a reduction in the frequency of clinical symptoms (67). In a double-blinded, randomized, prospective, investigational phase III study clinical trial involving 262 non hospitalized COVID-19 male patients (NCT04446429), the non-steroidal antiandrogen proxalutamide resulted in a reduction rate of hospitalization. Although such evidence provide support to the hypothesis that estrogen and androgen status are key players in COVID-19 pathogenesis and potential therapeutic targets, clinical evidence is limited by the small sample size and/or the observational nature of the findings. Seventeen clinical trials are registered on clinicaltrials.gov using as investigational product estrogen receptors agonists/modulators or anti-androgenic treatments in COVID-19 patients. Clinical trials are needed to define the role of such treatments for preventing COVID-19 severity and complications.

CONCLUSIONS

The interaction of endocrine factors linked to gender provides a mechanism to explain at least in part the greater severity of COVID-19 in males compared to females. Androgen to estrogen balance may modulate virus-host interaction and immune response as estrogen enhance anti-viral defences and immune activity while androgen displays immunosuppressive action (**Figure 1**).

This leads not only to a greater immunity to virus infection observed in women compared to men but also may highlights a different response to vaccines between genders. Therefore, sex hormones status and other gender-related factors (biological and behavioural) may further modulate the risk of severe disease conferred by other risk factors such as ageing and cardiometabolic diseases. Whether sex steroids can provide a therapeutic option for COVID-19 is still unknown. Taken together, these data suggest that gender should be taken into account to optimize treatment outcomes for women and men towards gender-based personalized medicine.

AUTHOR CONTRIBUTIONS

RS and FT conceived the review and wrote the first draft of the manuscript. NN and SF participated in the conception of the review and revised it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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The Association Between COVID-19 and Thyroxine Levels: A Meta-Analysis

Yiru Chen[†], *Xiuneng Li*[†], *Yu Dai and Jingjing Zhang*[*]

National Clinical Research Center for Metabolic Diseases, Metabolic Syndrome Research Center, Key Laboratory of Diabetes Immunology, Ministry of Education, and Department of Metabolism and Endocrinology, The Second Xiangya Hospital of Central South University, Changsha, China

Objectives: Recently, a number of reports have described the potential relationship between COVID-19 and thyroid hormones, but the results were conflicting. We performed a meta-analysis to evaluate the effect of the severity of COVID-19 on thyroid-related hormones and the effect of thyroid-related hormones on the outcome of COVID-19 in order to try to confirm the association between the serum levels of free triiodothyronine (FT3), free thyroxine (FT4) and thyroid stimulating hormone (TSH) and the severity or mortality of coronavirus-19 patients.

Methods: The methodology was already registered in the International Prospective Register of Systematic Reviews (PROSPERO) database, and the protocol number is CRD42021269246. Systematic searches were carried out on the Cochrane Library, Embase, PubMed and Web of Science databases on November 15, 2021. We set up the literature search strategy based on the following keywords: [(T3 OR FT3 OR triiodothyronine) or (T4 OR FT4 OR thyroxine) or (TSH or thyrotropin)] and (COVID-19 OR SARS-CoV-2), without time restrictions.

Results: Twenty studies satisfied the inclusion/exclusion criteria and were included in the meta-analysis. A total of 3609 patients were enrolled in the study. From the analysis of the included studies, the incidence of thyroid-related hormone abnormalities was higher in patients with severe COVID-19, and the serum levels of FT3 and TSH were lower than those of patients with nonsevere COVID-19. However, the difference in the FT4 levels was not significant. Similar characteristics were shown between survivors and nonsurvivors. In addition, the outcomes of the meta-analysis showed that patients with abnormal thyroid-related hormones had greater mortality.

Conclusions: Low FT3 serum levels, low FT4 serum levels and low TSH serum levels may increase the mortality of COVID-19 patients during admission. On the other hand, the higher the severity level of COVID-19, the higher the probability of decreases in the FT3, FT4, TSH levels.

Keywords: FT3, FT4, TSH, COVID-19, mortality

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Eleonore Fröhlich,
Medical University of Graz, Austria
Giusy Elia,
University of Pisa, Italy

***Correspondence:**
Jingjing Zhang
Doctorzhangjj@csu.edu.cn

[†]*These authors have contributed equally to this work*

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1 INTRODUCTION

The outbreak of COVID-19 pneumonia has had a great impact on the global community, and it has challenged the capacity of health care systems in all countries. Existing studies have revealed that SARS-COV-2 could influence the glucose and lipid levels and the blood pressure through metabolic and endocrine pathways (1–3) in which angiotensin-converting enzyme 2 (ACE2) plays a key role. ACE2 was originally found to be a functional receptor for the SARS coronavirus in 2003 (4), and it is highly expressed in the thyroid gland in humans (5), which is one of the potential mechanisms by which COVID-19 leads to thyroid dysfunction.

The hormones involved in the hypothalamus-pituitary-thyroid axis include thyrotropin-releasing hormone (TRH), thyroid stimulating hormone (TSH), free triiodothyronine (FT3) and free thyroxine (FT4). Currently, the relationship between thyroid gland function and COVID-19 remains unclear. In 2020, in Runmei Zou's study, it was found that 27.52% of patients with COVID-19 had thyroid disease (6). Moreover, in a study based on severe cases in the UK, the proportion of thyroid follicular epithelial cells that were damaged was 22.2% (7). It is currently believed that COVID-19 has a direct effect on thyroid function and the thyroid hormone levels through the hypothalamus-pituitary-thyroid axis and can also affect the thyroid gland by autoimmune diseases through cytokines (8). It has been found that the TSH levels are negatively correlated with the mortality of COVID-19 in patients with normal FT3 and FT4 levels (9). The TSH and FT4 levels were low in confirmed COVID-19-positive patients during admission, and they returned to normal levels when the patient recovered (10). The meta-analysis of M. Llamas has shown that the level of FT3 is closely related to the severity of COVID-19 (11). The correlation between the thyroid hormone level and mortality, severity and prognosis of patients with COVID-19 still need to be systematically described. This meta-analysis focuses on these problems and aims to guide the clinical classification and treatment of patients with COVID-19, and the effect of the thyroid hormone levels in COVID-19 patients with previously normal thyroid function was assessed.

2 MATERIALS AND METHODS

2.1 Protocols and Registration

Our material and methods were based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (12), which have already been registered in the International Prospective Register of Systematic Reviews (PROSPERO) database, and the protocol number is CRD42021269246.

2.2 Eligibility Criteria

All original peer-reviewed research publications were taken into consideration. We developed the following criteria to select eligible studies: (I) population:patients with COVID-19; (II) intervention: no; (III) comparator/control: ICU patients and non ICU patients were compared with each other; (IV) outcomes: studies in which the serum levels FT3, FT4 or TSH of the different groups (severe *vs*. nonsevere or survivor *vs*. nonsurvivor) in the form of mean ± SD or median with interquartile range (IQR) were available, the in-hospital mortality data of COVID-19 patients with low FT3 or FT4 or TSH serum levels were available, or the occurrence rate data of low FT3, FT4 and TSH levels in severe and patients with nonsevere COVID-19 were available or in survivors and nonsurvivors could be obtained; (V) study design: clinical studies.

Patients in the study who were hospitalized with a confirmed COVID-19 diagnosis were defined as having certain COVID-19 severities in accordance with the Clinical Guidelines for the Diagnosis and Treatment of COVID-19 in China (6th Edition). Studies involving patients with a history of thyroid disease or patients receiving or receiving treatment with a potential impact on thyroid function were excluded. An NTIS state was defined as patients with FT3<3.3 pmol/L, whose FT4 level was low or normal and with TSH levels of 0.35-4.8 mIU/L.

2.3 Information Sources and Search Strategy

We systematically searched the Cochrane Library, Embase, PubMed and Web of Science databases in November 2021 without time restrictions during the search for all published articles related to both thyroid-related hormones and COVID-19. The literature search strategy was based on the following keywords: [(T3 OR FT3 OR triiodothyronine) or (T4 OR FT4 OR thyroxine) or (TSH or thyrotropin)] and (COVID-19 OR SARS-CoV-2 OR 2019 novel coronavirus). In addition, to identify articles other than those found in the electronic databases, a further manual search of studies meeting our inclusion criteria was also performed. Two independent reviewers (YC and XL) performed the first step of the title/abstract screening and the second step of full-text assessment in the search process, and any disagreement that arose during this process was discussed until an agreement was reached.

2.4 Study Selection

After obtaining the list of all relevant articles, we removed duplicate articles and nonclinical research and also excluded a series of studies with poor correlations. Two reviewers (YC and XL) independently selected eligible studies for inclusion by reading the titles and abstracts. Disagreements were resolved by reaching a consensus or with the help of a third reviewer (YD).

2.5 Data Extraction

According to the inclusion/exclusion criteria, the full texts of all potentially qualified studies were independently reviewed by two reviewers (YC) and (XL). Disagreements were addressed through discussion. If a consensus could not be reached, a third reviewer (YD) resolved the disagreements. Information including the author, country, type of study, sample size, mean or median age, sex ratio, population and NOS scores was extracted from the selected studies. All of the extracted data were tabulated.

2.6 Quality Assessment of Studies

All the articles included in this meta-analysis were evaluated by the Newcastle Ottawa Scale (NOS) (13). We scored all 20 studies from the perspectives of the study type, inclusion criteria of COVID-19 patients, sample size, follow-up time, index detection, and comparability between the experimental group and the control group. Articles with a score of 6 or more were defined as high-quality articles. All of the authors reached a consensus on the disagreement on the quality of the studies through discussion and consultation.

2.7 Statistical Analysis

All data of this meta-analysis were analyzed in RevMan (version 5.4) and Stata (version 15.1). For all included studies, the risk ratio (RR) and 95% confidence interval (Cl) were used to measure the correlation between the TSH, FT3, and FT4 levels

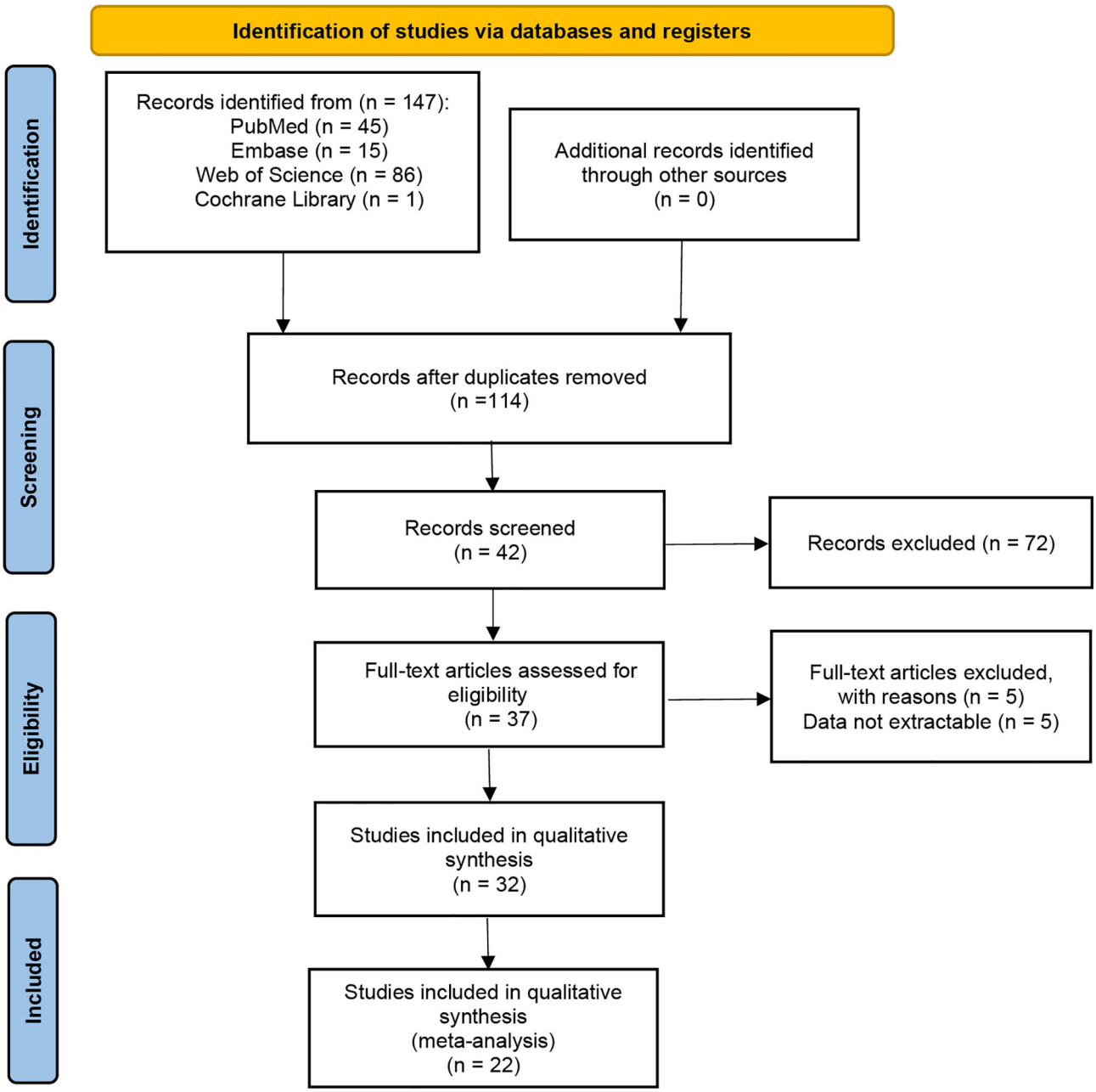

FIGURE 1 | Flow diagram of the study selection process.

and the mortality and severity of COVID-19 patients. The I^2 value and P value were used to evaluate heterogeneity. If the I^2 was less than 50% and if the P value was less than 0.05, there was no heterogeneity in the included studies. In addition, sensitivity analyses were used to ensure that the method we adopted was scientific (**Supplementary Figures 1–10**).

3 RESULTS

3.1 Search Results and Studies Characteristics

147 studies were identified in the literature search, and twenty retrospective studies satisfying our inclusion/exclusion criteria were involved in the meta-analysis, as shown in **Figure 1**. The involved studies had a total of 3609 patients. Sufficient details of the involved studies are shown in **Table 1**.

3.2 Quality Assessment

The quality of the included studies was assessed by the Newcastle-Ottawa Scale (NOS). The NOS scale is mainly composed of the sample selection, exposure and comparability between the experimental group and the control group in the studies. The scores of all the extracted studies scored with the NOS scale were higher than 6, indicating the high quality of the included studies. The risk of bias is described in **Table 2**.

3.3 Meta-Analysis

A total of 20 articles were included in this meta-analysis. The basic characteristics and the scores of these studies assessed with the NOS scale are described in **Table 1**. A total of 3609 COVID-19 hospitalized patients were included in our study, and the patients ages ranged from 15 to 92 years old (14). Among the 20 studies in this meta-analysis, eight studies were conducted in China, three in Italy, two in the United Kingdom and the other seven in Greece, Turkey, India, Brazil, Korea, Spain and Israel. Three of them were published in 2020 and 17 in 2021. Among the 20 studies, there were 2083 male patients, accounting for 57.72% of the included patients. All sensitivity analyses are available in the **Supplementary Materials** (**Supplementary Figures 1–10**).

3.4 Primary Outcomes

3.4.1 Thyroid-Related Hormones Levels and Survival Status of COVID-19 Patients

High heterogeneity existed in both the analysis of the association between FT3 and the survival state of COVID-19 patients (I^2 = 91%, P < 0.01) and the analysis of the association between TSH and the survival state of COVID-19 patients (I^2 = 88%, P < 0.01) (**Figures 2A, C**), indicating that the included articles in these analyses failed to provide reliable information for further analysis. However, the overall data showed that survivors had a higher FT4 level than nonsurvivors (SMD = - 0.37 95% CI,

TABLE 1 | Description of eligible studies reporting the association between thyroid-related hormones and COVID-19.

NO.	Author	Country	Type of Study	Sample Size	Age	Male	Population
1	Andrea Lania (14)	Italy	retrospective study	287	66 (27–92)	193	patients with COVID-19
2	Baldelli et al. (15)	Italy	retrospective study	66	60.8 ± 17.0 58.4 ± 12.1	34	with COVID-19 pneumonia/ICU patients
3	Campi et al. (16)	Italy	retrospective study	144	68.1 ± 14.67	97	patients with COVID-19
4	Chen et al. (17)	China	retrospective study	50	48.4 ± 13.7	33	patients with COVID-19
5	Gao et al. (18)	China	retrospective study	100	61.4 ± 15.2 63.2 ± 13.4	52	patients with non-severe COVID-19 severe or critically ill patients with COVID-19
6	Gong et al. (9)	China	retrospective study	150	69.5 (IQR:61-79)	81	patients with COVID-19
7	Güven and Gültekin (19)	Turkey	prospective study	250	68 (IQR:54- 78)	157	patients with COVID-19 in non-ICU and patients with COVID-19 in ICU.
8	Khoo et al. (10)	UK	cohort observational study	456	66.1± 16.0 63.8± 19.3	271	patients with COVID-19 and patients without COVID-19
9	Lui et al. (20)	Hong Kong, China	cohort study	191	53.5 ± 17.2	99	patients with COVID-19
10	Lui et al. (21)	Hong Kong, China	prospective study	367	54(IQR:38-65)	172	patients with COVID-19
11	Schwarz et al. (22)	Israel	retrospective study	54	Unknown	37	patients with COVID-19
12	Lang et al. (23)	China	retrospective study	127	66 (53–71)	62	patients with COVID-19
13	Vassiliadi et al. (24)	Greece	cohort observational study	196	59.3 ± 18.3	130	patients with COVID-19 and patients without COVID-19
14	Zou et al. (6)	China	retrospective study	149	47 (36, 61.5)	71	patients with COVID-19
15	Chen et al. (25)	China	retrospective study	274	62.0(44.0-70.0)	171	patients with COVID-19
16	Dutta et al. (26)	India	retrospective study	236	54(15-91)	159	patients with COVID-19
17	Beltrão et al. (27)	Brazil	retrospective study	245	62(49-74.5)	100	patients with COVID-19
18	Ahn et al. (28)	Korea	retrospective study	119	64.3 ± 16.8	62	patients with COVID-19
19	Ballesteros Vizoso et al. (29)	Spain	retrospective study	78	59 ± 12 68 ± 12	55	patients with COVID-19
20	Clarke et al. (30)	UK	prospective study	70	55.9 ± 13	47	patients with COVID-19

TABLE 2 | Quality scores of included studies using newcastle-ottawa scale.

NO.	First author	Year	Selection	Comparability	Outcome	NOS
1	Andrea Lania (14)	2020	**	*	***	6*
2	Baldelli, R (15)	2021	****	**	***	9*
3	Campi, I (16)	2021	***	**	**	7*
4	Chen, M (17)	2021	****	**	***	9*
5	Gao, W (18)	2021	****	**	***	9*
6	Gong, J (9)	2021	****	**	***	9*
7	Güven, M (19)	2021	***	*	***	7*
8	Khoo, B (10)	2021	***	**	***	8*
9	Lui, David Tak Wai (1) (20)	2021	****	**	***	9*
10	Lui, David Tak Wai (2) (21)	2021	***	**	***	8*
11	Schwarz,Y (22)	2021	****	**	***	9*
12	Shan Lang (23)	2021	****	**	***	9*
13	Vassiliadi, Dimitra (24)	2021	***	**	***	8*
14	Runmei Zou (6)	2020	****	**	***	9*
15	Tao Chen (25)	2020	***	**	***	8*
16	Aditya Dutta (26)	2021	****	**	***	9*
17	Beltrão FEL (27)	2021	****	**	***	9*
18	Jiyeon Ahn (28)	2021	***	**	***	8*
19	Ballesteros Vizoso MA (29)	2021	****	**	***	9*
20	Clarke SA (30)	2021	****	**	***	9*

Selection:
** :Meet the one item in the NOS selection section.*
*** :Meet the two items in the NOS selection section.*
**** : Meet the three items in the NOS selection section.*
***** :Meet the four items in the NOS selection section.*
Comparability:
** :The comparability of the study cohort design was of medium quality.*
*** :The comparability of the study cohort design was of high quality.*
Outcome:
** :Meet the one item in the NOS outcome section.*
*** :Meet the two items in the NOS outcome section.*
**** : Meet the three items in the NOS outcome section.*

-0.50 - -0.24), which is shown in **Figure 2B**. Sensitivity analyses were also performed to explore the potential sources of heterogeneity (**Supplementary Figures 1**–**3**).

3.4.2 Thyroid-Related Hormones Levels and Severity of COVID-19

As shown in **Figure 3**, high heterogeneity existed in both the analysis of the association between FT3 and the severity of COVID-19 patients (I^2 = 88%, P < 0.01) and the analysis of the association between TSH and the severity of COVID-19 patients (I^2 = 93%, P < 0.01) (**Figures 3A, C**), indicating that the included articles in these analyses failed to provide reliable information for further analysis. The heterogeneity of the correlation analysis between FT4 and the severity of COVID-19 patients cannot also be ignored (I^2 = 53%) (**Figure 3B**). Sensitivity analyses were also performed to explore the potential sources of heterogeneity (**Supplementary Figures 6**–**8**).

3.4.3 Probability of Low Thyroid-Related Hormones Levels and Severity of COVID-19 Patients

The results of this meta-analysis of the probability of low thyroid-related hormone levels and the severity of COVID-19 patients are shown in **Figure 4**. In general, the probability of low FT3 (RR = 3.75 95% CI, 2.09-6.73), FT4 (RR = 1.53 95% CI, 0.64-3.64) and TSH (RR = 3.54 95% CI, 2.06-6.07) levels was associated with a more severe COVID-19 disease. The statistical results showed that there was no significant heterogeneity among the studies. The sensitivity analysis also indicated the stability of our results (**Supplementary Figures 9**, **10**).

3.4.4 Mortality in COVID-19 Patients With Non-Thyroidal Illness Syndrome (NTIS) and Without NTIS

The heterogeneity test results of the three studies included in this study were calculated as X^2 = 1.40, df = 2, I^2 = 0% and P = 0.30 in the Q-test, and the test showed no significant heterogeneity among the three records. The risk ratio of the three records was 11.64, 95% CI (4.88, 27.78), and the results were distinct (Z = 5.53, P < 0.01), revealing that the mortality of COVID patients with NTIS was higher than that of non-NTIS patients (**Figure 5**).

3.4.5 Mortality in COVID-19 Patients With Low TSH and Normal TSH

The heterogeneity test results of the three studies included in this study were calculated as X^2 = 0.48, df = 2, I^2 = 0% and P =0.79 in the Q-test, and the results showed that there was no significant heterogeneity among the three records. The risk ratio of the three records was 1.96, 95% CI (1.47, 2.61), and the results were distinct (Z = 4.63, P < 0.01), revealing that the mortality of

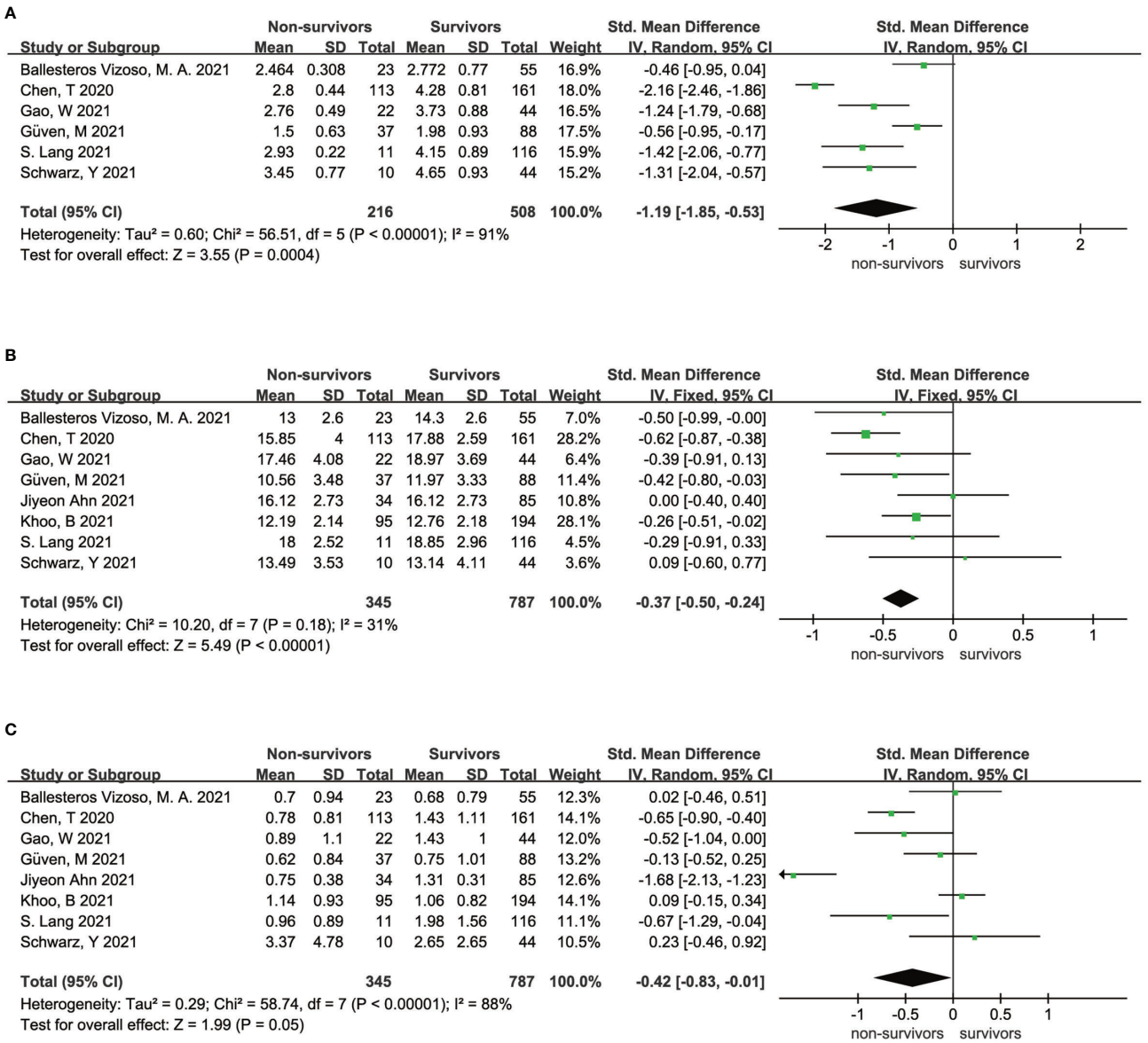

FIGURE 2 | Forest plot comparing the FT3 serum levels **(A)**, FT4 serum levels **(B)**, and TSH serum levels **(C)** between the survivors and nonsurvivors.

COVID patients with low TSH levels was higher than that of patients with normal TSH levels (**Figure 6**).

4 DISCUSSION

There is little literature in the world on the relationship between thyroxine and the COVID-19 virus thus far. Existing studies have revealed complex interactions between the thyroid gland and viruses through hormones and signaling molecules (31). However, the effect of the COVID-19 virus on the level of thyroid hormone and the mechanisms involved remain unclear.

Our meta-analysis mainly indicates that low FT4 levels may be associated with adverse outcomes (**Figure 2B**) and severe COVID-19 (**Figure 3B**). Low FT3 serum levels may also increase the degree of severity of COVID-19 (**Figure 3A**). Correspondingly, we found that NTIS (**Figure 5**) or low TSH (**Figure 6**) serum levels might also increase the mortality of COVID-19 patients and that patients with severe COVID-19 had a higher probability of low thyroid-related hormone levels (**Figure 4**). The data above may be due to the "cytokine storm" induced by SARS-COV-2 infection, which leads to the development of autoimmune thyroiditis (32) and thus supports the role of FT3 and FT4 as prognostic biomarkers in COVID-19

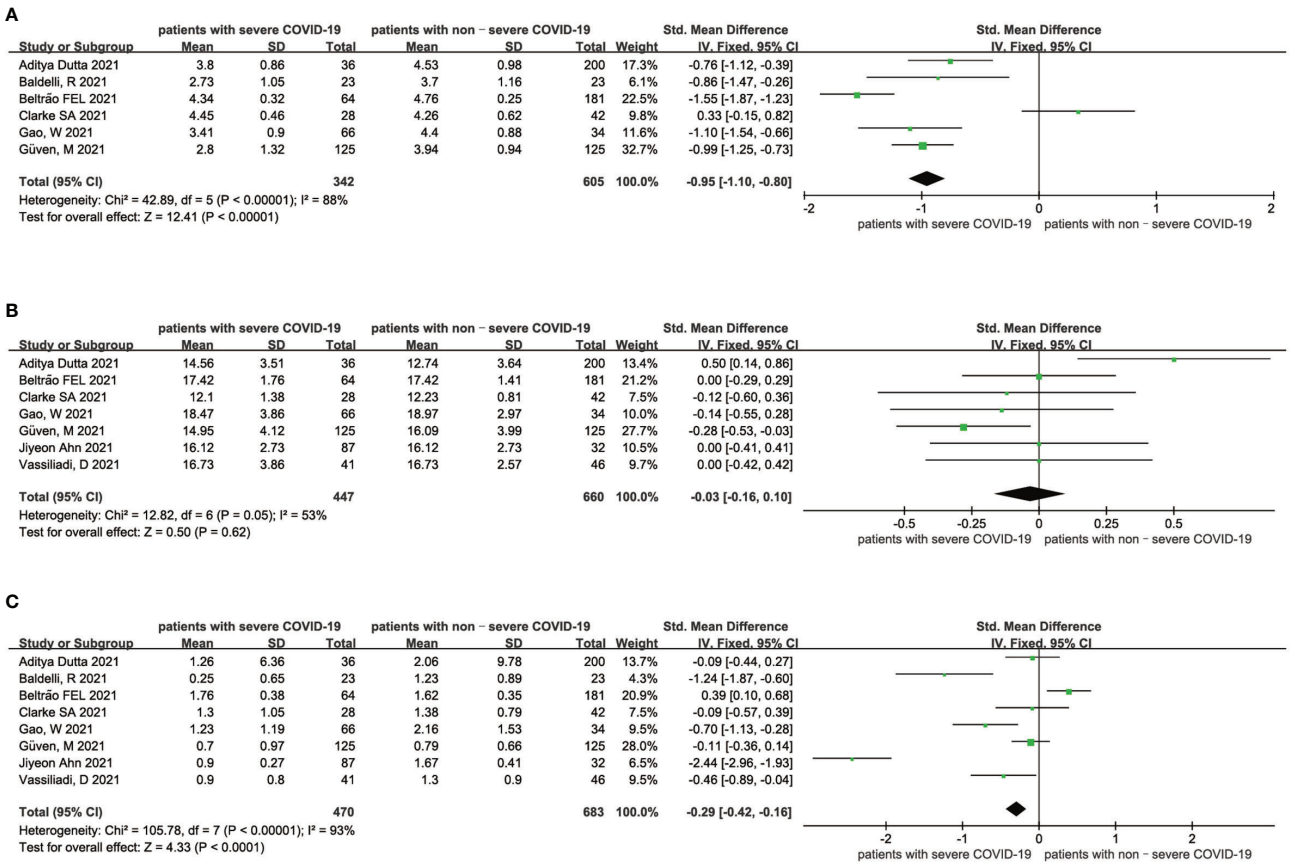

FIGURE 3 | Forest plot comparing the FT3 serum levels **(A)**, FT4 serum levels **(B)**, and TSH serum levels **(C)** between the severe patients and nonsevere patients with COVID-19.

patients. Also, the severity of SARS-COV-2 might be the dominant determinant of thyroid dysfunction (33).

The number of patients included in this study is limited, as few relevant studies in the world could be obtained, which leads to insufficient evidence currently supporting the interaction between thyroid-related hormones and the COVID-19 virus. Changes in the iodothyronine deiodinase levels, TSH secretion, the binding of thyroid hormone to plasma proteins, the transport of thyroid hormones in the peripheral tissues, and changes in the thyroid hormone receptor activity are all likely to contribute to the changes in serum levels of thyroid-related hormones in COVID-19 patients, but this needs further investigation. The severity of COVID-19 begins with the binding of the spike protein, which is on the surface of the virus, to the ACE2 receptor on the surface of the tissue cell (34). ACE2 is widely expressed in arteriovenous endothelial cells of many organs, especially in the thyroid gland (19). Studies have shown that the destruction of the thyroid gland (HPT axis) by SARS-COV-2 involves thyroid disease and the changes in related hormones (33). There are two possible mechanisms to explain the changes in the hypothalamic-pituitary-thyroid (HPT) axis in COVID-19 patients (33). First, the abnormal systemic inflammatory-immune responses caused by SARS-COV-2 (severe acute respiratory syndrome coronavirus 2) infection causes an indirect effect on the HPT axis. The presence of SARS-COV-2 RNA in the serum and plasma of COVID-19 patients, as well as the expression of ACE2 by the hypothalamus and pituitary gland, support this theory (5, 35). Second, the virus directly effects the thyroid gland. SARS-COV-2 attacks the lungs as well as other organs, including the thyroid gland (7, 36). Vojdani and coworkers also provide molecular evidence that SARS-COV-2 antibodies react with the thyroid gland (37). In addition, disruption of thyroid follicles and parafollicular cells was clearly observed in autopsies of patients who died of COVID-19 (38), which is a typical histopathological feature of thyroid injury. Molecular analysis of thyroid surgical specimens showed that thyroid follicular cells expressed ACE2 (39), suggesting that the thyroid gland is vulnerable to SARS-COV-2 damage once the patient is infected.

Abnormalities of thyroid function may represent an isolated change, but they can also be a precursor to autoimmune polyglandular syndrome or endocrine disorders (40). On the one hand, patients with severe COVID-19 do not have the typical characteristics of patients with subacute thyroiditis and instead have reduced white blood cell levels (41). However, SARS-COV-

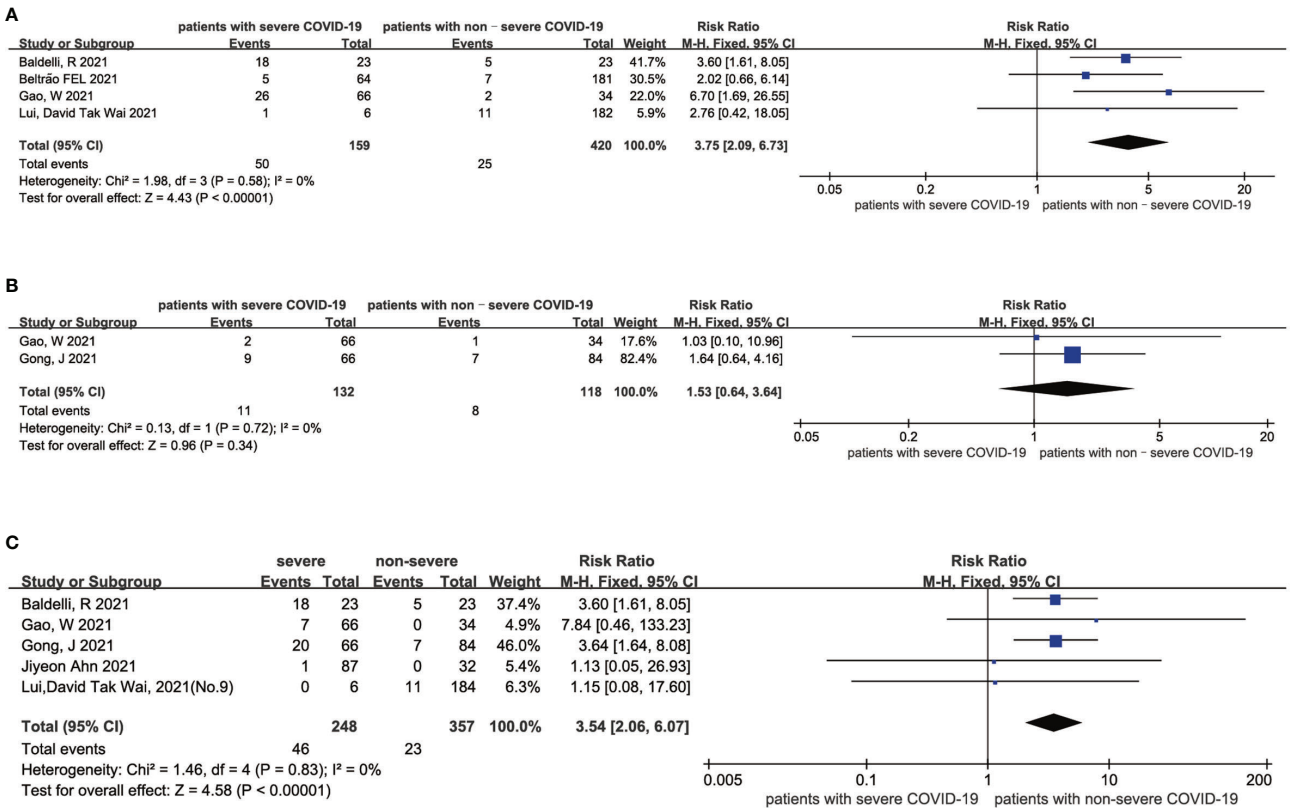

FIGURE 4 | Forest plot comparing the probability of low FT3 **(A)**, low FT4 **(B)**, and low TSH **(C)** between the severe patients and nonsevere patients with COVID-19.

2-induced thyroid dysfunction is consistent with syndromes of NTIS. Our meta-analysis suggests that such COVID-19 patients require intensive care and are at risk for thyrotoxicosis (**Figure 4**). On the other hand, studies have suggested that hyperthyroidism or hypothyroidism might increase the risk of developing a severe course of COVID-19 (42), as SARS-COV2 is able to enter the host cell by ACE-2. Therefore, an abnormal thyroid function would increase the burden of cardiovascular (43, 44) and psychiatric (45) comorbidities. Although the World Health Organization (WHO) did not recommend systematic thyroid function tests for hospitalized COVID-19 patients in March 2020 (12), the proportion of patients with severe COVID-19 who have abnormal serum-associated thyroxine levels is higher than patients with severe COVID-19 who did not have abnormal serum thyroxine levels according to the results of our study, and it is necessary to conduct thyroid function tests for COVID-19 patients, especially those admitted to the emergency room or intensive care unit (ICU), to avoid worsening outcomes (42). For severe or critically ill patients, low FT3 and TSH levels could be regarded as a type of adaptation to NTIS caused by major stress conditions such as systemic viral diseases, which could include SARS (13).

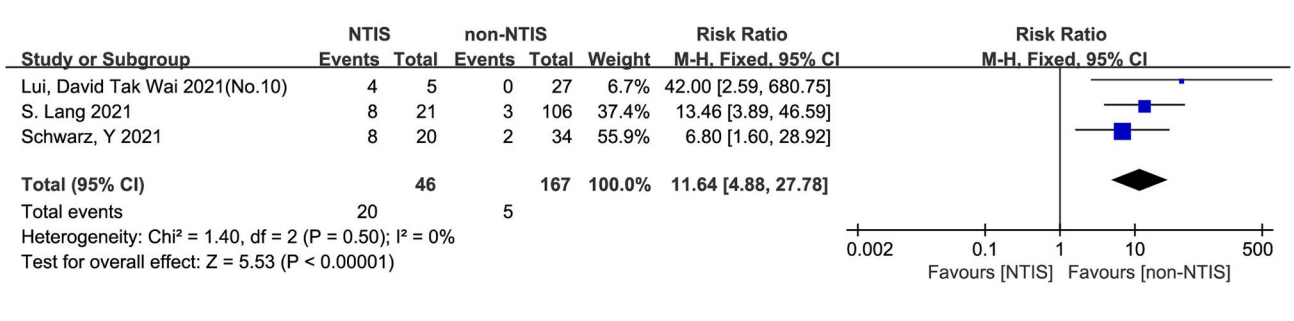

FIGURE 5 | Forest plot for all studies comparing the mortality in the NTIS and non-NTIS patients with COVID-19.

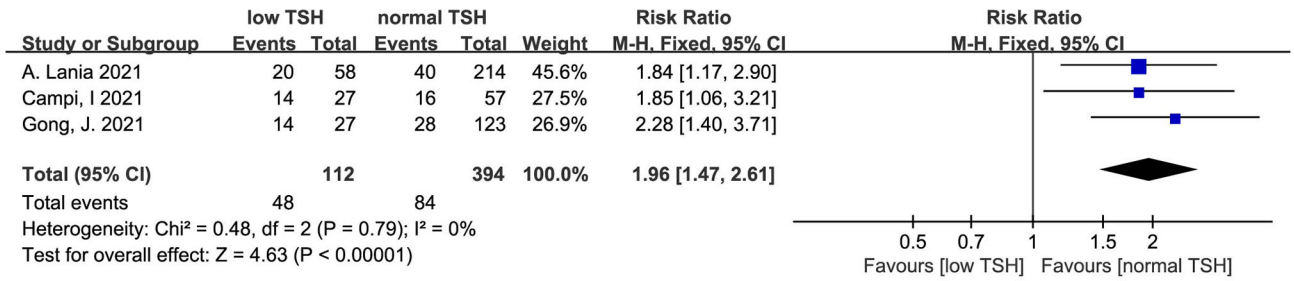

FIGURE 6 | Forest plot for all studies comparing the mortality in the COVID-19 patients with low TSH levels and normal TSH levels.

5 STRENGTH AND LIMITATIONS

A total of 20 studies were included in this meta-analysis, and these were mainly retrospective studies. We searched the PubMed, EMBASE, Web of Science, and Cochrane Library databases. A total of 3069 patients were included, including patients with nonsevere COVID-19 and patients with severe COVID-19, and the study included survivors and nonsurvivors. We summarized the relationship between TSH, FT3, FT4 and COVID-19 through scientific sorting and meta-analysis. To our knowledge, this is the first study to comprehensively summarize the relationship between levels thyroid-related hormones and COVID-19 by using a meta-analysis.

Our meta-analysis has some limitations, which must be carefully considered when interpreting the results. There were some heterogeneities among the studies included in this meta-analysis, and the sample size of the included studies was relatively insufficient after reasonable sorting. The current research regarding the relationship between thyroid-related hormones and COVID-19 is limited. Some of the included studies did not exclude the influence of other related factors (such as renal disorders) when examining thyroid function.

6 CONCLUSIONS

In summary, our study revealed that low serum levels of thyroid-related hormones may increase the mortality of COVID-19 patients during admission, and the higher the severity of COVID-19 is, the higher the probability of a decrease in the FT3, FT4, and TSH levels. Our findings could provide clinical guidance for thyroid function detection in patients with severe COVID-19. Further randomized controlled trials are needed to confirm these findings.

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

Conceptualization: YC. Methodology: YC and XL. Software: YC and XL. Validation: YC and XL. Formal analysis: YC and XL. Investigation: YC and XL. Data curation: YC, XL, and YD. Writing—review and editing: YC, XL, and YD. Visualization: JZ. Supervision: YD and JZ. Funding acquisition: JZ. 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/fendo.2021.779692/full#supplementary-material

Supplementary Figure 1 | | Sensitivity analysis for the FT3 serum levels between the survivors and nonsurvivors.

Supplementary Figure 2 | Sensitivity analysis for the FT4 serum levels between the survivors and nonsurvivors.

Supplementary Figure 3 | Sensitivity analysis for the TSH serum levels between the survivors and nonsurvivors.

Supplementary Figure 4 | Sensitivity analysis for the effect of NTIS on the mortality of COVID-19 patients.

Supplementary Figure 5 | Sensitivity analysis for the effect of low TSH serum levels on the mortality of COVID-19 patients.

Supplementary Figure 6 | Sensitivity analysis for the FT3 serum levels between the patients with severe and nonsevere COVID-19.

Supplementary Figure 7 | Sensitivity analysis for the FT4 serum levels between the patients with severe and nonsevere COVID-19.

Supplementary Figure 8 | Sensitivity analysis for the TSH serum levels between the patients with severe and nonsevere COVID-19.

Supplementary Figure 9 | Sensitivity analysis for the effect of severity on the probability of low FT3 in COVID-19 patients.

Supplementary Figure 10 | Sensitivity analysis for the effect of severity on the probability of low TSH in COVID-19 patients.

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ORIGINAL RESEARCH
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Patients With Autoimmune Thyroiditis Present Similar Immunological Response to COVID-19 BNT162b2 mRNA Vaccine With Healthy Subjects, While Vaccination May Affect Thyroid Function: A Clinical Study

Stavroula A. Paschou[1], Vangelis Karalis[2], Theodora Psaltopoulou[3], Vasiliki Vasileiou[4], Ioanna Charitaki[3], Tina Bagratuni[3], Vassiliki Ktena[4], Fotini Papandroulaki[4], Sentiljana Gumeni[5], Georgia N. Kassi[4], Ioannis P. Trougakos[5], Evangelos Terpos[3†] and Meletios A. Dimopoulos[3†]*

[1] Endocrine Unit and Diabetes Center, Department of Clinical Therapeutics, Alexandra Hospital, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece, [2] Faculty of Pharmacy, School of Health Sciences, National and Kapodistrian University of Athens, Athens, Greece, [3] Department of Clinical Therapeutics, Alexandra Hospital, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece, [4] Department of Endocrinology, Alexandra Hospital, Athens, Greece, [5] Department of Cell Biology and Biophysics, Faculty of Biology, National and Kapodistrian University of Athens, Athens, Greece

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Jeff M. P. Holly,
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Dilia Giuggioli,
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***Correspondence:**
Meletios A. Dimopoulos
mdimop@med.uoa.gr

[†]These authors share senior authorship

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Background: This is the first study, that aimed: a) to compare immune response, namely the kinetics of neutralizing antibodies (Nabs), after vaccination with BNT162b2 mRNA vaccine (Comirnaty, Pfizer/BioNTech) between patients with autoimmune thyroiditis and controls, and b) to investigate changes in thyroid function in healthy subjects with no history of thyroid dysfunction before and after vaccination with BNT162b2 mRNA vaccine (Comirnaty, Pfizer/BioNTech).

Methods: The entire study consisted of two sub-studies. In the first sub-study, NAbs levels after BNT162b2 mRNA vaccination were compared between 56 patients with autoimmune thyroiditis and 56 age and gender-matched healthy controls from the day of the first dose until a period of up to three months after the second dose. In the second sub-study, thyroid hormones (T3, T4, TSH) and thyroid auto-antibodies levels (anti-TG, anti-TPO) of 72 healthy subjects with no history of thyroid disease were examined before (D1) and one month after completion of the second dose (D50).

Results: Among patients with autoimmune thyroiditis, the median neutralizing inhibition on D22, immediately before second dose, was 62.5%. One month later (D50), values increased to 96.7%, while three months after the second dose NAbs titers remained almost the same (94.5%). In the healthy group, median NAbs levels at D22 were 53.6%. On D50 the median inhibition values increased to 95.1%, while after three months they were

89.2%. The statistical analysis did not show significant differences between two groups (p-values 0.164, 0.390, 0.105 for D22, D50 and three months). Regarding changes in thyroid function, the mean value for T4 before vaccination was 89.797 nmol/L and one month after the second dose was 89.11 nmol/L (p-value=0.649). On D1 the mean T3 value was 1.464 nmol/L, which dropped to 1.389 nmol/L on D50 (p-value = 0.004). For TSH, mean levels were 2.064 mIU/ml on D1 and fell to 1.840 mIU/ml one month after the second dose (p-value=0.037). Despite decrease, all thyroid hormone levels remained within the normal range. No changes were found for anti-TPO or anti-TG.

Conclusions: This study provided evidence that patients with autoimmune thyroiditis present similar immunological response to COVID-19 BNT162b2 mRNA vaccine (Comirnaty, Pfizer/BioNTech) with healthy subjects, while vaccination may affect thyroid function.

Keywords: autoimmune thyroiditis, vaccination, COVID-19, immune response, SARS–CoV–2

INTRODUCTION

Thyroid disorders are very common and affect more than 10% of the adult population in total (1), while the prevalence of undiagnosed thyroid dysfunction is very high too (1, 2). Autoimmune thyroiditis or Hashimoto's thyroiditis, also known as chronic lymphocytic thyroiditis, is a specific autoimmune disease in which the thyroid gland is gradually destroyed. It is characterized by the presence of thyroid autoantibodies, such as against thyroid peroxidase (anti-TPO) or thyroglobulin (anti-TG). Autoimmune thyroiditis is indeed the most usual thyroid problem nowadays, leading often to subclinical or clinical hypothyroidism (1, 3).

Since the coronavirus disease 2019 (COVID-19) pandemic outbreak, interesting data have been published on the possible thyroid complications of COVID-19 (4–6), including mainly decrease in triiodothyronine (T3) and thyroid stimulating hormone (TSH) levels, as well as subacute thyroiditis (7–12). During this challenging period, physicians strived not only to treat new cases but also to support patients with chronic disorders. Vaccination for SARS–CoV–2 is the most powerful and promising tool against the pandemic. Several questions have been raised about the safety and efficacy of vaccines in patients with existing medical problems, including those with autoimmune thyroiditis.

Scientific endocrine societies have reported early that COVID-19 vaccines are safe and recommended that all endocrine patients should be vaccinated, including those with autoimmune thyroiditis (13, 14). However, no data exist so far regarding the immunological response to COVID-19 vaccination of these patients. On the other hand, several cases of thyroid dysfunction following SARS-CoV-2 vaccination, with the majority of vaccines available, have also been described (15–29). The main complication reported is subacute thyroiditis (15–26), while there are also few cases of Graves' disease (27–29). Taking into consideration the vaccination of millions of people worldwide, these cases represent very rare conditions. On the other hand, thyroid dysfunction may be under-reported and no robust data deriving from a properly performed study exist so far regarding the possible changes of thyroid function after vaccination against SARS-CoV-2.

This study had two aims: a) to compare immune response, namely the kinetics of neutralizing antibodies (Nabs), against SARS-CoV-2 after vaccination with BNT162b2 mRNA vaccine (Comirnaty, Pfizer/BioNTech) between patients with autoimmune thyroiditis and healthy subjects, and b) to investigate any changes in thyroid function in healthy subjects with no history of thyroid dysfunction before and after vaccination with BNT162b2 mRNA vaccine (Comirnaty, Pfizer/BioNTech).

PATIENTS AND METHODS

Clinical Setting

The study was carried out at the Department of Clinical Therapeutics, Alexandra Hospital, School of Medicine, National and Kapodistrian University of Athens after approval from the relevant Ethical Committee. The entire clinical part followed the Helsinki Declaration and the International Conference on Harmonization for Good Clinical Practice. All subjects provided informed consent prior to participation in the study. The primary inclusion criteria for this trial were vaccination with the BNT162b2 mRNA vaccine (Comirnaty, Pfizer/BioNTech), being over the age of 18, and being able to sign informed permission. The key exclusion criteria were active malignant disease, use of immunosuppressive medications, and end-stage renal disease. Subject information was kept private in compliance with the General Data Protection Regulation. All names were kept anonymous. To prevent the patient from being identified, names were removed immediately after collecting and replaced with a random number.

The entire study was divided into two sub-studies. The first sub-study followed participants from the day of the first vaccination until a period of up to three months after the second dose. Specifically, NAbs levels after vaccination were

compared between 56 patients with known autoimmune thyroiditis (positive anti-TPO or/and anti-TG) but otherwise healthy and 56 healthy controls with no history of thyroid disease, who were age and gender-matched with patients. In the second sub-study, thyroid hormones [total triiodothyronine (T3), total thyroxine (T4), thyroid stimulating hormone (TSH)] and thyroid auto-antibodies levels (anti-TG, anti-TPO) of 72 healthy subjects with no thyroid disease or such history were examined before (D1) and one month after completion of the second dose (D50).

Neutralizing Antibodies Measurement

Blood was collected on the following days: D1 before the first vaccine, D8, D22 (the day of the second immunization right before the injection), D50, and 3 months afterwards. Serum was extracted and stored at -80°C within 4 hours of blood collection. The FDA-approved cPassTM SARS-CoV2 Nabs Detection Kit was used to measure SARS-CoV-2 neutralizing antibodies (GenScript, Piscataway, NJ, USA).

Thyroid Hormones Assays

Blood was collected on the following days: D1 before the first vaccine and one month after completion of the second dose (D50). T3, T4, TSH, anti-TG and anti-TPO were measured with electrochemiluminescence during the same day for all blood samples (Roche Cobas for anti-TPO, Siemens Immulite for the rest).

Statistical Analysis

The statistical analysis began with descriptive criteria and estimation of dispersion metrics. A normality test was performed prior to statistical comparisons between two or more groups. To determine the normality of the data distribution, the Shapiro-Wilk test was used. If the nominal normality hypothesis is denied, the data is regarded to not follow the normal distribution. In circumstances where the data was determined to have a normal distribution, parametric approaches, specifically the independent t-test, were used to compare two independent groups (e.g., healthy subjects vs. thyroid patients). When comparing two groups, such as before and after immunization, the paired t-test was used. Non-parametric methods were utilized for future statistical analysis when the data distribution departed from normality. The Mann Whitney U test was employed for two independent group comparisons, such as determining the gender effect. The Wilcoxon signed-rank test was employed for pairwise group comparisons, such as neutralizing antibody levels between two occasions. The effect of age and gender on the difference in immune response between thyroid and healthy persons was also evaluated using general linear models with NAbs levels as the response variable and either analysis of variance (ANOVA) or Kruskal-Wallis. For comparisons of nominal characteristics, such as the correlation between the binary variable anti-TG or anti-TPO and vaccination, chi-square analysis was performed. Body mass index (BMI) was calculated for each subject based on the height and weight measurements. According to the BMI estimates, each participant was classified into one of the BMI groups, namely: underweight (BMI < 18.5), normal weight (BMI: 18.5 - 24.9), overweight (BMI: 25 - 29.9), obese (BMI ≥ 30).

To better understand and illustrate the changes in hormone levels before and after vaccination, their differences were also estimated by subtracting the values before vaccination (i.e., at D1) from those at D50. Therefore, new variables were created: Diff_T3, Diff_T4, Diff_TSH, Diff_anti-TPO and Diff_anti-TG. In addition, the calculation of hormone differences allowed further investigation of the relationship between the difference measures and the levels of T3, T4, TSH, anti-TPO, anti- TG. This was to investigate whether there was some kind of relationship between the hormone levels and the degree of their decline. The bivariate Pearson correlation coefficient was estimated to express the degree of their relationship.

In this study, it was also investigated whether or not the decrease in hormone levels could be associated with the adverse events after vaccination. Adverse events after vaccination were related to local effects (e.g., pain or swelling at the vaccination site, restriction of hand movement), fatigue, arthralgias/myalgias/chills/fever, headache, dizziness/sleepiness, allergies (such as itching, runny nose, redness), anaphylaxis, and others that did not fall into any of the above categories. The association between adverse events and hormone levels was examined using parametric or nonparametric comparative methods, as described above. Due to the limited sample size, the study could only be conducted with the occurrence of adverse events (i.e., yes/no) and no further analysis could be done regarding the type of adverse event, frequency, etc.

In all cases in this study, the type I error (significance level) was set at 5% and a result was considered significant if the estimated p-value (p) was less than the significance level. All statistical analysis was performed in Python v.3.9.2.

RESULTS

Baseline Characteristics

The entire study consisted of two sub-studies.

In the first study, NAbs levels after a specific vaccination were compared between 56 patients with autoimmune thyroiditis and 56 healthy controls (**Table 1**).

The majority of the subjects were women, namely 91.1% in the thyroid group and 89.3% in the healthy group. The median age of the two groups was 52.0 years (thyroid patients) and 51.0 years (healthy controls). The distribution of BMI values was also similar in both groups. About half of the subjects were of normal weight, fewer were overweight, and only a few belonged to either the underweight or obese group.

In the second study, hormone levels of 72 healthy subjects with no history of thyroid disease were examined before and after vaccination (**Table 2**). The median age was 45 years and almost three quarters of the participants were women, while half of all 72 subjects were of normal weight.

Neutralizing Antibodies Titers: Thyroid Patients vs. Healthy Controls

Figure 1 shows the percentage inhibition of NAbs on the day of the second dose, one month later (i.e., D50 after the start of the

TABLE 1 | Characteristics of patients with autoimmune thyroiditis and healthy controls participating in the first sub-study.

1st Sub-study	Patients with autoimmune thyroiditis	Healthy controls
Sample size	56	56
Men	5 (8.9%)	6 (10.7%)
Women	51 (91.1%)	50 (89.3%)
Age (median) years	52.0	51.0
BMI (median) kg/m^2	24.3	23.9
Underweight (n, %)	1 (1.8%)	2 (3.6%)
Normal weight (n, %)	29 (51.8%)	31 (55.4%)
Overweight (n, %)	17 (30.4%)	16 (28.6%)
Obese (n, %)	9 (16.1%)	7 (12.5%)

n, number of subjects; BMI, body mass index. Values in parentheses refer to percentages. Statistical analyses were performed to compare the demographics of the two groups. Separate normality tests for each characteristic and group showed that age followed a normal distribution (p-value = 0.598 for patients, p-value = 0.708 for controls), but BMI did not (p-value = 0.004 for patients, p-value = 0.023 for controls). The independent t-test for age showed no statistically significant difference (p = 0.323) and for BMI the Mann-Whitney test also showed no difference (p = 0.101).

TABLE 2 | Characteristics of healthy subjects in whom thyroid levels were measured before (D1) and one month after the second vaccination (D50).

2nd Sub-study	
Sample size	72
Men	19 (26.4%)
Women	53 (73.6%)
Age (median) years	45.0
BMI (median) kg/m^2	24.5
Underweight (n, %)	5 (6.9%)
Normal weight (n, %)	36 (50%)
Overweight (n, %)	25 (34.7%)
Obese (n, %)	6 (8.3%)

n, number of subjects; BMI, body mass index. Values in parentheses refer to percentages.

study), and three months after the second dose. Among patients with autoimmune thyroiditis, the median neutralizing inhibition on D22, immediately before vaccination, was 62.5%, while 6 individuals (8.33%) had inhibition values below the threshold of 30%. There were also 18 individuals (25.0%) with values corresponding to very high protection (NAbs titers above 75%). One month later (day 50), median inhibition values had increased to 96.7%, while three months after the second dose, NAbs titers had remained almost the same (94.5%). In the group of healthy subjects, median NAbs levels at day 22 were 53.6% and only three of them (5.1%) had levels below the lower limit of 30%. On day 50, the median inhibition values increased to 95.1%, while after three months they were 89.2% (**Figure 1**).

Normality test showed that NAbs values were not normally distributed (for all three cases p-values < 0.05), for this reason Mann-Whitney test was used for the comparisons between thyroid and healthy control groups. The statistical comparison did not show significant differences between the two groups on any day; the p-values were 0.164, 0.390 and 0.105 for D22, D50 and three months, respectively. Thus, it can be considered that there is no difference in the immune response after vaccination between patients with autoimmune thyroiditis and healthy subjects. The kinetics of NAbs follow the same pattern, increasing at D50 and slowly decreasing from that point on.

The possible influence of other factors such as age and gender has also been studied. It was found that gender had no significant influence on any of the measurement days (D22, D50, three months). The p-values of gender contribution in Kruskal-Wallis model were 0.237, 0.434 and 0.488 for D22, D50 and three months respectively. It is possible that the limited number of males (26.4% and 11.9% for the thyroid and control groups, respectively) hindered the detection of such differences.

Age was found to play a significant role in neutralizing antibody values. At D22 (i.e., three weeks after the first dose), age statistically significantly (p = 0.002 < 0.05) affected SARS Cov-2 inhibition by neutralizing antibodies and contributed with a negative sign (-0.480) to the model, suggesting that with increasing age, NAbs levels become lower. Similar results were observed at D50, where age again made a significant contribution (p = 0.043) with a negative sign (-0.169). However, three months after the second dose, no significant contribution of age was observed, suggesting that it does not play a role in the decrease of NAbs. This feature is also confirmed by the fact that age contributes more to the expression of NAbs values at D22 than at D50. In fact, the model constant (in absolute terms) is larger at D22 (i.e., -0.480) than the estimate at D50 (-0.169). This finding suggests that the influence of age gradually decreases as we move away from the day of vaccination.

Thyroid Function Before vs. After Vaccination

The second purpose of the study was to evaluate thyroid function in a healthy group of individuals before and after vaccination. We studied in purpose healthy individuals, in order to avoid biases derived from autoimmunity or levothyroxine treatment. In this case, thyroid hormones (T3, T4, TSH) and thyroid auto-antibodies (anti-TG, anti-TPO) were measured on D1 (immediately before vaccination) and on D50 (i.e., one month after the second administration of the vaccine). The normal ranges are: T3 0.84-2.6 nmol/L, T4 58-161 nmol/L, TSH 0.4-4 mIU/ml, anti-TPO <34 IU/ml, anti-TG <40 IU/ml.

The hormone levels are shown in **Figure 2**.

Paired tests were performed to compare T3, T4, and TSH levels between D1 (before vaccination) and D50 (one month after the second dose). All three characteristics (T3, T4, TSH) were normally distributed at both days (D1, D50) according to the Shapiro-Wilk test. The p-values at D1 were 0.699, 0.756 and 0.551 for T3, T4 and TSH, respectively. For D50, the corresponding p-values were: 0.959, 0.849, 0.125. Therefore, the parametric paired t-test was used for the comparisons and the "mean" estimates were used instead of the "median" values to describe the profiles.

On D1, the mean T3 value was 1.464 nmol/L, which dropped to 1.389 nmol/L on D50. This decrease proved to be statistically significant (p-value = 0.004 < 0.05). For T4, the mean value for all subjects before vaccination was 89.797 nmol/L and one month after the second dose the mean value was 89.11 nmol/L, which is not a significant difference (p-value = 0.649 > 0.05). For TSH, mean levels were 2.064 mIU/ml on D1 and fell to 1.840 mIU/ml one month after the second dose. This decrease was also found to be significant at the 5% level (p-value = 0.037). Despite the

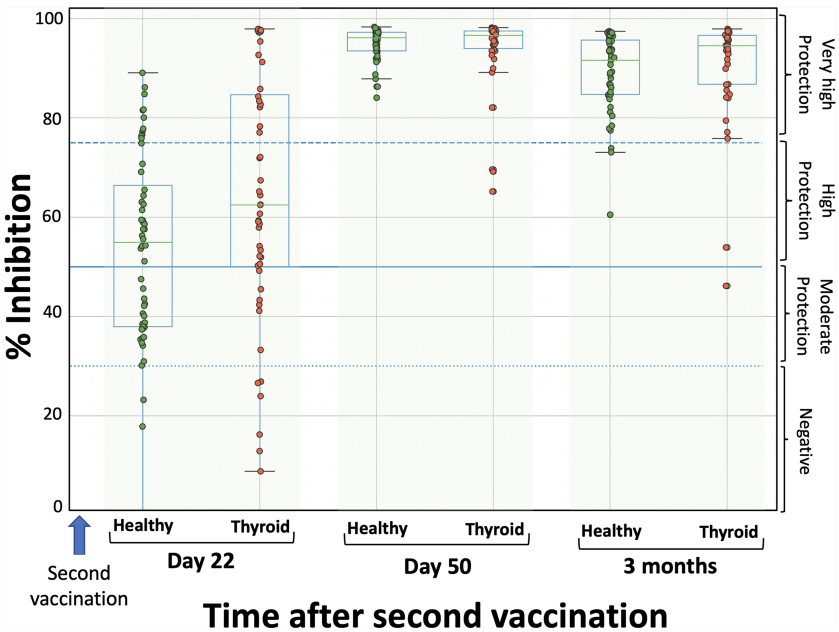

FIGURE 1 | Inhibition (%) of SARS-CoV-2 binding to the human host receptor angiotensin converting enzyme in patients with autoimmune thyroiditis (red) and healthy controls (green) on the day of the second vaccination (D22), one month later (D50) and three months after the second vaccination. The boxplot boundaries show the distribution's quartiles, whereas the superimposed dots represent individual levels of Nabs inhibition. The dashed lines represent the boundary levels of inhibition, which are 30%, 50%, and 75%.

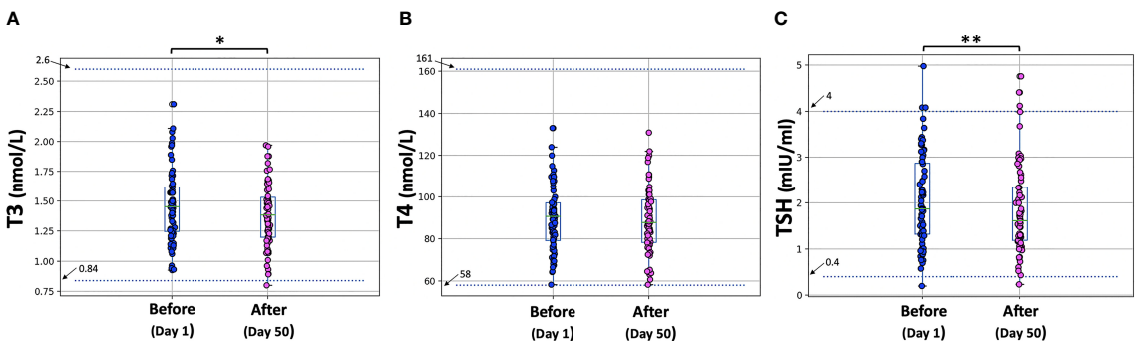

FIGURE 2 | T3, T4, and TSH levels before the first vaccine dose (D1) and one month after the second vaccination (D50). Asterisks indicate statistically significant differences (p-value < 0.05) between the two comparison groups (pre- and post-vaccination) for T3 (*) and TSH (**). The boxplot boundaries show the distribution's quartiles, while the superimposed dots represent the individual values of Nabs inhibition.

decrease in T3 and TSH, all thyroid hormone levels remained within the normal range.

To better examine and present the decrease in hormone levels before and after vaccination, the differences between subjects (for T3, T4, and TSH) were further estimated. These individual differences are shown graphically in **Figure 3**. **Figure 3** shows that the mean intrasubject difference is negative in all cases, i.e., all three types of hormone levels decrease after vaccination. However, this decrease was statistically significant only for T3 and TSH. In all subplots of **Figure 3**, normal distribution of the differences can be observed. Indeed, normality tests with the Shapiro-Wilk criterion resulted in p-values of 0.951, 0.167, 0.200 for the differences in T3, T4, and TSH, respectively.

By incorporating the concept of differences in hormone levels, the analysis could continue with an examination of possible relationships between individual "differences" in T3, T4, and TSH and hormone levels per se (i.e., blood T3, T4, and TSH levels). This was done to examine if there was some sort of relationship between hormone levels and degree of decline. The bivariate Pearson correlation coefficient was estimated for all

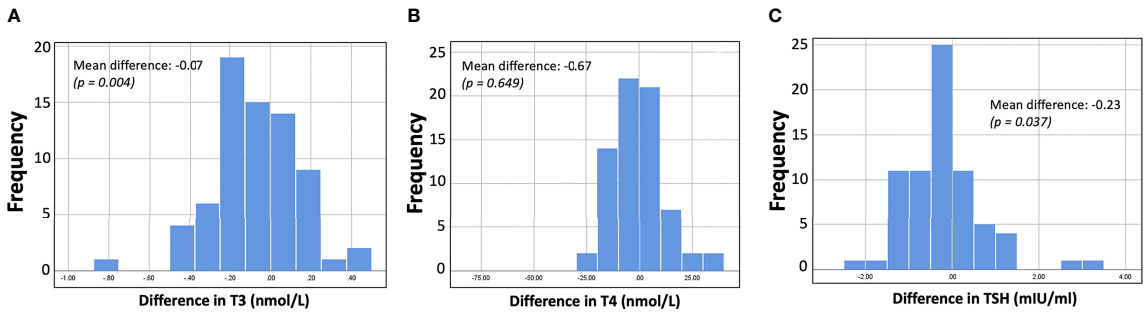

FIGURE 3 | Histograms showing the distribution of differences (i.e., hormone levels after minus before vaccination). For all three hormones (T3, T4, TSH) the values decrease after vaccination, but for T3 and TSH the difference was considered significant at the 5% level.

pairs, but no linear correlation was found in any of them. The highest correlation (Pearson correlation coefficient equal to 0.237) was observed for the relationship between Diff_T3 and T3 values on D22. However, even in this case the correlation was not significant.

The levels of anti-TPO antibodies before and after vaccination were also assessed by pairwise comparisons. However, the difference was found to be not statistically significant (p-value = 0.722). Anti-TG responses were also examined for their association with vaccination. Chi-square analysis assessed the association between anti-TG and vaccination, but no significant changes were found (p = 0.989).

We also examined whether or not the decrease in hormone levels could be associated with the occurrence of adverse events due to vaccination. In general, no statistically significant association (p-values > 0.05) was found between the decrease in hormones and occurrence of an adverse event.

DISCUSSION

No differences in the immunological response after vaccination with BNT162b2 mRNA vaccine (Comirnaty, Pfizer/BioNTech) between patients with autoimmune thyroiditis and healthy subjects, specifically on the day of the second dose (D22 from the start of the study), one month later (D50), and three months after the second dose, were observed. Among patients with autoimmune thyroiditis, the median neutralizing inhibition on D22 and immediately before second dose was 62.5%, one month later (D50) increased to 96.7%, while three months after the second vaccination NAbs titers had remained almost the same (94.5%). The statistical comparison did not show significant differences between patients with autoimmune thyroiditis and healthy controls on any day. The kinetics of NAbs follow the same pattern, increasing at D50 and slowly decreasing from that point on. To our knowledge this is the first study that investigated the immunological response of patients with autoimmune thyroiditis against any COVID-19 vaccine. These findings are of great importance, as Hashimoto's thyroiditis is a very usual clinical problem worldwide nowadays, affecting around 10% of the adult population (1, 2).

Patients and controls were age and gender matched; however, we investigated the impact of these parameters in immunological response in general. Gender had no significant influence on any of the measurement days, while age was found to play a significant role in NAbs values. At D22, age statistically significantly affected the response, suggesting that with increasing age, NAbs levels become lower. Similar results were observed at D50, where age again made a significant negative contribution. However, three months after the second dose, no significant contribution of age was observed, suggesting that the influence of age gradually decreases as we move away from the day of vaccination. This is in accordance with previous findings that indicate a negative effect of age on immunological response (30).

Another finding of this study was the decrease of T3 and TSH levels after vaccination with BNT162b2 mRNA vaccine. Specifically, T3 and TSH mean levels statistically significantly decreased from D1 to D50, while T4 levels remained stable. These were observed after 4 weeks since second dose and 7 weeks since first dose. We measured thyroid hormones in purpose at this time point, as thyroid axis needs few weeks for functional changes. To better examine the decrease in hormone levels before and after vaccination, the differences between subjects (for T3, T4, and TSH) were further estimated. The mean intrasubject difference was negative in all cases; this decrease was statistically significant only for T3 and TSH too, confirming the initial findings. We need to note that despite the decrease, all thyroid hormone levels remained within the normal range. No significant changes were found for anti-TPO or anti-TG auto-antibodies.

The question about possible changes in thyroid function is crucial. Several cases of thyroid dysfunction following SARS-CoV-2 vaccination, with the majority of vaccines available, have been described (15–29), including BNT162b2 mRNA vaccine (22–26). The main complication reported is subacute thyroiditis (15–26), while there are also few cases of Graves' disease (27–29). However, no robust data deriving from a properly performed study existed so far. Decrease in T3 and TSH levels has been reported in patients affected by COVID-19 during first days (31) or weeks after the disease (5–7). The possible thyroid dysfunction after COVID-19 vaccination cannot be completely explained. One hypothesis could

be the influence of systemic immune-mediated post-vaccination inflammatory response on the thyroid gland, leading to T3 reduction, and/or on pituitary gland, leading to TSH reduction and indirect effects on thyroid function. A second potential explanation could imply an underlying nonthyroidal illness syndrome or euthyroid sick syndrome, which is often caused by illness. It is characterized by normal or low serum TSH concentration and low T3 concentration, accompanied by a normal or low T4 concentrations. This is an adaptive body mechanism to recover from, critical mainly, illness (32). However, we need to note that despite the decrease, all thyroid hormone levels remained within the normal range. Moreover, when we examined whether or not the decrease in hormone levels could be associated with the occurrence of adverse events due to vaccination, no statistically significant association was found. On top, if such thyroid hormones changes remain for longer time needs further investigation.

To our knowledge, this is the first study that investigated the immunological response of patients with autoimmune thyroiditis against any COVID-19 vaccine. Moreover, this is the first research regarding the possible changes of thyroid function after vaccination against SARS-CoV-2 in the context of a clinical study. Two separate sub-studies were performed with different populations, in order to properly investigate the two aims. We studied in purpose healthy individuals in the second sub-study, in order to avoid possible biases derived from autoimmunity or levothyroxine replacement treatment. A limitation of the study is the relatively small sample size of the two sub-studies. This small sample size may lead to low statistical power and sensitivity in detecting differences in some variables being compared. Moreover, although patients and controls were age- and sex-matched, the unequal number of males and females (the vast majority were women) could potentially influence the results on the role of sex.

In conclusion, this study provided evidence that patients with autoimmune thyroiditis present similar immunological response to COVID-19 BNT162b2 mRNA vaccine (Comirnaty, Pfizer/BioNTech) with healthy subjects, while vaccination may affect thyroid function, namely decrease TSH and T3 levels.

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, Alexandra Hospital, National and Kapodistrian University of Athens. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SP designed the protocol, participated in the collection of the data and wrote the manuscript. VKa performed the statistical analysis. TP revised the manuscript. VV participated in the collection of the data. IC participated in the collection of the data. TB participated in the collection of the data. VKt participated in the collection of the data. FP participated in the collection of the data. SG participated in the collection of the data. GK revised the manuscript. IT participated in the collection of the data and revised the manuscript. ET designed the protocol, participated in the collection of the data and revised the manuscript. MD designed the protocol, participated in the collection of the data and revised the manuscript. All authors approved the final version of the article.

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Can We Cryopreserve the Sperm of COVID-19 Patients During the Pandemic?

Yongming Wu[1], *Xiaoxue Zhang*[2,3], *Zhiqiang Wang*[1] *and Xinyi Xia*[2*]

[1] Human sperm bank, the First Affiliated Hospital of Guangxi Medical University, Nanning, China, [2] COVID-19 Research Center, Institute of Laboratory Medicine, Jinling Hospital, Nanjing University School of Medicine, The First School of Clinical Medicine, Southern Medical University, Nanjing, Jiangsu, China, [3] Jiangsu Province Key Laboratory for Molecular and Medical Biotechnology, College of Life Science, Nanjing Normal University, Nanjing, China

An extreme strain has been placed on healthcare facilities in the COVID-19 era. Initial stage of the pandemic, national and international societies for reproductive medicine suggested the suspension of new IVF treatments and non-essential cryopreservation of gametes. Accordingly, the demands of cryopreservation of semen with COVID-19 patients also was suspended by some of cryobanks to protect staff and patients from unnecessary viral exposure at the acute stage. However, the pandemic may stay with us longer than expected. In addition, there will be some male COVID-19 patients with cancer or critically illness who needs to cryopreserve their semen before medical treatments, otherwise they might loss the chance of getting their own offspring. In this document, we summarize available evidence to deepen and expand awareness of feasibility of sperm cryopreservation and propose some suggestions to help cryobanks carry out sperm preservation procedure for COVID-19 male patients.

Keywords: COVID-19, SARS-CoV-2, semen, cryobanks, cryopreservation

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University of Catania, Italy

***Correspondence:**
Xinyi Xia
xinyixia@nju.edu.cn

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INTRODUCTION

Up to the end of June, 2021, the outbreak of Corona Virus Disease 2019(COVID-19), which has lasted for more than for a year and a half, is so prevalent around the world. Although epidemiologists' forecasts and timelines vary, they all agree on COVID-19 is here to stay, and the future depends on a lot of unknown (1). At present, national measures to reduce person to person transmission have succeeded in de-escalating of COVID-19 pandemic crisis, but the development of the epidemic in most countries is still far from optimistic. Globally, infections with SARS-CoV-2 virus are continuously rising with mounting numbers of deaths. At the time of writing, more than 160 million confirmed COVID-19 cases and over 4.0 million confirmed deaths have been reported (2). SARS-CoV-2 mainly affects the lungs, but emerging evidence suggests that the virus is also capable of infecting other organs, such as heart, kidney and human reproductive organs.

After the World Health Organization announced the onset of the SARS-CoV-2 pandemic, several fertility societies worldwide responded by recommending that fertility clinics should suspend the new IVF treatment, for patients who have the demands of fertility preservation, freezing gametes is recommended (3, 4). With the accumulation of data and experience, cryobanks have re-opened step wisely, but their activities, including semen cryopreservation, still be restricted

to some extent. Some of sperm cryobanks only accept asymptomatic patients who are about to undergo radio- and/or chemotherapy while the COVID-19 patients who are keen to access fertility preservation were curbed. Because we still can't answer following questions (with incomplete data) as yet: (1) Whether SARS-CoV-2 is present in the semen of COVID-19 patients? (2) Can the strategies of mitigating SARS-CoV-2 cross-contamination risk be established at cryopreservation stage. (3) Can SARS-CoV-2 from frozen semen be eliminated effectively by repeated washing to lessen infectivity?

The prudent measures may be the safest strategy at the stage to minimize the risks related to SARS-CoV-2 during the pandemic. However, over one year of inactivity, an inevitable issue is a backlog of COVID-19 patients with cancer or critically illness. Notably, compared with women, men are more vulnerable to infection in the outbreak, especially those of reproductive age, and their mortality of COVID-19 is also higher (5–8). Therefore, it is a significant subject for specialists to assess the necessity of semen cryopreservation while also developing safe and effective measures to meet the fertility preservation demands of COVID-19 patients.

THE EFFECT OF SARS-COV-2 ON MALE REPRODUCTIVE FUNCTION

Emerging evidence indicate that the changes of pathological structure and proteomics in the testicular tissue (9–11), disorders of sex hormones (12–15), damage to spermatogenesis (16, 17), and decreased sperm quality of COVID-19 patients (12, 18). These studies mentioned above suggest that SARS-CoV-2 can adversely affect multiple reproductive organs at least in a short term. The main mechanisms of SARS-CoV-2 impacting male fertility potential can be summarized as follows: (a) SARS-CoV-2 may lead to impairment of the blood–testis barrier and attack the germ cells directly (19); (b) SARS-CoV-2 affect the activity of the hypothalamic–pituitary–testicular (HPT) axis and lead to dysfunction in release of reproductive endocrine hormones (20); (c) Possible inflammatory responses and oxidative stress induced by SARS-CoV-2 disrupt the reproductive system (21); (d) the fever caused by infection interferes with normal reproductive physiology (22). Of note, the mechanisms mentioned above usually coexist and have a synergistic effect on impairing male reproductive function (23).

POSSIBILITY OF SARS-COV-2 VIRUS IN SEMEN, EPS AND TESTIS

To date, more than 27 viruses (HIV, mumps, zika, among others) have been found in semen. Some may be particularly persistent, like the Zika virus detected in the semen of asymptomatic men for up to 1 year after healing (24). Researchers also try to determine whether SARS-CoV-2 is present in semen of COVID-19 patients. The conclusion provides an especially important reference basis for sexual partner, semen processing, sperm cryopreservation and assisted reproductive technology (ART). For cryobanks, if there is active SARS-CoV-2 in semen, high attention should be paid to semen analysis and sperm preservation, as staff would be at great risk of infection. Fortunately, although there is still controversy concerning the presence of SARS-CoV-2 in human semen, available data increasingly appears to indicate the absence of SARS CoV-2 in semen (**Table 1**). Conversely, few studies have shown that viral RNA can be detected in semen from SARS-CoV-2 positive patients (18, 34). Considering that the distal urinary and reproductive tracts overlap in males, the RNA detected in semen may be just a residual of urinary shedding, which could lead to false-positive results. Thus, from the little data available, the risk that SARS-CoV-2 might be transmitted through semen seems fairly low in COVID-19 patients. What to need to be pointed out is, these studies used small sample sizes and examined confirmed cases of COVID-19 during recovery, the possibility that SARS-CoV-2 is present in semen cannot be completely ruled out. Taking the factor into account, large-scale and multi-center studies are needed to draw convincing conclusions about the presence of SARS-CoV-2 in semen.

ACE2 and transmembrane serine protease 2 (TMPRSS2) are highly expressed by the epithelium of the human prostate. Thus, it is possible that SARS-CoV-2 may be affect the prostate and the virus could infiltrate into the prostatic secretion. As an essential component of semen, prostatic secretion is secreted by the prostate which can be collected by prostatic massage. In this review, there are two studies on the EPS, with a total of 71 samples (29, 33). However, according to these research results, SARS-CoV-2 RNA were not detected in all EPS samples. These results indicate that the virus may not exist in EPS and further supports that there is little possibility of SARS-CoV-2 in the semen of COVID-19 patients.

Due to expressing the ACE2 receptor, a target for SARS-CoV-2 infection, the testes were also thought to be the target of SARS-CoV-2. Whereas growing evidence for the presence of the viral particles in the testicular biopsies from patients infected with SARS-CoV-2 is highly limited. To date, several studies (10, 17, 37–40) reported testicular histology outcomes in COVID-19 patients. In these studies, testicular/epididymal pathological analysis were performed on deceased COVID-19 patients, and at least 5 testicular samples positive for SARS-CoV-2 particles were identified. However, these studies analyzing SARS-CoV-2 in testicular biopsies have based on deceased COVID-19 patients, which may be limit the explanation whether there were SARS-CoV-2 viral particles in predominantly mild COVID-19 patients. It is necessary to further study the pathological histology of testis in mild and moderate COVID-19 patients to determine whether SARS-CoV-2 can be detected in this population.

THE FEASIBILITY OF SPERM CRYOPRESERVATION

From the current point of view, the control of the COVID-19 epidemic will take a long time. At this stage, the fertility preservation center needs to update part of its working procedures to prevent the outbreak of COVID-19 from

TABLE 1 | Summary of detection of SARS-CoV-2 in the male reproduction system.

Number	Study	Sample size (n)	Stage of Disease	Reported positive results of SARS-CoV-2 detection			
				Testis biopsies	Semen	Prostate/ EPS	Other samples
1	(Guo, Zhao et al., 2021) (25)	23	recovered:11 recent infection:12	ND	0/23	ND	ND
2	(Holtmann, Edimiris et al., 2020) (26)	20	recovered men:18; acute stage:2	ND	0/20	ND	ND
3	(Li, Xiao et al., 2020) (17)	29	recovered:23 deceased:6	ND	0/23	ND	ND
4	(Ma, Xie et al., 2021) (12)	12	recovered men:11; Treatment stage:1	ND	0/12	ND	ND
5	(Pan, Xiao et al., 2020) (27)	34	recovered:34	ND	0/34	ND	ND
6	(Kayaaslan, Korukluoglu et al., 2020) (28)	16	Acute Stage	ND	0/16	ND	ND
7	(Ruan, Hu et al., 2021) (29)	74	recovered	ND	0/70	0/61	0/74 (urine)
8	(Rawlings, Ignacio et al., 2020) (30)	6	recovered	ND	0/6	ND	ND
9	(Burke, Skytte et al., 2021) (31)	18	Symptomatic:15 Asymptomatic:3	ND	0/18	ND	ND
10	(Paoli, Pallotti et al., 2020) (32)	1	recovered	ND	0/1	ND	0/1 (urine)
11	(Zhang, Wang et al., 2020) (33)	10	positive nasal swab for SARS-CoV-2:3 nasal swab for SARS-CoV-2 turned negative:7	ND	ND	0/10	ND
12	(Li, Jin et al., 2020) (34)	38	recovered: 23 acute stage :15	ND	6/38	ND	ND
13	(Gacci, Coppi et al., 2021) (18)	43	recovered	ND	1/43	ND	2/43 (urine)
14	(Machado, Barcelos Barra et al., 2021) (35)	15	no symptoms: 2 mild symptoms:13	ND	1/15	ND	ND
15	(Temiz MZ et al., 2021) (36)	20	before treatment:10 after treatment:10	ND	0/20	ND	ND
16	(Song, Wang et al., 2020) (37)	13	recovered:12 deceased:1	0/1	0/12	ND	ND
17	(Yang, Chen et al., 2020) (10)	12	deceased	0/12	ND	ND	ND
18	(Bian and Team 2020) (38)	NR	deceased	+ (NR)	ND	ND	ND
19	(Achua, Chu et al., 2021) (39)	6	deceased	1/6	ND	ND	ND
20	(Ma, Guan et al., 2021) (40)	5	deceased	2/5	ND	ND	ND

ND, not determined indicates that assays were not performed; NR, no specific data reported; +, results were positive.

within (41). Moreover, in case of emergency, it is also necessary to face the fertility preservation demands of COVID-19 patients, who may be in the incubation phase, recovered phase, and even acute phase of critical illness. Although ART are being preferably cancelled or postponed during this pandemic, fertility preservation is an emergency requirement, as patients undergoing genotoxic treatments may induce transient or permanent sterility. Even if fertility preservation centers do not plan to cryopreserve sperm for COVID-19 patients, they will encounter people who need fertility preservation in high-risk environments. Therefore, cryobanks should make necessary preparations to ensure that they have the ability to cryopreserve sperm. At least professional consultation and advice should be formulated to meet the individual requirements of such patients.

The presence of virus in semen is not new to researchers, who have long known that semen may contain various viruses (42). During semen processing, laboratory operators are at high risk of transmission for the direct exposure to semen samples (43, 44). Sperm obtained from patients with viral illnesses, such as human immunodeficiency virus (HIV) infection and hepatitis, must be treated with special precautions to reduce exposure of the non-infected partner and cross-contamination of reproductive tissue within the laboratory (45). In practice, many laboratories have set up good safety protection systems and methods to lessen virus particles in semen. Although most studies have shown no detectable virus in ejaculate of COVID-19 patients, considering the special characteristics of SARS-CoV-2 transmission, we should not relax our vigilance, strengthening of precaution during semen handling procedures is still crucial.

Another serious concern is the potential cross-contamination during cryostorage stage, as most microorganisms can survive for a long time in the ultra-low temperature of LN_2 (46). There has been controversy about the research results of virus cross-contamination in ART cryobanks. Bielanski and colleagues clearly showed an absence of cross-contamination from infected semen and embryo straws to non-infected samples stored in the same LN2 tanks; furthermore, they reported no virus contamination in embryos vitrified in sealed cryovials or straws (47, 48). Cobo and colleagues also failed to detect the

presence of viral RNA or DNA sequences in LN_2 used for oocyte or embryo vitrification in patients with seropositive for HIV, hepatitis C virus, and hepatitis B virus (49). For COVID-19-positive men, given that only very low titres of SARS-CoV-2 have been detected in non-respiratory sites, some studies consider the risk of significant virus shedding into semen is low (50). However, the possibility of cross-contamination between cryopreserved sperm samples during storage in LN2 is difficult to determine in the phase where this "low" risk is estimated merely (51). Now that most viruses are able to survive in LN_2, contamination of other samples by virus invasion through flowing LN_2 into broken or poorly closed cryovials/tubes is possible (52). Hence, cryobanks must be aware of the possible presence of SARS-CoV-2 in cryopreserved sperm and LN_2, and take effective measures to minimize the aforementioned risk. If cryobanks plan to offer sperm cryopreservation for COVID-19 patients, some suggestions based on expert opinion informed by the literature should be followed.

- Managers of the cryobanks should be very prudent, and invite health authorities, including reproductive ethics committees, to evaluate their own conditions and facilities while providing regulatory standards (53).
- It is essential to establish the suitable precaution procedures and conduct strict training for the staff. If possible, dedicated areas should be set up to receive COVID-19 patients and collect samples (54, 55).
- Face-to-face interactions should be minimized with COVID-19 patients. Video conferencing, telephone and other online consultations can be used to collect the patient's epidemiological history and assess the possible hidden risks (53). Meeting COVID-19 patients who want to fertility preservation, andrologists should give corresponding suggestions based on the decision path for sperm crypreservation of COVID-19 patients by managers of the cryobanks (**Figure 1**).
- For recovered patients, considering the persistence and half-life of SARS-CoV-2 in the body, it is recommended that sperm cryopreservation could be provided after 3 months in non-emergency situations (56). Especially, referred to patients with long COVID-19, 6-month interval or more should be suggested after the typical symptoms disappear.
- If recovered patients present with any suspected clinical of COVID-19 symptoms at cryopreservation stage, the cryobanks should initiate emergency procedures to diagnose whether they are COVID-19 recurrences, and discuss how to dispose of cryopreserved sperm. It should be noted that the sperm cryopreservation of patients with reinfection should be postponed.
- Urgently, such as COVID-19 inpatients with cancer who need fertility preservation, the cryobanks should invite the reproductive ethics committee to convene a meeting to fully evaluate the safety before starting the cryopreservation procedure.
- In andrology laboratories, safety cabinet class II t is recommended when handling semen of COVID-19 patients (57). One should take extra-care while dealing with semen. Once the semen is examined and handled, all single-use materials should be discarded in individual bins and disposed of immediately.
- In view of results of studies on SARS-CoV-2 in semen of COVID-19 patients have been controversial, SARS-CoV-2 testing of semen should be considered before cryopreservation. Based on 56 recommendations, RT-PCR assays was the index test more recommended for the diagnosis of SARS-CoV-2 (58).
- In the case of sperm cryopreservation, high-security cryovials should be used for all COVID-19 positive males. Cryovials should be stored independently with warnings labels.
- For unwashed semen samples or those awaiting viral test results, using of a separate, vapour-phase storage is recommended to minimize risk (59).
- Considering that SARS-CoV-2 may be present in other tissue, direct freezing sperm obtained by surgery should be avoided. Repeated washing and viral test procedures should be observed before cryopreservation (41).
- In the worst case of a positive semen obtained by patient with no further opportunity of sampling, sperm-washing procedures such as double-density gradient followed by swim-up can be used to dilute virus present before cryopreservation (60).
- Do not use COVID-19 positive males' sperm until there is no evidence to prove the safety of these samples. When the cryopreserved sperm can be used in ART, the risk of transporting the samples between centers and the safety of the personnel working in the laboratory during thawing and handling should be considered (61).

DISCUSSION

Despite worldwide efforts to prevent and control the COVID-19 pandemic, SARS CoV-2 is still widespread in many countries and regions. As any emergent disease, numerous studies have been carried out to better understand characteristics of the virus and its short-and long-term repercussions on health status. So far, studies have strongly shown that SARS-CoV-2 can cause impairment of male fertility. The conclusion poses a distinctive problem to the cryobanks about how to carry out male fertility preservation during the pandemic. On the one hand, among so many COVID-19 patients, some do have the requirement of fertility preservation, otherwise they may never get their own offspring. Therefore, the health authorities should be fully aware of the fertility preservation demands of COVID-19 patients, and organize experts to issue the possibility of fertility preservation (62). Under the consensus formulated by experts and the suggestions recommended in the present article, the cryobanks could develop detailed preventive and operating procedures to carry out male fertility preservation for COVID-19 patients.

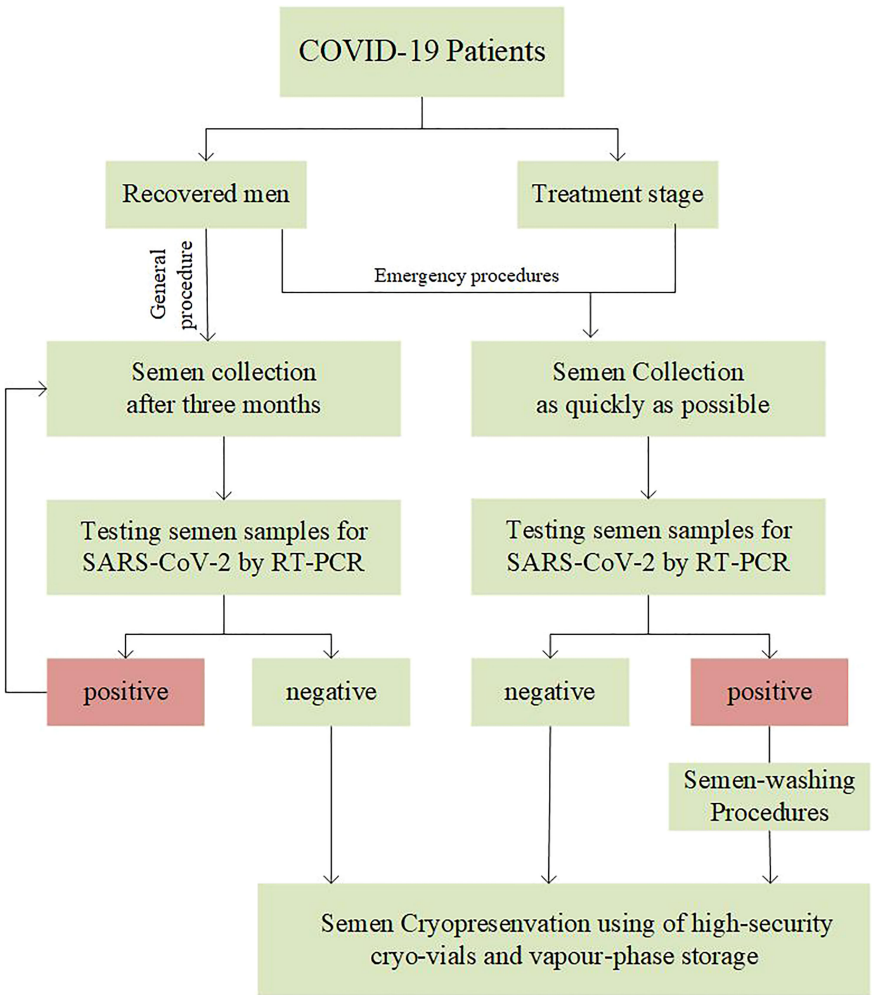

FIGURE 1 | A decision path for sperm cryopreservation of COVID-19 patient.

AUTHOR CONTRIBUTIONS

YW and XX conceived the review. YW, XZ, ZW wrote and reviewed the paper. All authors contributed to the article and approved the submitted version.

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TMPRSS2 Expression and Activity Modulation by Sex-Related Hormones in Lung Calu-3 Cells: Impact on Gender-Specific SARS-CoV-2 Infection

*Donatella Treppiedi[1], Giusy Marra[1], Genesio Di Muro[1,2], Rosa Catalano[1], Federica Mangili[1], Emanuela Esposito[1,3], Anna Maria Barbieri[1], Maura Arosio[1,4], Giovanna Mantovani[1,4] and Erika Peverelli[1,4]**

[1] Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy, [2] PhD Program in Endocrinological Sciences, University Sapienza of Rome, Rome, Italy, [3] PhD Program in Experimental Medicine, University of Milan, Milan, Italy, [4] Endocrinology Unit, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

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Alexandra Kautzky-Willer,
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Instituto Nacional de Medicina
Genómica (INMEGEN), Mexico

***Correspondence:**
Erika Peverelli
erika.peverelli@unimi.it

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Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Although males and females are at equivalent risk of infection, males are more prone to develop a higher severity disease, regardless of age. The factors that mediate susceptibility to SARS-CoV-2 and transmission are still under investigation. A potential role has been attributed to differences in the immune systems response to viral antigens between males and females as well as to different regulatory actions played by sex-related hormones on the two crucial molecular effectors for SARS-CoV-2 infection, TMPRSS2 and ACE2. While few and controversial data about TMPRSS2 transcript regulation in lung cells are emerging, no data on protein expression and activity of TMPRSS2 have been reported. Aim of the present study was to search for possible modulatory actions played by sex-related hormones on TMPRSS2 and ACE2 expression in Calu-3 cells, to test the effects of sex-steroids on the expression of the 32kDa C-term fragment derived from autocatalitic cleavage of TMPRSS2 and its impact on priming of transiently transfected spike protein. Cells were stimulated with different concentrations of methyltrienolone (R1881) or estradiol for 30 h. No difference in mRNA and protein expression levels of full length TMPRSS2 was observed. However, the 32 kDa cleaved serine protease domain was increased after 100 nM R1881 (+2.36 ± 1.13 fold-increase vs control untreated cells, $p < 0.05$) and 10 nM estradiol (+1.90 ± 0.64, fold-increase vs control untreated cells, $p < 0.05$) treatment. Both R1881 and estradiol significantly increased the activating proteolytic cleavage of SARS-CoV-2 Spike (S) transfected in Calu-3 cells (+1.76 ± 0.18 and +1.99±,0.76 increase in S cleavage products at R1881 100nM and 10 nM estradiol treatment, respectively, $p < 0.001$ and $p < 0.05$ vs control untreated cells, respectively). Finally, no significant differences in ACE2 expression were observed between hormones-stimulated cells and untreated control cells. Altogether,

these data suggest that both male and female sex-related hormones are able to induce a proteolityc activation of TMPRSS2, thus promoting viral infection, in agreement with the observation that males and females are equally infected by SARS-CoV-2.

Keywords: SARS-CoV-2, Spike, TMPRSS2, androgen, estradiol

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1). Even though the majority of people infected with SARS-CoV-2 are asymptomatic or present with mild symptoms and do not require hospitalization, in a subset of patients the clinical features may progress to acute respiratory distress syndrome (ARDS) and cardiac injury. Since the beginning of the pandemic, COVID19 has caused hundreds of thousands of deaths worldwide. Epidemiological studies have identified major risk factors for developing severe symptoms such as age, obesity, diabetes, hypertension, respiratory or cardiovascular diseases and sex (2–6). Although the percentage of confirmed cases has been reported to be equal among men and women, for every 14 males confirmed cases that have died from COVID-19 there are only 10 female (https://globalhealth5050.org/the-sex-gender-and-covid-19-project/the-data-tracker/) (Global Health 5050, 2021). This evidence has suggested the importance of considering sex gender as a critical variable in the clinical research to defeat SARS-CoV-2 pandemic. There are at least two possible mechanisms by which androgens may determine clinical outcomes in COVID-19. The first plausible mechanism is related to androgen-driven immune modulation. The second possibility refers to differences between males and females in the expression levels and genetic variants in angiotensin-converting enzyme 2 (ACE2) receptor and cellular serine protease TMPRSS2, the two crucial genes for viral infection (7, 8).

Indeed, as other SARS-CoV, SARS-CoV-2 cells entry and infection rely on recognition and attachment of the viral spike (S) glycoprotein to the ACE2 transmembrane protein on host cells and engagement of the TMPRSS2 protease for S protein priming (8–13). Briefly, entry depends on binding of the surface unit, S1, of the S protein to ACE2 receptor, that in turn promotes viral attachment to the plasma membrane of target cells. In addition, S protein priming by cellular proteases is required. The S protein cleavage at the S1/S2 and the S2' sites allows fusion of viral and cellular membranes, a process specifically driven by the S2 subunit (8). TMPRSS2 is made as a precursor protein (zymogen) of 70 kDa which undergoes autoproteolytic activation releasing the 32 kDa fragment containing the protease domain into the extracellular space (14, 15).

Both ACE2 and TMPRSS2 have been suggested to be implicated in the modulation of susceptibility to SARS-CoV (16, 17) and SARS-CoV-2 as well (8). These genes are predicted to mediate sex-related effects: ACE2 is located on the X chromosome, whilst TMPRSS2 gene has a 15 bp androgen response element (ARE) and is also regulated by estrogen stimulation (18–20). TMPRSS2 is largely expressed in epithelial cells of the respiratory, gastrointestinal, and urogenital tract (21, 22). In prostate cancer cells, TMPRSS2 is strongly upregulated in response to androgens (14, 19, 23). Although no changes in the TMPRSS2 transcript was observed in lung cell lines and lung from mice upon androgen stimulation and inhibition of androgen receptor (24, 25), the expression of TMPRSS2 in the lung was found slightly increased in males (7) and significantly upregulated by androgens in A549 lung cells (26). However, so far, no data about the impact of sex-related hormones on the status of activation of TMPRSS2 are available in lung cells.

In the preset study we investigated the effects of sex-related hormones on ACE2 and TMPRSS2 expression and proteolytic activation.

MATERIALS & METHODS

Cell Culture and Cell Transfection

The non-small-cell lung cancer cell line Calu-3 (ATCC HTB-55) was kept in culture with Eagle's Minimum Essential Medium (EMEM, ATCC, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and pen/strep (Life Technologies, Carlsbad, CA, USA) at 37°C and 5% CO_2 in a humidified atmosphere.

For experiments, cells were seeded in lipophilic hormones-free complete medium consisting of Eagle's Minimum Essential Medium supplemented with 2% charcoal-stripped bovine serum (CSS) (Merck, Darmstadt, Germany) and antibiotics. Estradiol (17β-estradiol) and R1881, also known as methyltrienolone, were purchased from Abmole Bioscience (Houston, TX, USA) and used as estrogen receptor and androgen receptor agonists, respectively. Expression vector coding for wild-type SARS-CoV-2 Spike (SARS-CoV-2 S) was kindly provided by Prof. Elisa Vicenzi (IRCCS San Raffaele Hospital, Milan, Italy) and transiently transfected in Calu-3 cells using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) as transfection reagent, according to the manufacturer's instructions.

Quantitative Real-Time RT-PCR

The RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) was used to extract total RNA from Calu-3 cells. Total RNA concentration and purity were measured using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Whaltam, MA, USA). RNA integrity was assessed by 1% agarose gel electrophoresis. Total RNA (1 μg) was reverse transcribed with RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Whaltam, MA, USA). qRT-PCR was carried out using the SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA) following the instructions of the manufacturer, in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA,

USA). Specific primers were designed for human TMPRSS2, aromatases (CY19A1), androgen receptor and estrogens receptors. All reactions were performed in triplicate and media of CT values was determined. GAPDH was used as housekeeping gene for the normalization of target genes using the Bio-Rad CFX Manager software.

Western Blot Analysis of TMPRSS2, ACE2 and Spike

Cells were seeded in 6-well plates at a cell density of 2×10^5/well in EMEM supplemented with 2% CSS and antibiotics. Cells were maintained in this lipophilic hormones-free medium for 7 days, with two medium changes, at day 4 and at day 7, before the start of treatments. For transfection experiments, cells were transfected with plasmid encoding SARS-CoV-2 S at day 5 and left incorporating plasmidic DNA for 2 days before the start of treatments. For each experiment, cells were treated at day 7 with estradiol (10 nM and 100 nM) or R1881 (10 nM and 100 nM) for 30 h in CSS medium. Fresh stimuli were re-added at 24 h of treatments. Doses of estradiol and R1881 as well as time of stimulation were chosen on the bases of preliminary time/concentration dependent experiments (**Supplementary Figure 1**) and literature data (14, 23). Cells were then lysed with lysis buffer. After quantification by BCA assay, 30 µg of total proteins extracted were separated on SDS/polyacrylamide gels and transferred to a nitrocellulose filter. TMPRSS2 antibody was from Santa Cruz Biotechnology (Dallas, TX, USA) and diluted at 1:200, Spike antibody was from GeneTex (Irvine, CA, USA) and used at 1:1000 dilution, ACE2 antibody was from R&D Systems (Minneapolis, MN, USA) and used at 1:1000. The incubation of primary antibodies was carried out at 4°C for 18 h, whilst secondary antibodies anti-mouse or anti-rabbit (Cell Signaling Technology, Danvers, MA, USA) were used at 1:2000 and incubated at room temperature for 1 h. The anti-GAPDH antibody (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) used for normalization was diluted 1:4000 and incubated for 1 h at room temperature. ChemiDOC-IT Imaging System (UVP, Upland, CA, USA) was used to detect chemiluminescence and NIH ImageJ software to analyse the intensity of the bands.

Immunofluorescence

Calu-3 cells were seeded in 6-well plates at the density of $2x10^5$ cells/well in EMEM supplemented with 2% CSS and antibiotics. Cells were maintained in this lipophilic hormones-free medium for 7 days, with two medium changes at day 4 and at day 7, before start of treatments. For the experiments, cells were treated at day 7 with estradiol (10nM) or R1881 (100nM) for 30 h in CSS medium. Fresh stimuli were re-added after 24 h. After the treatment, cells were trypsinized, counted and re-seeded on 13-mm poly-L-lysine coated coverslips at the density of 1.25×10^5 cells/well in 24-well plates and grown at 37°C for 18 h. The following day, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at room temperature, washed three times with PBS, and incubated for 1 h at room temperature with blocking buffer (5% FBS, 0.3% Triton™X-100, in PBS).

For immunofluorescence analysis of TMPRSS2 and ACE2 in Calu-3 cells, rabbit anti-TMPRSS2 (1:100, Proteintech, Germany GMBH) and rabbit anti-ACE (1:100, Proteintech, Germany GMBH) antibodies were used and incubated o/n at 4°C. Anti-rabbit Alexa Fluor™ -546-conjugated secondary antibody (1:500, ThermoFisher Scientific, CA, USA) was incubated at room temperature for 1 h. Antibodies were diluted in Antibody Diluent Reagent Solution (Life Techologies, ThermoFisher, CA, USA). Coverslips were mounted on glass slides with EverBrite™ Hardset Mounting Medium with DAPI (Biotium, Fremont, CA, USA) for subsequent observation at fluorescence microscope. The NIH ImageJ software was used to merge single channel images.

Statistical Analysis

The results are expressed as the mean ± SD. To assess the significance between two series of data, ANOVA with Student's t test was used. Statistical analysis was performed by GraphPad Prism 7.0 software and $P < 0.05$ was accepted as statistically significant.

RESULTS

R1881 and Estradiol Promote TMPRSS2 Serine Protease Activation Without Affecting Total TMPRSS2 Expression

Our clone of Calu-3 cells was previously tested for endogenous expression of TMPRSS2, ACE2, androgen receptor, estrogen receptor and aromatase (CY19A1). Calu-3 cells endogenously expressed all the mentioned genes with the exception of CY19A1 (data not shown). To evaluate whether sex-related hormones could differentially modulate TMPRSS2 expression and activity in Calu-3 cells, cells were first subjected to lipophilic hormones-deprivation for 1 week. Western blot analysis showed that under this culture condition TMPRSS2 expression was reduced compared with a not lipophilic hormones-deprived condition (**Figure 1A**). Cells were then stimulated with different concentrations of methyltrienolone (R1881) or estradiol to selectively test their effects on TMPRSS2 expression and activity. Preliminary experiments with different concentrations and incubation times were performed (**Supplementary Figures 1A-D**). No difference in mRNA and protein expression levels of full length TMPRSS2 was observed after R1881 and estradiol treatments (**Figures 1B, C**). Rather, TMPRSS2 auto proteolytic cleavage was promoted by both R1881 and estradiol since a significant increase in the intensity of the bands corresponding to the cleaved 32kDa TMPRSS2 fragment has been observed in cells exposed to 100 nM R1881 (+2.36 ± 1.13 fold-increase vs control untreated cells, $p < 0.05$) and 10 nM estradiol (+1.90 ± 0.64, fold-increase vs control untreated cells, $p < 0.05$), (**Figure 1C**). The effect exerted by R1881 was achieved at higher concentration compared to estradiol, although without a significative statistically difference.

To test a possible effect of R1881 and estradiol on TMPRSS2 intracellular localization, we performed immunofluorescence

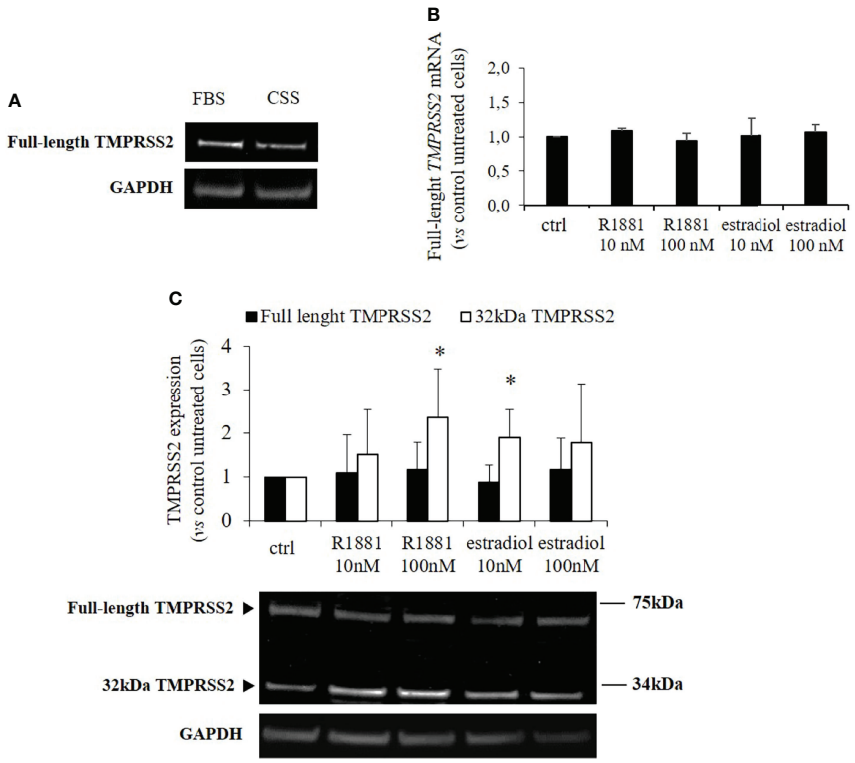

FIGURE 1 | TMPRSS2 expression in response to stimulation with R1881 and estradiol. **(A)** Representative Western blot showing TMPRSS2 expression in Calu-3 cells cultured in complete medium or FBS-CSS medium for one week. **(B)** Analysis of TMPRRS2 mRNA expression in Calu-3 cells stimulated for 30 h with the indicated concentration of R1881 and estradiol in FBS-CSS medium. **(C)** Representative Western blot and densitometrical analysis of bands corresponding to the full-length form of TMPRSS2 and the 32kDa cleaved fragment containing the protease domain. Proteins were extracted from Calu-3 cells treated with the indicated concentration of R1881 and estradiol in FBS-CSS medium for 30 h. GAPDH was used as housekeeping gene for normalization. Experiments were performed in triplicate and results are expressed as mean ± SD. * = $p < 0.05$ vs control untreated cells.

experiments. Our data showed that in basal condition TMPRSS2 was localized in the cytoplasm and plasma membrane, and no difference in its localization was observed after treatment with estradiol or R1881 (**Supplementary Figure 2**).

R1881 and Estradiol Modulate Spike Protein Priming

Then we asked whether the increase in TMPRSS2 serine protease domain release mediated by sex-related hormones could result in enhanced SARS-CoV-2 Spike protein priming in Calu-3 cells. Indeed, TMPRSS2 activity is required for proteolytic processing of SARS-CoV-2 Spike at the S1/S2 and the S2' sites in cell lines and subsequent SARS-CoV-2 infection of lung cells. To this purpose, Calu-3 cells kept in lipophilic hormone-deprived medium were transiently transfected with plasmid encoding SARS-CoV-2 Spike and then stimulated for 30 h with different concentrations of R1881 or estradiol. Western blot experiments on proteins from cell extracts showed significant increase in the expression levels of spike cleavage products after both 100 nM R1881 (+1.76 ± 0.18, $p < 0.001$ vs control untreated cells) and 10 nM estradiol (+1.99±,0.76 $p < 0.05$ vs control untreated cells) treatments (**Figure 2**). No statistically significative difference in the effects exerted by R1881 and estradiol was found.

ACE2 Expression Is Not Affected by R1881 and Estradiol Cells Incubation

SARS-CoV-2 Spike employs ACE2 as the entry receptor for host infection of target cells. We tested any possible effects of estradiol and R1881 on its expression. Western blot analysis was performed on Calu-3 cells deprived of lipophilic hormones for 1 week and subsequently stimulated with R1881 or estradiol at different doses. As reported in **Figure 3**, no significant differences in ACE2 expression were observed between hormones-stimulated cells and untreated control cells.

Immunofluorescence experiments showed that ACE2 was localized in the cytoplasm and plasma membrane in both basal condition and after estradiol or R1881 treatment (**Supplementary Figure 2**).

DISCUSSION

An unquestionable feature of the ongoing COVID-19 pandemic is the male bias towards the developing of a severe disease despite the

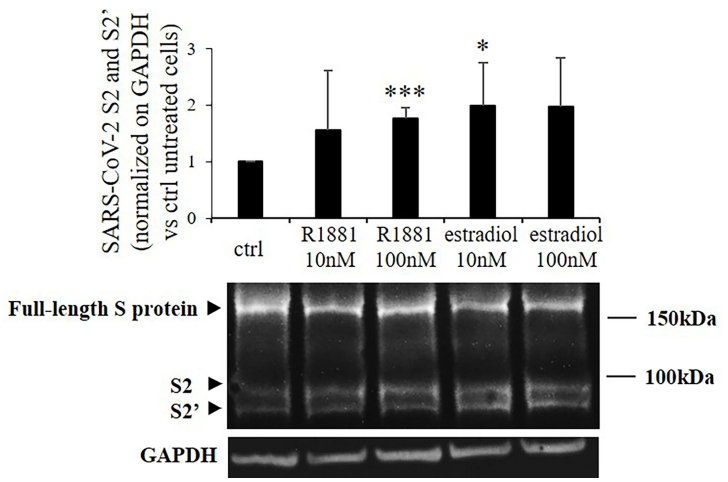

FIGURE 2 | SARS-CoV-2 Spike cleavage in response to stimulation with R1881 and estradiol. Representative Western blot image and densitometrical analysis of bands corresponding to SARS-CoV-2 Spike cleavage products (S2 and S2') in Calu-3 cells transfected with SARS-CoV-2 S plasmid and stimulated with the indicated concentration of R1881 and estradiol for 30 h in FBS-CSS medium. GAPDH was considered as housekeeping gene and was used for normalization. Experiments were replicated three times and results are expressed as mean ± SD. *, $p < 0.05$, ***, $p < 0.001$ vs control untreated cells.

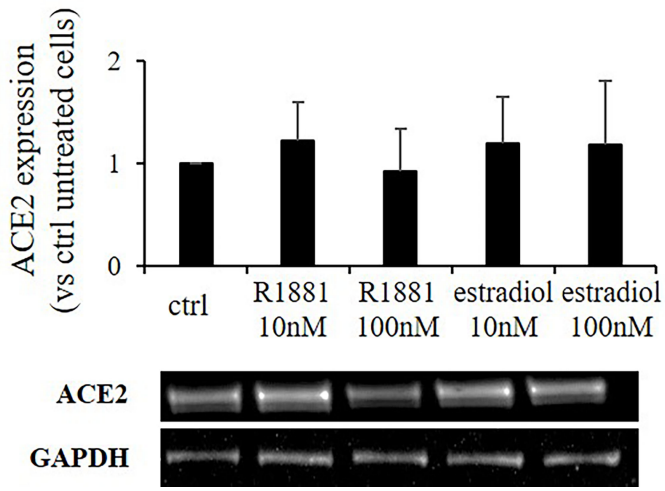

FIGURE 3 | R1881 and estradiol do not influence ACE2 protein expression. Representative Western blot image and densitometrical analysis showing ACE2 protein expression in Calu-3 cells stimulated or not with the indicated concentration of R1881 and estradiol for 30 h in FBS-CSS medium. GAPDH was considered as housekeeping gene and was used for normalization. Experiments were replicated three times and results are expressed as mean ± SD.

same proportion of males and females infected with SARS-CoV-2. It is well known that immune responses to infection present with noteworthy differences between genders and are likely to be driving factors behind the significant sex-bias observed worldwide (27). Among the other biological factors involved, attention has been pointed on possible effects of sex-steroids on genetic regulation of TMPRSS2 and ACE2 expression, the two critical host cell mediators for the spread of SARS-CoV-2. In this regard, though several data documented an androgen-mediated upregulation of TMPRSS2 in the prostate cancer cells (19, 23), only a slight increase in TMPRSS2 transcript has been found in lung males compared to females (7). Other few and controversial observations have been made in lung cells (24, 26). However, a possible modulation of TMPRSS2 protein expression and activity by sex-related hormones in lung cells has not been investigated yet.

Therefore, in the present study we used lung Calu-3 cells to test androgen and estradiol effects on the modulation of TMPRSS2 and ACE2 expression levels, that in turn would

influence SARS-CoV-2 host cell entry. As TMPRSS2 is an androgen responsive gene (7, 19, 20) but it is also regulated by estrogen (18) we first searched for possible changes in TMPRSS2 expression levels in Calu-3 cells stimulated with androgen or estrogen. At first, we could not find any substantial differences in TMPRSS2 mRNA levels, being this result in line with previous studies in lung cells (24) and human lung tissues showing a substantial equal expression patterns of the TMPRSS2 gene between males and females at different ages (7). Accordingly, we could not find differences in the expression levels of the full length 70kDa TMPRSS2 protein but, interestingly, we observed a statistically significant upregulation of TMPRSS2 cleavage upon both androgen and estrogen treatments. Similarly, treatment with mibolerone induced increase in the TMPRSS2 cleavage product in prostate cancer cells (14). Since TMPRSS2 cleavage is a consequence of its own catalytic activity (14, 15), our data suggested that both androgen and estrogen hormones could stimulate the protease function in Calu-3 cells, although a TMPRSS2 enzymatic activity assay could have provided a more definitive answer. Even if observed at a higher concentration, the effect exerted by R1881 seemed more pronounced if compared to estradiol. In Calu-3 cells, SARS-CoV-2-S was already shown to be a TMPRSS2 substrate (8). Here we demonstrated that, as a consequence of its functional activation promoted by androgen and estrogen hormones, TMPRSS2 significantly contributed to efficient proteolytic processing of Spike, generating the subunits S1 and S2. Given that our subclone of Calu-3 cells does not express aromatase we can assume that the increased TMPRSS2 cleavage observed in androgen treated cells was due to the action of androgen itself rather than its conversion into estradiol.

Up to date, several clinical trials are testing direct TMPRSS2 inhibitors as well as modulators of the sex-related hormones pathways indirectly acting on TMPRSS2 activity (28). Nevertheless, results from a meta-analysis conducted to evaluate the effect of androgen deprivation therapy (ADT) on COVID-19 in patients with prostate cancer failed to show a protective action against the risk of infection, hospital admission and mortality in these patients (29).

The S1 subunit of spike contains the ACE2 receptor binding domain, whereas the S2 subunit is anchored to the virus membrane and harbours the fusion machinery. We asked whether sex-related hormones could interfere with ACE2 expression in lung cells, as already seen in other tissues. Indeed, estrogen in atrial tissue has been shown modulate the local renin-angiotensin system *via* upregulation of ACE2 (30). Moreover, ACE2 has been identified as an AR-regulated target in certain types of cells in the lungs (24) and in prostate cancer cells, where both mRNA and protein expression of ACE2 were strongly upregulated by R1881 (25). However, we did not observe any difference in ACE2 expression levels in Calu-3 cells stimulated with estradiol or R188. Similarly, no difference was found in human lung ACE2 transcripts between males and females (7, 31). In our case, it is possible to speculate that the absence of considerable changes in ACE2 expression levels observed upon stimulation with hormones might be due to the abundant levels of this receptor in Calu-3 cells compared to other cell lines (25, 32). TMPRSS2 and HAT proteases can mediate ACE2 proteolytic cleavage in order to augment SARS-S-driven entry (33). Specifically, the 13-kDa fragment of ACE2 has been detected in HEK293 cells transiently transfected with TMPRSS2 or HAT, being this efficiency of ACE2 cleavage dependent on the proteases expression level (33). Here, we also searched for the production of cleaved fragments of ACE2, but none of them were detectable nor under basal condition neither after treatment with hormones. Thus, it is possible to hypothesize that TMPRSS2 levels in Calu-3 cells were not sufficient enough to promote the C-terminal processing of ACE2.

Overall, our data are in agreement with the lack of difference in the percentage of males and females infected with SARS-CoV-2 globally reported (27). However, why males are exposed to higher odds of both intensive therapy unit (ITU) admission and death compared to females is a still an unsolved question and the mechanism underlying the gender disparity in COVID-19 outcomes is expected to be multifactorial. Thus, understanding of the factors that modulate susceptibility to SARS-CoV-2 remains crucial for controlling disease transmission and health consequences.

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

Conceptualization, EP and GioM; investigation, DT, GiuM, GDM, RC, FM, EE, AB; writing—original draft preparation, DT; writing-review and editing, EP and GioM; supervision, MA. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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EDITED BY
Jeff M. P. Holly,
University of Bristol, United Kingdom

REVIEWED BY
Luca Giovanelli,
University of Milan, Italy
Nazareth Novaes Rocha,
Fluminense Federal University,
Brazil

*CORRESPONDENCE
Jingjing Zhang
Doctorzhangjj@csu.edu.cn

†These authors have contributed equally to this work

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Associations between COVID-19 infection and sex steroid hormones

Zixin Cai[1†], Jiaxin Zhong[1†], Yingling Jiang[2] and Jingjing Zhang[1*]

[1]National Clinical Research Center for Metabolic Diseases, Metabolic Syndrome Research Center, Key Laboratory of Diabetes Immunology, Ministry of Education, and Department of Metabolism and Endocrinology, The Second Xiangya Hospital of Central South University, Changsha, China, [2]Department of Metabolism and Endocrinology, The Affiliated Zhuzhou Hospital Xiangya Medical College CSU, Zhuzhou, China

Aims: Coronavirus disease 2019 (COVID-19) is caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and within a few months of the first outbreak, it was declared a global pandemic by the WHO. The lethal virus SARS-CoV-2 is transmitted through respiratory droplets and enters host cells through angiotensin-converting enzyme 2 (ACE-2) receptors. ACE-2 receptors are highly expressed in many tissues, including testes. Therefore, the objective of this study was to summarize the available literature regarding the correlation between sex hormone levels and COVID-19.

Methods: The PubMed, Web of Science, Embase, and Cochrane Library databases were reviewed systematically through August 2022 for studies comparing sex hormone levels between different patient groups: COVID-19 versus no COVID-19, more severe versus less severe COVID-19, and non-survivors versus survivors. Various types of clinical research reporting sex hormone levels, including free testosterone (FT), luteinizing hormone (LH), follicle-stimulating hormone (FSH), 17β-oestradiol (E_2), the oestradiol-to-testosterone ratio (E_2/T), prolactin (PRL), and sex hormone-binding globulin (SHBG), were included. Random- or fixed-effects models were used to calculate weighted mean differences (WMDs) and 95% confidence intervals (CIs). Heterogeneity among the studies was assessed by the I^2 index, and data analyses were performed using meta-analysis with Stata version 12.0.

Results: Twenty-two articles that included 3369 patients were ultimately included in the meta-analysis. According to analysis of the included studies, patients with COVID-19 had significantly low T/LH, FSH/LH, and SHBG levels and high levels of LH, and E_2/T, but their levels of FT, FSH, PRL, E_2, and progesterone were not affected. Publication bias was not found according to funnel plots and Egger's regression and Begg's rank correlation tests.

Conclusion: Low T/LH, FSH/LH, and SHBG serum levels and high LH, and E_2/T levels may increase the risk of COVID-19. Additionally, the greater is the clinical severity of COVID-19, the higher is the probability of increases in LH, and E_2/T serum levels and decreases in T/LH, FSH/LH, and SHBG levels. COVID-19 may

have unfavourable effects on gonadal functions, which should be taken seriously by clinicians. Routine monitoring of sex hormone levels might help clinicians to evaluate disease severity in patients with COVID-19.

KEYWORDS

COVID-19, sex hormones, meta-analysis, predict, WMD

Introduction

In early December 2019, the outbreak of coronavirus disease 2019 (COVID-19) quickly progressed to a pandemic, bringing severe challenges to global health; this disease can be transmitted through respiratory droplets or by direct contact (1). As of September 8th, 2021, there were more than 4,582,338 deaths (2). In addition, vulnerable individuals may experience a variety of serious and life-threatening complications, including acute respiratory distress syndrome (ARDS), sepsis, coagulopathy, disseminated intravascular coagulation (DIC), acute kidney injury (AKI), and multiorgan dysfunction (3–5). Thus, a better understanding of clinical risk factors that can distinguish between severe and non-severe cases or between a high and low risk of death is vital for improving therapeutic interventions.

Currently, studies have indicated that sex differences are present in the clinical outcomes of COVID-19 patients (6). Sex disaggregated data indeed showed that men had higher rates of mortality than women (7). In particular, the mortality rates of men are 2.4 times higher than those of women, thus indicating sex-specific differences in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-associated sequelae (8). Angiotensin-converting enzyme 2 (ACE2) and TMPRSS2 are critical factors for virus transmission. SARS-CoV-2 is mainly transmitted *via* respiratory droplets and enters human cells through ACE-2 receptors (9). In addition, expression pattern analysis of ACE2 in adult human testes indicated that ACE2 is mainly distributed in spermatogonia and Sertoli and Leydig cells, suggesting that the human testis is susceptible to SARS-CoV-2 (10). TMPRSS2 is predominantly expressed in spermatogonia and spermatids. In a retrospective study, a large proportion of the total patients with COVID-19 were male (11). Consequently, concern has been raised about whether SARS-CoV-2 may affect the male reproductive system. It is assumed that SARS-CoV-2 may have a negative impact on the male reproductive tract; however, results are inconsistent.

Method

Search strategy

To find all studies that evaluated the association between sex hormone levels and the risk of COVID-19, two of the authors (ZC and JZ) independently searched the PubMed, Embase, Web of Science, and Cochrane Library databases through August 2022. The search terms included the following key words: (a) COVID-19; (b) testosterone (FT), luteinizing hormone (LH), follicle-stimulating hormone (FSH), 17β-oestradiol (E_2), the oestradiol-to-testosterone ratio (E_2/T), prolactin (PRL), progesterone and sex hormone-binding globulin (SHBG). The reference lists of relevant reviews or included studies were also manually searched to identify relevant articles.

Study selection

Using the PECO/PICO (population, exposure/intervention, comparison/control, and outcome) strategy, we included the studies that met the following criteria in the study.

- Populations: subjects participating in studies that assessed the impact of sex hormone levels on COVID-19.
- Exposure/Intervention: presence or absence of COVID-19
- Comparison: sex hormone levels
- The outcome of the study: sex hormone levels
- Exclusion criteria
- Studies without full text
- *In vitro* and animal studies
- Data of interest were not presented
- Abstracts, commentary articles, reviews, meta-analyses, editorials and conference presentations

Data extraction and quality assessment

Two of us (ZC, JZ) independently extracted the data using a standardized data extraction form. Extracted information included the following: (1) the characteristics of the study, including the first author, year of publication, study design, and country; (2) basic characteristics of the population, including the sample size, mean age, and sex ratio; and (3) sex hormone indicators, including FT, LH, FSH, E_2, E_2/T, PRL, progesterone and SHBG. The weighted mean difference (WMD) and corresponding 95% confidence intervals (CIs) as well as I^2

were extracted. For studies reporting only the median ± interquartile range (IQR), we converted these values into the mean and standard deviation (SD) (12). Any dissenting opinions were resolved through discussion and consensus. Quality assessment of the nonrandomized comparative studies was performed with the Newcastle–Ottawa scale (NOS) (13).

Statistical analysis

Statistical analyses were carried out by STATA (Version 12.0; STATA Corporation, College Station, TX, USA) software. Fixed-effects or random-effects models were adopted according to the heterogeneity of the studies (I^2 < 50%, fixed-effects models; I^2 > 50%, random-effects models). The WMD with the random-effects model (DerSimonian–Laird method) and 95% CI were applied for continuous data. Sensitivity analysis was conducted by excluding one study each time through influence analysis to assess the stability of the results. Heterogeneity among the included studies was assessed with the I^2 statistic. I^2 values above 70% were considered to indicate the presence of extreme heterogeneity. The potential evidence of publication bias was assessed using a funnel plot, Egger's regression and Begg's rank correlation tests. If publication bias was confirmed, it was corrected using Duval's trim-and-fill method used the properties of the funnel plot. Subgroup analysis was conducted based on the severity of the disease or age and country. Statistical significance was determined with a two-tailed $p < 0.05$.

Results

Study selection

Following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines, we included 22 studies that involved 3369 patients and satisfied our inclusion/exclusion criteria for meta-analysis. The preliminary literature search resulted in 5218 articles, and 756 studies remained after exclusion due to duplication. After scanning the titles and abstracts, we obtained 52 studies by excluding an additional 346 studies. After reading the full texts and review articles, we further excluded 30 studies that did not report sex hormone levels. Finally, 22 studies (14–30) (19, 31–33) were included in our analysis. The process of study identification and selection is shown in Figure 1.

Description of included studies

In total, there were 3369 patients: 8 studies from Turkey (930 patients), 7 from Italy (975 patients), 5 from China (773 patients), and 1 each from France (118 patients), and the USA (152 patients). The patient ages ranged from 18 to 73 years old. Overall, there were nine cohort studies, six case–control studies, and seven cross-sectional studies. Virtually all respondents were male, with only four studies including female respondents. The articles were published during the period from 2020 to 2021. According to the NOS scores, all studies were deemed to be of high quality. The characteristics

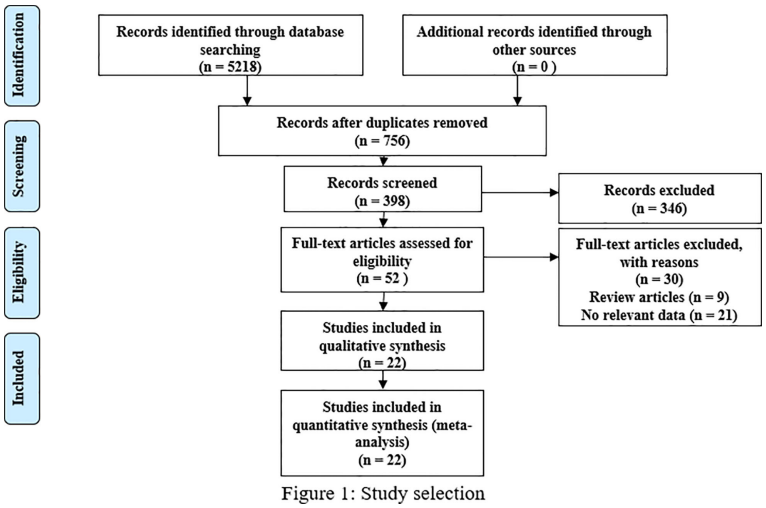

Figure 1: Study selection

FIGURE 1
Flow of study selection.

of the 22 eligible studies and their NOS scores are summarized in Table 1.

Overall analysis

Results of the meta-analysis of free testosterone levels in the COVID-19 group and controls

Overall, we found a milieu of clinical androgenicity (sexual function, bone density and haematopoiesis) resulting from the interaction between polyQ polymorphism in the androgen receptor (AR) and serum testosterone levels. In particular, the calculated FT correlated better than total testosterone (TT) with all relevant clinical parameters of androgenicity, and androgenicity was reduced when the AR showed a large number of CAG repeats. We identified 3 studies published from 2020 to 2022 (three cohort studies) that presented results on FT levels and COVID-19, including a total of 194 subjects. Subgroup analyses were stratified by disease severity (patients with COVID-19 vs. controls; patients with severe COVID-19 vs. those without severe COVID-19. In 1 study that measured levels of FT in patients with COVID-19 or controls, the aggregated WMD was 0.03, with a 95% CI of 0.01 and 0.05. Based on 3 studies that compared patients with severe COVID-19 vs. those without severe COVID-19, the pooled WMD was -0.08 (95% CI: -0.17 to 0.02) (Table 2 and Figure 2). Egger's test showed that no publication bias existed among the included studies ($p > 0.05$). The publication bias analysis showed that the funnel plot was nearly symmetrical (Figure 7A). The results of the sensitivity analyses indicated that the conclusions were robust (Figure 8A). The above results revealed that patients with COVID-19 have no significance FT levels than subjects without COVID-19.

Results of the meta-analysis of FSH levels in the COVID-19 group and controls

We identified 12 studies published from 2020 to 2021 (four case–control studies, five cohort studies, and three cross-sectional studies) that presented results on FSH levels and COVID-19. The 12 studies included a total of 3257 subjects. The effect size from the random-effects model showed no significant changes in FSH levels (pooled WMD: 0.60, CI: -0.14 and 1.35) (Figure 3). The heterogeneity test results found obvious heterogeneity ($I^2 = 89.1\%$, $p = 0.000$). Sensitivity analysis confirmed that no individual study influenced the overall results.

Subgroup analyses were stratified by sex (male, female), study design (cohort, case–control and cross-sectional) and country (Italy, Turkey and China). In 1 study with females that assessed levels of FSH in patients, the aggregated WMD was 19.65, with a 95% CI of -1.12 and 40.42. From 11 studies with males, the pooled WMD was found to be 0.58 (95% CI: -0.16 to 1.32). In 4 studies with a cohort design that assessed the levels of FSH in patients, the aggregated WMD was 2.05 with a 95% CI of 1.03 and 3.07. From 3 studies using a case–control design, the pooled SDM was found to be 0.02 (95% CI: -0.30 to 0.34). From 3 studies with a cross-sectional design, the pooled WMD was found to be 0.22 (95% CI: -0.93 to 1.38). Subgroup analysis was performed according to country, and decreased FSH levels were found in Turkey but not in Italy or China (pooled WMD: 1.21, CI: 0.24 and 2.19 for Turkey; pooled WMD: -1.13, CI: -3.15 and 0.88 for Italy; pooled WMD: -0.08, CI: -0.85 and 0.69 for China) (Table 2). Egger's test showed that no publication bias was present among the included studies ($p > 0.05$). The publication bias analysis showed that the funnel plot was nearly symmetrical (Figure 7B). The results of the sensitivity analyses indicated that the conclusions were robust (Figure 8B). The above results revealed that patients with COVID-19 have higher FSH levels than individuals without COVID-19.

Results of the meta-analysis of LH levels in the COVID-19 group and controls

We identified 13 studies published from 2020 to 2021 (four case–control studies, five cohort studies, and four cross-sectional studies) that presented results on LH levels and COVID-19. The 13 studies included a total of 3288 subjects. The effect size from the random-effects model showed a significant increase in LH levels (pooled WMD: 0.92, CI: 0.12 and 1.72) (Figure 4). The heterogeneity test results found obvious heterogeneity ($I^2 = 93.4\%$, $p = 0.000$). Sensitivity analysis confirmed that no individual study influenced the overall results.

Subgroup analyses were stratified by sex (male, female), disease severity (patients with COVID-19 vs. controls; patients with severe COVID-19 vs. those without severe COVID-19), age (younger than 50 years old vs. older than 50 years old), and study design (cohort, case–control and cross-sectional). In 1 study that measured levels of LH in female patients with COVID-19 or controls, the aggregated WMD was 11.30, with a CI of -7.88 and 30.48. From 12 studies that compared male patients with COVID-19 or those without COVID-19, the pooled WMD was 0.90 (95% CI: 0.10 to 1.71). In 7 studies that measured the levels of LH in patients with COVID-19 or controls, the aggregated WMD was 1.42 with a CI of 0.21 and 2.63. From 6 studies that compared patients with severe COVID-19 with those without severe COVID-19, the pooled WMD was found to be -0.48 (95% CI: -1.59 to 0.63). In 5 studies with a cohort design that assessed the levels of LH in patients, the aggregated WMD was 1.11 with a CI of 0.06 and 2.16. From 4 studies with a case–control design, the pooled WMD was found to be 1.57 (95% CI: 0.33 to 2.82). From 4 studies that used a cross-sectional design, the pooled SDM was found to be -0.17 (95% CI: -0.93 to 1.38). In a subgroup

TABLE 1 Studies included in the meta-analysis.

Number	Author	Year	Country	Type of study	Sample size	Female/Male	Age (Mean±SD)	BMI (Mean±SD)	Sex hormone	NOS	Short description
1	M. Infante	2021	Italy	Cross-sectional	59	Only Male	Survivors:64.10 ± 13.02 Non-survivors:68.23 ± 12.74	Survivors: 28.03 ± 1.95 Non-survivors: 28.5 ± 3.0	TT, E2, E2/T, Progesterone, PRL	8	The study aimed to test the correlation between serum levels of sex hormones [total testosterone, estradiol (E2), estradiol to testosterone (E2/T) ratio, progesterone), prolactin and 25-hydroxyvitamin D [25(OH)D]and markers of inflammation, coagulation and sepsis at admission in hospitalized men with COVID-19.
2	Abdullah Gul	2021	Turkey	Cross-sectional	29	Only Male	COVID-19 patients:31.21±5.48	COVID-19 patients: 27.05±2.34	FSH, LH, TT, PRL	8	The aim of the study has been to investigate the long-term effects of SARS-CoV-2 infection (COVID-19) and its relative treatment on male reproductive health.
3	Erdem Koç	2021	Turkey	Cross-sectional	21	Only Male	COVID-19 patients:32 ±6.30	COVID-19 patients: 25.62±2.12	FSH, LH, TT	7	The study aimed to evaluate the effect of COVID-19 on the semen parameters and sex-related hormone levels in infertile men.
4	Ling Ma	2021	China	Case-control	392	Only Male	patients with COVID-19:39.33±7.74 Control: 38.66±6.02	-	TT, FSH, LH, T/LH, FSH/LH	9	It's the first report about semen assessment and sexhormone evaluation in reproductive-aged male COVID-19 patients.
5	Mustafa Zafer Temiz	2020	Turkey	Cross-sectional	30	Only Male	Control:36.64±9.63 COVID-19 patients Before treatment:38.00 ±8.28 COVID-19 patients After treatment:37.00±8.69	Control: 26.57 ± 2.71 COVID-19 patients Before treatment:25.55 ± 2.08 COVID-19 patients After treatment :26.55 ± 1.14	FSH, LH, PRL, TT, T/LH, FSH/LH, PRL/T	8	The study investigated whether there is a male reproductive system coronavirus disease-2019 (COVID-19) phenomenon.
6	Hui Xu	2020	China	Cohort	61	Only Male	COVID-19 patients: 57.33±14.62 Control: 60.91±13.27	COVID-19 patients: 25.1±2.8 Control: 26.9±3.6	TT, FT, FSH, LH, PRL, E2, T/LH	7	The study aimed to assess whether SARS-CoV-2 infection can affect sex-related hormones and testicular function in recovering patients. In males infected with SARS-CoV-2, most sex-related hormones (T, FSH and LH levels) remain within the normal reference ranges after recovery from COVID-19, and no significant associations were observed between T level and disease duration or severity .
7	Marta Camici	2021	Italy	Case-control	48	Only Male	COVID-19 patients: 50.66±12.60 Control:50.33±11.03	-	TT, SHBG	8	The study aimed to investigate the association between sex hormones and the severity of coronavirus disease 2019 (COVID-19). A low level of testosterone was found to be a marker of clinical severity of COVID-19.
8	Andrea Salonia	2021	Italy	Case-control	567	Only Male	COVID-19 patients:57.66±12.66 Healthy Control:44.33 ±12.66	COVID-19 patients:27.9±4.24 Healthy Control:25.4 ±1.93	FSH, LH, TT, E2	9	The study aimed to assess: (a) circulating sex steroids levels in a cohort of 286 symptomatic men with laboratory-confirmed COVID- 19 at hospital admission compared to a cohort of 281 healthy men; and (b) the association between serum testosterone levels (tT), COVID- 19, and clinical outcomes.

(Continued)

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TABLE 1 Continued

Number	Author	Year	Country	Type of study	Sample size	Female/Male	Age (Mean±SD)	BMI (Mean±SD)	Sex hormone	NOS	Short description
9	Sezgin Okçelik	2020	Turkey	Case-control	44	Only Male	from 18 to 50 years	-	FSH, LH, TT	7	The study aimed to evaluate the testicular damage caused by COVID-19, Testosterone levels seem to decrease during acute COVID-19 infection, especially in the patient group with viral pneumonia.
10	Andrea Salonia	2021	Italy	Cohort	121	Only Male	COVID-19 patients: 57.00±12.00	-	FSH, FSH, TT, E2	8	The study aimed to assess total testosterone levels and the prevalence of total testosterone still suggesting for hypogonadism at 7-month follow-up in a cohort of 121 men who recovered from laboratory-confirmed COVID-19.
11	Giulia Rastrelli	2020	Italy	Cohort	31	Only Male	Transferred to IM :61.50±9.14 In charge in RICU:62.83 ±48.15 Transferred to ICU/ deceased :73.00±27.84	-	TT, SHBG, LH	7	The study aimed to estimate the association between T level and SARS-CoV-2 clinical outcomes (defined as conditions requiring transfer to higher or lower intensity of care or death) in a cohort of patients admitted in the respiratory intensive care unit (RICU).
12	Selahittin Çayan	2020	Turkey	Cohort	221	Only Male	Asymptomatic group:34.83 ± 12.51 IMU group:44.54 ± 17.63 ICU group:56.8 ± 18.57	Asymptomatic group:23.87±3.6 IMU group:23.62±3.6 ICU group:24.18±2.83	FSH, LH, TT, Prolactin, E2	8	The study aimed to investigate effect of serum total testosterone and its relationship with other laboratory parameters on the prognosis of coronavirus disease 2019 (COVID-19) in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infected male patients.
13	Sandeep Dhindsa	2021	USA	Cohort	152	COVID-19 patients :152 (62/90)	Severe COVID-19: Men :68 ± 11; Women:68±14 Without Severe COVID-19: Men: 55 ± 15; Women:51 ±19	Severe COVID-19: Men: 26.7±6.0; Women:32.6±9.3 Without severe COVID-19: Men: 30.0±8.0 ; Women:34.3 ±8.1	TT, E2, E2/T	8	The study aimed to investigate the association of concentrations of serum testosterone, estradiol, and insulinlike growth factor 1 (IGF-1, concentrations of which are regulated by sex hormone signaling) with COVID-19 severity.
14	Anna Beltrame	2022	Italy	Cohort	120	COVID-19 patients :120 (52/68)	50 years and over, stratified by sex and outcome.	-	TT, E2, Progesterone	7	The study investigated sex hormone levels and their association with outcomes in COVID-19 patients, stratified by sex and age.In males, higher testosterone seems to be protective against any considered outcome.
15	Ting Ding	2020	China	Cross-sectional	78	Only Female	younger than 60 years of age	-	E2, AMH, LH, TT, FSH, FSH/LH、PRL	7	The study aimed to find the factors that potentially protect females from COVID-19, Menopause is an independent risk factor for female COVID-19 patients.
16	Ahmet Emre Cinislioglu	2021	Turkey	Cohort	450	Only Male	COVID-19 patients:64.9±11.6 Control:67.2±13.6	COVID-19 patients: 25.9±3.8 Control:26.4±3.1	TT, FSH, LH, T/L	8	The study aimed to investigate the relationship of serum testosterone with other laboratory parameters on the prognosis of coronavirus disease-19 (COVID-19) in male patients with COVID-19 diagnosis.
17	Shufa Zheng	2021	China	Cross-sectional	61	Only Male	Non-ICU:50.03±18.87 ICU:63.26±17.54	-	TT	8	The purpose of this study is to analyze the relationship between testosterone changes and disease severity in male patients with COVID-19 and to compare the differences in

(Continued)

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TABLE 1 Continued

Number	Author	Year	Country	Type of study	Sample size	Female/Male	Age (Mean±SD)	BMI (Mean±SD)	Sex hormone	NOS	Short description
											transcriptome expression in patients with different testosterone levels.
18	Emre Urhan	2021	Turkey	case-control	54	COVID-19 patients : 43 (19/24) Control : 11 (5/6)	COVID-19 patients:44.28±10.76 Control:44.18±12.41	COVID-19 patients:31.04±5.92 Control:29.92±3.29	PRL	8	The study investigated the pituitary functions three to seven months after acute COVID-19 infection.
19	Stefano Salciccia	2020	Italy	Cross-sectional	29	Only Male	None O2 assistance:57.00±58.60 Invasive O2 assistance:63.66±40.69	-	TT	7	The study aimed to evaluate whether serum TT levels among a cohort of 29 COVID-19 men at the time of hospital admission were associated with the need for "invasive" oxygenation strategy. and may allow for patient monitoring and predict disease outcome.
20	Tugce Apaydin	2022	Turkey	Cohort	81	Only Male	Mild-moderate COVID-19 patients:43.16±28.94 Severe COVID-19 patients:46.33±25.82	-	FSH, LH, TT, FT, SHBG	8	The study aimed to evaluate the acute and chronic effects of coronavirus disease 2019 on gonadal functions.
21	Ling Ma	2020	China	Case-control	181	Only Male	Men with COVID-19: 38.33±6.03 Age-matched healthy men:38.00±4.51	-	TT, FSH, LH, PRL, T/LH, FSH/LH, AMH, E2, T/E2	9	This study provides the first direct evidence about the influence of medical condition of COVID-19 on male sex hormones, alerting more attention to gonadal function evaluation among patients recovered from SARS-CoV-2 infection, especially the reproductive-aged men.
22	Kamila Kolanska	2021	France	Cohort	118	Only Female	COVID-19 positive:35.7±4.2 COVID-19 negative:34.5±4.5	COVID-19 positive: 23.1±3.7 COVID-19 negative:24.3±5.5	AMH	9	The aim of this prospective study was to evaluate the effect of mild COVID-19 infection on the ovarian reserve of women undergoing an assisted reproductive technology (ART) protocol.

10.3389/fendo.2022.940675

TABLE 2 Sex hormone levels.

Sex hormone parameters	Subgroup	Study	Number of patients	WMD	95% CI	I2
FT	COVID-19 VS Control	1	61	0.03	(0.01, 0.05)	0.00%
	Severe VS non-severe	3	133	-0.08	(-0.17, 0.02)	70.97%
FSH	Cohort	4	1249	2.05	(1.03, 3.07)	72.30%
	Case-control	3	838	0.02	(-0.3, 0.34)	0.00%
	Cross-sectional	3	178	0.22	(-0.93, 1.38)	41.30%
	Italy	2	809	-1.13	(-3.15, 0.88)	93.20%
	Turkey	6	1658	1.21	(0.24, 2.19)	87.70%
	China	4	790	-0.08	(-0.85, 0.69)	45.50%
	Male	11	3409	0.58	(-0.16,1.32)	89.50%
	Female	1	78	19.65	(-1.12,40.42)	0.00%
LH	COVID-19 VS Control	7	1737	1.42	(0.21, 2.63)	95.30%
	Severe VS non-severe	6	1031	-0.48	(-0.59, 0.63)	64.50%
	Cohort	5	880	1.11	(0.06, 2.16)	84.20%
	Case-control	4	1230	1.57	(0.33, 2.82)	93.70%
	Cross-sectional	4	209	-0.17	(-1.42, 1.08)	51.70%
	Younger than 50 years old	6	841	1.28	(0.06, 2.5)	90.90%
	Old than 50 years old	7	2447	0.66	(-0.43, 1.75)	94.40%
	Male	12	3461	0.9	(0.10, 1.71)	93.70%
	Female	1	78	11.3	(-7.88, 30.48)	0.00%
PRL	Cohort	2	360	1.13	(0.16, 2.10)	0.00%
	Case-control	3	294	2.26	(-13.60, 18.13)	99.20%
	Cross-sectional	3	156	0.12	(-0.91, 1.15)	0.70%
	Male	6	739	0.68	(-0.01,1.37)	0.00%
	Female	1	78	-0.77	(-5.28,3.74)	0.00%
E2	COVID-19 VS Control	4	857	6.49	(0.27, 12.7)	90.30%
	Severe VS non-severe	5	548	0.51	(-3.22, 4.25)	61.90%
	Non-survivor VS survivor	2	179	7.36	(-7.98, 22.69)	85.90%
	Male	9	1729	0.15	(-0.16,0.46)	87.60%
	Female	2	182	-5.36	(-32.44,21.71)	79.60%
E2/T	COVID-19 VS Control / Severe VS non-severe	3	330	0.89	(-2.17, 3.96)	76.10%
Progesterone	Severe VS non-severe	3	377	0.01	(-0.05, 0.07)	73.40%
	Male	2	195	0.24	(-0.48,0.97)	76.10%
	Female	2	182	-0.02	(-0.98,0.42)	75.20%
SHBG	COVID-19 VS Control / Severe VS non-severe	3	160	-8.69	(-17.20, -0.18)	0%
T/LH	COVID-19 VS Control / Severe VS non-severe	4	1778	-1.1	(-1.42, -0.79)	85.30%
FSH/LH	COVID-19 VS Control / Severe VS non-severe	3	651	-0.66	(-0.74, -0.57)	0.00%

analysis according to age, LH levels were found to be significantly higher only in patients younger than 50 years old but not in those older than 50 years old (pooled WMD: 1.28, CI: 0.06 and 2.5 for younger than 50 years old; pooled WMD: 0.66, CI: -0.43 and 1.75 for older than 50 years old) (Table 2). Egger's test showed that no publication bias was present among the included studies ($p > 0.05$). The publication bias analysis showed that the funnel plot was nearly symmetrical (Figure 7C). The results of the sensitivity analyses indicated that the conclusions were robust (Figure 8C). The above results revealed that patients with COVID-19 present higher LH levels than subjects without COVID-19.

Results of the meta-analysis of PRL levels in COVID-19 and control groups

We identified 8 studies published from 2020 to 2021 (three case–control studies, two cohort studies, and three cross-sectional studies) that presented results on PRL levels and COVID-19. The 8 studies included a total of 810 subjects. The effect size from the fixed-effects model showed a significant increase in PRL levels (pooled WMD: 0.65, CI: -0.03 and 1.33) (Figure 5).

Subgroup analyses were stratified by sex (male, female), study design (cohort, case–control and cross-sectional). In 1 study with females that detected levels of PRL in patients, the aggregated WMD was -0.77, with a CI of -5.28 and 3.74. From 6 studies

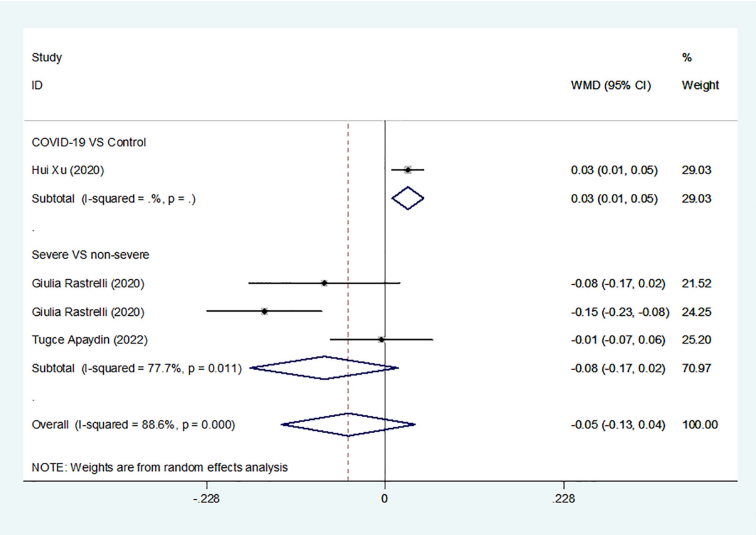

FIGURE 2
FT levels in people with COVID-19 versus those without COVID-19.

involving males, the pooled WMD was 0.68 (95% CI: -0.01 to 1.37). In 2 studies with a cohort design that detected the levels of PRL in patients, the aggregated WMD was 1.13 with a CI of 0.16 and 2.10. From 3 studies with a case–control design, the pooled WMD was found to be 2.26 (95% CI: -13.60 to 18.13). From 3 studies that used a cross-sectional design, the pooled WMD was found to be 0.12 (95% CI: -0.91 to 1.15). Egger's test showed that no publication bias existed among the included studies ($p > 0.05$). The publication bias analysis showed that the funnel plot was nearly symmetrical (Figure 7D). The results of the sensitivity analyses indicated that

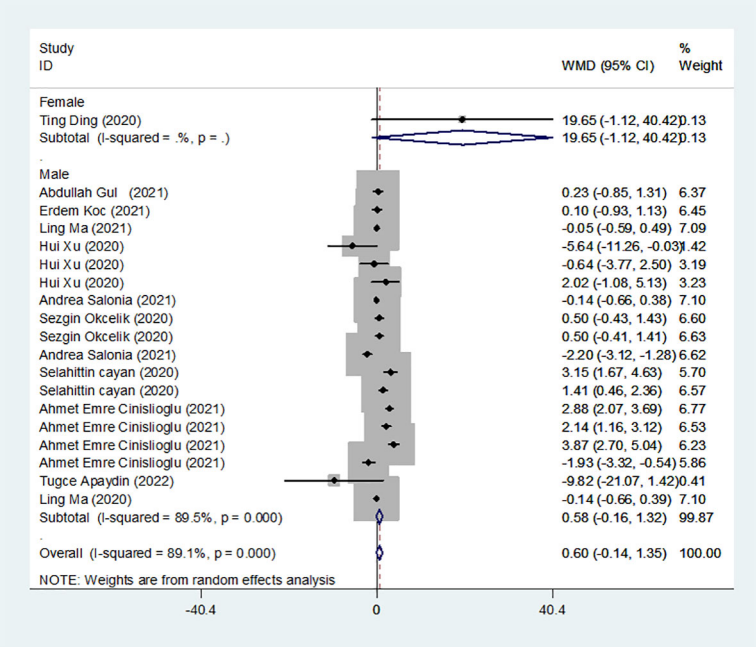

FIGURE 3
FSH levels in people with COVID-19 versus those without COVID-19.

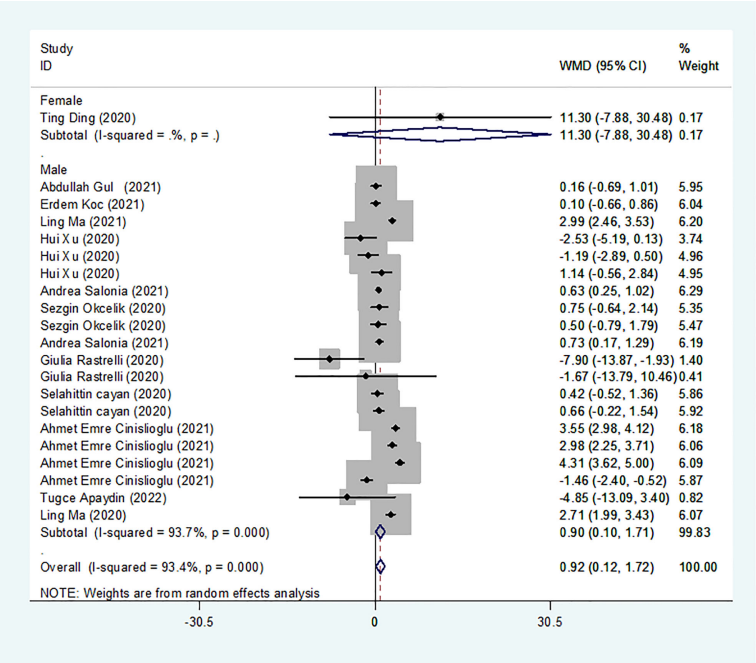

FIGURE 4
LH levels in people with COVID-19 vsersus those without COVID-19.

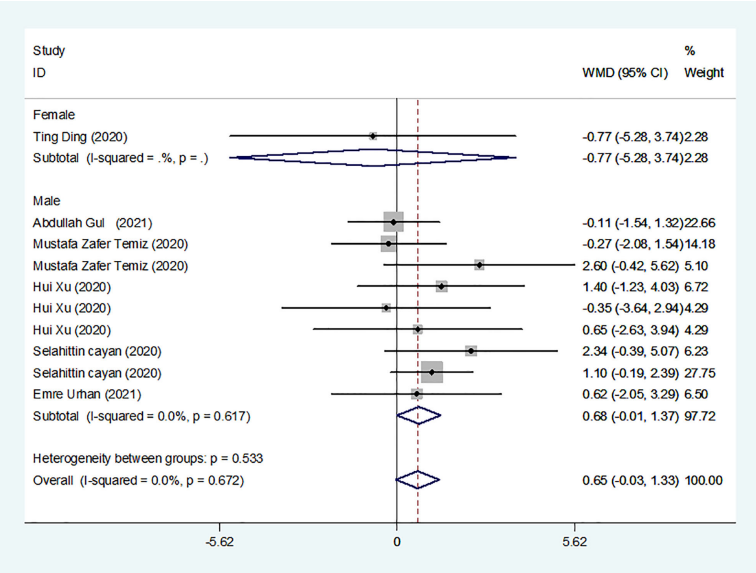

FIGURE 5
PRL levels in people with COVID-19 versus those without COVID-19.

the conclusions were robust (Figure 8D). The above results revealed that patients with COVID-19 present no significant changes in PRL levels compared to subjects without COVID-19.

Results of the meta-analysis of E_2 levels in the COVID-19 group and controls

We identified 10 studies published from 2020 to 2021 (three case–control studies, four cohort studies, and two cross-sectional studies) that presented results on E_2 levels and COVID-19. The 10 studies included a total of 1584 subjects. The effect size from the random-effects model showed no significant difference in PRL levels (pooled WMD: 0.88, CI: -2.19 and 3.95) (Figure 6). The heterogeneity test results found obvious heterogeneity (I^2 = 77.6%, p = 0.000).

Subgroup analyses were stratified by sex (male, female), disease severity (patients with COVID-19 vs. controls; patients with severe COVID-19 vs. those without severe COVID-19; non-survivors vs. survivors). In 2 studies that measured levels of E_2 in female patients with COVID-19 or controls, the aggregated WMD was -5.36, with a CI of -32.44 and 21.71. Based on 7 studies comparing male patients with and without COVID-19, the pooled WMD was found to be 0.89 (95% CI: -2.17 to 3.95). In 4 studies that measured the levels of E_2 in patients with COVID-19 or controls, the aggregated WMD was 6.49 with a CI of 0.27 and 12.7. From 5 studies that compared patients with severe COVID-19 with those without severe COVID-19, the pooled WMD was found to be 0.51 (95% CI: -3.22 to 4.25). Non-survivors vs. survivors were assessed in only 2 studies, with a WMD value of 7.36 (95% CI: -7.98 to 22.69). Egger's test showed that no publication bias existed among the included studies (p > 0.05). The publication bias analysis showed that the funnel plot was nearly symmetrical (Figure 7E). The results of the sensitivity analyses indicated that the conclusions were robust (Figure 8E). The above results revealed that patients with COVID-19 present lower E_2 levels than subjects without COVID-19.

Results of the meta-analysis of progesterone, SHBG, T/LH, FSH/LH, and E_2/T levels in the COVID-19 group and controls

We identified 3 studies published from 2020 to 2021 that presented results on progesterone levels and COVID-19. The 3 studies included a total of 196 subjects and 181 COVID-19 patients. The effect size from the random-effects model showed a significant increase in progesterone levels (pooled WMD: 0.01, CI: -0.05 and 0.07) (Table 2). The heterogeneity test results found obvious heterogeneity (I^2 = 73.4%, p = 0.000).

We identified 3 studies published from 2020 to 2021 that presented results on SHBG levels and COVID-19. The 3 studies included a total of 120 subjects and 61 COVID-19 patients. The effect size from the random-effects model showed a significant increase in SHBG levels (pooled WMD: -8.69, CI: -17.20 and

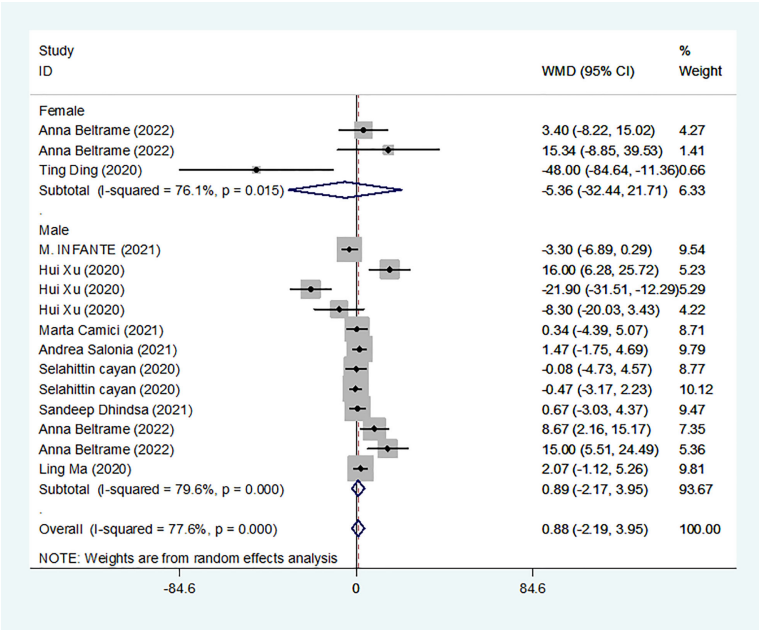

FIGURE 6
E_2 levels in people with COVID-19 versus those without COVID-19.

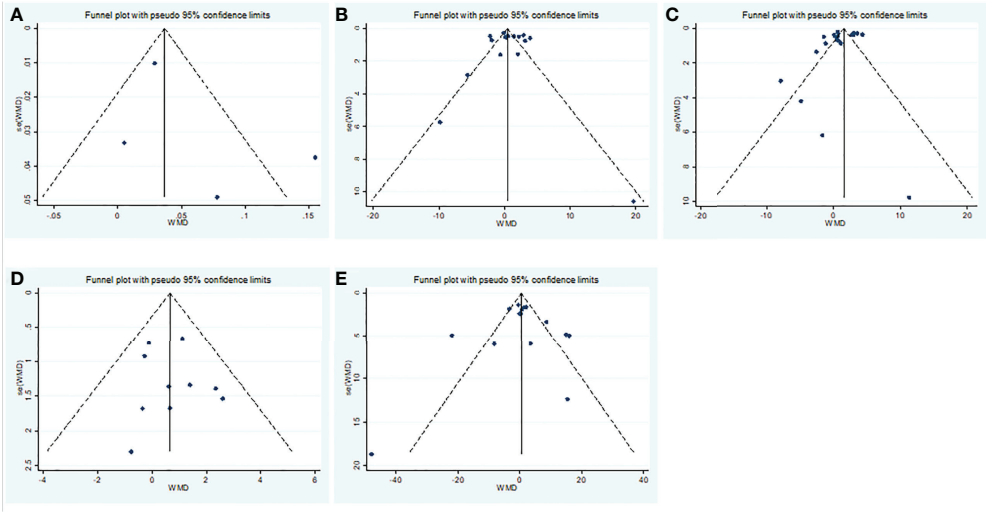

FIGURE 7
Publication bias funnel plots of the WMD for **(A)** TT, **(B)** FSH, **(C)** LH, **(D)** PRL, **(E)** E_2 and COVID-19.

-0.18) (Table 2). The heterogeneity test results found obvious heterogeneity (I^2 = 0%, p = 0.000).

We identified 4 studies published from 2020 to 2021 that presented results on T/LH levels and COVID-19. The 4 studies included a total of 874 subjects and 1088 COVID-19 patients. The effect size from the random-effects model showed a significant increase in T/LH values (pooled WMD: -0.95, CI: -1.36 and -0.55) (data not shown). The heterogeneity test results found obvious heterogeneity (I^2 = 93.4%, p = 0.000). Sensitivity analyses were performed and showed that the study type was the main factor impacting the results (pooled WMD: -1.10, CI: -1.42 and -0.79) (Table 2).

We identified 3 studies published from 2020 to 2021 that presented results on FSH/LH levels and COVID-19. The 3 studies included a total of 434 subjects and 217 COVID-19 patients. The effect size from the random-effects model showed a significant increase in FSH/LH values (pooled WMD: -0.66, CI: -0.74 and -0.57) (Table 2). The heterogeneity test results found no heterogeneity (I^2 = 0%, p = 0.000).

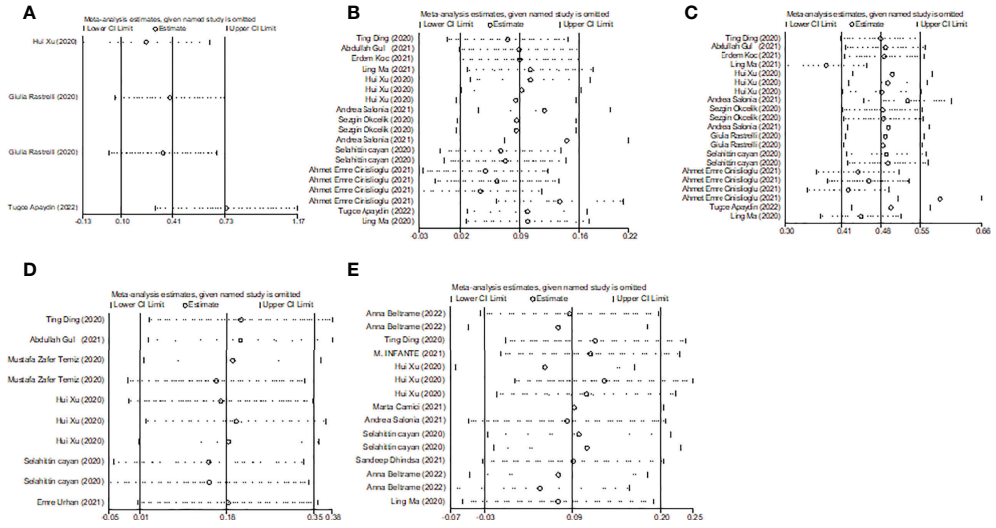

FIGURE 8
Sensitivity analyses of the WMD for **(A)** TT, **(B)** FSH, **(C)** LH, **(D)** PRL, **(E)** E_2 and COVID-19.

We identified 3 studies published from 2020 to 2021 that presented results on E_2/T levels and COVID-19. The 3 studies included a total of 144 subjects and 186 COVID-19 patients. The effect size from the random-effects model showed a significant increase in E_2/T values (pooled WMD: 0.40, CI: 0.18 and 0.63) (Table 2). The heterogeneity test results found no heterogeneity (I^2 = 26.4%, p = 0.000).

Discussion

Association between COVID-19 and sex hormone levels

To the best of our knowledge, our meta-analysis is the first to systematically assess the potential correlation between COVID-19 and sex hormone levels. In total, we included 22 studies for estimation of the effect size for sex hormone levels. Overall, patients with COVID-19 exhibited a significant change in the levels of sex hormones. Specifically, COVID-19 patients showed a significant decrease in the levels of T/LH, FSH/LH, and SHBG hormones. In contrast, COVID-19 patients displayed a significant increase in the levels of LH, and E_2/T. There were no significant changes in levels of FT, FSH, PRL, E_2, or progesterone. Sensitivity analysis and subgroup analyses both identified an obvious association between sex hormone levels and the risk of COVID-19.

Low SHBG levels seem to correlate with worse COVID-19 prognosis (Table 2). SHBG levels are known to be inversely related to obesity and insulin-resistance (34). Increasing evidence demonstrates that obesity is reversely associated with the development of COVID-19 (35). Thus, low plasma SHBG level predicts the severity of COVID-19.

Notably, this is closely linked to the infection-related failure of homeostatic HPG axis feedback to compensate for weakened AR signalling (36). Production of testosterone by testicular Leydig cells is tightly regulated by the hypothalamic-pituitary-gonadal (HPG) axis, forming a homeostatic negative feedback loop (37). Secondary hypogonadism involves pathology of the pituitary or hypothalamus, leading to disturbance in the HPG axis and subsequently in reduced testosterone (38). In addition, the overall milieu of clinical androgenicity (sexual function, bone density and haematopoiesis) results from interaction between polyQ polymorphism in the androgen receptor (AR) and serum testosterone (T) levels. The AR gene, located on the X chromosome, encodes a member of the receptor group that binds to and mediates the actions of androgens (39). AR gene polymorphisms and testosterone level may enhance the behaviours involved in obtaining and maintaining high social status and reproductive success in men (40). Therefore, HPG axis insufficiency and androgen receptor polymorphisms are jointly involved in testosterone function. According to the present meta-analysis, higher gonadotropin concentrations (LH) may increase COVID-19 risk and severity, whereas FT was relatively lower in these patients. These results suggest a decrease in peripheral organ function and a compensatory increase in central function. While, a compensatory increase in central function" which may be nonetheless insufficient due to the "infection-related failure of homeostatic HPG axis feedback.

Many risk factors associated with the progression of COVID-19 to a severe and critical stage have been identified, including old age, male sex, underlying comorbidities such as hypertension, diabetes, obesity, chronic lung disease, heart, liver and kidney diseases, and tumours, clinically apparent immunodeficiencies, local immunodeficiencies such as early type I interferon secretion capacity, and pregnancy. Sex hormones such as oestrogen and testosterone as well as sex chromosome complement likely contribute to sex differences in blood pressure (BP) and cardiovascular disease (CVD). At the cellular level, differences in cell senescence pathways may contribute to increased longevity in women and may also the limit organ damage caused by hypertension. In addition, many lifestyle and environmental factors - such as smoking, alcohol consumption and diet - may influence BP and CVD in a sex-specific manner. Evidence suggests that cardioprotection in women is lost under conditions of obesity and type 2 diabetes mellitus. Treatment strategies for hypertension and CVD that are tailored according to sex may lead to improved outcomes for affected patients. We also found that FSH and LH levels to be significantly increased in female COVID-19 patients. However, in male patients, only LH levels were significantly increased, and there was no significant difference in FSH expression. Treatment strategies for COVID-19 that are tailored according to sex may lead to improved outcomes for patients. This also reflects the different manifestations of sex in disease.

Underlying mechanisms of COVID-19 effects on sex hormone levels

In COVID-19, SARS-CoV-2 may directly act on ACE2-positive spermatogonia, Sertoli cells and Leydig cells, resulting in disruption of spermatogenesis and male gonadal function (41).

Furthermore, in addition to the direct damage to the testes by viruses, other factors, such as fever, inflammation, and dysregulation of the hypothalamic-pituitary-gonadal axis (HPG axis), may also play a role in testosterone secretion or sperm production (42).

During viral infection, virus-induced inflammation leads to systemic or local production of cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interferon (IFN) and monocyte chemoattractant protein 1 (MCP-1). These cytokines are harmful to testicular cells. For example, IL-6 inhibits the

differentiation of Leydig cells. In addition, IFN-γ has been shown to suppress the expression of the rate-limiting enzyme steroidogenic acute regulatory protein (StAR) to inhibit testosterone production (43–45). In addition, COVID-19 has also been reported within the central nervous system, including increased antidiuretic hormone secretion (46). The emotional, physical, or psychological stresses and pain associated with infections can affect the hypothalamohypophyseal axis (47). Thus, abnormalities in the hypothalamic-pituitary axis and abnormalities in LH secretion rhythm may also be a possible cause.

Aromatase is the enzyme responsible for the conversion of testosterone and androstenedione into E_2 and oestrone, respectively. In men, aromatase is expressed in the testes (mainly in Leydig cells) as well as in a number of extragonadal sites, including adipose tissue, bone, the breasts and the brain (48). The upregulation of aromatase enzyme production in adipose tissue during critical illness (as a possible consequence of the excessive production of proinflammatory cytokines) may promote the conversion of testosterone to oestradiol (49–52).

Inflammatory mediators contribute to the suppression of the gonadal axis, and an increased metabolic clearance rate of testosterone may be the underlying cause of lower testosterone levels (53, 54).

There was no significant difference in the values of oestradiol in patients with COVID-19. The probable cause is that a potential upregulation of aromatase enzyme production in adipose tissue during COVID-19, possibly due to inflammatory cytokines, is likely to increase the conversion of testosterone to oestradiol.

Strengths and limitations

The advantages of this study lie in the study design, which included cohort studies, cross-sectional studies, and case-control studies. To the best of our knowledge, our meta-analysis comprising 22 studies is the largest and first meta-analysis to evaluate the relationship between sex hormone levels and COVID-19. There are, however, also a number of disadvantages. First, the present meta-analysis had substantial heterogeneity across studies, which might be due to differences in study design and inconsistencies in baseline characteristics. Second, none of the studies were RCTs, so any causal pathway conclusions should be treated with appropriate caution. Third, many factors may have an impact on sex hormone levels, including genetic polymorphisms, age, other health conditions, sun exposure behaviour, and season. We thus cannot rule out that potential risk factors might have influenced our results. Finally, there is no specific cut-off point for sex hormone levels, which is important for clinical relevance. In the future, we will consider additional confounding factors to obtain more reliable and repeatable results.

Conclusion

Overall, this study demonstrated that changes in sex hormone levels, such as decreased T/LH, FSH/LH, and SHBG levels and elevated LH, and E_2/T levels, were strongly correlated with the severity and prognosis of COVID-19. We suggest that clinicians should be aware of the changes in sex hormone parameters of COVID-19 patients and seek guidance for treatment.

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

JinZ coordinated the study. ZC had the idea for the study, along with JiaZ, YJ and JinZ, contributed to the study design, literature search, figures, statistical analysis, data synthesis of outcomes and drafted and edited the final paper. All authors critically revised the report. All members have confirmed and agreed to submit the 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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EDITED BY
Richard Ivell,
University of Nottingham,
United Kingdom

REVIEWED BY
Constantin-Cristian Topriceanu,
University College London,
United Kingdom
Carmen Pheiffer,
South African Medical Research Council, South Africa

*CORRESPONDENCE
Guanghui Li
liguanghui@ccmu.edu.com

†These authors have contributed equally to this work

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Maternal and infant outcomes in women with and without gestational diabetes mellitus in the COVID-19 era in China: Lessons learned

Wei Zheng[1,2†], Jia Wang[1,2†], Kexin Zhang[1,2], Cheng Liu[1,2], Li Zhang[1,2], Xin Liang[1,2], Lirui Zhang[1,2], Yuru Ma[1,2], Ruihua Yang[1,2], Xianxian Yuan[1,2] and Guanghui Li[1,2*]

[1]Division of Endocrinology and Metabolism, Department of Obstetrics, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing, China, [2]Beijing Maternal and Child Health Care Hospital, Beijing, China

Aims: The global COVID-19 pandemic has required a drastic transformation of prenatal care services. Whether the reformulation of the antenatal care systems affects maternal and infant outcomes remains unknown. Particularly, women with gestational diabetes mellitus (GDM) are among those who bear the greatest brunt. Thus, this study aimed to evaluate the impact of COVID-19 lockdown during late pregnancy on maternal and infant outcomes in women stratified by the GDM status in China.

Study design: The participants were women who experienced the COVID-19 lockdown during late pregnancy (3185 in the 2020 cohort) or not (2540 in the 2019 cohort) that were derived from the Beijing Birth Cohort Study. Maternal metabolic indicators, neonatal outcomes, and infant anthropometrics at 12 months of age were compared between the two cohorts, stratified by the GDM status.

Results: Participants who experienced COVID-19 lockdown in late pregnancy showed lower gestational weight gain than those in the control cohort. Nevertheless, they displayed a worse metabolic profile. COVID-19 lockdown during pregnancy was associated with higher glycosylated hemoglobin (HbA1c) (β= 0.11, 95% CI = 0.05–0.16, q-value = 0.002) and lower high density lipoprotein cholesterol level (HDL-C) level (β=−0.09, 95% CI = −0.14 to −0.04, q-value = 0.004) in women with GDM, adjusted for potential confounders. In normoglycemic women, COVID-19 lockdown in late pregnancy was associated with higher fasting glucose level (β= 0.10, 95% CI = 0.08–0.12, q-value <0.0001), lower HDL-C level (β=−0.07, 95% CI = −0.08 to −0.04, q-value <0.0001), and increased risk of pregnancy-induced hypertension (adjusted OR=1.80, 95%CI=1.30–2.50, q-value=0.001). The fasting glucose level decreased less from early to late pregnancy in women who experienced COVID-19 lockdown than in the controls, regardless of the

GDM status. The HDL-C has risen less with COVID-19 lockdown in the normoglycemic subgroup. In contrast, no significant differences regarding neonatal outcomes or infant weight were found between the two cohorts.

Conclusion: Experiencing the COVID-19 lockdown in pregnancy was associated with worse maternal metabolic status but similar neonatal outcomes and infant weight.

KEYWORDS

the COVID-19 pandemic, lockdown, gestational diabetes mellitus, pregnancy outcome, offspring outcome

Introduction

The coronavirus disease 2019 (COVID-19) pandemic is rampant worldwide and has challenged the healthcare system (1). Emergency measures such as social distancing, reallocating medical resources, and adapting medical strategies have been implemented to curb the unprecedented crisis (2). These contingency strategies have disrupted the original order of medical services and brought difficulties to the health management of vulnerable populations such as pregnant women (3).

Cases of pneumonia with unknown causes emerged in Wuhan, China, in December 2019. Following the pandemic evolution and lockdown of Wuhan on 23 January 2020, the first-level public health emergency response was launched in many provinces, districts, and cities including Beijing in China. After more than three months of strict prevention and control, Beijing has changed the level of public health emergency response from first-level to second-level from 30 April 2020, and adjusted prevention and control strategies accordingly. Pregnant women with metabolic disorders are among those who bear the greatest brunt of the crisis (3). Gestational diabetes mellitus (GDM) is one of the most common pregnancy complications affecting about 14% of pregnant women (4), profoundly impacting the short-term and long-term health of both mothers and their offspring (5). While desirable glycemic control during pregnancy can reduce the risk of future type 2 diabetes and dyslipidemia in mothers (6), neonatal adiposity and childhood obesity in their offspring (7, 8), and thereby has important implications for breaking the intergenerational transmission of metabolic diseases. However, the unprecedented COVID-19 pandemic has posed challenges to regular prenatal check-ups during pregnancy and blood glucose monitoring for pregnant women with GDM (9).

In addition, pregnant women during the COVID-19 pandemic experienced heightened anxiety levels (10, 11). Restrictions during the COVID-19 pandemic, including social distancing, isolation, and home confinement, also substantially impacted dietary habits and physical activity (12, 13). The above factors may significantly influence both maternal and neonatal outcomes of pregnant women (7). A previous study by Ghesquière et al. has reported that the COVID-19 pandemic lockdown may result in poor glycemic control in women with GDM (14). However, there is a lack of data to comprehensively evaluate the impact of COVID-19 and the temporary measures on maternal and infant outcomes of women with and without GDM.

Therefore, this study aimed to examine the influence of COVID-19 lockdown during late pregnancy on the maternal and infant outcomes stratified by the maternal GDM status.

Material and methodsStudy design and settings

The study population was selected from the ongoing Beijing Birth Cohort Study conducted in the Beijing Obstetrics and Gynecology Hospital (registration number ChiCTR2200058395). The trained researchers recruited singleton pregnant women without pre-gestational diabetes mellitus (PGDM), including type 1 diabetes and type 2 diabetes, chronic hypertension or cardiovascular diseases at their first visit to the hospital at 6-12 weeks gestation. We excluded twin pregnant women since their

Abbreviations: COVID-19, coronavirus disease 2019; GDM, gestational diabetes mellitus; LGA, large for gestational age; PGDM, pre-gestational diabetes mellitus; HbA1c, Glycosylated hemoglobin; OGTT, oral glucose tolerance test; GI, glycemic index; IADPSG, International Association of Diabetes and Pregnancy Study Groups; PIH, pregnancy-induced hypertension; GWG, gestational weight gain; IOM, Institute of Medicine; LBW, low birth weight; SGA, small for gestational age; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; NICU, neonatal intensive care unit.

maternal metabolic status and neonatal outcomes differed from singleton pregnancies. Our sample size is not enough for subgroup analysis in twin pregnancies. The participants were followed monthly until delivery, and their offspring were followed until 12 monthsonths. In this study, we selected 3029 pregnant women who received a 75 g oral glucose tolerance test (OGTT) for GDM diagnosis at 24–28 weeks of gestation between 23 January 2020 (the date of the lockdown of Wuhan and the implementation of first-level public health emergency response in Beijing) and 31 July 2020 and delivered during this period as the exposed study population. Accordingly, 3582 women who received the OGTT and deliver in the same period in 2019 (before the COVID-19 outbreak) were selected as the historical control population.

The study was approved by the Ethics Committee of the Beijing Obstetrics and Gynecology Hospital in China (2017-KY-015-01). Written informed consent was obtained from all participants.

Health management before and during the COVID-19 pandemic

Pregnant women in the unexposed 2019 cohort received prenatal health check-ups every month in the first and second trimesters and every two weeks in the third trimester in the hospital. Women diagnosed with GDM attended the hospital-based "one-day diabetes clinic". They spent a whole day in the hospital on theory learning and practice at this visit. In addition to the theoretical classes mentioned above, they also had a standard low glycemic index (GI) diet, attended aerobics classes, and practiced self-blood glucose monitoring. They were also required to visit the diabetes doctors every two weeks until delivery.

The frequency of prenatal health check-ups has dropped notably since the lockdown of Hubei Province on 23 January 2020, China. Traditional glucose management has been switched to telehealth-oriented management. Therefore, women with GDM in the 2020 cohort received a combination of remote and face-to-face glycemic management after the diagnosis of GDM. The intervention included online videos "Management of GDM", "Dietary Guidance", "Exercise Therapy", and "Self-glucose Monitoring". Wechat groups were also built for communication between diabetes doctors, nurses, and women with GDM smartphones. They were also required to meet the diabetes doctors if their blood glucose levels did not achieve the treatment goal.

Measurements

Baseline characteristics were collected at recruitment. Anthropometric measurements were collected by trained researchers. Bodyweight before pregnancy was self-reported. Clinical information, including the history of pregnancy, medical history, family history, pregnancy complications, and pregnancy outcomes, were collected from the medical record. Anthropometrics of the offspring at 12 monthsonths of age was measured by the primary child healthcare physician.

Definition of the variables

GDM was diagnosed according to standards proposed by the Obstetrics Subgroup, Chinese Medical Association, which is numerically equivalent to the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria. The diagnosis was made if any measurement met or exceeded these threshold values at a 75 g OGTT at 24–28 weeks of gestation: 0h ≥5.1 mmol/L, 1 h glucose ≥10.0 mmol/L, and 2 h glucose ≥8.5 mmol/L (15). The treatment goals of fasting glucose and glycated hemoglobin (HbA1c) in women with GDM in late pregnancy were: Fasting glucose ≤5.3 mmol/L and HbA1c <5.5% (15). The cut-off value for neonatal hypoglycemia requiring intervention was <2.6 mmol/L (16).

Pregnancy-induced hypertension (PIH) was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg that first appeared after 20 weeks of gestation. Preeclampsia was defined as PIH accompanied by any one of the following: (1) urine protein quantification ≥0.3 g/24 h, or urine protein/creatinine ratio ≥0.3, or random urine protein ≥ (+); (2) Without proteinuria, but accompanied by relevant target organ complications: the heart, lungs, liver, kidney, or other vital organs; or abnormal changes in the blood, digestive, nervous systems, placenta or fetal development, etc. (17).

Gestational weight gain (GWG) was classified as insufficient GWG, adequate GWG, and excessive GWG according to the Institute of Medicine (IOM) criteria (18). Gestational age <37 weeks was defined to be preterm birth. Neonatal birth weight <2500 g or ≥4000 g was defined as a low birth weight (LBW) or macrosomia, respectively. LGA and small for gestational age (SGA) were defined according to the criteria proposed by Villar et al. (19). Weight for age z-score, length for age z-score, and weight for length z-score at 12 months was calculated according to the World Health Organization Child Growth Standards (20).

Statistical analysis

Pregnancy complications and infant outcomes were compared between the 2020 and 2019 cohorts stratified by GDM status. The baseline characteristics, GWG, and maternal and infant outcomes were compared by an unpaired Student t-test for continuous variables conforming to a normal distribution and by Mann-Whitney U test for continuous data

without normal distribution. The chi-square test was used for comparison of categorical variables. In addition, we used the q-value that represents the False discovery rate-adjusted P-value when evaluating the maternal and offspring outcomes to control type I error due to multiple comparisons.

Subsequently, the differences in metabolic indicators between the two groups, as well as metabolic changes from the first to the third trimester between the two groups, were evaluated using logistic regression models for binary outcomes and generalized linear models with fixed effects for continuous outcomes. The models were adjusted for age, pre-pregnancy body mass index (BMI), gravidity, parity, glucose level during OGTT, family history of hypertension and diabetes using enter selection. All analyses were conducted using SAS 9.4.

Results

As shown in Figure 1, the 2020 cohort and 2019 cohort initially screened 3029 and 3582 participants. After excluding participants with PGDM or chronic hypertension or without complete information, 321 women with GDM and 2219 women without GDM in the 2020 cohort, and 396 women with GDM and 2789 women without GDM in the 2019 cohort were included in the analyses, respectively. As shown in Table 1, most baseline characteristics were comparable between the two cohorts, except that the participants in the 2020 cohort showed lower fasting glucose levels and higher low-density lipoprotein cholesterol (LDL-C) levels in the first trimester than those in the 2019 cohort both irrespective of the GDM status.

There were significant differences in GWG between the two cohorts (Table 2). Women in the 2020 cohort showed lower total GWG than women in the 2019 cohort, irrespective of the GDM status. Further analysis revealed that GWG before OGTT was similar between the two cohorts, while GWG after OGTT was lower in the 2020 cohort than in the 2019 cohort in women with GDM.

Notable differences in the metabolic indicators were also observed between the two cohorts. As indicated in Table 3, women in the 2020 cohort showed higher fasting glucose and a lower high-density lipoprotein cholesterol (HDL-C) level in the third trimester than in the control cohort. Concordantly, fasting glucose level has decreased less, and HDL-C has risen less from the first to the third trimester in women of the 2020 cohort than in the 2019 cohort (Table 3). For women without GDM, the prevalence of PIH was higher in the 2020 cohort than in the 2019 cohort. For women with GDM, the proportion of HbA1c ≥5.5% (above the treatment target value) in the third trimester was 48.98% vs. 36.43% (p = 0.002) in the 2020 and 2019 cohort, respectively.

The differences regarding HDL-C level in the third trimester, and changes in fasting glucose and HDL-C level throughout pregnancy between the two cohorts, as well as the difference in HbA1c level between the two cohorts in the GDM subgroup, remained significant after adjustment for potential confounders by the multivariate analysis (Table 4).

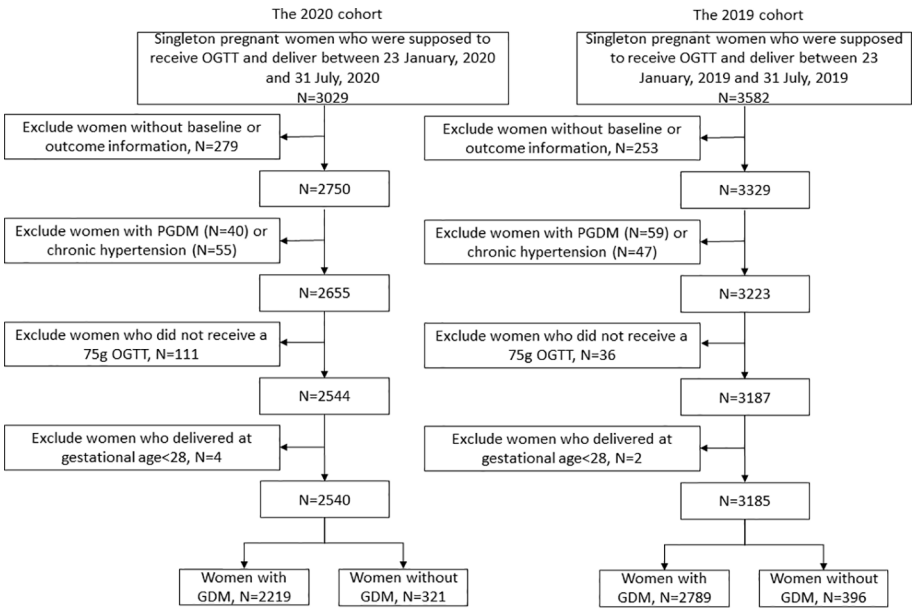

FIGURE 1
Study flow chart.

TABLE 1 Baseline characteristics of the participants.

	Women with GDM			Women without GDM		
	The 2020 cohort	The 2019 cohort	p-value*	The 2020 cohort	The 2019 cohort	p-value*
N	321	396	–	2219	2789	
Age, year (mean ± SD)	34.1 ± 4.9	33.9 ± 4.2	0.3	32.6 ± 3.8	32.0 ± 3.7	<0.0001
First pregnancy, n(%)	163 (50.78)	164 (41.41)	0.01	1143 (51.51)	1475(52.89)	0.3
Primipara, n(%)	236 (73.52)	273 (68.94)	0.2	1693 (76.30)	2134(76.51)	0.8
Adverse pregnancy history, n(%)	89 (27.73)	133 (33.59)	0.09	595 (26.81)	667(23.92)	0.02
Pre-pregnancy BMI, kg/m^2 (mean ± SD)	22.6 ± 3.3	22.8 ± 3.2	0.2	21.40 ± 2.94	21.31 ± 2.86	0.3
Family history of diabetes, n(%)	77 (23.99)	79 (19.95)	0.2	223 (10.05)	267(9.57)	0.6
Family history of hypertension, n(%)	80 (24.92)	82 (20.71)	0.2	385 (17.35)	504(18.07)	0.5
Metabolic indicators in the first trimester						
Fasting glucose, mmol/L (mean ± SD)	4.71 ± 0.35	4.87 ± 0.36	<0.0001	4.54 ± 0.34	4.70 ± 0.33	<0.0001
TC, mmol/L (mean ± SD)	4.37 ± 0.71	4.40 ± 0.71	0.7	4.20 ± 0.68	4.25 ± 0.68	0.06
TG, mmol/L (mean ± SD)	1.28 ± 0.54	1.32 ± 0.56	0.2	1.04 ± 0.44	1.03 ± 0.42	0.2
HDL-C, mmol/L (mean ± SD)	1.45 ± 0.27	1.49 ± 0.31	0.3	1.53 ± 0.28	1.53 ± 0.30	0.6
LDL-C, mmol/L (mean ± SD)	2.45 ± 0.64	2.35 ± 0.60	0.04	2.24 ± 0.610	2.20 ± 0.58	0.007
Gestational week of measurements in the first trimester, week (mean ± SD)	8.45 ± 1.40	8.68 ± 1.71	0.2	8.82 ± 1.55	8.57 ± 1.49	0.0002
Glucose levels during OGTT						
0h, mmol/L (mean ± SD)	4.85 ± 0.56	4.83 ± 0.54	0.6	4.30 ± 0.31	4.33 ± 0.32	0.001
1h, mmol/L (mean ± SD)	9.89 ± 1.54	9.84 ± 1.50	0.4	6.94 ± 1.63	6.92 ± 1.64	0.3
2h, mmol/L (mean ± SD)	8.61 ± 1.48	8.49 ± 1.32	0.4	6.00 ± 1.33	6.07 ± 1.30	0.03
Gestational week of OGTT, week (mean ± SD)	25.83 ± 1.66	25.47 ± 1.31	0.004	25.14 ± 1.46	24.86 ± 1.14	<0.0001

*p-value was calculated by Student t-test or Mann-Whitney U test for the continuous variables and chi-square test for the categorical variables.
BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; OGTT, oral glucose tolerance test.

TABLE 2 Changes in body weight during pregnancy in 2019 and 2020 cohort.

	Women with GDM			Women without GDM		
	The 2020 cohort	The 2019 cohort	p-value*	The 2020 cohort	The 2019 cohort	p-value*
Total GWG, kg (mean ± SD),	9.73 ± 5.81	11.34 ± 6.59	0.0006	12.70	13.99	<0.0001
GWG category according to IOM criteria			0.02			<0.0001
Insufficient GWG, n(%)	185 (57.63)	187 (47.22)		858 (38.67)	774 (27.75)	
Appropriate GWG, n(%)	91 (28.35)	138 (34.85)		859 (38.71)	1214 (43.53)	
Excessive GWG, n(%)	45 (14.02)	71 (17.93)		502 (22.62)	801 (28.72)	
GWG before OGTT, kg (mean ± SD)	7.57 ± 3.81	7.97 ± 4.00	0.2	–	–	
GWG category before OGTT according to IOM criteria			0.4	–	–	
Insufficient GWG, n(%)	75 (26.32)	78 (22.48)		–	–	
Appropriate GWG, n(%)	112 (39.30)	134 (38.62)		–	–	
Excessive GWG, n(%)	98 (34.39)	135 (38.90)		–	–	
GWG after OGTT, kg (mean ± SD)	2.12 ± 4.12	2.93 ± 3.44	0.007	–	–	
GWG category after OGTT according to IOM criteria			0.047	–	–	
Insufficient GWG, n(%)	205 (71.93)	230 (66.28)		–	–	
Appropriate GWG, n(%)	33 (11.58)	65 (18.73)		–	–	
Excessive GWG, n(%)	47 (16.49)	52 (14.99)		–	–	

*p-value was calculated by Mann-Whitney U test for the continuous variables and chi-square test for the categorical variables.
GWG, gestational weight gain; IOM, Institute of Medicine.

TABLE 3 Comparison of maternal outcomes between 2019 and 2020 cohort.

	Women with GDM			Women without GDM		
	The 2020 cohort	The 2019 cohort	q-value*	The 2020 cohort	The 2019 cohort	q-value*
PIH, n (%)	12 (3.74)	15 (3.79)	1	91 (4.10)	67 (2.40)	0.01
Preeclampsia, n (%)	27 (8.41)	22 (5.56)	0.2	87 (3.92)	79 (2.83)	0.06
Caesarean section, n (%)	158 (49.22)	176 (44.44)	0.4	844 (38.04)	954 (34.21)	0.009
Metabolic indicators in the third trimester						
Fasting glucose, mmol/L (mean ± SD)	4.60 ± 0.51	4.51 ± 0.57	0.03	4.30 ± 0.37	4.21 ± 0.39	<0.0001
Fasting glucose>5.3 mmol/L, n (%)	30 (9.74)	21 (6.05)	0.2	25 (1.17)	24 (0.98)	0.5
HbA1c, % (mean ± SD)	5.46 ± 0.34	5.35 ± 0.42	<0.0001	–	–	–
HbA1c≥5.5%, n (%)	144 (48.98)	122 (36.43)	0.01	–	–	–
TC, mmol/L (mean ± SD)	6.33 ± 1.12	6.29 ± 1.15	0.9	6.52 ± 1.10	6.48 ± 1.11	0.2
TG, mmol/L (mean ± SD)	3.26 ± 1.29	3.39 ± 1.71	0.5	2.94 ± 1.05	2.98 ± 1.11	0.5
HDL-C, mmol/L (mean ± SD)	1.72 ± 0.33	1.81 ± 0.36	0.004	1.84 ± 0.35	1.90 ± 0.36	<0.0001
LDL-C, mmol/L (mean ± SD)	3.32 ± 0.94	3.24 ± 0.94	0.4	3.55 ± 0.98	3.54 ± 0.98	0.5
Gestational week of measurements in the third trimester	34.2 ± 1.1	34.1 ± 1.1	0.2	34.1 ± 0.9	34.0 ± 1.0	0.009
Changes in metabolic indicators from early to late pregnancy						
△Fasting glucose, mmol/L (mean ± SD)	-0.11 ± 0.51	-0.36 ± 0.56	<0.0001	-0.24 ± 0.42	-0.49 ± 0.42	<0.0001
△TC, mmol/L (mean ± SD)	1.95 ± 0.97	1.88 ± 0.98	0.4	2.31 ± 0.92	2.23 ± 0.95	0.01
△TG, mmol/L (mean ± SD)	1.98 ± 1.13	2.06 ± 1.46	0.9	1.90 ± 0.89	1.94 ± 0.95	0.2
△HDL-C, mmol/L (mean ± SD)	0.26 ± 0.28	0.33 ± 0.31	0.02	0.31 ± 0.29	0.37 ± 0.31	<0.0001
△LDL-C, mmol/L (mean ± SD)	0.86 ± 0.91	0.89 ± 0.87	1	1.31 ± 0.89	1.34 ± 0.91	0.2
Insulin treatment, n (%)	61 (19.00)	63 (15.91)	0.4	–	–	–

*The q-value represented the False discovery rate-adjusted P-value calculated by unpaired Student t-test or Mann-Whitney U test for the continuous variables and chi-square test for the categorical variables.
PIH, pregnancy-induced hypertension; HbA1c, glycosylated Hemoglobin; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

On the other hand, most neonatal outcomes, including the prevalence of macrosomia, LBW, LGA, and SGA, were comparable between the two cohorts according to the adjusted models (Table 5). The weight of the offspring at 12 months and the proportions of the offspring with weight/length for age z-score and weight for length z-score <-1 or >1 at 12 months were similar between the two cohorts. Nevertheless, infants born to normoglycemic women in the 2020 cohort showed lower length at 12 months than those in the 2019 cohort(Table 5).

Discussion

This study indicated that women who experienced COVID-19 lockdown during late pregnancy showed features of metabolic disorders, including higher blood glucose levels and lower HDL-C levels than the historical controls, regardless of the GDM status. These results have raised concerns regarding the potential influence of the COVID-19 pandemic on the metabolic health of pregnant women. On the other hand, we did not find the effect of COVID-19 lockdown on the risk of adverse neonatal outcomes or abnormal weight for age at 12 months of the infants despite less GWG during pregnancy in women with GDM, although its influence on the long-term growth and development and metabolic health of the offspring needs to be further clarified.

The COVID-19 pandemic is a serious threat to human health (1, 21). Previous evidence has revealed the impact of the COVID-19 pandemic on adverse pregnancy and neonatal outcomes, including increased risk of preeclampsia, preterm birth, and stillbirth (3, 22–24). A population-based study by Gurol-Urganci et al. revealed that COVID-19 infection was associated with higher rates of fetal death, preterm birth, preeclampsia, and emergency cesarean delivery (25). Rodo et al. also reported that the COVID-19 pandemic might affect the maternal, newborn, and child health and nutrition in fragile and conflict-affected settings through literature review (26). A recent study by Ghesquière et al. revealed worse glycemic control in women with GDM during the COVID-19 lockdown (14). Consistent with the previous findings, we found the influence of COVID-19 lockdown on adverse maternal metabolic health in women with and without GDM.

The COVID-19 pandemic has posed dramatic changes to many aspects of our lives (3). Thus, it is unlikely to attribute the disturbed metabolism in pregnant women to any particular cause (3). One of the possible reasons is the restricted prenatal

check-ups and transition from face-to-face intervention to remote glycemic control during the pandemic, as described in the methods section (3, 9). Another potentially important factor affecting metabolism during pregnancy was stress resulting from the COVID-19 pandemic (27). Pregnant women had increased anxiety due to the risk of infection in the infants, isolation and social distance, and deteriorated economic conditions during the pandemic (11, 28). It has been reported that psychological stress was positively associated with glucose levels in pregnant women (29). Furthermore, several studies have reported that the isolation measures at the time of the COVID-19 pandemic were associated with unhealthy dietary habits and reduced physical activity (12, 30), which are critical factors affecting metabolic health (31).

In this study, pregnant women during the COVID-19 lockdown gained less weight than the historical controls, despite the worsened metabolic indicators. These results are contrary to the classical concept that GWG is positively associated with glucose level (32). A common misconception regarding glycemic management is that energy restriction has been given undue weight, and the diet quality is underemphasized (33), while face-to-face consultation by the doctor may improve diet quality (34). These results warn us that our current telehealth-oriented health management still needs improvement. The adaption of healthcare in pregnant women and especially the glycemic control in women with GDM to the "new normal" in the era of COVID-19 has become an important task (35).

Nevertheless, we did not observe an increased risk of adverse newborn or infant outcomes in women with GDM who experienced COVID-19 lockdown during pregnancy. In comparison, the COVID-19 lockdown in late pregnancy has been associated with lower offspring length at 12 months in normoglycemic women, which is a less reliable anthropometric than body weight at that age. To the authors' knowledge, this is the first study to investigate the influence of COVID-19 lockdown during pregnancy on offspring growth, although the investigated outcomes were limited to weight and length.

Results from this study provide valuable insights into health management during pregnancy in the COVID-19 era, both in the field of research and clinical application. The major strength of the current study is that we comprehensively evaluated the association between COVID-19 lockdown during late pregnancy and maternal and infant outcomes stratified by GDM status. This study also went a step further by following the offspring until 12 months of age. There are also certain limitations in this study. Firstly, this study used a historical control group to evaluate how the COVID-19 pandemic affects maternal and infant outcomes. Different characteristics between the two groups may exaggerate or attenuate the influence of the pandemic on study outcomes. Therefore, we conducted

TABLE 4 Metabolic differences between the 2020 and 2019 cohort by multivariate analysis.

	Women with GDM			Women without GDM		
Continuous variables	**β**	**95% CI**	**q-value***	**β**	**95% CI**	**q-value***
Metabolic indicators in the third trimester						
Fasting glucose, mmol/L	0.08	0.01~0.16	0.1	0.10	0.08~0.12	<0.0001
HbA1c, %	0.11	0.05~0.16	0.002	–	–	
TC, mmol/L	0.09	-0.16~0.19	0.9	0.03	-0.03~0.10	0.4
TG, mmol/L	-0.19	-0.42~0.04	0.2	-0.02	-0.08~0.04	0.6
HDL-C, mmol/L	-0.09	-0.14~-0.04	0.004	-0.07	-0.08~-0.04	<0.0001
LDL-C, mmol/L	0.08	-0.07~0.22	0.4	0.01	-0.05~0.07	0.8
Changes in metabolic indicators from early to late pregnancy						
△Fasting glucose, mmol/L	0.24	0.16~0.33	<0.0001	0.24	0.22~0.27	<0.0001
△TC, mmol/L	0.06	-0.08~0.21	0.9	0.09	0.03~0.14	0.002
△TG, mmol/L	-0.11	-0.31~0.10	0.4	-0.04	-0.10~0.01	0.2
△HDL-C, mmol/L	-0.06	-0.11~-0.02	0.2	-0.06	-0.08~-0.04	<0.0001
△LDL-C, mmol/L	-0.02	-0.15~0.11	0.9	-0.02	-0.07~0.03	0.8
Categorical variables	**aOR**	**95% CI**	**p-value***	**aOR**	**95% CI**	**p-value***
PIH	0.94	0.41~2.13	0.9	1.80	1.30~2.50	0.001
Preeclampsia	1.42	0.78~2.58	0.4	1.37	1.00~1.89	0.09
Fasting glucose in the third trimester>5.3 mmol/L	1.68	0.92~3.07	0.2	1.28	0.72~2.26	0.5
HbA1c in the third trimester≥5.5%	1.71	1.22~2.40	0.004	–	–	–

*Regression coefficients for metabolic indicators in the third trimester and aOR for the categorical variables were calculated adjusted for age, pre-pregnancy BMI, gravidity, parity, glucose level during OGTT, family history of hypertension, and family history of diabetes; regression coefficients for changes of metabolic indicators were adjusted for age, pre-pregnancy BMI, gravidity, parity, family history of hypertension, and family history of diabetes.
HbA1c, glycosylated Hemoglobin; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; PIH, pregnancy induced hypertension; BMI, body mass index; OGTT, oral glucose tolerance test.

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TABLE 5 Comparison of offspring outcomes between 2019 and 2020 cohort.

	Women with GDM				Women without GDM			
	The 2020 cohort	The 2019 cohort	q-value*	Adjusted q-value#	The 2020 cohort	The 2019 cohort	q-value*	Adjusted q-value#
Neonatal outcomes								
Gestational age, week (mean ± SD)	38.5 ± 1.7	38.5 ± 1.7	0.8	0.9	38.9 ± 1.5	39.0 ± 1.4	0.1	0.1
Preterm birth, n(%)	23(7.17)	34(8.59)	0.8	0.9	106(4.78)	114(4.09)	0.4	0.3
Neonatal birthweight, g (mean ± SD)	3301 ± 517	3313 ± 510	0.8	0.9	3343 ± 461	3337 ± 432	0.5	0.8
Macrosomia, n(%)	20(6.23)	29(7.32)	0.9	0.9	145(6.53)	167(5.99)	0.6	0.8
LGA, n(%)	51(16.14)	75(19.28)	0.8	0.9	351(15.93)	384(13.82)	0.1	0.2
LBW, n(%)	14(4.36)	17(4.29)	1	0.9	67(3.02)	77(2.76)	0.7	0.8
SGA, n(%)	7(2.22)	10(2.57)	1	0.9	55(2.50)	71(2.56)	0.9	0.8
NICU admission, n(%)	26(8.10)	31(7.83)	1	0.9	–	–		
Blood glucose, mmol/L (mean ± SD)	3.92(0.72)	3.88(0.76)	0.8	0.9	–	–		
Blood glucose<2.6 mmol/L, n(%)	5(2.60)	7(2.57)	1	1	–	–		
Anthropometrics at 12 months								
weight at 12 months, kg (mean ± SD)	9.78 ± 1.04	9.93 ± 0.97	0.8	0.8	9.91 ± 1.04	9.98 ± 1.9	0.4	0.2
Length at 12 months, cm (mean ± SD)	76.65 ± 2.81	76.69 ± 2.66	1	0.9	76.68 ± 2.60	76.94 ± 2.70	0.03	0.01
Weight for age z-score at 12 months, (mean ± SD)	0.38 ± 0.89	0.47 ± 0.84	0.8	0.9	0.51 ± 0.86	0.55 ± 0.89		0.2
Weight for age category, n(%)			1	0.9			0.6	0.5
Weight for age z-score<-1	16(6.18)	12(4.86)			62(3.77)	73(4.18)		
-1≤Weight for age z-score≤-1	180(69.50)	171(69.23)			1130(68.78)	1168(66.93)		
Weight for age z-score>1	63(24.32)	64(25.91)			451(27.45)	504(28.88)		
Length for age z-score at 12 months, (mean ± SD)	0.70 ± 1.09	0.66 ± 1.06	0.8	0.9	0.73 ± 1.02	0.83 ± 1.06	0.03	0.01
Length for age category, n(%)			0.8	0.8			0.2	0.06
Length for age z-score<-1	17(6.56)	9(3.67)			66(4.02)	70(4.04)		
-1≤Length for age z-score≤-1	143(55.21)	146(59.59)			982(59.81)	972(56.06)		
Length for age z-score>1	99(38.22)	90(36.73)			594(36.18)	692(39.91)		
Weight for length z-score at 12 months, (mean ± SD)	0.11 ± 0.89	0.25 ± 0.87	0.8	0.8	0.26 ± 0.89	0.26 ± 0.90	0.8	0.9
Weight for length category, n(%)			0.9	0.9			0.8	0.8
Weight for length z-score<-1	27(10.42)	17(6.94)			127(7.73)	129(7.44)		
-1≤Weight for length z-score≤-1	195(75.29)	191(77.96)			1239(75.46)	1323(76.30)		
Weight for length z-score>1	37(14.29)	37(15.10)			276(16.81)	282(16.26)		

*The q-value represented the False discovery rate-adjusted P-value calculated by unpaired Student t-test or Mann-Whitney U test for the continuous variables and chi-square test for the categorical variables.
#p-value was calculated adjusted for age, maternal height, pre-pregnancy BMI, gravidity, parity, glucose level during OGTT, family history of hypertension, and family history of diabetes.
LGA, large for gestational age; LBW, low birth weight; SGA, small for gestational age; NICU, neonatal intensive care unit.

multivariate analyses to adjust for potential confounders. Secondly, we did not investigate the participants' psychosocial stress, dietary intake, or physical activities. Therefore, it is uncertain how these factors may affect metabolic status. Furthermore, the effect of the COVID-19 lockdown on the long-term health of the offspring remains unclarified.

We should also be aware that the impact of the COVID-19 pandemic on maternal and offspring health may vary greatly between countries, depending on the severity of the outbreak, medical resources, health management strategies, regional economic conditions, and the maternal educational level as well (7, 31, 36–38). All these factors may modify the influence of the COVID-19 pandemic on maternal and infant outcomes. Thus, the focus and strategies for health management during pregnancy in different regions should be tailored to local conditions.

Conclusions

In summary, our study showed similar neonatal and infant outcomes, less GWG, and a worse overall metabolic profile in GDM and non-GDM pregnant women in the COVID-19 era compared to the historical control group. It is unclear whether these findings can be generalized to other populations due to variations in the

severity of the pandemic, response measures to the outbreak, efforts in health management, etc. Despite these uncertainties, the results from our study provided essential references for health management in women with different glucose statuses in the protracted battle against the 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 authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Beijing Obstetrics and Gynecology Hospital in China. The patients/participants provided their written informed consent to participate in this study.

Author contributions

WZ, data curation, methodology, formal analysis, funding acquisition, writing-original draft; KZ, investigation, data curation, writing-original draft; JW, investigation, methodology, validation, writing-review; CL, investigation, methodology, writing-editing; LZ, investigation, resources, writing-review; XL, data curation, investigation; LRZ, data curation, investigation; YM, data curation, investigation; RY, data curation, investigation; XY, methodology, validation; GL, conceptualization, project administration, writing – review. 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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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.982493/full#supplementary-material

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EDITED BY
Jeff M. P. Holly,
University of Bristol, United Kingdom

REVIEWED BY
Enrique Rodilla,
Hospital de Sagunto, Spain
Theocharis Koufakis,
Aristotle University of Thessaloniki, Greece

*CORRESPONDENCE
Mykola Khalangot
nikhalangot@ukr.net

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RAAS inhibitors are associated with a better chance of surviving of inpatients with Covid-19 without a diagnosis of diabetes mellitus, compared with similar patients who did not require antihypertensive therapy or were treated with other antihypertensives

Mykola Khalangot[1,2]*, Nadiia Sheichenko[3], Vitaly Gurianov[4], Tamara Zakharchenko[2], Victor Kravchenko[2] and Mykola Tronko[1,2]

[1]Endocrinology, Shupyk National Healthcare University of Ukraine, Kyiv, Ukraine, [2]Epidemiology of Endocrine Diseases, V. P. Komisarenko Institute of Endocrinology and Metabolism, Kyiv, Ukraine, [3]Infectious Diseases Hospital, Kostiantynivka, Ukraine, [4]Healthcare Management, Bohomolets National Medical University, Kyiv, Ukraine

Purpose: The effect of renin-angiotensin-aldosterone system (RAAS) inhibitors in combination with COVID-19 and diabetes mellitus (DM) remains unknown. We assessed the risk of death in COVID-19 inpatients based on the presence or absence of DM, arterial hypertension (AH) and the use of RAAS inhibitors or other antihypertensives.

Methods: The results of treatment of all adult PCR-confirmed COVID-19 inpatients (n = 1097, women 63.9%) from 02/12/2020 to 07/01/2022 are presented. The presence of DM at the time of admission and the category of antihypertensive drugs during hospital stay were noted. Leaving the hospital due to recovery or death was considered as a treatment outcome. Multivariable logistic regression analysis was used to assess the risk of death. Patients with COVID-19 without AH were considered the reference group.

Results: DM was known in 150 of 1,097 patients with COVID-19 (13.7%). Mortality among DM inpatients was higher: 20.0% *vs*. 12.4% respectively (p=0.014). Male gender, age, fasting plasma glucose (FPG) and antihypertensives were independently associated with the risk of dying in patients without DM. In DM group such independent association was confirmed for FPG and treatment of AH. We found a reduction in the risk of death for COVID-19 inpatients without DM, who received RAAS inhibitors compared with the corresponding risk of normotensive inpatients, who did not receive antihypertensives: OR 0.22 (95% CI 0.07–0.72) adjusted for age, gender and FPG.

Conclusion: This result raises a question about the study of RAAS inhibitors effect in patients with Covid-19 without AH.

KEYWORDS

COVID-19, RAAS inhibitors, mortality, diabetes mellitus, angiotensin-converting-enzyme inhibitors (ACEi), angiotensin II receptor blockers (ARBs)

Introduction

The renin-angiotensin-aldosterone system (RAAS) signaling and angiotensin-converting enzyme 2 (ACE2) have been implicated in the pathogenesis of COVID-19 because ACE2 – an enzyme that physiologically counteracts RAAS activation, is a functional receptor for SARSCoV-2 – the virus responsible for the Covid-19 pandemic (1). RAAS inhibitors may increase ACE2 expression, leading to understandable concerns about their potential danger to patients with Covid-19 (2, 3). Nevertheless, circulating levels of ACE2 in serum of type 2 diabetes mellitus (DM) patients are rather low (4), so the effect of RAAS inhibitors in combination with COVID-19 and DM remains unknown. It has also been suggested that direct and indirect loss of ACE2 through binding of SARS-CoV-2 to ACE2 partially causes the systemic manifestations of COVID-19 (5). A recent observational patient register-based study revealed that prior treatment of hypertensive patients with RAAS inhibitors, rather than increasing the risk, may actually confer some protection against in-hospital mortality (6). However, the effect of RAAS inhibitors on Covid-19 outcomes was previously evaluated in comparison with the effects of other antihypertensives but not with the absence of AH (7), it remains unknown how this effect is related to the presence of DM.

Materials and methods

The study was conducted by analyzing the archives of one of the infectious disease hospitals of Ukraine (Kostiantynivka, Donetsk region). The archive of this hospital was recently already used to analyze mortality risk factors for Covid-19 patients, treated in 2020 (8). In 2021, patients with clinical symptoms similar to Covid-19 were hospitalized at the Infectious Diseases Hospital, which provides care to 961,000 residents. Diagnosis of Covid-19, selection of patients for hospitalization, clinical examinations and treatment were performed according to relevant national standards (9), which were updated according to WHO recommendations. Details of hospitalization criteria are shown in Supplement Table 1. The diagnosis of Covid-19 was confirmed by PCR. Electronic dataset was developed by Komisarenko Institute of Endocrinology and Metabolism, Kyiv, Ukraine. The results of treatment of all adult PCR - confirmed COVID-19 inpatients (n = 1,097, women 64.0%) from 02/12/2020 to 07/01/2022 are presented.

Standard clinical and anthropometric characteristics of patients (fasting plasma glucose, peripheral capillary oxygen saturation (SpO2); white blood cells (WBC); body mass index (BMI); systolic and diastolic blood pressure, were measured. Arterial hypertension (AH) category was defined as systolic/diastolic blood pressure of 140/90 mm Hg and above or hypotensive treatment. BMI was determined as the body weight (kg) divided by the height (m) squared (kg/m^2). The presence of a documented diagnosis of diabetes mellitus (based on GP/family doctor's references and/or use of medication) at the time of admission, myocardial infarction/revascularization history, and the category of antihypertensive drugs for the treatment of hypertension during hospital stay were considered. The antihypertensive drugs were categorized as follows: RAAS inhibitors – Angiotensin-converting-enzyme inhibitors (ACEi) or angiotensin II receptor blockers (ARBs); Calcium channel blockers; Other hypotensives. Leaving the hospital due to recovery or death was considered as a dichotomous treatment outcome.

Statistical analysis has been performed using MedCalc® Statistical Software version 20.110 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2022). Shapiro-Wilk test was used to check the law of distribution for the quantitative data. All of the variables were not normally distributed, thus median (Me) and interquartile range (Q_I – Q_{III}) were calculated. Pairwise comparisons were performed by the Mann–Whitney test. Frequency (%) was calculated for the qualitative data. Fisher's exact test was used to compare frequencies. Logistic regression analysis was applied to evaluate the effects of the independent variables on the risk of death. Stepwise method was used to find the best fitting model which described the relationship between the risk of death and a set of independent variables in a multifactor logistic regression model. Odds ratios (OR) with 95% confidence interval (95% CI) were calculated to evaluate the effect of risk factors on the result of treatment. Patients with COVID-19 without AH were considered the reference group. The diagnostic performance of the logistic regression models was evaluated using Receiver Operating Characteristic (ROC) curve analysis. The area under the ROC curve (AUC) and its 95% CI were calculated. In all of the tests the p value < 0.05 was considered significant.

Results

At the time of admission to the Infectious Diseases Hospital, diabetes was known in 150 of 1,097 patients with COVID-19 (13.7%). The share of women in the group of individuals with diabetes was 68.0%, while among individuals without diabetes it was 63.4%, i.e., there is no statistically significant difference in gender distribution in these groups (p = 0.314). Glycemia and phenotypic characteristics in the diabetes group differed from those without such a diagnosis at the

time of admission: moderate hyperglycemia and obesity were present in most people with diabetes. Age, FPG, BMI and blood pressure were higher in the diabetes group. The history of myocardial infarction, coronary revascularization, and stroke was also more common in the diabetes group. However, body temperature and oxygen saturation, i.e., characteristics that reflect the course of COVID-19, did not differ in these groups (Table 1).

People with diabetes were almost twice as likely to be treated with positive pressure ventilation (PPV) and mortality among them was also higher than in the group without diabetes: 20.0% *vs* 12.4% respectively. There were twice as less people without hypertension in the group with diabetes as in the group without diabetes. The use of antihypertensives also differed: ACEIs/ARBs was used by 25.3% of persons in the diabetes group and only 9.4% in the non-diabetes group. Steroid hormones for the treatment of COVID-19 were used in most patients of both groups, the structure of their use differed, but not significantly. Insulin was used to treat 37.3% of patients diagnosed with diabetes at the time of admission (Table 2).

Male gender, age, blood glucose, systolic blood pressure, body mass index (continued variables) were positively associated with the risk of dying in hospitalized patients with COVID-19 who were not diagnosed with diabetes mellitus. The presence of hypertension in the absence of any treatment or antihypertensive treatment with drugs that did not belong to the categories of ACEIs/ARBs or Calcium channel blockers was also associated with a risk of death. There was a statistical tendency (p = 0.060) mortality risk reduction in patients with COVID-19 taking ACEIs/ARBs. Dexamethasone treatment increased the chances of death (Table 3).

Stepwise method (entering variable if $p < 0.1$, removing variable if $p > 0.2$) was used to find the best fitting model that described the relationship between the risk of death and a set of independent variables in a multifactor logistic regression model. Four variables were selected: gender, age, FPG and treatment of arterial hypertension (AH). The area under the curve of this model (Supplementary Figure 1) AUC = 0.78 (95% CI 0.75 - 0.81), indicating an average-strength association between the risk of death and four selected variables.

If gender, age, FPG, and antihypertensive categories were considered within the same model (Table 4), the trend became statistically significant (p = 0.013) and indicated a multiple reduction in the chances of death for patients treated with ACEIs/ARBs: OR = 0.22 (0.07–0.72). The risks associated with male gender, age, FPG, and other AH treatments did not change significantly from one-factor estimates.

In 150 patients with diabetes and COVID-19, evaluation using one-way logistic regression models confirmed a positive association between age, FPG, BMI, and antihypertensive treatment with drugs that did not belong to the ACEIs/ARBs or calcium channel blockers and the likelihood of death (Table 5). Within the joint model (age, FPG, AH treatment), there was no association between ACEIs/ARBs and the chances of death (Table 6).

The category of "participants that were apparently hypertensive but did not get antihypertensive treatment" (AH, no AH treatment) included COVID-19 inpatients, who have not previously received antihypertensive treatment and their maximum blood pressure was often borderline for the presence of arterial hypertension: the median

TABLE 1 Some characteristics of patients with COVID-19 depending on their "diabetes mellitus" diagnosis at the time of admission to the infectious disease hospital.

Characteristics	No Diabetes n=947 (600 women)	Diabetes n=150 (102 women)	p
Age, all (yrs)	61.7 (49.5–69.7)	67.1 (60.6–71.9)	**<0.001**
Age, women (yrs)	63.0 (51.1–70.1)	67.9 (61.6–72.2)	**<0.001**
Age, men (yrs)	60.3 (44.9–69.3)	66.0 (52.2–70.7)	**0.022**
FPG (mmol/L)	5.6 (4.8–6.6)	9 (6.9–12.4)	**<0.001**
Saturation O2 (%)	89 (86–95)	92 (86–95)	0.174
Systolic BP (mmHg)	130 (120–140)	135 (130–148)	**<0.001**
Diastolic BP (mmHg)	80 (75–85)	80 (78–90)	**<0.001**
Body temperature (°C)	37.9 (37.4–38.5)	37.8 (37.2–38.5)	0.101
BMI, (kg/m^2)[1]	29.4 (25.9–32.7)	32.7 (28.7–36.7)	**<0.001**
BMI, women (kg/m^2)	29.2 (25.7–32.6)	33.2 (27.9–36.9)	**<0.001**
BMI, men (kg/m^2)	29.8 (26.2–32.7)	31.8 (29.9–33.7)	**<0.001**
Stroke history	8 (0.8)	6 (4.0)	**0.007**
MI/revascularization history	6 (0.6)	6 (4.0)	**0.003**
AH	354 (37.4)	97 (64.7)	**< 0.001**

FPG, fasting plasma glucose; BMI, body mass index; BP, blood pressure; MI, myocardial infarction; AH, arterial hypertension.
Data are medians and inter quartile ranges (Q_I-Q_{III}) or n and %. The comparison was based on the Mann-Whitney test.
[1]BMI data were only for 852 "No Diabetes" patients and 130 "Diabetes" patients.
Bold values denote statistical significance at the P < .05 level.

TABLE 2 Distribution of some treatments and mortality of patients with COVID-19 depending on their "diabetes mellitus" diagnosis at the time of admission to the infectious disease hospital.

		No Diabetes n=947	Diabetes n=150	p
Treatment of arterial hypertension (AH)	no AH and no AH treatment	593 (62.6)	53 (35.3)	**<0.001**
	AH, no AH treatment	140 (14.8)	22 (14.7)	
	ACEIs/ARBs	89 (9.4)	38 (25.3)	
	Calcium channel blockers	38 (4.0)	20 (13.4)	
	Other hypotensives	87 (9.2)	17 (11.3)	
Insulin		–	56 (37.3)	–
Steroids	no steroids	407 (43.0)	62 (41.3)	**0.048**
	Dexamethasone	488 (51.6)	80 (53.3)	
	dexamethasone + methylprednisolone	27 (2.9)	0 (0.0)	
	methylprednisolone	24 (2.5)	8 (5.3)	
PPV		62 (6.5)	18 (12.0)	**0.026**
Lethal outcomes		117 (12.4)	30 (20.0)	**0.014**

ACEIs, Angiotensin-converting-enzyme inhibitors; ARBs, angiotensin II receptor blockers; PPV, positive pressure ventilation. Data are n and %. The comparison of the two groups was performed according to Fisher's exact test or the chi-square test.
Bold values denote statistical significance at the P < .05 level.

TABLE 3 Risks of death of COVID-19 inpatients without diabetes mellitus (n=947) estimated using one-way logistic regression models.

Factors		Model coefficient, b ± m	p	OR (95% CI)
Gender	women	Reference		
	men	0.41 ± 0.20	**0.039**	1.51 (1.02–2.23)
Age, yrs		0.060 ± 0.009	**<0.001**	1.06 (1.04–1.08)
FPG, mmol/L		0.18 ± 0.05	**<0.001**	1.20 (1.09–1.32)
Systolic BP, mm Hg		0.017 ± 0.006	**0.002**	1.02 (1.01–1.03)
Diastolic BP, mm Hg_		0.006 ± 0.010	0.522	–
BMI, kg/m^2		0.046 ± 0.021	**0.032**	1.05 (1.00–1.09)
Treatment of arterial hypertension (AH)	no AH and no AH treatment	Reference		
	AH, no AH treatment	0.54 ± 0.27	**0.045**	1.72 (1.01–2.92)
	ACEIs/ARBs	-1.13 ± 0.60	0.060	–
	Calcium channel blockers	-0.23 ± 0.62	0.704	–
	Other hypotensives	1.63 ± 0.26	**<0.001**	5.11 (3.05–8.55)
Steroids	no steroids	Reference		
	Dexamethasone	0.74 ± 0.22	**0.001**	2.10 (1.36–3.23)
	Dexamethasone + methylprednisolone	0.95 ± 0.53	0.072	–
	Methylprednisolone	0.48 ± 0.64	0.451	–

FPG, fasting plasma glucose; BMI, body mass index; AH, arterial hypertension; ACEi, Angiotensin-converting-enzyme inhibitors; ARB's, angiotensin II receptor blockers.
Bold values denote statistical significance at the P < .05 level.

TABLE 4 Analysis of a multifactor logistic regression model for predicting the risk of death for patients without diabetes.

Factors		Model coefficient, b ± m	p	OR (95% CI)
Gender	women	Reference		
	men	0.52 ± 0.23	**0.022**	1.67 (1.08–2.61)
Age, yrs		0.063 ± 0.010	**<0.001**	1.07 (1.05–2.61)
FPG, mmol/L		0.12 ± 0.05	**0.026**	1.12 (1.01–1.24)
Treatment of arterial hypertension (AH)	no AH and no AH treatment	Reference		
	AH, no AH treatment	0.23 ± 0.29	0.435	_
	ACEIs/ARBs	-1.53 ± 0.61	**0.013**	0.22 (0.07–0.72)
	Calcium channel blockers	-0.70 ± 0.64	0.274	–
	Other hypotensives	1.23 ± 0.29	**<0.001**	3.41 (1.94–5.98)

FPG, fasting plasma glucose; AH, arterial hypertension; ACEi, Angiotensin-converting-enzyme inhibitors; ARB's, angiotensin II receptor blockers.
Bold values denote statistical significance at the P < .05 level.

was equal to 140/90 mmHg and did not exceed the corresponding indicator in groups of hypotensive treatment (Supplement Table 3).

Discussion

We are presenting the results of an observational study on the outcomes of all COVID-19 inpatients (n = 1097) treated in one of the infectious diseases hospitals of Ukraine in 2021.

The group of COVID-19 inpatients (n = 150) with known diabetes mellitus at the time of admission, represented within this cohort, was expected to show not only higher mortality and PPV demand, but also more frequent use of ACEIs/ARBs *vs.* inpatients without such a diagnosis. However, concerns about the detrimental effects of ACEIs/ARBs on patients with COVID-19, which were declared at the beginning of the pandemic (2), were not justified, i.e. we did not find the expected increase in mortality associated with these drugs. Moreover, we found that the use of RAAS inhibitors for the treatment of hypertension in patients with COVID-19 who did not have DM was associated with a reduced risk of death. Within the logistic regression model, which also takes into account age, sex and plasma glucose levels, the use of ACEIs/ARBs (but not other

TABLE 5 Risks of death of COVID-19 inpatients with diabetes mellitus (n=150) estimated using one-way logistic regression models.

Factors		Model coefficient, b ± m	p	OR (95% CI)
Gender	women	Reference		
	men	0.26 ± 0.43	0.541	–
Age, yrs		0.047 ± 0.021	**0.023**	1.05 (1.01–1.09)
FPG, mmol/L		0.11 ± 0.05	**0.018**	1.12 (1.02–1.22)
Systolic BP, mm Hg		0.017 ± 0.009	0.060	–
Diastolic BP, mm Hg		0.021 ± 0.020	0.300	–
BMI, kg/m^2		0.094 ± 0.040	**0.020**	1.10 (1.02–1.19)
Treatment of arterial hypertension (AH)	no AH and no AH treatment	Reference		
	AH, no AH treatment	-1.32 ± 1.09	0.223	–
	ACEIs/ARBs	-1.13 ± 0.60	0.060	–
	Calcium channel blockers	0.88 ± 0.62	0.156	–
	Other hypotensives	2.08 ± 0.62	**0.001**	8.03 (2.36–27.3)
Steroids	no steroids	Reference		
	Dexamethasone	0.30 ± 0.53	0.486	–
	Methylprednisolone	-0.41 ± 1.12	0.713	–

FPG, fasting plasma glucose; BMI, body mass index; AH, arterial hypertension; ACEIs, Angiotensin-converting-enzyme inhibitors; ARBs, angiotensin II receptor blockers.

TABLE 6 Analysis of a multifactor logistic regression model for predicting the risk of death for patients with diabetes.

Factors		Model coefficient, b ± m	p	OR (95% CI)
Age, yrs		0.043 ± 0.022	0.058	1.04 (1.00–1.09)
FPG, mmol/L		0.13 ± 0.05	**0.016**	1.14 (1.02–1.26)
Treatment of arterial hypertension (AH)	no AH and no AH treatment	Reference		
	AH, no AH treatment	-1.26 ± 1.11	0.255	–
	ACEIs/ARBs	-0.26 ± 0.64	0.688	–
	Calcium channel blockers	0.71 ± 0.65	0.277	–
	Other hypotensives	1.98 ± 0.682	**0.001**	7.24 (1.93–27.2)

FPG, fasting plasma glucose; AH, arterial hypertension; ACEIs, Angiotensin-converting-enzyme inhibitors; ARBs, angiotensin II receptor blockers.

antihypertensive drugs) is associated with a significant decrease in the chances of dying: OR 0.22 (0.07–0.72), patients without AH were considered as the reference group. RAAS inhibitors did not affect the mortality of COVID-19 inpatients with DM.

In 2020, Italian researchers who observed COVID-19 outcomes in a small cohort of hypertensives (n = 133) concluded that chronic use of RAAS inhibitors does not negatively affect the clinical course of COVID-19 in hypertensive patients. A significantly lower risk of admission to intensive care was observed in COVID-19 positive subjects chronically treated with ACEIs/ARBs as compared with other hypertensive patients, whereas the rates of hospitalization, oxygen therapy, noninvasive ventilation, and death did not differ between the 2 groups (10). Recently, British researchers who analyzed outcomes of 9,197 hospitalized patients with Covid-19 informed that the use of RAAS inhibitors tended to have a protective effect for in-hospital mortality in fully adjusted models (OR 0.88, 95% CI 0.78,0.99). The variables used in these fully adjusted models included age, sex, diabetes, chronic kidney disease, chronic obstructive pulmonary disease, obesity and known heart failure (6). Chinese researchers using the propensity score-matched analysis of 1128 hospitalized COVID-19 patients with hypertension, demonstrated a lower risk of COVID-19 mortality in patients who received ACEI/ARB versus those who did not (adjusted hazard ratio, 0.37 [95% CI, 0.15–0.89] (11).

Our risk assessment model was less complex because it did not include the presence of chronic diseases other than hypertension and was implemented separately for patients without DM and with known DM. In addition, we assessed the risk of death for RAAS inhibitors not comparing with any other antihypertensive drugs as in the mentioned studies (10, 11), but in comparison with the corresponding risk for normotensive patients.

The hypothesis that RAAS inhibitors may prevent COVID-19 deaths in hospitalized, hypertensive patients has been previously published by Spanish researchers based on the SEMI-COVID-19 cohort registry data (12). This benefit seems to equalize the risk of treated patients to the risk of non-hypertensive patients. But in our study, RAAS treatment reduces the risk of death beyond the risk of normotensives as the reference group. Rodilla et al, 2020 (12) were probably the first to describe a reduction in the risk of death in hospitalized patients with COVID-19 treated with RAAS inhibitors, which does not contradict our data on the probable protective role of RAAS inhibitors. But we failed to prove the independent nature of the effect of arterial hypertension on mortality in COVID-19 inpatients. The National Cohort Study in England investigated 19,256 COVID-19–related intensive care unit admissions and revealed that patients with type 2 diabetes were at increased risk of mortality independently of hypertension (13).

Today there are still many controversies in the relationship between hypertension and COVID-19. This concerns the predictive value of hypertension, the effect of blood pressure levels, the impact of previously known and newly diagnosed hypertension, and the effect of antihypertensive therapy on the severity and outcomes in COVID-19 patients (14).

The lack of consideration of the degree of frailty/comorbidity of the participants is an important limitation of our study. The authors will try to overcome this limitation as soon as the humanitarian situation in the country normalizes. Another limitation of our study is the fact that we compared outcomes of patients, hospitalized with COVID-19 depending on whether the DM diagnosis was known prior to admission. This means that the group of COVID-19 inpatients without DM could contain persons with unknown diabetes. Stress-induced acute hyperglycemia is commonly observed in critical illness (15), therefore diagnosing DM during acute illnesses and infections is considered undesirable. Besides, the compared groups have undeniable anthropometric, biochemical, and other differences (Table 1) which confirms the validity of categorization we have applied. The question of qualitative sufficiency of Covid-19 inpatients with known DM group (n=150) to confirm or refute the null hypothesis (type II errors) was resolved positively (see Supplementary Table 2 and Supplementary Figure 3).

Thus, we found a very significant reduction in the risk of death for hospitalized patients with COVID-19 without DM, who received RAAS inhibitors compared with the corresponding risk of normotensive COVID-19 inpatients who did not receive antihypertensive treatment. Attention should be paid to the lack of influence of RAAS inhibitors on the risk of mortality of COVID-19 inpatients with DM. A recent retrospective multicentre European study (16) revealed no association between mortality and renin-angiotensin-aldosterone system inhibitor therapy in adults with diabetes admitted to hospital with COVID-19.

We hypothesize that the decrease in circulating levels of ACE2 resulting from both DM and COVID-19 is too great for RAAS

inhibitors to fully overcome, that is why the positive effect of RAAS inhibitors may be absent in patients with COVID-19. Despite the opinion that effects of RAAS inhibitors on ACE2 in humans are still uncertain (1), their treatment potential for Covid-19 is rather positive (17, 18). We believe that our results not only confirm the safety of RAAS inhibitors in hypertensive patients with Covid-19, but also raise a question about the study of their therapeutic effect in patients with Covid-19 without arterial hypertension. The long-term positive experience of using RAAS inhibitors in normotensive patients with diabetic kidney disease (19) is well known. Thus, we speculate that ACE2-based regulation strategies may become one of the most promising approaches for future therapies and improve disease prognosis in COVID-19. Thus, medications that were thought to spread the pandemic and increase mortality may have therapeutic potential not only for individuals with AH, but also normotensive patients with COVID-19. Interestingly, an almost similar situation arose with Sodium-glucose co-transporter 2 inhibitors (SGLT2i) - a new class of oral, glucose-lowering agents used for the management of type 2 diabetes (T2D). At the beginning of the COVID-19 pandemic, SGLT2i was not recommended to be included for the treatment of T2D COVID-19 patients due to fears of the development of euglycemic diabetic ketoacidosis (DKA), a potentially fatal clinical entity (20). Recently, Greek researchers (21) hypothesized that SGLT2i might have a role in the future management of COVID-19 and raised a question: do SGLT2i have the potential to improve COVID-19-related outcomes in people with or even without diabetes? To answer this question they are counting on results of two ongoing, randomized-controlled trials (RCTs) (21). It is reasonable to introduce RCTs for studying COVID-19-related effects of RAAS inhibitors as well.

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 study was approved by the ethics committee of the Institute of Endocrinology and Metabolism (National Academy of Medical Sciences of Ukraine) protocol # 35/4-KE from 01/04/2021. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MK and MT conceived the study design. NS participated in data collection. MK, NS, TZ and VK participated in data analysis and interpretation. VG carried out the final statistical analysis. 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1077959/full#supplementary-material

SUPPLEMENTARY FIGURE 1
ROC-curve of the four-factor (gender, age, FPG and treatment of AH) model of the risk of death for patients without diabetes (95% CI is presented). The criterion value is corresponding with the Youden index.

SUPPLEMENTARY FIGURE 2
ROC-curve of the three-factor model (age, FPG and treatment of AH) of the risk of death for patients with diabetes (95% CI is presented). The criterion value is corresponding with the Youden index.

SUPPLEMENTARY FIGURE 3
Central (solid line) and non-central (dotted line) distribution to protocol of power analyses.

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EDITED BY
Gesthimani Mintziori,
Aristotle University of Thessaloniki, Greece

REVIEWED BY
Moises Leon Juarez,
Instituto Nacional de Perinatología (INPER), Mexico
Rabia Anwar,
University Health Network (UHN), Canada

*CORRESPONDENCE
M.V. Surekha
surekha_mv@yahoo.com
mullapudi.surekha781@gmail.com
Gargi Meur
gargimeur@icmr.gov.in

†These authors have contributed equally to this work and share senior authorship

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Impact of COVID-19 during pregnancy on placental pathology, maternal and neonatal outcome – A cross-sectional study on anemic term pregnant women from a tertiary care hospital in southern India

M. V. Surekha[1*†], N. Suneetha[2], N. Balakrishna[3], Uday Kumar Putcha[1], K. Satyanarayana[1], J. J. Babu Geddam[4], Pagidoju Sreenu[4], B. Tulja[4], Raja Sriswan Mamidi[5], Guy A. Rutter[6,7,8] and Gargi Meur[9*†]

[1]Pathology and Microbiology Division, Indian Council of Medical Research-National Institute of Nutrition, Hyderabad, India, [2]Obstetrics & Gynecology Department, Government Area Hospital, Nampally, Hyderabad, India, [3]Department of Statistics, Apollo Hospitals Educational and Research Foundation (AHERF), Hyderabad, India, [4]Clinical Epidemiology Division, Indian Council of Medical Research-National Institute of Nutrition, Hyderabad, India, [5]Clinical Division, Indian Council of Medical Research-National Institute of Nutrition, Hyderabad, India, [6]Centre of Research of Centre Hospitalier de l'Université de Montréal (CRCHUM), Faculty of Medicine, University of Montreal, Montreal, QC, Canada, [7]Section of Cell Biology and Functional Genomics, Imperial College London, London, United Kingdom, [8]Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, Singapore, [9]Cell Biology Division, Indian Council of Medical Research-National Institute of Nutrition, Hyderabad, India

Background: SARS-CoV-2 infection during pregnancy may cause adverse maternal, neonatal and placental outcomes. While tissue hypoxia is often reported in COVID-19 patients, pregnant women with anemia are suspected to be more prone to placental hypoxia-related injuries.

Methods: This hospital-based cross-sectional study was conducted between August-November 2021, during COVID-19 second wave in India. Term pregnant women (N=212) admitted to hospital for delivery were enrolled consecutively. Since hospital admission mandated negative RT-PCR test for SARS-CoV-2 virus, none had active infection. Data on socio-demography, COVID-19 history, maternal, obstetric, and neonatal outcomes were recorded. Pre-delivery maternal and post-delivery cord blood samples were tested for hematological parameters and SARS-CoV-2 IgG. Placentae were studied for histology.

Results: Of 212 women, 122 (58%) were seropositive for SARS-CoV-2 IgG, but none reported COVID-19 history; 134 (63.2%) were anemic. In seropositive women, hemoglobin (p=0.04), total WBC (p=0.009), lymphocytes (p=0.005) and neutrophils (p=0.02) were significantly higher, while ferritin was high, but not significant and neutrophils to lymphocytes (p=0.12) and platelets to lymphocytes ratios (p=0.03) were lower. Neonatal outcomes were similar. All RBC parameters

and serum ferritin were significantly lower in anemic mothers but not in cord blood, except RDW that was significantly higher in both, maternal (p=0.007) and cord (p=0.008) blood from seropositive anemic group compared to other groups. Placental histology showed significant increase in villous hypervascularity (p=0.000), dilated villous capillaries (p=0.000), and syncytiotrophoblasts (p=0.02) in seropositive group, typically suggesting placental hypoxia. Maternal anemia was not associated with any histological parameters. Univariate and multivariate logistic regression analyses of placental histopathological adverse outcomes showed strong association with SARS-CoV-2 seropositivity but not with maternal anemia. When adjusted for several covariates, including anemia, SARS-CoV-2 seropositivity emerged as independent risk factor for severe chorangiosis (AOR 8.74, 95% CI 3.51-21.76, p<0.000), dilated blood vessels (AOR 12.74, 95% CI 5.46-29.75, p<0.000), syncytiotrophoblasts (AOR 2.86, 95% CI 1.36-5.99, p=0.005) and villus agglutination (AOR 9.27, 95% CI 3.68-23.32, p<0.000).

Conclusion: Asymptomatic COVID-19 during pregnancy seemed to be associated with various abnormal placental histopathologic changes related to placental hypoxia independent of maternal anemia status. Our data supports an independent role of SARS-CoV-2 in causing placental hypoxia in pregnant women.

KEYWORDS

COVID-19, SARS-CoV-2, pregnancy, cord blood, placenta, anemia

1 Introduction

Coronavirus disease -2019 (COVID-19) pandemic has recently affected the whole world. The first case was reported from Wuhan (China) in the year 2019 (1) and India reported its first case on 30th January 2020 (2). Pregnant women are among the more vulnerable groups to be adversely affected by COVID-19 due to their physiological immunodeficiency (3, 4). While many studies have attempted to evaluate the effects of COVID-19 on the common population (5, 6), but till date only a limited number (7–9) focused on pregnant women. COVID-19 infection was reported to cause increase in preterm birth rates, varying from 5 to 41% (7–9)in different study population. Preterm rupture of membranes (PROM) was another frequent finding in COVID-19 during pregnancy with rates being 6.1, 20.7 and 26.5% in three different studies (8–10). A retrospective observational study from India found higher incidence of severe oligohydramnios and cesarean section during the second wave, while high frequency of preterm deliveries (24-27%) and low birth weight during both waves (11), which was also observed by others in maternal COVID-19 (12). Neonatal deaths or stillbirths were found occasionally, especially in cases with severe or critical disease (13–15), while intrauterine growth retardations were also frequently reported (16). However, majority of the studies reporting on neonatal outcomes found no serious adverse outcomes in neonates born to SARS-CoV-2-positive mothers (17–19).

Entry of SARS-CoV-2 virus into the cells trigger cascades of immune responses, prompting production of pro-inflammatory cytokines (Interferon 1/IFN1, Tumor necrosis factor-alpha/TNF-α, Interleukins/IL-16,33,25) and activation of $CD4^+$ and $CD8^+$ T lymphocytes (20). One study also reported presentation of anemia with decreased hemoglobin (Hb) levels and pathologically increased ferritin levels in COVID-19 in their subjects (21). Anemia affects about 50% of pregnant women in developing nations and is an indirect major cause of mortality in mothers (22). Prevalence of anemia in pregnant women in India was reported to be 52.2%, and 53.2% in Telangana state as per the latest National Family Health Survey-5 (2019-21) (23). Despite a high burden of anemia in pregnant women, there were almost no studies reporting on the effects of COVID-19 in pregnant women, fetus and newborns, as pre-existing anemia could be an additional complicating factor. Tissue hypoxia is one of the common adverse effects of COVID-19 being reported, which is caused by a hypercoagulable state. Anemia is presumed to be another potential independent risk factor for tissue hypoxia pertaining to lower hemoglobin and lower physiological capacity for oxygen transport, when faced with increased demand, such as placental tissue.

The placenta forms an important mediator for the transfer of nutrients and oxygen from mother to fetus. Diverse maternal conditions, leading to morphological changes in the organ can influence the placenta's functions (24). Maternal SARS-CoV-2 infection and its effect on the placenta and the fetus has been a major concern. A few studies investigated placental histopathology in COVID-19 patients, where histomorphological and ultrastructural changes were reported (25–27), but none of these studies could come up with specific features or hallmark changes in the placenta. Further, impact of maternal COVID-19 on pregnancy and neonatal outcomes in a population with high anemia burden remains largely unknown.

We undertook this study during the waning phase of the second wave of COVID-19 in India to understand if asymptomatic/mild COVID-19 during pregnancy was associated with any adverse maternal and fetal outcomes, as we suspected that SARS-CoV-2 infection might seriously compromise the peripheral oxygen supply to placenta and induce tissue hypoxia.

2 Materials and methods

2.1 Study design and recruitment of subjects

This cross-sectional study was conducted in a government maternity hospital in Hyderabad city of Telangana state. Consecutive pregnant women (N=212) in labor, admitted for delivery in the hospital, and who were willing to participate were enrolled after taking written informed consent. As per the COVID-19 protocol followed by the hospital during the pandemic, all women were required to undergo compulsory RT-PCR screening test for SARS-CoV-2 viral RNA at the time of admission and were admitted onlyif tested negative. Hence, all our participants, by default, were negative for any active infection during delivery.None had received any COVID-19 vaccine, as the immunization program for pregnant women did not start during the study period. Women were enrolled between August 2021 and November 2021, and only in the first half (till 2 pm) of the weekdays. The study was carried out according to 'The Code of Ethics of the World Medical Association (Declaration of Helsinki)' after obtaining approval from Institutional Ethical Committee (IEC) of ICMR-NIN as well as the maternity hospital.

Sample size was calculated by assuming the prevalence of SARS-CoV-2 in pregnancies as 15%, with a 95% confidence level, 80% power, which was 195. All women aged between 18–49 years, at 37-40 weeks of gestation, who were either primi or multiparous, and who did not receive any treatment for viral infections in their last trimester of pregnancy, were allowed to take part in the study. Those suffering from kidney disease, rheumatoid disease, diabetes mellitus, hypertension, acquired immune deficiency syndrome (AIDS), on immunosuppressant drugs, and who had complicated conditions such as ectopic pregnancy and hydatidiform mole were not included in the study.

2.2 Data collection

Complete epidemiologic history, clinical signs, symptoms, obstetric and immunization history, and outcome data of the participants were collected through a structured pre-tested questionnaire (Supplementary-Annexure I). The information on fever, cough, cold and other symptoms of COVID-19 were self-reported by the subject. Gestational age was calculated from the first reported day of last menstrual period (LMP). A digital balance (SECA scale, SECA robusta 813, Hamburg, Germany) was used to record the weight of the mothers to the nearest 100 g. Height was measured using a stadiometer to the nearest 0.1 cm (SECA 213 portable stadiometer). Maternal body mass index (BMI) was calculated using weight and height at baseline (kg/m^2). Newborns were weighed without diapers and their crown-heel length was measured to the nearest 0.1cm using an infantometer (SECA). APGAR (appearance, pulse, grimace, activity, and respiration) scores at 1 min and 5 min were recorded by the attending neonatologist.

2.3 Blood sample collection and processing

Venous blood sample (5 ml) was drawn from all the subjects by venipuncture from the antecubital vein before delivery under strict aseptic conditions. After delivery, 5 ml of cord blood was collected by trained study personnel in the labor room in vacutainers (Beckton Dickinson, USA) with ethylene diaminetetraacetate (EDTA).For hematological parameters,whole blood was analyzed within six hours of collection in an automated hematoanalyzer (ADVIA 120, Seimens, Germany) for hemoglobin (Hb), packed cell volume (PCV), red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width coefficient of variation percentage (RDW-CV %), platelets distribution width (PDW) and mean platelet volume (MPV).The serum from both maternal and cord blood was separated by centrifugation for 15 minutes at 1000 x *g* at room temperature and aliquots were stored at -20°C till further analysis.Anti-SARS-CoV-2 IgG was estimated in all serum samples by Enzyme-linked Immunosorbent Assay (ELISA) using Covid Kavach ELISA kit (ICMR-NIV, Pune and Zydus Diagnostics, Ahmedabad, India) following manufacturer's instructions (Supplementary- Annexure II) using an ELISA reader (Model No: BioTek Synergy HT, USA).Serum ferritin was measured in all samples by ELISA (Calbiotech, El Cajon, CA, USA).

2.4 Placental analysis

The placentae after delivery were immediately placed in adequate volume of 10% neutral buffered formalin and brought for grossing. The placentas were fixed for 72 hours, and examined for gross abnormalities and measurements. They were cut at 1cm intervals and into 3 mm thick tissue sections. Three sections from the placenta parenchyma, one section from membranes and two cross sections of cord were studied from each placenta. After overnight processing of the tissues in a tissue processor (Automated Vacuum tissue processor, Shadon Excelsior ES, by Thermo Scientific Fisher, Ramsey, USA) these sections were embedded in paraffin (Histocentre 3, Shandon, Thermo electron corporation, Fischer Scientific, Singapore) and tissue blocks were prepared. Each block was cut to 4-5µm thick sections on a microtome (Leica RM 2155,Nussloch, Germany) and stained (Sakura Tissue Tek DRS Autostainer, Finetek, Europe) with hematoxylin and eosin stain (H&E), mounted on glass slides and examined under Nikon Eclipse E800 microscope (Nikon Instruments, Florida, USA). Histopathological analysis was done according to the Amsterdam protocol (28). The H&E stained sections were studied for histopathological findings like inflammation, vascularity, infarcts, calcification, vasculitis etc. and were counted for statistical analysis. Chorangiosis was diagnosed based on the criteria laid down

by Altshuler (29). Grading of amniochorionitis was done as follows: Grade1- focal areas of neutrophils infiltration, Grade 2- neutrophils infiltration in 50% of the section, Grade 3-neutrophils infiltrating >80% of the section. Vascularity of villi was graded as normal chorionic villi that contain <5 capillaries in 10 high-power microscopic fields, and larger numbers are defined as hypervascularity; Grade 1 : 5 to 7 capillaries per villi, Grade 2 : 7 to 10 capillaries per villi, and Grade 3 : hypervascularity also known as chorangiosis is characterized by >10 capillaries in more than 10 terminal chorionic villi in several areas of the placenta. Dilated blood vessels were graded as follows: Grade 1- mildly dilated and Grade 2- moderate to severely dilated blood vessels with large lumina occupying more than 80% of the villi. Number of syncytiotrophoblasts were graded as, Grade 1 - normal with few scattered syncytiotrophoblasts, Grade 2 - increased number with the formation of syncytial knots. Fibrin deposition was graded as, Grade 1 - 10-20% of the section, Grade 2 - 20-50% of the section, Grade 3 - >50% of the section.

2.5 Statistical analyses

IBM SPSS (version 24.0, SPSS Inc. IBM, USA) was used for all statistical analysis. Mean and standard deviation (SD) were calculated for all continuous variables and proportions were calculated for qualitative variables. Mean values of blood and histopathological variables of mothers were compared based on their SARS-CoV-2 antisera positivity and hemoglobin (Hb) status using *t* test or Mann Whitney U test, whenever assumptions of equality of variances and normality were violated. Chi-square test was performed to study the association between categorical variables and outcomes. Spearman rank correlation coefficients were calculated to study the relationships between maternal and cord blood parameters including SARS-CoV-2 IgG. ANOVA was done for comparing multiple groups with or without COVID-19 and anemia. Univariate and multivariate logistic regression were done and models were built to identify risk factors for placental deformities. Odds ratio (OR) were calculated for potential independent variables including SARS-CoV-2 infection and anemia, and other covariates for different placental histopathologic outcomes as dependent variable. Results were considered statistically significant when p value was <0.05.

3 Results

3.1 Sociodemographic and maternal variables and neonatal outcome among SARS-CoV-2 antibody seropositive and seronegative groups

Of the total 212 term pregnant women enrolled during the study period, 122 (58%) were positive for SARS-CoV-2 antisera, while 90 (42%) were negative. Through self-reporting by the subjects, it was found that none had developed any symptom of COVID-19, such as cough, cold, or fever during their entire gestational period to indicate if they were infected. Hence, we concluded that those who developed SARS-CoV-2 antisera had asymptomatic COVID-19. Of the 212, 63.2% (n=134) women were anemic. As shown in Table 1, comparison of seropositive and negative women found that they were of almost comparable age (24.11 vs. 23.58 yr), mean body weight (62.66 vs 60.98 kg) and BMI (26.45 vs 24.72 kg/m^2), but more women in positive group were in overweight category (65.5 vs. 76.7%). While maternal education level was comparable between the groups, a significant difference was noted in monthly family income, being lower in the positive group (p=0.04), and more women belonged to the community of other backward class (OBC) (p=0.06). About 92% (n=194 of 210) women had >=4 antenatal checkups, >94% (n=199 of 210) had two Tetanus Toxoid injections, and >98% (n=207 of 210) had taken 100 tablets of IFA (Iron and folic acid supplementation) supplementation during their pregnancy course(dose: 100 mg Fe and 500 µg folic acid) and there was no difference between the seropositive and negative groups. The mean gestational age between the two groups was similar (38.22 vs 38.73 weeks).

None of the 212 participants had weakness, dyspnoea, palpitation, or chest pain, and none showed signs of koilonychia and jaundice, but two women had developed pedal edema and three had pallor. Among obstetric outcomes, of the 212 pregnancies, there were cases of oligohydramnios (n=51), post-dated pregnancy (n=12), gestational hypertension (n=2), cephalopelvic disproportion (n=12), premature rupture of membranes (PROM) (n=22), placenta previa (n=1), Rh-ve pregnancy (n=8), meconium-stained liquor (MSL) (n=5) and pre-eclampsia (n=3). There were 3 cases of still births and 7 cases of spontaneous abortions.

Among the neonatal outcomes (Table 1), birth weight (2.85 vs. 2.87 g), APGAR scores at 1 min (6.20 vs. 6.21) and 5 min (9.43 vs. 9.5), gestational age were not different for newborns of seropositive and negative mothers. However, newborn length was lesser in seropositive group when compared to the negative group and the difference was significant (p=0.006).

3.2 Hematological variables based on SARS-CoV-2 antibody in maternal and cord blood

Hematological parameters in SARS-CoV-2 antibody seropositive and negative mothers and cord blood specimens were compared (Table 2). Maternal hemoglobin (p=0.04), WBC (p=0.009), MCHC (p=0.04), absolute lymphocyte (p=0.005), and absolute neutrophils (p=0.02) were significantly higher in seropositive mothers while, NLR (p=0.12) and PLR (p=0.03) were lower in seropositive mothers. Unlike maternal hematology parameters, only RDW (p=0.02) and PDW (p=0.005) were significantly different between the two groups in cord blood, while remaining parameters, although showed some difference, but did not vary significantly. Serum ferritin level was similar between seropositive and negative samples for both maternal and cord blood. While SARS-CoV-2 IgG level correlated well between maternal and cord blood samples (Figure S1), there were few exceptional cases where antibody was detected either in mother

10.3389/fendo.2023.1092104

TABLE 1 Socio-demography, maternal features and neonatal birth outcomes of the study participants based on SARS-CoV-2 serological status.

Maternal characteristics (N=210)		SARS-CoV-2 serological status		*p*
		Positive (n=122)	Negative (n=88[#])	
Maternal age (yr)	Mean (SD)	24.11 (3.65)	23.58 (3.19)	0.25
Weight (kg)	Mean (SD)	62.66 (9.89)	60.98 (10.47)	0.23
BMI category	<18.5, n (%)	1 (0.9)	4 (4.6)	0.29
	18.5-23, n (%)	26 (22.4)	26 (29.9)	
	>=23, n (%)	89 (76.7)	57 (65.5)	
Educational status	No school, n (%)	16 (13.1)	15 (16.7)	0.69
	Up to secondary, n (%)	97 (79.5)	70 (77.8)	
	Degree, n (%)	9 (7.4)	5 (5.6)	
Occupation	Working, n (%)	4 (3.3)	2 (2.2)	0.63
	Housemaker, n (%)	117 (95.9)	85 (95.5)	
Family monthly income (INR[$])	<5000, n (%)	40 (32.8)	16 (17.8)	**0.04**
	5000-10,000, n (%)	64 (52.5)	61 (67.8)	
	10,000-50,000, n (%)	18 (14.8)	12 (13.3)	
	>50,000, n (%)	0	1 (1.1)	
Community	SC, n (%)	1 (0.8)	4 (4.4)	0.06
	ST, n (%)	6 (4.9)	5 (5.6)	
	OBC, n (%)	85 (69.7)	60 (66.7)	
	OC, n (%)	18 (14.8)	19 (21.1)	
	Others, n (%)	12 (9.8)	2 (2.2)	
Placental weight (g)	n (mean ± SD)	122 (410.84 ± 84.31)	90 (420.60 ± 78.49)	0.39
Neonatal outcome[$]				
Newborn weight (kg)	n (mean ± SD)	100 (2.85 ± 0.47)	76 (2.87± 0.43)	0.82
Newborn length (cm)	n (mean ± SD)	97 (48.65 ± 2.65)	68 (49.77 ± 2.40)	**0.006**
Gestation age (weeks)	n (mean ± SD)	102 (38.22 ± 1.05)	78 (38.73 ± 5.82)	0.38
APGAR score 1 min	n (mean ± SD)	122 (6.20 ± 3.01)	92 (6.21± 3.11)	0.99
APGAR score 5 min	n (mean ± SD)	100 (9.43 ± 0.68)	74 (9.51± 0.58)	0.39

For quantitative variables, comparison of mean for normally distributed dataset was done by parametric t test, and otherwise non-parametric Mann-Whitney U test. For categorical variables, Chi-square test was done.[#]Maternal variables in were missing for 2 cases and mean was calculated of n=210, unless specified otherwise. [$]For some cases, few parameters were missing in data records, due to staff shortage at labor room on certain days. SD, standard deviation; SC, scheduled caste; ST, scheduled tribe; OBC, other backward class; OC, open category; APGAR, Appearance, Pulse, Grimace, Activity, and Respiration; UC, umbilical cord; INR- Indian Rupee. [$]1 USD ~ 75 INR as in the August-Nov, 2021. Values in bold signifies p<0.05.

or in cord blood. Spearman correlations (Table S1) performed between maternal and cord blood that showed significant positive correlation for presence of IgG antibodies (Figure S1) in SARS-CoV-2 seropositive mother's blood and seropositive cord blood (r=0.42, 95% CI 0.26-0.56, 2-tailed $p<0.0001$) and for monocytes % (r=0.37, $p<0.001$), but not for Hb (r=0.15, p =0.12), WBCs (r=0.06, p=0.51), absolute lymphocyte count (r=0.07, p=0.46) and absolute monocyte count (r=0.10, p=0.29). Other parameters including absolute neutrophil count (r = -0.04, p=0.69), NLR (r=-0.06, p=0.50) and PLR (r=-0.18, p=0.05) showed negative correlations between SARS-CoV-2 antisera positive mother and COVID-19 antisera positive cord blood but the correlation was significant only for PLR.

3.3 Hematological and other variables based on SARS-CoV-2 antisera and maternal anemia

We compared hematological parameters of maternal and cord bloodsamples between SARS-CoV-2 antibody positive and negative groups, based on maternal anemia status and shown only the parameters that were significantly different by one-way ANOVA (Table 3). We found, among the seropositive group, there was a difference of about 2.5 g/dl in hemoglobin between anemic and non-anemic group, while in seronegative group the difference was about 3.5 g/dl. *Post-hoc* multiple comparison between and within the groups for hemoglobin showed that mean hemoglobin level was

TABLE 2 Comparison of haematological parameters in SARS-CoV-2 antisera positive and negative maternal and cord blood samples.

#Parameters	Maternal blood			Cord blood		
	Positive (n=122)	Negative (n=90)	*p*	Positive (n=134)	Negative (n=65)	*p*
	Mean (SD)	Mean (SD)		Mean (SD)	Mean (SD)	
Hemoglobin (g/dl)	10.26 (1.91)	9.64 (2.48)	**0.04**	11.87 (2.36)	11.92 (2.38)	0.87
Ferritin (ng/ml)	41.6 (62.52)	44.0 (65.42)	0.253	107.88 (78.57)	124.23 (93.49)	0.11
WBC (10^3/µl)	11.01 (3.29)	9.83 (3.23)	**0.009**	9.33 (3.86)	10.01 (11.41)	0.55
RBC (10^6/µl)	4.03 (0.64)	3.87 (0.86)	0.12	3.62 (0.68)	3.62 (0.67)	0.99
PCV (%)	32.62 (5.29)	30.92 (7.25)	0.06	36.88 (6.80)	37.19 (6.97)	0.75
MCV (fl)	81.15 (8.78)	79.78 (8.21)	0.25	102.11 (7.33)	102.99 (5.46)	0.35
MCH (pg)	25.52 (3.67)	24.87 (3.43)	0.19	32.86 (2.97)	33.00 (2.27)	0.73
MCHC (g/dl)	31.35 (1.69)	30.87 (1.81)	**0.04**	33.55 (14.98)	32.045 (1.23)	0.37
Plt (10^3/µl)	252.42 (84.5)	232.5 (80.20)	0.08	138.02 (95.62)	124.47 (93.65)	0.32
Lymph.absol	2.44 (0.90)	2.07 (0.96)	**0.005**	3.90 (2.36)	3.48 (2.08)	0.18
Lymphocyte (%)	23.02 (8.03)	22.37 (11.83)	0.63	40.49 (15.36)	39.72 (18.05)	0.74
Mono.absol.	0.35 (0.26)	0.30 (0.15)	0.09	0.91 (1.78)	0.69 (0.28)	0.19
Monocyte (%)	3.22 (3.24)	3.45 (2.60)	0.59	7.94 (4.15)	8.21 (2.97)	0.62
Neutro.absol.	8.25 (3.01)	7.35 (2.55)	**0.02**	4.44 (2.15)	4.32 (2.38)	0.70
Neutrophil (%)	72.39 (13.55)	73.85 (13.71)	0.44	48.54 (13.77)	47.08 (13.62)	0.46
NLR	3.69 (1.75)	4.07 (1.78)	0.12	1.37 (1.19)	1.19 (0.67)	0.22
PLR	109.87 (40.80)	124.56 (54.86)	**0.03**	37.97 (27.95)	33.43 (25.43)	0.25
RDW (%)	15.64 (3.04)	15.63 (3.74)	0.98	15.23 (1.74)	14.73 (0.93)	**0.02**
MPV (fl)	6.81 (1.07)	6.82 (1.10)	0.95	7.49 (1.40)	7.45 (1.30)	0.85
PDW (%)	16.90 (1.05)	17.07 (0.92)	0.24	18.38 (0.98)	18.85 (1.19)	**0.005**

#The mean values were compared between SARS-CoV-2 seropositive and negative subjects by parametric t test for normally distributed data set, or otherwise non-parametric Mann-Whitney U test was done. A p value <0.05 was considered significant and given in bold. BMI, body mass index; WBC, white blood cells count; RBC, red blood cells count; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular hemoglobin concentration; PCV, packed cell volume; RDW, red cell distribution width, Plt, platelets; MPV, mean platelets volume; PDW, platelet distribution width; Lymp.absol, absolute lymphocyte count; Mono.absol, absolute monocyte count, Neutro.absol, absolute neutrophil count; NLR, neutrophils to lymphocyte ratio; PLR, platelets to lymphocytes ratio; IgG, Immunoglobulin; fl, femtoliter; dl, decilitre; pg, picogram.

significantly different between the anemic and normal subjects in our cohort. We observed that among the non-anemic subjects, COVID-19 did not alter hemoglobin level. However, within the anemic subjects, hemoglobin level was significantly different (~0.6 g/dl) between seropositive and negative groups. Serum ferritin level was significantly lower in seronegative anemic women when compared to seronegative non-anemic group (p=0.004), however, serum ferritin level was found to be raised in the anemic seropositive group (43.9 vs. 27.8 ng/ml), maybe due to infection, when compared to the seronegative anemic group (Table 3). In seropositive mothers with anemia, all the red cell parameters, including hemoglobin, RBC, PCV, MCV, MCH, and MCHC were significantly lower than in non-anemic mothers (Table 3). Among other parameters WBC, neutrophils, lymphocytes were significantly lower while PLR and RDW were higher in anemic mothers. Maternal variables such as age, height, weight, and BMI were not significantly different between and within groups. Obstetric variables, such as systolic and diastolic blood pressure, oxygen saturation % (SpO2), pulse rate, respiratory rate and heart rate were analyzed and found to be similar among the groups. Cord blood examination showed, except for RDW and PDW, almost similar hematological features between the groups, and while most of the red cell and white blood cell parameters were lower in anemic conditions, but the difference was not significant. RDW percentage, which describes the variability in red cell size, were generally higher in the anemic mothers, irrespective of SARS-CoV-2 serological status, however, in the cord blood, we noticed significantly higher variation in red cell size in anemic and seropositive group compared to others. There was no difference in cord blood hemoglobin, WBC, RBC, absolute lymphocytes, neutrophils, monocytes, and other hematological parameters. We failed to notice any difference in newborn parameters, such as weight, gestational age, APGAR scores at 1 min and 5 min, except neonatal length, which was slightly lower for those born to anemic and seropositive mothers compared newborns of other groups. Further *post hoc* multiple comparison, showed significant difference of -1.642 cm between the seropositive and negative groups within anemic mothers, but none within non-anemic mothers.

TABLE 3 Comparison of haematological parameters in SARS-CoV-2 antisera positive and negative mothers and cord blood samples based on anaemia status of the mother.

Maternal blood (N=212)					
Parameters	SARS-CoV-2 antisera positive		SARS-CoV-2 antisera negative		*p*
	Anaemic (n=72)	Normal (n=50)	Anaemic (n=62)	Normal (n=28)	
Haemoglobin (g/dl)	9.17 (1.73)^{a,b,c}	11.83 (0.64)^{a,d}	8.57 (2.22)^{b,d,e}	12.02 (0.79)^{c,e}	**0.000**
Ferritin (ng/ml)	43.9 (80.27)	44.2 (38.13)	27.8 (44.85)^{a}	71.2 (84.26)^{a}	**0.000**
WBC (10^3/µl)	10.57 (3.31)^{a}	11.66 (3.18)^{b}	9.10 (2.92)^{a,b,c}	11.43 (3.33)^{c}	**0.000**
RBC (10^6/µl)	3.90 (0.75)^{a,b,}	4.23 (0.36)^{a,d}	3.67 (0.94)^{d,e}	4.31 (0.37)^{b,e}	**0.000**
PCV (%)	30.05 (5.37)	36.32 (1.91)	28.11 (6.97)	37.16 (2.24)	**0.000**
MCV (fl)	77.60 (8.86)	86.26 (5.61)	76.74 (7.39)	86.50 (5.51)	**0.000**
MCH (pg)	23.71 (3.30)	28.14 (2.37)	23.46 (2.96)	27.99 (2.11)	**0.000**
MCHC (g/dl)	30.49 (1.56)	32.58 (0.93)	30.36 (1.31)	31.99 (2.25)	**0.000**
Lymph.absol	2.44 (1.05)	2.44 (0.65)	1.89 (0.84)	2.47 (1.09)	**0.002**
Neutro.absol	7.8 (2.9)	8.87 (3.14)	6.91 (2.38)	8.31 (2.67)	**0.003**
PLR	114.19 (46.47)^{a}	103.65 (30.21)	133.54 (58.89)	104.69 (38.62)	**0.003**
NLR	3.57 (1.75)^{a}	3.85 (1.76)	4.19 (1.82)	3.81 (1.69)	0.255
RDW (%)	16.26 (2.95)^{a,b}	14.77 (2.98)^{a,c}	16.22 (4.20)^{c,e}	14.34 (1.97)^{b,e}	**0.007**
Cord blood (N=200)$^{\$}$					
	SARS-CoV-2 antisera positive		**SARS-CoV-2 antisera negative**		***p***
	Anaemic$^{\#}$(n=70)	**Normal (n=49)**	**Anaemic$^{\#}$(n=56)**	**Normal (n=25)**	
RDW (%)	15.51 (2.15)^{a,b,c}	14.83 (0.76)^{a}	14.78 (0.84)^{b}	14.63 (1.14)^{c}	**0.008**
PDW (%)	18.28 (0.94)^{a,b}	18.54 (1.02)	18.81 (1.19)^{a}	18.93 (1.24)^{b}	**0.015**
Newborn outcome (N=165)$^{\$}$					
	Anaemic$^{\#}$ (n=58)	**Normal (n=39)**	**Anaemic$^{\#}$ (n=44)**	**Normal (n=24)**	***p***
Newborn length (cm)	48.52 (2.79)^{a}	48.85 (2.43)	50.16 (2.32)^{a}	48.75 (2.09)	**0.009**

The mean values were compared between groups and within groups by One-way ANOVA. Only variables where F values were significant by ANOVA are shown here. Same alphabets in the superscript are given to denote significant difference within these groups upon Bonferroni's post hoc multiple comparison. $^{\#}$Groups based on maternal anemia status of the mother. $^{\$}$12 of 212 cord blood samples had clots and analysis failed. Total missing data of a few parameters of the newborn outcome is n=47, of which fetal/neonatal death accounted for n=10. white blood cells count, RBC, red blood cells count; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular hemoglobin concentration; PCV, packed cell volume; RDW, red cell distribution width; PDW, platelet distribution width; Lymp.absol, absolute lymphocyte count; Neutro.absol, absolute neutrophil count; PLR, platelets to lymphocytes ratio; NLR, neutrophil to lymphocytes ratio. Values in bold signifies p<0.05.

3.4 Placental variables

Mean placental weight (Table 1) was relatively lower in seropositive group but the difference was not significant. Histopathological features were studied in all the 212 placentae received. Figure 1 shows a typical histology of placentae from seronegative and non-anaemic healthy mothers, where the normal sized chorionic villi are seen lined by normal syncytiotrophoblasts and cytotrophoblasts and with the stroma showing normal fetal capillaries. When compared between the groups, acute mild chorioamnionitis was observed to be significantly higher in the membranes of seropositive placentae. Most of the umbilical cords (UC) were histologically normal in both seropositive and negative groups. With respect to placenta proper, histological features, like mild to severe degree of hypervascularity or increased vascularity of villi (increased number of fetal capillaries/villus) (Figures 2A–C), increased dilatation of capillaries in the villi (Figures 3A–C) and increased number of syncytiotrophoblasts with the formation of syncytial knots (Figure 4) and increased fibrin deposition (Figures 5A–C) were observed in seropositive placentae. Other features like villitis (Figure S2), vasculitis involving blood vessels in the chorionic villi, intervillous hemorrhage (Figure S3), infarcts in the decidua as well as the villi, smaller terminal villi indicating accelerated villous maturation (Figures S4, S5), calcification, decidual inflammation, villous agglutination and avascular fibrosed villi (Figure S5) were also found to be significantly more in the seropositive placentae.

Table 4 shows the difference in various histopathological findings between the placentae of seropositive and seronegative mothers and the association was tested using Chi-square test. There were significant increase in hypervascularity of villi of grade 3

10.3389/fendo.2023.1092104

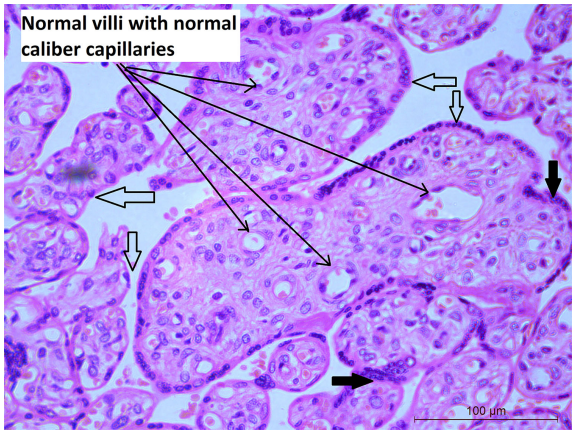

FIGURE 1
Histology of villi from normal placenta: Microphotograph shows normal chorionic villi (empty bold arrows) from placenta of mother who was COVIID-19 negative and non-anemic. The villi are lined by trophoblast cells (black bold arrows) with few scattered normal capillaries in the stroma of the villi (long black arrows). Original magnification, ×20.

category (28 vs. 11%), dilated blood vessels in the villi of grade 2 (84.9 vs. 36.3%), number of syncytiotrophoblasts (84.8 vs. 69.2%), intervillous haemorrhage (40.3 vs. 15.4%), degeneration of trophoblast lining (39.5 vs. 5.5%) and villus agglutination (52.5 vs. 17.6%) in placental sections from seropositive group when compared to the negative group.

We further analyzed between anemic mothers within seropositive and negative groups based on maternal anemia status (Table 5) to find out that chorangiosis (p=0.000), dilated blood vessels (p=0.000), syncytiotrophoblasts ((p<0.01), accelerated villous maturation were significantly higher (p=0.02) in seropositive anemic mothers. We tested but found no difference in fibrin, villitis, karyorrhexis, infarcts, fibrosed, avascular villi, premature villi and other parameters, while weaker association was found for calcification and decidual inflammation,

3.5 Multivariate logistic regression analysis of risk factors for abnormal placental findings

Among placental histopathological findings, logistic regression models (Table 6) were developed for moderate to severe chorangiosis, dilated blood vessels, syncytiotrophoblasts, agglutination of villus and calcification (data not shown) as dependent variables. The predictors or independent variables were included in the models were based on either their significant association in bivariate analyses or based on assumptions of their potential to influence the outcome. In all the models, SARS-CoV-2 seropositivity was an independent risk factor for increased chorangiosis (AOR=8.74; 95% CI 3.51 - 21.76, p<0.000), dilated blood vessels (AOR=12.7;95% CI 5.46 - 29.75,p<0.000), syncytiotrophoblasts (AOR=2.86; 1.36 - 5.99, p<0.005), villus

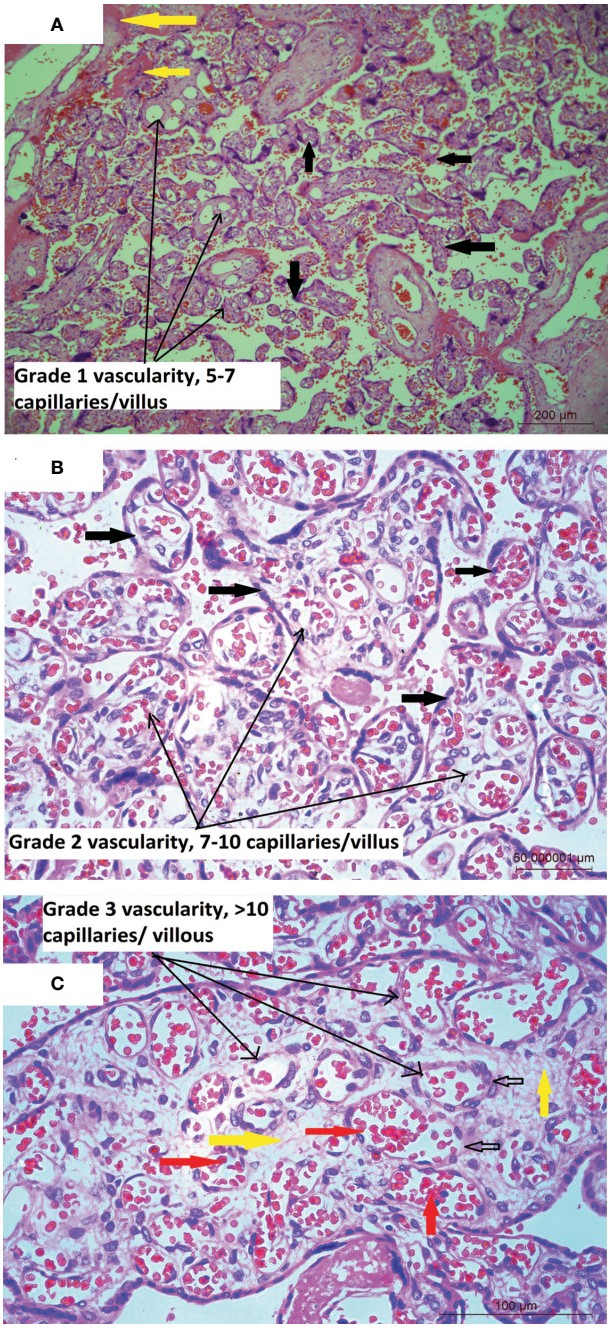

FIGURE 2
(A) Placental histology showing increased number of blood vessels: Microphotograph from placenta of COVID-19 positive mother shows chorionic villi (bold black arrows) with mild increase in the number of capillaries (5-7/villus) in the stroma (long black arrows), with normal amount of fibrin (bold yellow arrows). Original magnification, ×10. **(B)** Microphotograph shows chorionic villi from placenta of COVID-19 positive mother. The villi are marked in bold black arrows, are lined with trophoblast cells and show moderate or Grade 2 increase in the number of capillaries (7-10/villus) containing red blood cells (RBCs) in the stroma (long black arrows) of majority of the villi. Original magnification, ×20. **(C)** Microphotograph shows chorionic villus from placenta of COVID-19 positive mother showing Grade 3 increase in the number of capillaries per villus in the stroma (bold yellow arrows) of the villi (long black arrows), bold black empty arrows point to capillaries lined by endothelial cells and containing RBCs (bold red arrows). Original magnification, ×20.

10.3389/fendo.2023.1092104

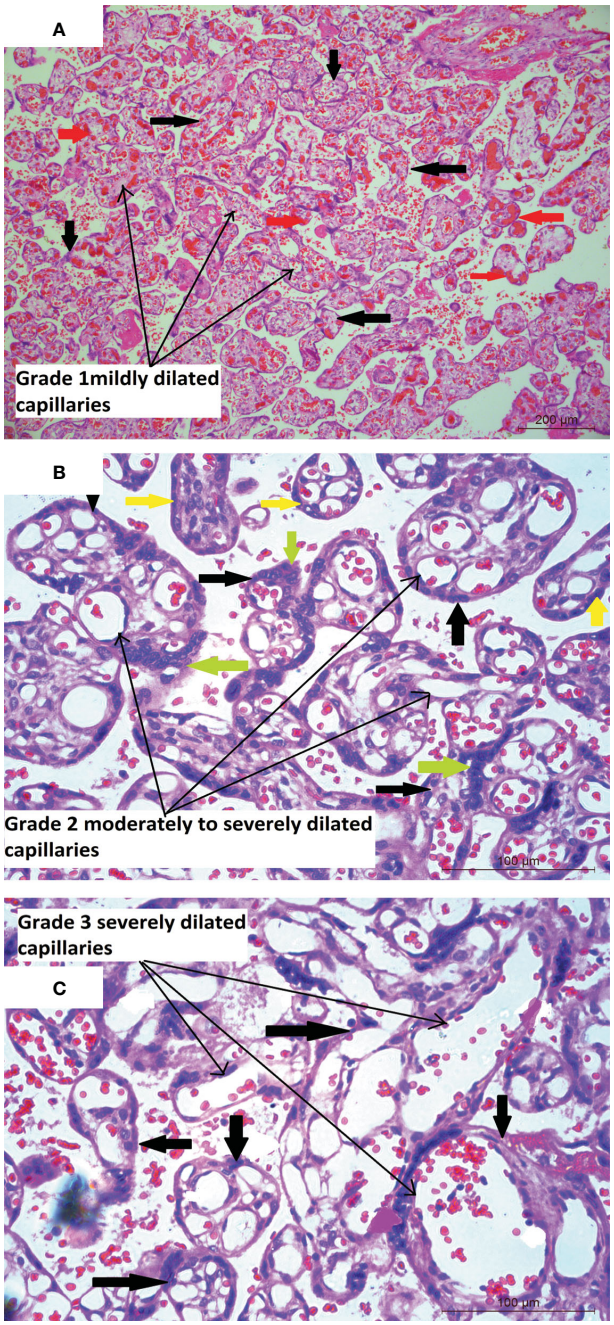

FIGURE 3
(A) Placental histology showing dilated blood vessels Microphotograph shows multiple chorionic terminal villi (bold black thick arrows) from placenta of COVID-19 positive mother with the stroma containing mildly dilated and congested capillaries (long black arrows). The congested capillaries with RBCs are marked in bold red arrows. Original magnification, ×10. **(B)** Microphotograph shows multiple chorionic terminal villi (bold black thick arrows) from placenta of COVID-19 positive mother with the stroma containing moderately to severely dilated capillaries (long black arrows) occupying most of the villus area. The villi are lined by syncytiotrophoblasts (bold green arrows) and cytotrophoblasts (bold yellow arrows). Original magnification, ×20. **(C)** Microphotograph shows chorionic terminal villi (bold black thick arrows) lined by syncytiotrophoblasts and cytotrophoblasts, from placenta of COVID-19 positive mother with extremely dilated fetal capillaries (long black arrows) occupying a major part to the whole of the villus area. Original magnification, ×20.

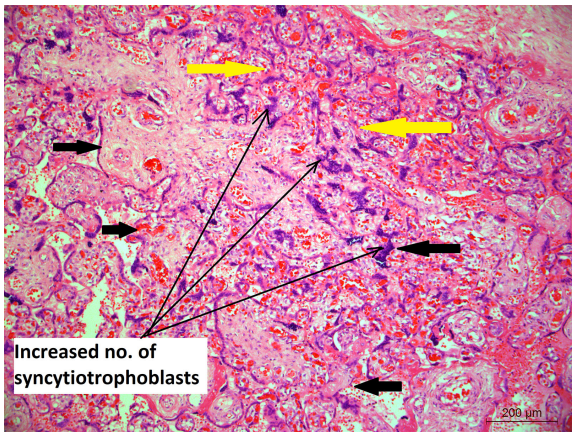

FIGURE 4
Placental histology showing increased number of syncytiotrophoblasts; Microphotograph shows chorionic villi (bold black thick arrows) from placenta of COVID-19 positive mother lined by increased number of multinucleated syncytiotrophoblasts (long black arrows). Moreover, the bold yellow arrows also show the area where the villi are closely placed indicating villus agglutination. Original magnification, ×10.

agglutination (AOR=9.27, 95% CI 3.68-23.32, $p<0.000$) and calcification (AOR=2.72; 95% CI 1.21-6.11, $p<0.015$) after adjustments for the other covariates. In separate models (data not shown) sociodemographic and maternal factors (maternal age, BMI, income, parity, gestational age), haematological factors (haemoglobin, WBC, PCV, MCV, RDW, Neutrophils, Lymphocytes, Monocytes, NLR, PLR) and birth weight were individually tested along with SARS-CoV-2 seropositivity, but none of the parameters contributed significantly. In addition, we will like to emphasize that haemoglobin did not emerge as a risk factor for any of these histopathological outcomes tested. Advancing gestational age showed a trend of increasing risk (AOR=8.97, $p=0.057$) for severe syncytiotrophoblasts, but was not significant for the other placental abnormalities. Low birth weight was found to be associated with villus agglutination (AOR=3.72, $p=0.012$). Abnormal WBC count (other than $4\text{-}12\times10^3/\mu l$) was associated with higher risk of severe chorangiosis (AOR=2.76, $p=0.031$), syncytiotrophoblasts (AOR=2.96, $p=0.041$) and increased agglutination of villus (AOR=2.33, $p=0.028$).

4 Discussion

In the first global wave of COVID-19, India saw its first outbreak between March, 2020 and February, 2021, later followed by the emergence of the Delta variant (B.1.617.2), responsible for India's deadly second wave following its emergence in February 2021 (16, 30). In the present study, we observed that, whilst all of the participants were RT-PCR negative for SARS-CoV-2 virus at the time of hospital admission for delivery during August-November, 2021, and whilst almost all of them self-reported to have never experienced COVID-19 like symptoms during pregnancy, but about 58% were carrying IgG antibodies against SARS-CoV-2.

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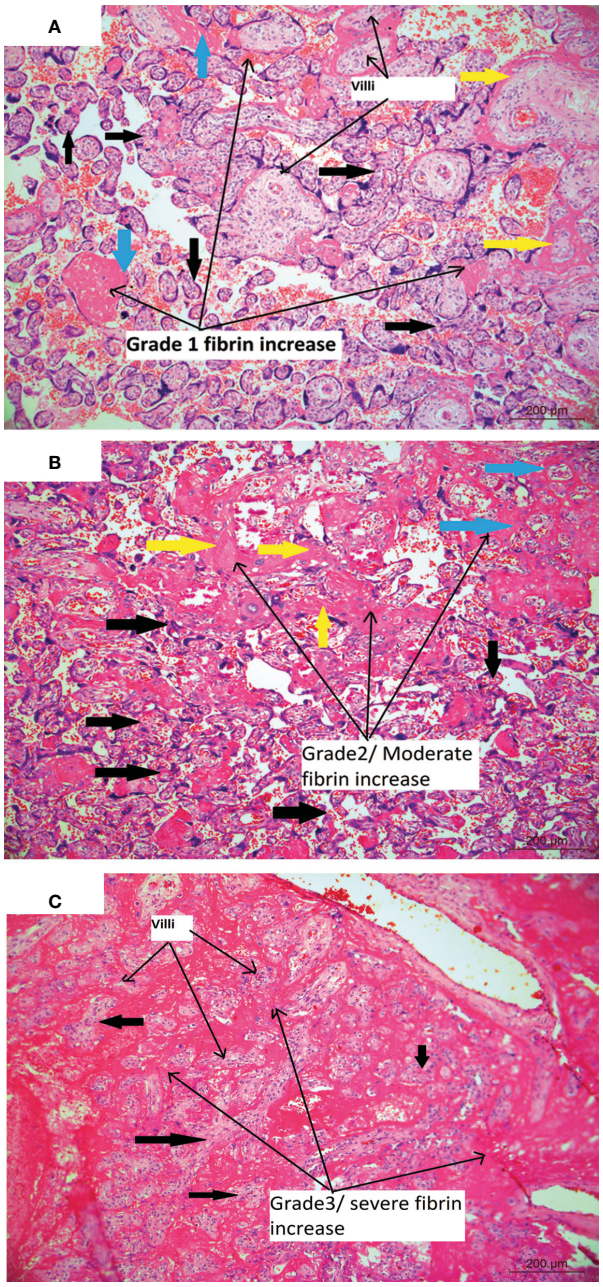

FIGURE 5
(A) Placental histology showing varied deposition of fibrin: Microphotograph shows chorionic villi (bold black thick arrows) from placenta of COVID-19 positive mother and with mild and grade1 increase in fibrin deposition in between (intervillous/blue bold arrows) and around the villi (perivillous/bold yellow arrows) (long black arrows) while the normal villi are shown in short black arrows. Original magnification, ×10. **(B)** Microphotograph showschorionic villi (bold black thick arrows) from placenta of COVID-19 positive mother and with moderate and grade2 increase in fibrin deposition in between (intervillous/bold yellow arrows) and around the villi (perivillous/bold blue arrows) and long black arrows. Original magnification, ×10. **(C)** Microphotograph shows multiple chorionic villi chorionic villi (bold black thick arrows) from placenta of COVID-19 positive mother, majority of which are surrounded by excessive amount of fibrin (intravillous and intervillous) (long black arrows) while the villi are shown in short black arrows. Original magnification, ×20.

The national sero-surveillance data of India showed SARS-CoV2 IgG prevalence as 24.1% [95% CI (23.0 - 25.3)] in the general population during December, 2020- January, 2021, before the vaccines were available (31). By June-July, 2021, this value had increased to 62.3% [95% CI (60.9 - 63.7)] in unvaccinated adults (including pregnant women). Vaccine roll out started for pregnant women in our country almost at the end of October 2021 (and our study cohort included pregnant women till November 2021) due to which we say that all the women were unvaccinated. Sero-prevalence was significantly higher among individuals who had received either one or two vaccine doses (81.0-89.8%) (32). A meta-analysis of all sero-prevalence data in India between March 2020 to August 2021 showed an overall pooled SARS-CoV-2 IgG sero-prevalence of 20.7% [95% CI (16.1 - 25.3)] in the first and 69.2% [95% CI (64.5 - 73.8)] in the second wave (33). Therefore, it is highly likely that majority of our subjects were infected by SARS-CoV2 during their gestational period, lying between November, 2020 and November, 2021, irrespective of the SARS-CoV2 variant involved. A number of serology studies globally reported a reduced immunological response (34) and rapid decay of the antibody (35) in asymptomatic cases or in mild COVID-19 infection (36).Others also reported a longer duration of IgG in circulation lasting beyond six months (37) to twelve months as in convalescent plasma donors having higher titer of antibodies (38). Since our study was conducted before COVID-19 vaccination drive for pregnant women in India, the presence of SARS-CoV-2 IgG only indicates asymptomatic infection in the past, most likely due to an infection in the preceding 9-months of gestational period. In another study, the authors reported that about 84% of their infected pregnant subjects were asymptomatic and such that the duration of the placental histopathological findings could not be determined (39).

The prevalence of COVID-19 infection in our study was much higher when compared to the studies by Bachani et al. (40) of 16.3%, and of Waghmare et al. (41) showing 12.3%, or of Singh et al. (42) reporting 4.83%. Since all of these studies estimated prevalence based on SARS-CoV-2 antigen and therefore active infection, and likely symptomatic patients only, the values are not comparable to our values of seropositivity. Our finding corroborates with other reports stating the fact that COVID-19 causes milder disease in pregnant women (40, 43–45). In our study population, however, birth weight and APGAR scores (appearance, pulse, grimace, activity, and respiration) of all neonates were within normal limits when compared between the seropositive and seronegative mothers, and no obvious adverse effect of COVID-19 was reported on these neonatal outcomes as reported earlier (40, 44, 45). A few other studies had reported lower birth weight in SARS-CoV-2 infected pregnant women (43, 46, 47) but it was found similar in the two groups in our participants.

Anemia has been stated to be one of the co-morbidities affecting COVID-19 pregnancies and in India anemia in pregnancy is very common. Earlier studies have reported development of anemia in COVID-19 patients and the patients presented with decreased Hb and increased ferritin levels (21, 48). However, in our study population, hemoglobin level was similar in seropositive and negative groups among non-anemic women as reported in a few

10.3389/fendo.2023.1092104

earlier studies (48, 49). Also, ferritin level in both maternal and cord blood were similar between the seropositive and negative groups, likely because none of our subjects were suffering from an acute infection during delivery. It was however, interesting to note a lower ferritin level in the anemic seronegative subjects, but showing higher trend in anemic seropositive subjects, which maybe due to a non-acute COVID-19 infection. This contrasts with other studies where asymptomatic women with RT-PCR positive acute infection had a significantly higher level of ferritin in COVID-19 positive group (266.4 µg/l and 40.5 µg/l, p=0.001) along with higher C-reactive protein compared to COVID-19 negative group (50).We observed higher leucocytosis, lymphocytosis, and neutrophilia in seropositive mothers similar to other studies (1, 51), but not all (48). Neutrophil lymphocyte ratio (NLR) and platelet lymphocyte ratios (PLR) were lower in seropositive mothers in our study, which could be due to the presence of both leucocytosis and lymphocytosis leading to their lower levels, while others have reported lymphopenia as the leading cause behind elevated NLR and PLR. Dixon JB et al. (52) have showed that WBC, neutrophils and lymphocyte counts increase with increasing BMI and decrease with age, while another study (53) reported age, race, and obesity to be significantly associated with the WBC count in healthy individuals. In the present study, the mean age of the participating pregnant mothers was less than 25 years and mean BMI was higher (>24 kg/m^2), which could be the reason for

TABLE 4 Comparison of placental histopathological features based on SARS-CoV-2 serological status of the mother.

Histological variable	Grade	Seropositive, n (%)	Seronegative, n (%)	p
Umbilical cord	Normal	118 (98.3)	90 (98.9)	1.0
	AI[#]	2 (1.7)	1 (1.1)	
Membranes (chorion and amnion)	Normal,	44 (36.7)	55 (60.4)	**0.01**
	AC[$] Grade1	59 (49.2)	30 (33)	
	AC Grade 2	12 (10)	5 (5.5)	
	AC Grade 3	5 (4.2)	1 (1.1)	
Hypervascularity of villi	Absent	3 (2.5)	17 (18.7)	**0.000**
	Grade1	10 (8.4)	28 (30.8)	
	Grade 2	72 (60.5)	36 (39.6)	
	Grade 3	34 (28.6)	10 (11)	
Dilated blood vessels in villi,	Normal	5 (4.2)	16 (17.6)	**0.000**
	Grade 1	13 (10.9)	42 (46.2)	
	Grade 2	101 (84.9)	33 (36.3)	
Number of syncytiotrophoblasts	Grade 1	18 (15.2)	28 (30.8)	**0.02**
	Grade 2	101 (84.8)	63 (69.2)	
Fibrin	Normal,	71 (59.7)	58 (63.7)	0.44
	Grade 1	18 (15.1)	18 (19.8)	
	Grade 2	21 (17.6)	11 (12.1)	
	Grade 3	9 (7.6)	4 (4.4)	
Villitis	Absent	106 (89.1)	88 (96.7)	0.06
	Present but focal	13 (10.9)	3 (3.3)	
Vasculitis	Absent	98 (82.4)	89 (97.8)	**0.000**
	Present	21 (17.6)	2 (2.2)	
Intervillous haemorrhage	Absent	71 (59.7)	77 (84.6)	**0.000**
	Present	48 (40.3)	14 (15.4)	
Infarcts	Absent	107 (89.9)	87 (95.6)	0.19
	Present	12 (10.1)	4 (4.4)	
Accelerated villous maturation	Absent	58 (48.7)	67 (73.6)	**0.000**

(Continued)

10.3389/fendo.2023.1092104

TABLE 4 Continued

Histological variable	Grade	Seropositive, n (%)	Seronegative, n (%)	*p*
	Present	61 (51.3)	24 (26.4)	
Calcification	Absent	71 (59.7)	68 (74.7)	**0.03**
	Present	48 (40.3)	23 (25.3)	
Decidual inflammation	Absent	97 (82.2)	86 (94.5)	**0.01**
	Present	21 (17.8)	5 (5.5)	
Degeneration of trophoblasts lining villi	Absent	72 (60.5)	86 (94.5)	**0.000**
	Present	47 (39.5)	5 (5.5)	
Villus agglutination	Absent	57 (47.5)	75 (82.4)	**0.000**
	Present	63 (52.5)	16 (17.6)	
Fibrosed avascular villi	Absent	104 (86.7)	88 (96.7)	**0.01**
	Present	16 (13.3)	3 (3.3)	

Chi-square test was performed to study the association between different histological variables and serological status. Significant difference ($p<0.05$) are given in bold. #AI, Acute inflammation-Infiltration with polymorphs. $AC, Amniochorionitis.

high WBC counts in our participants. NLR is known to be a steady marker for systemic inflammation, which is less affected by physiological conditions, increase in patients suffering from severe COVID-19 infection, and linked to a poor outcome (54). However, the data comparing NLR in pregnant mothers who are healthy with SARS-CoV-2 infected women are limited and results are inconsistent. In our population, we failed to notice striking difference in NLR among the groups, although in regression analysis, normal NLR seemed to offer protection from adverse histopathological events.

TABLE 5 Comparison of placental histopathological features based on SARS-CoV-2 serological status of the mother and maternal anemia.

Histological variable	Severity	SARS-CoV-2 positive		SARS-CoV-2 negative		*p*
		Anaemic n (%)	Normal n (%)	Anaemic n (%)	Normal n (%)	
Chorangiosis	Absent/mild (n=56)	9 (4.3)	4 (1.9)	26 (12.5)	17 (8.1)	**0.000**
	Moderate/severe (n=152)	61(29.3)	45 (21.6)	36 (17.3)	10 (4.8)	
DB vessels	Absent/mild (n=74)	10 (4.8)	6 (2.8)	37(17.8)	19 (9.1)	**0.000**
	Moderate/severe (n=134)	58 (27.9)	43 (20.7)	25 (12.0)	8 (3.8)	
Syncytiotrophoblasts	Absent/mild (n=74)	21(10.1)	11 (5.2)	26 (12.5)	16 (7.7)	**0.006**
	Moderate/severe (134)	49 (23.5)	38 (18.3)	36 (17.3)	11 (5.2)	
Villous degeneration	Absent (n=157)	43 (20.7)	29 (13.9)	58 (27.9)	27 (13)	**0.000**
	Present (n=51)	27 (13)	20 (9.6)	4 (1.9)	0	
IBV	Absent (n=185)	58 (27.9)	40 (19.2)	61 (29.3)	26 (12)	**0.006**
	Present (n=23)	12 (5.8)	9 (4.3)	1 (0.5)	1 (0.5)	
Intervillous hemorrhage	Absent (n=146)	41 (19.7)	30 (14.4)	51 (24.5)	24 (11.5)	**0.002**
	Present (n=62)	29 (13.9)	19 (4.3)	11 (5.2)	3 (1.4)	
Villus agglutination	Absent (n=132)	35 (16.7)	22 (10.6)	50 (23.9)	25 (11.9)	**0.000**
	Present (n=76)	36 (17.2)	27 (12.9)	12 (5.7)	1 (0.5)	
Small terminal villi	Absent (n=124)	28 (13.5)	30 (14.4)	44 (21.1)	22 (10.6)	**0.000**
	Present (n=84)	42 (20.2)	19 (9.1)	18 (8.6)	5 (2.4)	

Chi-square test was performed to study the association between different histological abnormalities with SARS-CoV-2 serology based on maternal anemia status. Dilated blood (DB) vessels, IBV inflammation around blood vessels. Values in bold signifies $p<0.05$.

10.3389/fendo.2023.1092104

TABLE 6 Bivariate and multivariate logistic regression models for SARS-CoV-2 serological status as a risk factor for placental abnormality.

Dependent variable	Independent variable				Model 1[a]			Model 2		
Placental abnormality	SARS-CoV-2 serology	COR	95% CI	*p*	AOR	95% CI	*p*	AOR	95% CI	*p*
Chorangiosis None/mild vs. moderate/severe	Negative	1			1			1		
	Positive	8.20	3.66-18.37	**0.000**	8.74	3.51- 21.76	0.000	8.04 [b]	3.55-18.24	**0.000**
Dilated blood vessels None/mild vs. moderate/severe	Negative	1			1					
	Positive	12.42	5.75-26.82	**0.000**	12.74	5.46 - 29.75	0.000			
Syncytiotropho blasts None/mild vs. moderate/severe	Negative	1			1			1		
	Positive	2.53	1.30-4.89	**0.006**	2.86	1.36-5.99	0.005	2.62[c]	1.34-5.14	**0.005**
Villus agglutination None/mild vs. moderate/severe	Negative	1			1			1		
	Positive	5.32	2.57-11.02	**0.000**	9.27	3.68 -23.32	0.000	5.46[d]	2.54-11.73	**0.000**

[a]Variables entered on step 1: Age, BMI, Income, Parity, GA, WBC, PCV, MCV, RDW, Neutrophils, Lymphocytes, Monocytes, NLR, PLR, birth weight, Haemoglobin, SARS-CoV-2 group.
[b]Variables entered on step 1: SARS-CoV-2 group and WBC.
[c]Variables entered on step 1: SARS-CoV-2 group and GA.
[d]Variables entered on step 1: SARS-CoV-2 group, GA, birth weight and WBC.
COR, crude odds ratio; AOR, adjusted odds ratio; CI, confidence interval; GA, gestational age; NLR, Neutrophil to Lymphocyte ratio; PLR, Platelet to Lymphocyte ratio, GA, gestational age. Values in bold signifies p<0.05.

To date, in placental histopathology in COVID-19 (25–27, 55, 56) specific histologic features or hallmarks of the disease have not been identified. Placental histopathology in our study revealed a few intriguing features which were difficult for us to decipher due to the paucity of literature. The predominant finding was extreme dilatation of villous vessels or the villous capillaries, which is one of the features of fetal vascular malperfusion (FVM), leading to extreme thinning of the villous matrix, distortion of the villous outline, and disruption of outer trophoblast layer, seen in both terminal and stem villi, present in mostly in COVID-19 seropositive cases, irrespective of maternal anaemia status. Capillary dilatation was accompanied by villous hypervascularity (increased number of capillaries per villus), in proportion to vessel dilation. Pathological evidence of FVM was earlier shown in many different studies (12, 25, 27, 44, 57–62), however, the severity of changes observed in our study was not seen in other studies.

Since both anemia and COVID-19 are known to be causes of maternal hypoxia, we wanted to examine the effect of maternal anemia on COVID-19-affected placentas or vice versa. In our study, a significant increase in syncytiotrophoblasts (STBs) and syncytial knots in villi was observed in placentas of anemic COVID-19 seropositive mothers, similar to earlier studies (59, 60). Syncytial knots usually develop in hypoxic placenta leading to increased terminal villi vessels as a compensatory response. Hypoxia due to maternal anemia has been reported to significantly increase terminal villi blood vessels (59, 60) and SARS-CoV-2 infection is also known to cause maternal hypoxia, probably due to a hypercoagulable state, leading to hypoperfusion in the placenta and subsequent hypoxic-ischemic injury to the placenta (63). However, the exaggerated villous capillary response observed in our study could probably be explained by placental hypoxia attributed to COVID-19 solely with limited contribution by anemia.Apart from hyper mature villi which were significantly observed in COVID seropositive anemic cases, all the remaining parameters, including dilated fetal vessels, although slightly increased number in COVID seropositive anemic cases, but were not significantly different from the non-anaemic group, thus pointing to a lesser effect of anemia on fetal vascularity in comparison to COVID-19.

We had also observed maternal vascular malformations (MVM), like perivillous fibrin, infarcts, agglutination of villi, accelerated villous maturation (compensatory change due to MVM, composed of short hyper mature villi for gestational age), intervillous hemorrhage significantly higher in seropositive mothers. With respect to FVMs and MVMs, there are conflicting reports in the literature. Prabhu (64) had reported a higher incidence of lesions about FVM in the COVID cases while other studies (27, 65) reported a higher frequency of MVMs than controls. However, both the studies dealt with placentas from preterm pregnancies in which MVM lesions are commonly known. In contrast, we had studied placentas from term deliveries.

The histological features of hypoxia we observed were moderate to severe increase (chorangiosis) as well as dilation of capillaries, indicating feto-vascular malformation in placental villi and these were present in seropositive placentas irrespective of anemia. What was of greater clinical concern in our study was the fact that women were asymptomatic while the hypoxia induced changes in the placenta suggests “silent hypoxia” and possibilities of fetus being exposed to chronic fetal hypoxia (24, 66–68) and adverse fetal outcomes like pre-term birth, stillbirth, hypoxia of the fetal brain. We did not notice any profound impact of COVID-19 in terms adverse neonatal outcomes, such as birth weight, gestational age, admissions to ICU and APGAR scores as reported in the earlier studies, maybe pertaining to milder infection. However, a long-term effect of fetal exposure to chronic subclinical hypoxia due to abnormalities in the placentas cannot be ruled out and will need longitudinal follow up studies of the affected children.

5 Conclusion

Although the prevalence of anemia was high in the present study, its effects on placentae were less prominent than that of SARS-CoV-2. The most intriguing and novel finding of the study was strong evidences that of maternal COVID-19 infection, which was otherwise asymptomatic, was being associated with increased placental damage, indicating histopathological features of placental hypoxia and thus possibilities of intrauterine fetal hypoxia. The long-term adverse consequences of this placental pathology to the fetal and neonatal growth and development can be understood only through follow-up studies.

6 Strengths and limitations

This is one of the largest studies on pregnant women from this region examining effects of COVID-19 during pregnancy and placental histopathology. The cohort was exposed to one of the deadliest waves of COVID-19 in India and in spite of asymptomatic infection they developed severe placental histopathology. However, there are a few limitations of the study. Firstly, our observation was made in a single center and therefore, the findings cannot be generalized for the whole of the Indian population. Secondly, the cross-sectional study design did not allow us to assess the exact time of the time of SARS-CoV-2 infection, while the use of a self-reporting by recall method also failed as cases were asymptomatic. Further, we did not assess IgM sero-prevalence and hence the time of infection could not be established. Finally, maternal hemoglobin was measured only once at the time of hospital admission and hence it was not possible to determine if COVID-19 worsened the anemia status during pregnancy.

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 Ethical Committee of National Institute of Nutrition (ICMR), registered with National Ethics Committee Registry for Biomedical and Health Research (NECRBHR), Department of Health Research, India. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MS was involved in conceptualization, methodology, validation, conducting research, writing manuscript, fund acquisition, and overall supervision. NS was involved in conducting research, recruitment of subjects and sample collection. NB performed the statistical analysis, PK was involved in the conception of the manuscript. KS performed hematological and histopathological work. JG helped in conducting the research. PS was involved biochemical analysis. BT in immunological analysis. RM was involved in conceptualization and data analysis. GR contributed to data analysis and edited the manuscript. GM was involved in sample procurement, validation of methodologies, data analysis, manuscript writing and editing. All authors contributed to the article and approved thesubmitted version.

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Conflict of interest

GR has received grant funding from and serves as a consultant for Sun Pharmaceuticals Inc.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1092104/full#supplementary-material

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EDITED BY
Jeff M. P. Holly,
University of Bristol,
United Kingdom

REVIEWED BY
Tope Oyelade,
University College London,
United Kingdom

*CORRESPONDENCE
Sara Momtazmanesh
✉ smomtazmanesh@gmail.com

†These authors have contributed equally to this work and share first authorship

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COVID-19 and MAFLD/NAFLD: An updated review

Ali Nowroozi[1,2†], Sara Momtazmanesh[1,2,3*†] and Nima Rezaei[1,2,3,4]

[1]School of Medicine, Tehran University of Medical Sciences, Tehran, Iran, [2]Network of Immunity in Infection, Malignancy and Autoimmunity (NIIMA), Universal Scientific Education and Research Network (USERN), Tehran, Iran, [3]Research Center for Immunodeficiencies, Pediatrics Center of Excellence, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran, [4]Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

The COVID-19 pandemic is ongoing and places a substantial burden on healthcare systems worldwide. As we further shed light on different disease characteristics, we identify more and more groups of people at higher risk of poor COVID-19 outcomes. Metabolic-associated fatty liver disease (MAFLD) (previously non-alcoholic fatty liver disease or NAFLD) is a common metabolic disorder characterized by fat accumulation and liver fibrosis. Given its close correlation with metabolic syndrome, an established risk factor for severe COVID-19, it is necessary to investigate its interplay with the novel coronavirus. In this study, we review the available data on COVID-19 prognosis, treatment and prevention options in patients with MAFLD, and the effect that the disease and the pandemic have on MAFLD care. Furthermore, we point out the gaps in the current literature to accentuate the work that needs to be done to improve MAFLD care during the pandemic and beyond.

KEYWORDS

COVID-19, MAFLD (metabolic associated fatty liver disease), NAFLD (non alcoholic fatty liver disease), NASH (non-alcoholic steatohepatitis), metabolic syndome, vaccine, SARS-CoV-2

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has posed a daunting challenge since late 2019, with approximately 600 million confirmed cases and 7 million deaths as of September 1st, 2022 (1). Early on, we learned that while respiratory symptoms may be predominant in COVID-19, the disease affects various organ systems, with gastrointestinal, cardiovascular, neurological, hematological, and renal involvement (2–10). With the growing knowledge of the disease, we learned that in addition to the acute phase, COVID-19 might induce multi-system long-term consequences (i.e., long COVID), such as fatigue, myalgia, psychological symptoms, and hepatitis (11–13).

Liver damage is one of the most important aspects of COVID-19, with elevated liver enzymes appearing in approximately 15–65% of patients in the acute phase (14, 15) and prolonged hepatobiliary complications in some cases (16, 17). In critically ill COVID-19 patients, pathological studies have revealed mild lobular and portal inflammation as well as moderate macrovesicular steatosis (18, 19). Direct viral cytotoxic effects, systemic inflammation, hypoxia, coagulopathy, and drug-induced liver injury are all potential causes of liver damage (4). Notably, viral RNA has been detected in liver samples, and SARS-CoV-2 isolated from liver tissue is infectious (20–22). Angiotensin-converting enzyme 2 (ACE2) is the primary viral receptor for SARS-COV-2. The host transmembrane serine protease 2 (TMPRSS2) is also crucial

for viral infectiousness. ACE2 and TMPRSS2 were found to be highly expressed in the liver. Cholangiocytes had the highest levels of ACE2 expression, followed by hepatocytes. Transmembrane serine protease 2 was found to be mainly expressed in cholangiocytes, hepatocytes, periportal liver sinusoidal endothelial cells, and erythroid cells. (23, 24). Interestingly, hypoxia and inflammatory conditions were found to upregulate ACE-2 expression (25, 26).

The liver injury could be more severe in patients with pre-existing chronic liver diseases. This can be partly explained by the increased expression of ACE2 in these patients (26–28). Non-alcoholic fatty liver disease (NAFLD), recently known as metabolic-associated fatty liver disease (MAFLD), is a spectrum of diseases ranging from simple steatosis with or without mild inflammation to a necroinflammatory subtype with the presence of hepatocellular injury (non-alcoholic steatohepatitis (NASH)) and cirrhosis (29, 30). NAFLD is the most common cause of chronic liver disease and is estimated to have affected a quarter of the global population (31, 32). Of note, given the role of cardiometabolic risk factors in the development and progression of the disease, two new position papers (29, 30) proposed the terminology of MAFLD instead of NAFLD in 2020 to better capture the pathophysiology of the disease (33, 34).

Though controversial, early reports during the pandemic indicated that patients with NAFLD have a greater risk of developing a more severe disease course (35–37). Given the association of MAFLD with other cardiometabolic risk factors, which are also well-established predictors of poor prognosis in COVID-19, it remains unclear whether MAFLD is merely associated with poor outcomes or plays a causal role. Moreover, not only can MAFLD influence the course of COVID-19, but it is also important to recognize the effects of the COVID-19 pandemic on the care of patients with MAFLD and the epidemiology of the disease. In addition, given the global scale of COVID-19 vaccination, the focus of research should shift to the safety and efficacy of COVID-19 vaccines in patients with MAFLD.

In this review, we provide a concise yet comprehensive overview of the interplay between MAFLD and the COVID-19 pandemic, focusing on the COVID-19 outcomes in patients with MAFLD, the impact of the COVID-19 pandemic on the care of patients with MAFLD and the epidemiology of the disease, and COVID-19 vaccination in patients with MAFLD (Figure 1). We also pave the way for future research by highlighting the current gaps in the field's knowledge.

2. COVID-19 in patients with MAFLD

Metabolic factors such as obesity and diabetes are established risk factors for severe COVID-19 (38). Hence, it is only logical to assume that MAFLD is associated with a worse prognosis for COVID-19 (39). While almost all studies show that patients with MAFLD are at a higher risk of severe disease, it is not yet well understood whether MAFLD-related changes can act as an independent prognostic factor and, if they do, to what extent they can impact the clinical course of COVID-19. The following sections review the various aspects of the MAFLD-COVID-19 interaction.

2.1. COVID-19 severity

According to an analysis of a Korean nationwide cohort, patients with NAFLD had a 35–41% increased disease risk of severe COVID-19 (40), and in another study, patients with MAFLD were four times more likely to acquire severe disease (adjusted odds ratio (OR) = 4.07, $p = 0.02$) (41). A study in Turkey (42) showed that patients with hepatosteatosis (HS) had significantly higher pneumonia severity scores compared with non-HS patients ($p < 0.001$). Among hospitalized SARS-CoV-2 infected patients with NAFLD, diabetes and advanced liver fibrosis were independent predictors of progression to severe disease (adjusted ORs = 8.26 and 11.06 ($p = 0.03$ for both), respectively) (43). Targher et al. (44) reported that patients with intermediate and high Fibrosis-4 (FIB-4) scores had more than four and five times higher risk of severe COVID-19, respectively, compared with patients without MAFLD. Even when adjusted for sex, obesity, and prior diabetes history, the odds of severe disease remained high (OR = 2.59, $p = 0.03$ for intermediate and OR = 4.04, $p = 0.02$ for high FIB-4 scores). Intermediate and high FIB-4 scores had a combined 3-fold increase in severe COVID-19 risk (adjusted OR = 2.95, $p < 0.005$). Similarly, in a study in Italy (45), the FIB-4 score < 1.45 was associated with lower disease severity (adjusted OR = 0.3, $p = 0.01$) and mortality (adjusted OR = 0.4, $p = 0.04$). Contrary to the previous results, do Amaral e Castro and colleagues (46) did not find an association between HS and worse COVID-19 outcomes, although HS was more common among patients with worse outcomes. Furthermore, one study (47) found increased risks of intensive care unit (ICU) admission and mortality with increasing liver fibrosis degree in univariable analysis, although they became insignificant when the risk was adjusted for other factors. A recent meta-analysis of 16 studies by Hayat et al. (48) showed a three-fold increase in severe COVID-19 risk in patients with MAFLD compared with controls. ICU admission was also more incident in patients with MAFLD; however, mortality was similar to the control group. Similar results were achieved in a 2021 analysis of adjusted risks (37), with an adjusted OR of 2.6 ($p < 0.001$) for severe disease, 1.66 ($p < 0.001$) for ICU admission, and 1.01 ($p = 0.96$) for mortality.

2.2. Hospitalization and recovery

Corapli et al. (42) observed that patients with HS were more likely to be admitted (65% vs. 48%, $p = 0.003$), with similar ward ($p = 0.93$) and ICU ($p = 0.50$) stay durations in HS and non-HS groups nonetheless. In a preprint (49), each additional year of having NAFLD/NASH was associated with an 86% increase in the risk of hospitalization ($p < 0.01$). An interesting result of this study was that when patients were adjusted for NAFLD/NASH, obesity decreased the chance of hospitalization by almost 60% ($p < 0.01$), pointing toward the important role of liver fibrosis in COVID-19 prognosis in obese patients. While using medications in the 3 months leading to the COVID-19 diagnosis did not result in less hospitalization, those who had undergone bariatric surgery were less likely to be admitted (OR = 0.22, $p < 0.05$). Furthermore, patients with NAFLD were much

Abbreviations: ACE2, angiotensin-converting enzyme 2; BMI, body mass index; COVID-19, coronavirus disease 2019; FIB-4, fibrosis-4; FLI, fatty liver index; HCC, hepatocellular carcinoma; HS, hepatosteatosis; ICU, intensive care unit; IFN-γ, interferon-γ; IL-6, interleukin-6; MAFLD, metabolic associated fatty liver disease; NAFLD, Non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OR, odds ratio; SARS-COV-2, severe acute respiratory syndrome coronavirus 2; TMPRSS2, transmembrane serine protease 2.

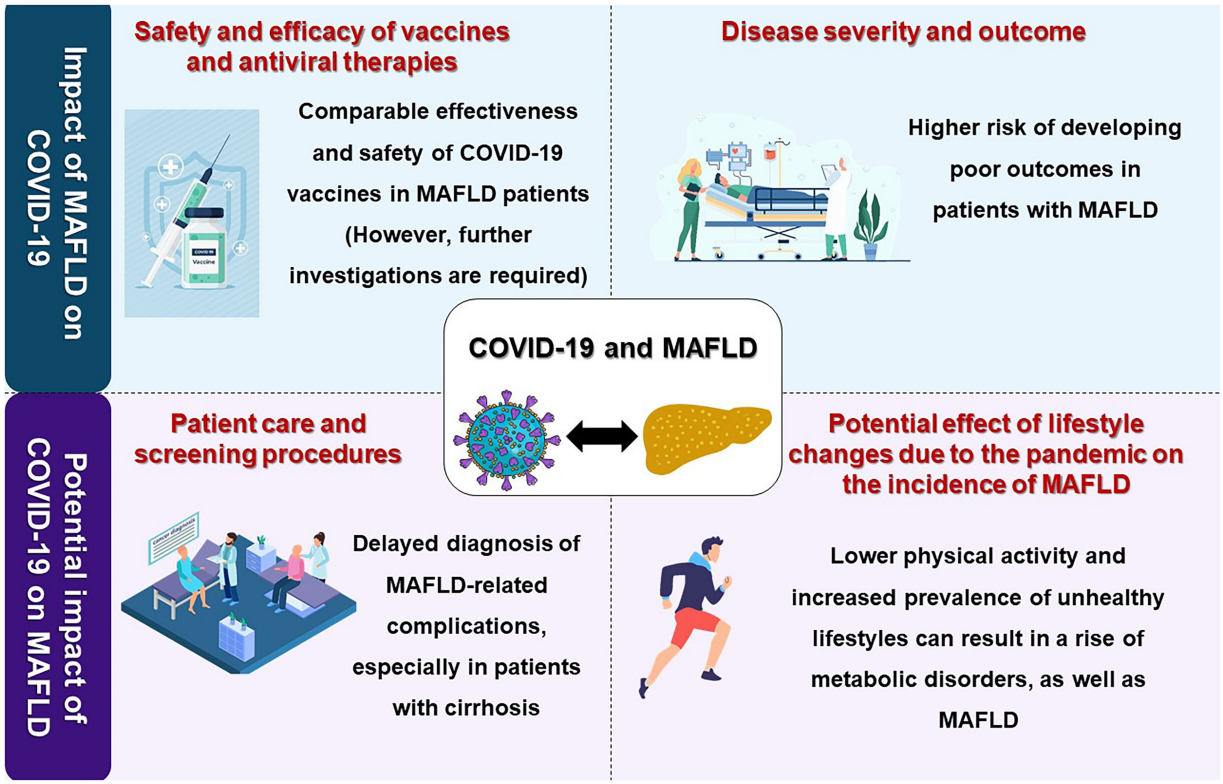

FIGURE 1
A visual summary of the interplay of the COVID-19 pandemic and metabolic-associated fatty liver disease (MAFLD). Image made using material provided by pch.vector on Freepik.

more likely to experience disease progression when hospitalized (OR = 6.4) (50). Additionally, these patients recover 36% slower ($p < 0.001$, based on time to or readiness for discharge) and are more likely to face pulmonary thromboembolism (OR = 2.15, $p = 0.04$) (51).

2.3. Indirect effects of fatty liver diseases

In addition to being an independent predictor, MAFLD appears to increase the effect of obesity on the prognosis of COVID-19. While obese patients are 1.5 times more likely to acquire severe disease (52), Zheng and colleagues (53) demonstrated that in patients with MAFLD, obesity was associated with six times higher risk for severe COVID-19. Moreover, prolonged viral shedding might also be present in this population (50).

Secondary sclerosing cholangitis (SSC) is a hepatic complication of COVID-19, with an incidence of 11.8% in patients with severe disease (54) and 2.0% among all hospitalized patients (55). Hartl et al. (55) found that among 10 hospitalized patients with COVID-19 who developed SSC, seven (70%) were because of NAFLD/NASH.

2.4. The potential underlying mechanisms for the impact of MAFLD on the prognosis of COVID-19

The reason behind these worse outcomes is a matter of debate, with several possible mechanisms involved. Some propose that MAFLD exacerbates COVID-19's cytokine storm by increasing the release of pro-inflammatory cytokines from the liver (44, 56). In contrast, others hypothesize that innate immunity diminishes with the liver's immune cell shift from pro-inflammatory M1 macrophages to regulatory M2 macrophages (57), leading to the deterioration of the patient's condition (50). A recent study (58) has confirmed both of these findings and demonstrated that patients with MAFLD expressed higher levels of some inflammatory cytokines [such as interleukin-6 (IL-6), which has been shown to play an important role in severe disease and its treatment (59)] and lower levels of interferon-γ (IFN-γ), which is crucial to macrophage activity. Another involved mechanism might be the upregulation of SARS-CoV-2 entry proteins (i.e., ACE2 and TMPRSS2) in obese patients with NASH (28). Furthermore, since fatty liver diseases are closely intertwined with metabolic syndrome, similar detrimental pathophysiological pathways are likely involved (42).

3. COVID-19 vaccination in patients with MAFLD

Few studies are available on how MAFLD affects COVID-19 vaccination outcomes.

3.1. Adverse reactions

Wang et al. (60) found that 24.9% of patients with MALFD who received the Sinopharm (BBIBP-CorV) vaccine (inactivated virus)

showed adverse reactions seven days post-inoculation, which is lower than the vaccine's phase 3 results (more than 40%) (61). In another study (62), patients receiving either Comirnaty (BNT162b2) or CoronaVac were divided into HS (those with moderate/severe hepatosteatosis) and control groups. Patients in the HS group showed fewer adverse reactions after the first and second doses of CoronaVac. In contrast, Comirnaty resulted in a higher rate of systemic reactions in the HS group after the first dose (58% vs. 39%, $p = 0.008$), especially fatigue (40% vs. 27%, $p = 0.07$), and also a higher rate of joint pain after the second dose (13% vs. 1%, $p < 0.001$).

3.2. Immunogenicity and effectiveness

In the study by Wang and colleagues (60) (Sinopharm vaccine), seroconversion was observed in 95.5% of the patients, which is comparable to the nearly 100 percent achieved in the phase 3 trial. Moreover, Cheung et al. (62) observed that on day 56 after the first dose, all cases receiving Comirnaty had achieved seroconversion with similar titer levels ($p = 0.68$). However, the best-responding cases (top 25% of virus microneutralization titer levels) were more prevalent in the control group ($p = 0.04$). All HS patients and all controls, except one, remained seroconverted on day 180 after the first dose, with similar titers ($p = 1.00$ for both). CoronaVac also produced similar seroconversion rates in HS and control groups on day 56 ($p = 0.13$); however, the geometric mean titer was lower in the HS group ($p = 0.02$). Similar to Comirnaty, the best responders were mostly from the control group ($p = 0.04$).

4. Effect of the COVID-19 pandemic on the care and incidence of MAFLD

NASH is one of the most common causes of cirrhosis and the second leading indication for a liver transplant. Patients with cirrhosis require prompt diagnosis and treatment of the relevant complications. Considering the annual cumulative hepatocellular carcinoma (HCC) incidence rate of 2.6% for NASH-related cirrhosis, these patients need routine screening for HCC (31). Notably, a multicenter investigation found a significantly decreased number of HCC diagnoses and an increased rate of HCC treatment delay compared to the same period in the previous year during a high prevalence of COVID-19 (63). Moreover, regular screening for esophageal varices, given the high risk of mortality, is also required in patients with NASH-related cirrhosis (64); however, during the COVID-19 pandemic, most screening procedures were delayed, which presumably has led to undiagnosed cases as we are recovering from the pandemic (65). One such example is screening endoscopy, which was recommended to be performed only in urgent circumstances by the pandemic guidelines. This has most likely resulted in missed esophageal varices due to delayed screening (66, 67), especially in earlier periods of the pandemic, though to the best of our knowledge, no studies have reported the corresponding data. The COVID-19 pandemic has also adversely affected transplantation activity and, in turn, affected the care of patients with NASH-related cirrhosis (68).

From another perspective, the COVID-19 pandemic caused considerable behavioral changes due to the restrictions, including lockdown, home confinement, and closure of sports facilities. Several studies have shown decreased physical activity and increased prevalence of unhealthy lifestyles, including increased dietary intake, decreased sleep, and increased smoking during COVID-19 lockdowns (69–71). A longitudinal investigation of NAFLD patients showed that the more active patients had lower physical activity than before during the lockdown, while inactive people had higher physical activity (72). Importantly, cohorts of patients with NAFLD showed that COVID-19 lockdown caused a significant increase in body weight, body mass index (BMI), insulin resistance, cholesterol levels, low-density lipoprotein (LDL) levels, and glucose levels. It also led to a reduction in high-density lipoprotein (HDL) levels alongside with progression of fatty liver (73–75). In addition, population-based analyses using the United States (US) national mortality records revealed that the steady increase in NAFLD mortality prior to the COVID-19 pandemic sped up during the pandemic (76). A recently published cohort study comparing patients before and after a COVID-19 lockdown grouped patients with NAFLD according to the level of physical activity. They found that the fatty liver index (FLI) increased in all groups after the lockdown. However, the elevation in the FLI was higher in the medium physical activity than in the low physical activity group (72). In addition to worsening metabolic risk factors and liver involvement in patients with NAFLD, such lifestyle changes may increase the likelihood of an increased incidence of NAFLD in the coming years as we recover from the pandemic, given the strong association of metabolic risk factors with NAFLD development and progression (32).

The COVID-19 pandemic, although catastrophic, provided and continues to provide many valuable lessons to experts and policymakers of all fields, especially medicine. First and foremost, healthcare providers must always anticipate a sudden crisis that halts delivering health services. Although the COVID-19 pandemic pressured organizations into identifying actions and developing protocols to counter the effect of this global crisis, the effort must be continuous and the results have to be updated regularly based on most recent evidence. Telemedicine is an example of a tool that has been extensively studied during the ongoing pandemic and shown to be effective (77, 78) and satisfactory (79); hence, we suggest healthcare facilities commence and test out different telemedicine approaches to identify the most suitable one for their use. Rapid re-initiation of medical practices during crises is of utmost importance; however, this must not result in the normalization of the ongoing calamity. While continuing care for some patients is necessary, some medical practices could be postponed with no or minimal adverse outcomes (80). Comprehensive crisis-management guidelines and protocols are required to stratify medical services based their delay capacity. Undoubtedly, in the unfortunate event of a similar disaster in the future, the world's response would be much more appropriate given the experience gained from COVID-19, just as it was for COVID-19 because of the previous outbreaks such as Middle East Respiratory Syndrome (MERS) and SARS (81).

5. Limitations and future directions

Current studies, as presented in this manuscript, provide strong evidence that patients with MAFLD tend to experience worse COVID-19 outcomes. However, literature is short of approaches to mitigate this risk. Consequently, to the best of our knowledge, no specific COVID-19 guidelines have been developed for patients with MAFLD. We believe the focus of future related studies should be on evaluating different care strategies in these patients.

Vaccination and specific antiviral treatments are the current trend in COVID-19 research. To the best of our knowledge, very few studies have evaluated COVID-19 vaccine effectiveness in patients with MAFLD and no studies have evaluated antiviral treatments (such as remdesivir and Paxlovid) in this population. Investigating these subjects in patients with MAFLD is of utmost importance, given the crucial role of the liver in drug metabolization.

Delay in health care and shift of resources toward managing COVID-19 is the pandemic's predominant impact on other diseases. No comprehensive analyses have yet been performed to check whether MAFLD prevalence and incidence have increased in this period. Furthermore, there are no studies investigating whether MAFLD complications such as esophageal varices and HCC have significantly increased, given the cardinal role of screening in their detection and prevention.

6. Conclusion

Almost 3 years after the emergence of COVID-19, we very well know that it can affect the liver, and patients with certain comorbidities, such as metabolic dysfunction, are at higher risk for severe disease or mortality. Herein, we concisely reviewed the substantial evidence supporting the mutual association between MAFLD and COVID-19. Patients with MAFLD are at a higher risk of poor outcomes during COVID-19, even after controlling for the confounding effect of the other metabolic abnormalities. COVID-19 caused drastic changes in human lifestyle, screening programs, and transplantation programs, which could adversely affect the care of patients with MAFLD, especially those with NAFLD cirrhosis, or even potentially increase the incidence rate of MAFLD in years to come. Assessment of the efficacy and safety of COVID-19 vaccines in this group of patients has garnered attention recently, with the wide global vaccination showing comparable results with the healthy population. Further investigations are required for development of guidelines for management of MAFLD during the COVID-19 pandemic, and assessment of efficacy of vaccination and antiviral therapies in this group of patients.

Author contributions

AN: conceptualization, investigation, writing—original draft, and writing—review & editing. SM: conceptualization, investigation, writing—original draft, writing—review & editing, and visualization. NR: conceptualization, writing—review & editing, and supervision. All authors contributed to the article and approved the submitted version.

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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EDITED BY
Jeff M. P. Holly,
University of Bristol, United Kingdom

REVIEWED BY
Neftali Eduardo Antonio-Villa,
National Institute of Cardiology Ignacio Chavez, Mexico
Max Carlos Ramírez-Soto,
University of San Martín De Porres, Peru

*CORRESPONDENCE
Rubio-Herrera MA
marubioh@gmail.com
Torrego-Ellacuría M
macarena.torrego@gmail.com

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Clinical and economic impact of COVID-19 on people with obesity in a Spanish cohort during the first pandemic peak

Torrego-Ellacuría M[1,2*], Rubio-Herrera MA[2,3*], González López-Valcárcel B[4], Fuentes-Ferrer ME[5,6], Martín V[7], Poyato F[8], Barber-Pérez P[4], Santucci C[4,9], Nuñez A[10], González-Pérez C[1,11] and Luaces M[1]

[1]Innovation Unit, Hospital Clínico San Carlos, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Madrid, Spain, [2]Department of Endocrinology and Nutrition, Hospital Clínico San Carlos, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Madrid, Spain, [3]Department of Medicine, Faculty of Medicine, Universidad Complutense de Madrid, Madrid, Spain, [4]Department of Quantitative Methods for Economics and Management, Universidad de Las Palmas de Gran Canaria, Las Palmas de Gran Canaria, Spain, [5]Department of Preventive Medicine, Hospital Clínico San Carlos, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Madrid, Spain, [6]Research Unit, Hospital Universitario Nuestra Señora de Candelaria, Santa Cruz de Tenerife, Spain, [7]Department of Market Access and Public Affairs, Novo Nordisk Pharma, Sociedad Anónima (SA.), Madrid, Spain, [8]Department of Clinical, Medical and Regulatory, Novo Nordisk Pharma, Sociedad Anònima (SA.), Madrid, Spain, [9]Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy, [10]Department of Critical Care, Hospital Clínico San Carlos, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Madrid, Spain, [11]Department of Hospital Pharmacy, Hospital Clínico San Carlos, Instituto de Investigaciòn Sanitaria del Hospital Clínico San Carlos (IdISSC), Madrid, Spain

Introduction: COVID-19 and obesity relationship has been extensively studied since the COVID-19 outbreak, proving obesity is a risk factor. This study aims to broaden the available information about this association and to evaluate the economic impact of obesity and the COVID-19 disease combination.

Methods: This retrospective study analyzed a sample of 3,402 patients admitted to a Spanish hospital with available body mass index (BMI) data.

Results: The prevalence of obesity was 33.4%. Patients with obesity showed a higher risk of hospitalization (OR 95% ConfidenceInterval [CI]=1.46; [1.24-1.73]; $p < 0.001$), which increased with the obesity degree (I: OR [95% CI]=1.28 [1.06-1.55], p =0.010; II: OR [95% CI]=1.58 [1.16-2.15], p =0.004; III: OR [95% CI] =2.09 [1.31-3.34], p =0.002). Patients with type III obesity had a significantly higher risk of intensive care unit (ICU) admission (OR [95% CI]= 3.30 [1.67-6.53]; p = 0.001) and invasive mechanical ventilation (IMV) need (OR [95% CI]= 3.98 [2.00-7.94]; p<0.001). The average cost per patient was remarkably higher in patients with obesity (p = 0.007), reaching an excess cost of 28.41% in the study cohort and rising to 56.5% in patients < 70 years. The average cost per patient increased significantly with the degree of obesity (p = 0.007).

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Discussion: In conclusion, our results suggest a strong association between obesity and adverse COVID-19 outcomes and higher expenditures in patients with both conditions.

KEYWORDS

COVID-19, obesity, invasive mechanical ventilation (IMV), intensive care unit (ICU), economic burden, obesity comorbidities, diabetes mellitus

1 Introduction

The coronavirus disease (COVID-19) pandemic has been the most notable global concern of this century. Since its outbreak at the end of 2019, researchers worldwide have made an enormous effort to investigate and draw a picture not only of the disease itself but also of its association with other common diseases. COVID-19 is a respiratory illness caused by infection with severe acute respiratory syndrome coronavirus (SARS-CoV-2). It may present as an asymptomatic, mild, moderate, or severe disease, depending on the patient baseline condition among other factors.

On the other hand, obesity is a metabolic, systemic, chronic, multifactorial disease leading to increased subcutaneous and visceral adipose tissue. It is well known that obesity promotes inflammation and it is associated with the appearance of comorbidities such as diabetes mellitus (DM), arterial hypertension (HT), and/or cardiovascular disease (CVD), strongly related with mortality in the general population (1, 2). Nevertheless, some authors have concluded that not all obese patients have a deteriorated metabolic profile, and they have described different obesity phenotypes referring to their metabolic status independent on the person weight; Metabolically Healthy Obesity (MHO) and Metabolically Obese Normal Weight (MONW). These findings received the name of "obesity paradox", which is still controversial and debate-generating (3–5). Obesity is present in approximately 13% of adults worldwide, and it continues to rise globally (6). The prevalence of obesity in the Spanish population is worryingly higher, rising to 21.6%, according to the ENPE study published in 2016 (7). The association between obesity and inflammation is well established, as the excess nutrients lead to activation of a metabolic signaling pathway which ends up causing activation of cytokines resulting in a low-grade inflammatory response (8). Clinical evidence has demonstrated that COVID-19 patients express a high level of cytokines, known as "cytokine storm", and present hyperinflammation, which could be the link between COVID-19 and obesity (9). Obesity already plays a significant role in the hospitalization and mortality rate in other respiratory diseases caused by viral infections, such as other coronaviruses pandemics, SARS and MERS in 2002 and 2012, respectively, or the H1N1 flu pandemic in 2009 (10, 11).

The first published data following the COVID-19 outbreak came from China and the United States, suggesting that age, male sex, obesity, HT, CVD, DM, and chronic kidney disease were factors associated with COVID-19 adverse outcomes (12–14). By 2023, the relationship between obesity and other chronic diseases and COVID-19 has been highly studied. Obesity has been determined to be a risk factor for COVID-19, and it is associated with adverse outcomes in terms of higher rates of hospital admission, intensive care unit (ICU) admission, and need for invasive mechanical ventilation (IMV) (15–17). Moreover, the prevalence of obesity seems to be higher among patients developing acute respiratory distress syndrome (ADRS) (16, 18, 19). The association between obesity and higher mortality in COVID-19 patients is still controversial, with clear evidence being found in many studies (12, 17, 20–25); but lacking a reliable association between both conditions in other investigations (15, 18, 26).

The high rates of hospitalization, ICU admission, and need for IMV occurring during the COVID-19 pandemic, added to the cost of antiviral and coadjuvant drugs, entail an enormous impact on public health expenditure (27). Clearly, these expenses may be higher if patients present other underlying diseases besides COVID-19. The presence of obesity as one of the most prevalent chronic diseases in western countries, and specifically in Spain, and the critical impact seen in Spain during the peak of the pandemic, led us to investigate the use of resources in patients with overweight and obesity, obtaining clear results about the excess cost derived from obesity (28, 29).

This study aimed to assess the clinical and economic impact of the COVID-19 disease in patients diagnosed with obesity alone or obesity along with three specific obesity-related comorbidities: DM, HT and CVD in a Spanish hospital. According to the reviewed literature and the clinicians expertise, we hypothesized the impact of the disease combination to be significantly higher than the impact of COVID-19 infection itself.

2 Materials and methods

2.1 Study design and patients

The OBESITY-COVID study is a retrospective, registry-type study including COVID-19 positive patients admitted to Hospital Clínico San Carlos in Madrid (Spain) during the first wave of the COVID-19 pandemic (from March 1, 2020 to June 30, 2020). The study was conducted in accordance with the Declaration of Helsinki, the study protocol was approved by the Ethics Committee of the participating hospital on 01/02/2021 and registered at https://www.isrctn.com/(ID ISRCTN11242213).

The cohort included 5,517 consecutive patients with a probable or certain diagnosis of COVID-19, with or without microbiological diagnostic confirmation, who had at least one visit to the emergency department (ED) during the study period. Patients met at least one of the following inclusion criteria: primary and/or secondary diagnosis codes (ICD-10) for clinically diagnosed COVID-19 (30); having a positive RT-PCR. Exclusion criteria included: being under 18 years of age, being admitted to a medicalized hotel and/or having an advanced or terminal disease. The study objectives were evaluated in the study cohort with assessed nutritional status (N=3,402) and all the analyses involving patient stratification according to body mass index (BMI) were done for a secondary cohort of 3,371 patients, since the quantitative BMI record was missing in 31 patients. Additionally, the study objectives were analyzed in a sub-cohort of patients aged less than 70 years within the overall cohort, considering N=1,871 patients with an assessed nutritional status and N=1,847 patients with quantitative BMI data.

2.2 Study endpoints

The primary endpoint was to calculate the frequency of obesity and obesity-related comorbidities, specifically DM, HT, and CVD among COVID-19 positive patients to further estimate the clinical impact and cost. The secondary objective was to investigate the association of obesity status, obesity degree and obesity-related comorbidities with the clinical outcomes of hospitalization, ICU hospitalization, need for IVM and mortality, and the outcome of healthcare cost based on resource utilization. Adjustments for secondary endpoints were based on the results of primary endpoint, including adjusting for demographic variables and the presence of DM, HT and CVD at the time of ED visit.

2.3 Data sources and variables

Demographic and clinical data used for this analysis were extracted from the BDCLIN_HCSC_COVID-19 database of the participating hospital, which integrates information from several hospital departments including the ED, microbiology department, and hospital pharmacy. CVD included coronary heart disease, stroke, hypertensive heart disease, inflammatory heart disease, rheumatic heart disease, transient ischemic attack, and other cardiovascular diseases such as tumors, cardiomyopathy, and heart valve diseases. BMI was extracted from the electronic health records at specialized care and/or primary care, in order to have the most updated measurement in each case. For each patient, the BMI value closest to the ED visit was chosen. Records older than 10 years were discarded. Based on their BMI (kg/m^2) records, patients were classified into non-obesity and obesity groups (this one additionally subclassified into obesity I, II or III), according to the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) criteria (Obesity: BMI≥30; Obesity I: BMI=30-34.9; Obesity II: BMI=35-39.9; Obesity III: BMI≥40). Data related to ED and hospital stay (conventional hospitalization and ICU) were obtained from the Minimum Basic Hospital Discharge Data Set 2020 and the ICU database. The need for IMV was collected using the related ICD-10-PCS. Mortality outcome was assessed using two different variables 1) 30-day mortality (from the first contact with the hospital to day 30) and in-hospital mortality (from the first contact with the hospital to the event of death occurring at any time during the patient's admission), analyzed both as a dichotomous yes/no variables. Thirty-day mortality was also analyzed as time to event (survival) using Kaplan-Meier analysis. Regarding cost variables, the categories of health expenditure considered were days in ED, length of stay in conventional hospitalization, length of stay in ICU, pharmacological treatment with a high economic impact, and prolonged IMV (having undergone tracheostomy procedure with IMV for more than 96 hours). For the length of stay in each inpatient unit, the total per patient was calculated, considering the number of admissions in each inpatient unit and stay. Additional costs of medication and procedures were included in the cost of each corresponding inpatient unit. The unit costs were obtained from the hospital departments and external sources (31). The cumulative cost per patient was calculated as the sum of all cost components, obtained by multiplying the unit cost of each health expenditure category by the patient's use of the corresponding resource.

2.4 Statistical analyses

The analyses were performed in the global cohort and in the subgroup of patients younger than 70 years aiming to decrease the age effect on COVID-19 morbidity and mortality, according to previous research (32, 33). All data processing and analysis were performed using the Statistical Package for the Social Sciences (IBM SPSS Statistics version 26, IBM Corp., Armonk, NY, USA) and Stata 17. All p-values lower than 0.05 were deemed statistically significant.

2.4.1 Analysis of clinical variables

Sociodemographic and clinical characteristics of patients and the use of healthcare resources were analyzed by descriptive statistics. Absolute and relative frequencies were used to describe categorical variables. The Clopper-Pearson exact method was used to calculate confidence intervals for the prevalence of obesity. Quantitative variables were summarized with their mean and standard deviation (SD) and those showing a skewed distribution were summarized with median and interquartile range (IQR). For the comparison of qualitative variables, the Chi-square test or Fisher's exact test were used, if necessary. Comparisons of means between two independent groups were performed by Student's t-test if the variables followed a normal distribution, or by the nonparametric Mann-Whitney U test for asymmetric variables. Comparisons of means between more than two independent groups were performed by analysis of variance (ANOVA), or by the nonparametric Kruskal-Wallis test for asymmetric variables. A multivariate logistic regression analysis was performed to assess the risk of hospitalization, risk of ICU admission, need for IMV and risk of mortality associated with obesity diagnosis, obesity degree and obesity-related comorbidities categories. For each of the outcome variables, crude odds ratios (OR) adjusted for age, sex, presence of HT, DM, and history of CVD were calculated. In each

table, the reference category is specified. The relationship between obesity and 30-day mortality was also explored by survival analysis using the Kaplan-Meier method, where the log-rank test was used to compare survival functions. Cox regression models were used to obtain the crude and adjusted (age, sex, presence of HT, DM, and history of CVD) effect of obesity on the 30-day mortality rate. The hazard ratio (HR) is presented as a measure of effect.

2.4.2 Analysis of economic variables

For the economic impact assessment, multivariate regression models were performed with log(cost) as the dependent variable, adjusted for covariates that could influence the cost (i.e., age, sex, country of birth, and comorbidities). To correct the selection bias caused by missing values of the variable of interest nutritional status, a missing data multiple imputation statistical procedure was designed (see Supplementary Material Tables S5, S6). An individual obesity status has been imputed to each patient with an unrecorded diagnosis of obesity, estimated from the information available in the sample. The procedure, which is applied to the whole sample and for the specific group of patients under 70 years of age, is as follows: A stratified random sample with replacement, of size 1,000 for the total sample and 800 for the under-70s, is drawn from the population of patients with known obesity status. It is stratified according to the quartiles of the age distribution of the missing group, so that its age composition is similar. A binary probit model (Table S6) of obesity is estimated whose predictors are age and its square and the dummies of sex, CVD, DM and HT, Latin American origin and other countries. The estimated equation is used to predict the probability of obesity of the missing patients, and the binary variable obese/non-obese is defined with a cut-off point of 0.5 on the predicted probability. This process (1-2) is repeated 100 times and the results of the 100 samples are recorded to obtain 100 cost estimates for both patients with and without obesity and their corresponding standard deviations.

3 Results

3.1 Study population

Between March 1, 2020 and June 30, 2020, 5,517 patients meeting the inclusion criteria were identified from the BDCLIN_HCSC_COVID-19 database, of whom 216 patients were excluded consensually from the analysis to minimize the effect of confounding factors, and 1,899 were excluded due to absence of BMI record. Therefore, 3,402 patients were evaluable for the study analysis (Figure 1). Table 1 shows the baseline demographic and clinical characteristics of patients included in the study cohort and in the subgroup of patients aged <70 years. Patient's baseline characteristics according to obesity degree and the presence or absence of obesity-related comorbidities are shown in Supplementary Tables S1, S4, respectively.

We found a 33.4% (95% CI, 31.8-35.0) prevalence of obesity among the evaluable COVID-19 patients. The rate by sex was 34.2%

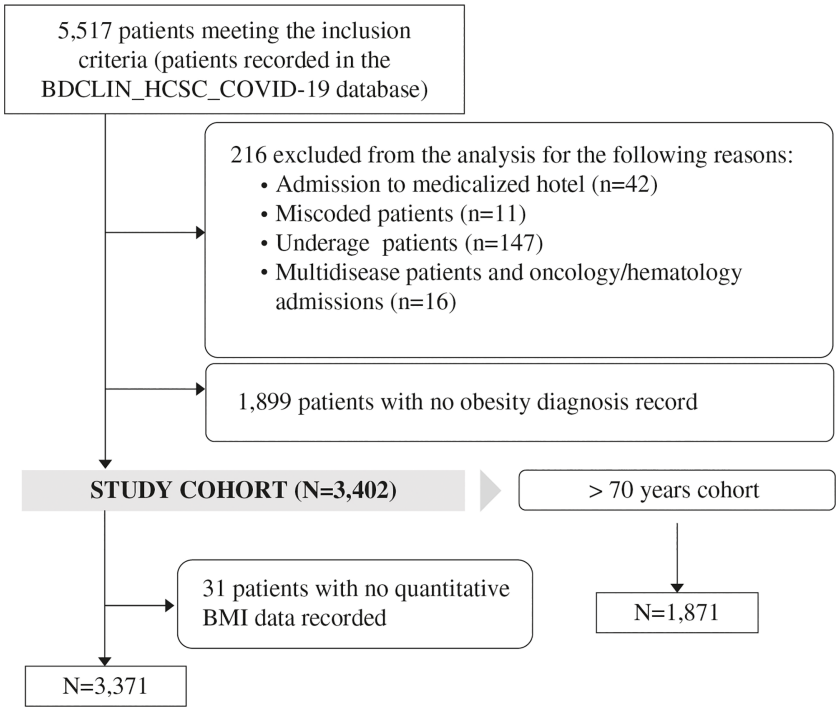

FIGURE 1
Patient inclusion flowchart.

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TABLE 1 Demographic and main clinical characteristics of the study cohort.

Baseline characteristics	Overall population (N=3,402)	Population <70 years old (N=1,870)	*p* value
Age (years), mean ± SD,	64.7 ± 18.8	50.4 ± 12.4	<0.001
Female sex, n (%)	1,786 (52.5)	1,058 (56.6)	0.0045
BMI (kg/m^2), mean ± SD*	28.3 ± 5.2	28.6 ± 5.6	0.055
Obesity degree*			<0.001
Obesity I, % (95% CI)	23.1 (21.7-24.6)	23.0 (23.1-24.9)	
Obesity II, % (95% CI)	6.9 (6.1-7.8)	8.0 (6.8-9.3)	
Obesity III, % (95% CI)	2.8 (2.3-3.4)	4.0 (3.1-4.9)	
Origin, n (%)			<0.001
Spanish	2,535 (74.6)	1,069 (57.1)	
Latin American	709 (20.8)	684 (36.6)	
Other countries	154 (4.5)	118 (6.3)	
Emergency room stay but no admission, n (%)	1,362 (40.0)	1,019 (54.5)	<0.001
Length of stay in ED (days/h), mean ± SD	1.7 ± 0.7	1.7 ± 0.7	<0.001
Length of stay in ED (days/h), median (IQR)	2 (1-2)	2 (1-2)	<0.001
Inpatient hospitalization (at least one admission), n (%)	2,040 (60.0)	852 (45.5)	<0.001
Length of inpatient hospitalization (days), median (IQR)	8 (4-16)	7 (4-5)	0.017
Readmission within 30 days of discharge from the first admission, n (%)	77 (2.3)	31 (1.7)	0.137
ICU admission, n (%)	153 (4.5)	121 (6.5)	0.002
Mechanical ventilation, n (%)	137 (4.0)	109 (5.8)	0.003

BMI, Body mass index; CI, confidence interval; ED, emergency department; ICU, intensive care unit; IQR, interquartile range; SD, standard deviation.
*The sample with available BMI records was 3,371 patients in overall population and 1847 in population <70 years old.

(32.0-36.5%) in females and 32.5% (30.2-34.8%) in males, with no significant differences between groups (p = 0.287). Patients with obesity were significantly younger than patients without obesity (63.79 ± 17.35 years vs. 65.18 ± 19.57 years; p = 0.035). Regarding the country of origin, the prevalence of obesity was significantly lower in Spanish patients (31.4%) than in patients from Latin America (39.8%) and other countries (37.0%) (p < 0.001).

In the population aged <70 years, the rate of obesity was significantly higher than in patients older than 70 years (35.7%, 95% CI, 33.5-37.9 vs. 30.6%, 95% CI, 28.3-32.9), (p = 0.002).

Several evaluable patients (40.2%) did not show any of the studied comorbidities (DM, HT and CVD). A total of 32.2% of patients had at least one comorbidity, and 20.4% and 7.3% had two or three comorbidities, respectively. Figure 2 shows the prevalence of obesity, independent comorbidities, and combined prevalence, defined as the coexistence of a diagnosed obesity status and at least one of the obesity-related comorbidities already defined in the study cohort. The most predominant combination was obesity and HT (19.2%). There were significant differences in the obesity rate based on the diagnosis of DM (43.6% vs 30.2%, p < 0.001) and HT (37.1% vs 29.5%, p < 0.001), but not with regard to CVD (33.1% vs 33.5%, p = 0.874). In the subgroup aged <70 years, 62.4% of patients had no comorbidities, 26.4% had at least one comorbidity, and 8.9% and 2.2% had 2 and 3 comorbidities, respectively. The prevalence was 14.5% DM, 30.0% HT and 6.4% CVD

3.2 Hospitalization and ICU admission

Table 2 shows the profile of patients in each inpatient unit: ED without admission, conventional hospitalization, and ICU. Overall, 60% of patients required hospitalization. Global comparisons among three groups were always significant for each variable (p < 0.001) Obesity was more prevalent in patients that required hospitalization (35.6%, 95% CI, 33.4-37.8) than in patients only visiting the ED (29.4%, 95% CI, 27.0-31.9; p < 0.001). The prevalence of obesity was also significantly higher in patients requiring ICU admission (41.2%, 95% CI, 33.3-49.4) than in non-admitted patients (35.6% 95% CI 33.4-37.8; p = 0.003). There were statistically significant differences in the distribution of obesity categories between the No admission and non-ICU admission groups (p = 0.02), between the No admission and Inpatient with ICU admission groups (p < 0.001), and between the Inpatient without ICU and Inpatient with ICU admission groups (p < 0.001) (Table 2).

3.3 Risk of hospitalization, ICU admission, IMV, and mortality

There were significant differences in the need for hospitalization according to obesity status (p < 0.001) and obesity categories (p =

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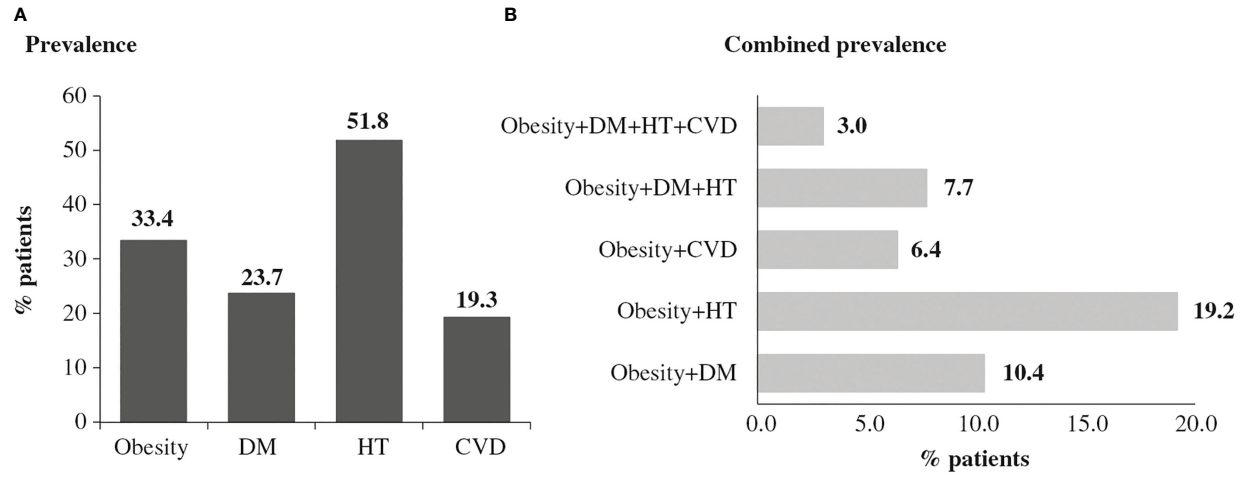

	95% CI
Obesity	31.8-35.0
DM	22.3-25.1
HT	50.1-53.5
CVD	17.9-20.6

	95% CI
Obesity+DM+HT+CVD	2.4-3.6
Obesity+DM+HT	6.8-8.6
Obesity+CVD	5.6-7.2
Obesity+HT	17.9-20.5
Obesity+DM	9.3-11.4

FIGURE 2
Obesity and obesity-related comorbidities prevalence (N=3,402). **(A)** Individual obesity and obesity-related comorbidities prevalence. **(B)** Combined obesity and obesity-related comorbidities prevalence.

TABLE 2 Profile of patients with COVID-19 according to inpatient unit.

Variable	No hospital admission (N=1,362)	Inpatient, non-ICU (N=1,887)	Inpatient, ICU admission (N=153)	*p* value *(No hospital admission vs. Inpatient, no ICU)*	*p* value *(Inpatient, no ICU vs. Inpatient ICU-admission)*	*p* value *(No hospital admission vs. Inpatient, no ICU)*
Age (years), mean ± SD	56.04 ± 18.74	71.31 ± 16.70	60.60 ± 11.17	**<0.001**	**0.002**	**<0.001**
Female sex, n (%)	815 (59.8)	923 (48.9)	48 (31.4)	**<0.001**	**<0.001**	**<0.001**
Non-obesity, % (95% CI)	70.6 (68.1-73.0)	65.4 (63.2-67.6)	58.8 (50.6-66.7)	<0.001	0.003	0.168
Obesity, % (95% CI)	29.4 (27.0-31.9)	35.6 (33.4-37.8)	41.2 (33.3-49.4)			
Obesity degree				0.020	**<0.001**	**<0.001**
Obesity I, % (95% CI)	20.7 (18.6-23.0)	24.8 (22.8-26.8)	24.2 (17.6-31.8)			
Obesity II, % (95% CI)	6.3 (5.1-7.7)	7.2 (6.1-8.5)	7.8 (4.1-31.8)			
Obesity III, % (95% CI)	2.4 (1.6-3.3)	2.6 (1.9-3.4)	9.2 (5.1-14.9)			
Obesity without obesity-related comorbidity, % (95% CI)	14.1 (12.3-16.1)	8.6 (7.4-10.0)	14.4 (9.2-21.0)	**<0.001**	**0.001**	0.054
Obesity with obesity-related comorbidity, % (95% CI)	15.3 (13.5-17.4)	27.0 (25.0-29.0)	26.8 (20.0-34.5)			

CI, confidence interval; ICU, intensive care unit; SD, standard deviation.
All variables were compared using the Chi-square test, except for the variable "age" which was compared in multiple comparison with ANOVA test.
Bold values stand for statistically significant differences.

10.3389/fendo.2023.1146517

0.007). The multivariate logistic regression analysis showed that patients with obesity had a higher risk of hospitalization than patients without obesity (OR [95% CI]=1.46;, [1.24-1.73] p < 0.001), and the risk of hospitalization increases with increasing obesity (type 1: OR[95% CI]= 1.28[1.06-1.55], p =0.010 < type II: OR[95% CI]= 1.58[1.16-2.15], p =0.004 < type III: OR[95% CI]= 2.09[1.31-3.34], p =0.002). There were significant differences in the need for ICU admission and IMV according to obesity categories (p < 0.001). In addition, patients with type III obesity had a significantly higher risk of ICU admission (OR[95% CI]: 3.30 [1.66-6.53]; p = 0.001) and IMV need (OR[95% CI]= 3.98[2.00-7.94] p < 0.001) as compared to those without obesity (Table 3). There were no significant differences in the need for ICU admission according to obesity condition (p = 0.168). There was a significant association between the need for IMV and obesity status (p = 0.032); however, no association between exposure and outcome variables was observed.

Results of the analyses on the combination of obesity status and the presence of obesity-related comorbidities are shown in Table 4. There were significant differences in the need for hospitalization according to comorbidity categories (p < 0.001). Patients with comorbidities (obese and non-obese) showed a higher risk of hospitalization as compared to patients without obesity and without any comorbidity. There were significant differences in the need for ICU admission (p = 0.018) and the need for IMV (p = 0.005) according to comorbidity categories; however, no association between exposure and outcome variables was observed. According to the Kaplan-Meier analysis, survival was significantly longer in patients without comorbidities, regardless of the presence of obesity (p < 0.001). However, the Cox regression model adjusting for age and sex showed that there was no association between comorbidity categories and survival.

Post hoc analyses in the subgroup of patients aged <70 showed similar results (Supplementary Materials Tables S2, 3). No significant associations were found with any of the outcome variables in patients over 70 years of age.

3.4 Mortality

The in-hospital mortality rate was significantly higher in patients without obesity than in those with obesity (22.2% vs. 17.8%; p=0.018). There were also significant differences in 30-day mortality from the first ED contact within the cohort of hospitalized patients (N=2,040) according to obesity status, with a higher mortality rate in non-obese patients (21.0% vs. 17.0%; p =

TABLE 3 Risk of hospitalization, ICU admission, IMV and mortality based on the presence of obesity and obesity degree in the overall population.

Overall population (N=3,402)	Obesity		Obesity I		Obesity II		Obesity III	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Hospitalization	**1.46 (1.24-1.73)**	**<0.001**	**1.28 (1.06-1.55)**	**0.010**	**1.58 (1.16-2.15)**	**0.004**	**2.09 (1.31-3.34)**	**0.002**
ICU admission	1.08 (0.76-1.53)	0.674	0.99 (0.66-1.49)	0.970	0.94 (0.49-1.80)	0.848	**3.30 (1.66-6.53)**	**0.001**
IMV	1.26 (0.87-1.81)	0.219	1.13 (0.74-1.73)	0.574	1.14 (0.59-2.21)	0.700	**3.98 (2.00-7.94)**	**<0.001**
Mortality (30-day)	1.03 (0.80-1.33)	0.834	0.93 (0.70-1.24)	0.617	1.26 (0.79-2.02)	0.352	1.56 (0.72-3.40)	0.261
Mortality (in-hospital)	0.99 (0.78-1.27)	0.961	0.89 (0.67-1.18)	0.425	1.15 (0.72-1.83)	0.569	1.56 (0.75-3.28)	0.238

CI, confidence Interval; ICU, intensive care unit; IMV, invasive medical ventilation; OR, Odds ratio.
Reference category: non-obesity. Results adjusted for age, sex, and comorbidities (DM, HT, and CVD).
The logistic regression analysis for the variables "ICU admission", "IMV" and "mortality" were calculated in the total number of patients requiring hospital admission (N=2,040).
Bold values stand for statistically significant differences.

TABLE 4 Risk of hospitalization, ICU admission, IMV and mortality based on the presence of comorbidities in the overall population.

Overall population (N=3,402)	Non-obesity with obesity-related comorbidity		Obesity without obesity-related comorbidity		Obesity with obesity-related comorbidity	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Hospitalization	1.85 (1.49-2.28)	<0.001	1.67 (1.30-2.14)	<0.001	2.29 (1.84-2.87)	<0.001
ICU admission	1.31 (0.78-2.21)	0.314	1.15 (0.64-2.05)	0.638	1.34 (0.80-2.25)	0.265
IMV	1.19 (0.69-2.08)	0.532	1.25 (0.69-2.28)	0.459	1.49 (0.87-2.54)	0.146
Mortality (30-day)	1.12 (0.74-1.69)	0.592	0.76 (0.37-1.58)	0.468	1.22 (0.79-1.87)	0.353
Mortality (in-hospital)	0.99 (0.68-1.47)	0.985	0.83 (0.44-1.58)	0.573	1.03 (0.69-1.54)	0.873

CI, confidence Interval; ICU, intensive care unit; IMV, invasive medical ventilation; OR, Odds ratio.
Reference categories: non-obesity without any obesity-related comorbidity. Results adjusted for age and sex Obesity-related comorbidities have been previously defined in this text as diabetes mellitus, arterial hypertension, and cardiovascular disease. The logistic regression analysis for the variables "ICU admission", "IMV" and "mortality" were calculated in the total number of patients requiring hospital admission (N=2,040).

10.3389/fendo.2023.1146517

0.029). The regression analysis showed no differences in the risk of in-hospital or 30-day mortality in patients with and without obesity, and neither across obesity degrees (Table 3). The Kaplan-Meier analysis showed a significantly higher survival in patients with obesity compared with non-obese patients (p = 0.032). However, neither of the groups reached the median OS at day 30 (Supplementary Material Figure S1). The results of the Cox regression model adjusting for age, sex and comorbidities show that there was no association between obesity status and overall survival (HR=1.05; 95% CI 0.84-1.30; p=0.669). The median 30-day OS was not reached in any obesity category (Supplementary Material Figure S2), and there were no significant differences in survival according to obesity categories (p = 0.329) and no association between obesity categories and survival was found.

Among patients under 70 years of age, no significant association was found with the mortality outcomes based on obesity status or categories (Table S2). The median 30-day OS was not reached in any of the groups (obesity vs. non-obesity) and no statistically significant differences were found (p = 0.365). Similarly, no obesity category reached the median 30-day OS and no statistically significant differences were found among categories (p = 0.329; Supplementary Material Figures S3, 4). The results of the Cox regression model adjusting for age, sex and comorbidities showed no association between obesity categories and survival (HR=1.31; CI 95% 0.46-3.70; p = 0.612).

3.5 Healthcare resource use and cost

The total average cost per patient for patients with obesity (N=3,402) was €10,805, substantially higher than the average cost per patient in patients without obesity (€8,418.30, p = 0.007). Table 5 shows the mean cost according to obesity status in the overall population and patients under 70 years, including cost for each healthcare resource use and the percentage excess cost associated with obesity status. The excess cost of obesity in the study cohort was 28.4%, reaching 56.5% in patients under 70 years of age. When the data was imputed in those patients with no available BMI record, the magnitude of the obesity excess cost is even higher (Table S6).

There were significant differences in the mean total cost by BMI category (p=0.007), the average cost increased with obesity degree (Figure 3). The lowest cost corresponds to the normal weight category (€ 6,964.42), while the highest cost corresponds to the grade III obesity category (€ 14,523.22). Moreover, the average cost per patient rises with the combination of obesity and two comorbidities (€ 12,676.61) or three OAC (€ 15,039.97) (Figure 4).

4 Discussion

The OBESITY-COVID study evaluated the prevalence of obesity and its associated comorbidities in a large cohort of patients attending the emergency service of a Spanish hospital during the first wave of the COVID-19 pandemic. Our results show a high prevalence of obesity (33.4%) and comorbidities (HT: 51.8%, DM: 23.7% and CVD: 19.3%) among COVID-19 patients in the participating center, an excess cost burden of obesity of 28%, and a higher risk of hospital admission, ICU admission and need for IMV for patients with obesity. However, we did not find a significant association between obesity and COVID-19 mortality. To our knowledge, this is the first Spanish study specifically evaluating the rate and association of obesity and comorbidities with costs in a large cohort of patients.

Our results confirmed obesity as a robust predictor of critical outcomes in COVID-19 patients (21, 24, 34–36). We found a higher rate of obesity in those patients requiring hospitalization (35.6%), and even higher in patients requiring ICU admission (41.2%). The odds of being hospitalized were significantly higher when the patients had obesity, and they increased with BMI, reproducing what has been described in large cohorts (37). Patients with BMI over 40 kg/m^2 had a higher risk of hospitalization, ICU admission and to receive IMV, regardless of age, sex and diagnosis of comorbidities. This increased odds for ICU admission in grade III obesity, without being significant for obese status, has been

TABLE 5 Average costs per COVID-19 patient according to obesity status.

	Overall population (N=3,402)			Population <70 years old (N=1,871)		
	Average cost Non-obese (€)	Average cost Obese (€)	Obesity excess cost (%)	Average cost Non-obese (€)	Average cost Obese (€)	Obesity excess cost (%)
Emergency department	316.9	329.6	4.0	301.0	321.6	6.8
Hospitalization	5,790.0	6,674.0	15.3	3,443.0	5,379.0	56.2
ICU admission	1,686.0	2,738.0	62.4	2,428.0	3,818.0	57.2
Medication	38.0	67.7	78.2	50.9	98.2	92.9
Tracheostomy procedures + IMV >96 h	650.7	995.4	53.0	846.7	1434.3	69.4
Total	8,418.3	10,805.0	28.4	7,070.0	11,051.0	56.3

ICU, intensive care unit; IMV, invasive mechanical ventilation.

10.3389/fendo.2023.1146517

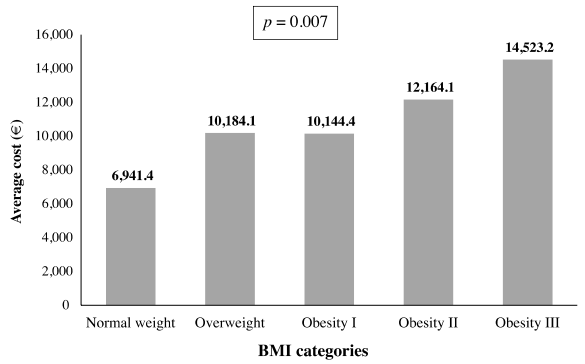

FIGURE 3
Average cost per patient according to BMI categories (N=3,371).

described in similar studies (24, 38). Previous studies used a cut-off point around 70 years of age to stratify the sample, finding a greater association in the need for hospitalization among patients under 65 years (37) or under 70 years of age (39). Other studies have found a more significant association when stratifying the sample below 60 years (40) or even 50 years (38, 41). Our study also describes a higher association between obesity and obesity degrees with the need for hospitalization in those under 70 years of age, with no significant association found in the age range over 70 years. In the need for ICU admission and the need for IMV, no differences were observed stratifying by age, which should be analysed considering that the average age of the patient admitted to the ICU is 60.6 years.

Both in-hospital mortality and 30-day mortality rates were significantly lower in patients with obesity, and we found no association between mortality and obesity in the multivariate analysis. These findings are in line with the results obtained in three relevant studies. One of them is the systematic review and meta-analysis of 120 studies, which could not find an association between obesity status and mortality in COVID-19 patients (26). Moreover, the HOPE-COVID-19 retrospective cohort registry, evaluating a similar-sized cohort of patients, suggested that BMI was not a mortality predictor (15). Finally, a study performed in Madrid with a cohort of patients receiving antiviral treatment for COVID-19 reported a relationship between obesity and the development of acute respiratory distress syndrome, but not between obesity and mortality (18). Nevertheless, some other large cohort studies and meta-analyses demonstrated a reliable link between obesity and mortality (12, 17, 20–25). Based on these controversial results, the potential association between obesity and mortality in COVID-19 patients deserves to be studied in greater depth.

This study showed a rate of obesity of 33.4%, which increases to 35.7% in patients younger than 70 years old. This percentage is substantially higher than the obesity prevalence found in the SIESTA cohort, representing almost all the Spanish territory (14.3%) (20), and in the study by Rodriguez-Gonzalez et al. (15.1%) (18), which included a cohort from the same region as ours. This might be due to differences in patient population, since the SIESTA cohort consisted of a random sample of patients clinically or microbiologically diagnosed with COVID-19, and many were asymptomatic patients not attending the ED, while all patients included in our study came from the ED. The obesity prevalence found in our cohort is within the range of that reported in larger studies, ranging from 25 to 42% (23, 25, 26, 29, 34, 35, 42). The obesity rate by sex and age range is comparable to that reported in the ENRICA study (43),which shows an increase in the prevalence of obesity over 65 years of age (44), suggesting that our cohort is representative of the Spanish population and that the results of our study could be aligned with it.

The DM rate found in our study (23.7%) is slightly higher than the range of prevalence previously reported for this condition in COVID-19 patients (17-22%) (18, 20, 26, 35, 42). Similarly, the HT rate (51.8%) observed in our study was slightly higher than that found in prior reports ranging from 32 to 45% (18, 20, 26, 35, 42). CVD prevalence in COVID-19 patients showed variability between studies (13% (35), 16% (26) and 31.4% (18)). Our data remains within this range (19.3%). In the subgroup of patients under 70 years of age, the prevalence of comorbidities was significantly lower than in the overall cohort.

We found that patients with comorbidities had a higher risk of hospitalization and those without comorbidities showed the highest OS, regardless of their BMI, in line with prior reports (14, 26, 35, 45). However, Spanish investigators could not find an association between the presence of DM and CVD and mortality in the SIESTA cohort (20).

Some studies carried out in Spain had evaluated resource utilization in cohorts of COVID-19 patients at different stages of the disease (18, 27) but, to our knowledge, none of the studies conducted so far has undertaken a comparative analysis between patients with and without obesity. As expected, our data show that the cost per patient was significantly higher in patients with obesity compared to patients without obesity, with an excess cost of 28.4% in the overall cohort and 56.5% in patients under 70 years of age. Additionally, there is a significant increase in the average cost per patient when the obesity degree increases, with the highest excess cost in subjects with grade III obesity. Furthermore, we observed a rising trend in the average cost when the number of comorbidities increases, but it was not statistically significant, likely because the number of patients decreases as the number of comorbidities increases.

This increase in health expenditure in patients with obesity has been previously reported. In a cohort of patients from 273 hospitals located in the U.S., overweight and obesity were associated with higher economic cost of inpatient care, with the highest excess cost

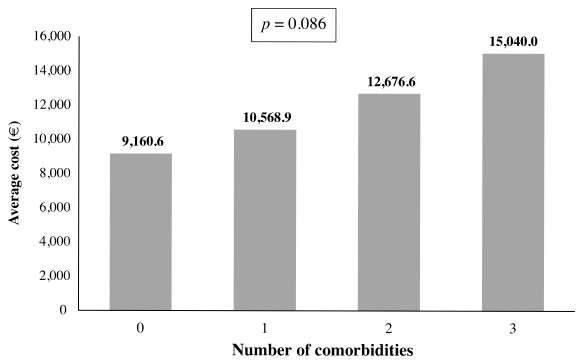

FIGURE 4
Average cost per patient according to number of comorbidities (N=3,402).

in patients with BMI over 45kg/m^2 (46). This increased cost associated with obesity status is also reported in a European study evaluating the costs of COVID-19 associated with obesity in the first 6 months of the pandemic (29). According to this study, the average costs for patients without obesity are aligned with ours (taking into account differences in the study timeframe, 6 months vs. 3 months), but the average cost for patients with obesity is substantially higher. Differences with this European study might come from differences in the study design (cost model estimation vs. single-center observational study). The economic excess cost associated with obesity, along with the increased risk of critical outcomes, reinforce the need to address obesity treatment as a health priority.

This investigation has some limitations. First, participation of a single center located in Madrid, which was one of the most severely stricken areas during the COVID-19 pandemic in Spain, may entail bias. Data such as risk for hospitalization, ICU admission and in-hospital mortality in obese patients cannot be extrapolated to other countries or other regions in Spain. Secondly, the timeframe in which this study was conducted corresponds to the pandemic first wave, from March to June 2020, a period in which emergency and inpatient activity, and especially ICU occupancy, were determined by the high occupancy demand of Madrid's community hospitals (46). Despite its limitations, this study provides valuable data from a large cohort of Spanish patients during the most critical period of the COVID-19 pandemic, assessing clinical and economic aspects of the disease.

We must consider these results carefully due to the rapid evolution of the pandemic since the outbreak at the end of 2019 to the current situation in 2023. The virus strain has been evolving and changing its virulence, the standard of care has changed according to scientific evidence and almost all of the population is fully vaccinated, therefore this must be borne in mind when comparing hospitalization, ICU admission and mortality rates as well as the cost analysis from 2020 to the present.

In conclusion, this study suggests a strong association between obesity and inpatient hospitalization, ICU admission, need for IMV in COVID-19 patients and economic excess cost. The risk for adverse outcomes and cost increases with obesity degree and the presence of comorbidities, regardless of obesity status. Likewise, obesity has been associated with poorer clinical outcomes and higher costs in other pandemics that occurred in this century or other noninfectious diseases, therefore we must promote the management of obesity as a health priority

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 Hospital Clínico San Carlos 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

T-EM, LM, R-HM, MV and PF contributed to conceptualization of the study; T-EM, F-FM. and GB contributed with the study methodology; F-FM, GB, B-PP and SC performed the statistical analysis; T-EM, R-HM, MV, PF and LM conducted the investigation; T-EM, NA, G-PC participated in resource searching; T-EM, NA, G-PC contributed to data curation; T-EM, MV, and R-HM wrote the original draft; T-EM, R-HM, LM participated in the supervision; T-EM, LM, R-HM, MV, PF, F-FM, GB and SC contributed to manuscript revision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

R-HM reports professional fees from Novo Nordisk, outside the submitted work. MV and PF are employees of Novo Nordisk.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1146517/full#supplementary-material

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EDITED BY
Jeff M. P. Holly,
University of Bristol, United Kingdom

REVIEWED BY
Asli Suner,
Ege University, Türkiye
Linfu Li,
Gannan Medical University, China

*CORRESPONDENCE
Xiu-fang Huang
879172531@qq.com
Kai Wang
827108317@qq.com
Shao-feng Zhan
zsfstone@126.com

†These authors share first authorship

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Network pharmacology and bioinformatics analysis identifies potential therapeutic targets of *Naringenin* against COVID-19/LUSC

Wen-yu Wu[1,2†], Xin Jiao[1,2†], Wen-xin Song[1,2], Peng Wu[1,2], Pei-qi Xiao[1,2], Xiu-fang Huang[2,3*], Kai Wang[2*] and Shao-feng Zhan[2*]

[1]The First Clinical Medical College of Guangzhou University of Chinese Medicine, Guangzhou, China, [2]The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China, [3]Lingnan Medical Research Center of Guangzhou University of Chinese Medicine, Guangzhou, China

Background: Coronavirus disease 2019 (COVID-19) is a highly contagious respiratory disease that has posed a serious threat to people's daily lives and caused an unprecedented challenge to public health and people's health worldwide. Lung squamous cell carcinoma (LUSC) is a common type of lung malignancy with a highly aggressive nature and poor prognosis. Patients with LUSC could be at risk for COVID-19, We conducted this study to examine the potential for naringenin to develop into an ideal medicine and investigate the underlying action mechanisms of naringenin in COVID-19 and LUSC due to the anti-viral, anti-tumor, and anti-inflammatory activities of naringenin.

Methods: LUSC related genes were obtained from TCGA, PharmGKB, TTD, GeneCards and NCBI, and then the transcriptome data for COVID-19 was downloaded from GEO, DisGeNET, CTD, DrugBank, PubChem, TTD, NCBI Gene, OMIM. The drug targets of *Naringenin* were revealed through CTD, BATMAN, TCMIP, SymMap, Chemical Association Networks, SwissTargetPrediction, PharmMapper, ECTM, and DGIdb. The genes related to susceptibility to COVID-19 in LUSC patients were obtained through differential analysis. The interaction of COVID-19/LUSC related genes was evaluated and demonstrated using STRING to develop a a COX risk regression model to screen and evaluate the association of genes with clinical characteristics. To investigate the related functional and pathway analysis of the common targets of COVID-19/LUSC and Naringenin, KEGG and GO enrichment analysis were employed to perform the functional analysis of the target genes. Finally, The Hub Gene was screened and visualized using Cytoscape, and molecular docking between the drug and the target was performed using Autodock.

Results: We discovered numerous COVID-19/LUSC target genes and examined their prognostic value in LUSC patients utilizing a variety of bioinformatics and network pharmacology methods. Furthermore, a risk score model with strong predictive performance was developed based on these target genes to assess the prognosis of LUSC patients with COVID-19. We intersected the therapeutic target

genes of naringenin with the LUSC, COVID-19-related targets, and identified 354 common targets, which could be used as potential target genes for naringenin to treat COVID-19/LUSC. The treatment of COVID-19/LUSC with naringenin may involve oxidative stress, anti-inflammatory, antiviral, antiviral, apoptosis, immunological, and multiple pathways containing PI3K-Akt, HIF-1, and VEGF, according to the results of the GO and KEGG enrichment analysis of these 354 common targets. By constructing a PPI network, we ascertained AKT1, TP53, SRC, MAPK1, MAPK3, and HSP90AA1 as possible hub targets of naringenin for the treatment of COVID-19/LUSC. Last but not least, molecular docking investigations showed that naringenin has strong binding activity in COVID-19/LUSC.

Conclusion: We revealed for the first time the pharmacological targets and potential molecular processes of naringenin for the treatment of COVID-19/LUSC. However, these results need to be confirmed by additional research and validation in real LUSC patients with COVID-19.

KEYWORDS

COVID-19, lung squamous cell carcinoma, *naringenin*, network pharmacology, bio informatics

1 Introduction

An acute respiratory infectious illness called COVID-19 is brought on by the SARS-CoV-2 virus (1). Coughing, a sore throat, fever, arthralgias, myalgias, exhaustion, and headache are among the usual COVID-19 symptoms. Acute respiratory distress syndrome (2, 3), shock (4, 5), metabolic acidosis (6), and multiple organ failure (7, 8) may develop in patients with comorbidities. As of November 28, 2022, with 636,440,663 confirmed COVID-19 cases and 6,606,624 deaths reported globally (9). Despite the development of many COVID-19 vaccines and the initiation of mass vaccinations, the number of infections is still continuously increasing (10). Additionally, several antiviral medications have been used to treat COVID-19, such as remdesivir, but they have not been generally adopted because of their high cost and requirement for intravenous administration (11). Studies have demonstrated that cancer patients, including those with lung cancer (12), esophageal cancer (13), colorectal cancer (14), and breast cancer (15), among others, are more susceptible to SARS-CoV-2 infection and have a higher fatality rate (16, 17). Therefore, it is crucial to screen beneficial, affordable, and widely accessible drugs against COVID-19. Lung cancer is one of the most common malignant tumors in humans and has the highest mortality rate worldwide, with 1.6 million fatalities per year (18, 19). The hospital served as the primary infection source during the early stages of the outbreak, and patients with lung cancer who were admitted there for antitumor therapy significantly increased their risk of COVID-19 (20). The majority of patients with lung cancer are immunosuppressed (21), and there is a pressing need for effective drugs to treat lung cancer and COVID-19 with few side effects.

Naringenin is a common dietary flavanone found in citrus fruits such as oranges, bergamots, lemons, and grapefrui (22). *Naringenin* has a molecular formula C15H12O5 and is chemically named 2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)4H-1-benzopyran-4-one (23). Pharmacologically, it has anticancer, antimutagenic, antioxidant, antiproliferative, and antiatherogenic activities (24). *Naringenin* is Commonly used for the treatments of diabetic (25), cognition deficits (26), bronchial pneumonia (27), nonalcoholic steatohepatitis (28, 29), and neurodegenerative diseases (30–32). Naringin possesses antiviral and anti-inflammatory properties, which include lowering viral replication and cytokine production, according to recent studies (33). By entering human cells through the angiotensin-converting enzyme 2(ACE2) receptor and Transmembrane Serine Protease 2(TMPRSS2) (34), SARS-CoV-2 can cause infection. When a virus infects a host, it causes the host to produce and release more inflammatory cytokines, which can boost immune activity and cause tissue damage (35). A growing body of research suggests that antiviral therapy may help treat COVID-19 symptoms as well as those that reduce inflammatory responses (36, 37). Through both transcriptional and post-transcriptional processes, *naringenin* can reduce the generation of inflammatory molecules (38). Macrophages have a crucial role in the pathogenesis of COVID-19 because they can detect infections, react to them, and create inflammatory cytokines and chemokines (39). Without affecting the toll-like receptor(TLR) cascade, *naringenin* decreased the generation of TNF and IL-6 by macrophages and T cells in animal experimental models (39). A recent study suggests that *Naringenin* can function as having the potential inhibitor of SARS-CoV-2 main protease, *naringenin* may be considered as potential for preventing CoV replication (40, 41). In addition, *Naringenin* exhibits the role of treatment of lung cancer by reducing tumor cell proliferation, migration, and invasion while increasing apoptosis (42, 43). As far as we can tell, naringin's molecular mechanisms and targeting have not been investigated in the treatment of COVID-19

in patients with LUSC. In this study, we examine the prognostic value of COVID-19-related genes in LUSC patients and further explore the potential anti-COVID-19/LUSC mechanisms of *naringenin* using network pharmacology and bioinformatics methods. Our findings offer some fresh perspectives on how *naringenin* works to treat COVID-19/LUSC. A clear graphical summary that detailed the entire study process was used in Figure 1.

2 Materials and methods

2.1 Ethics statement

Because the data were sourced from free databases, ethics committee approval was not necessary for this study.

2.2 *Naringenin* database building

PubChem (https://pubchem.ncbi.nlm.nih.gov/) comprises a wide range of chemical information from 750 data sources (44). The 2D structure, 3D structure, InChI, and canonical SMILES profiles of naringin were obtained from PubChem (44).

2.3 Identification of COVID-19/LUSC-associated genes

The transcriptome profiles of LUSC patients were downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/) on 19 November 2022, and the differentially expressed genes (DEGs) were screened and obtained using the 'limma' package of R-language(version 4.2.2) Bioconductor with P-value< 0.05 and |log2FC| >1 (45). DEGs were represented using volcano plots created with the R-language packages "ggpubr" and "ggthemes". PharmGKB (https://www.pharmgkb.org), NCBI Gene (https://www.ncbi.nlm.nih.gov/) (46), Therapeutic Target Database (TTD, http://db.idrblab.net/), GeneCards (https://www.genecards.org/), and Online Mendelian Inheritance in Man (OMIM, https://www.omim.org/) (47) were also used to collect LUSC related targets.

The COVID-19-related targets were identified by examining the transcriptome RNA-seq data of COVID-19 (GSE171110 and GSE179850) from the Gene Expression Omnibus database (GEO, https://www.ncbi.nlm.nih.gov/geo) (48). After normalization and removal of the batch effect by using the "sva" package, the difference analysis was conducted with the criteria of adjusted P-value< 0.05 and |log2FC| > 1 by using the "limma" package (45). Furthermore,

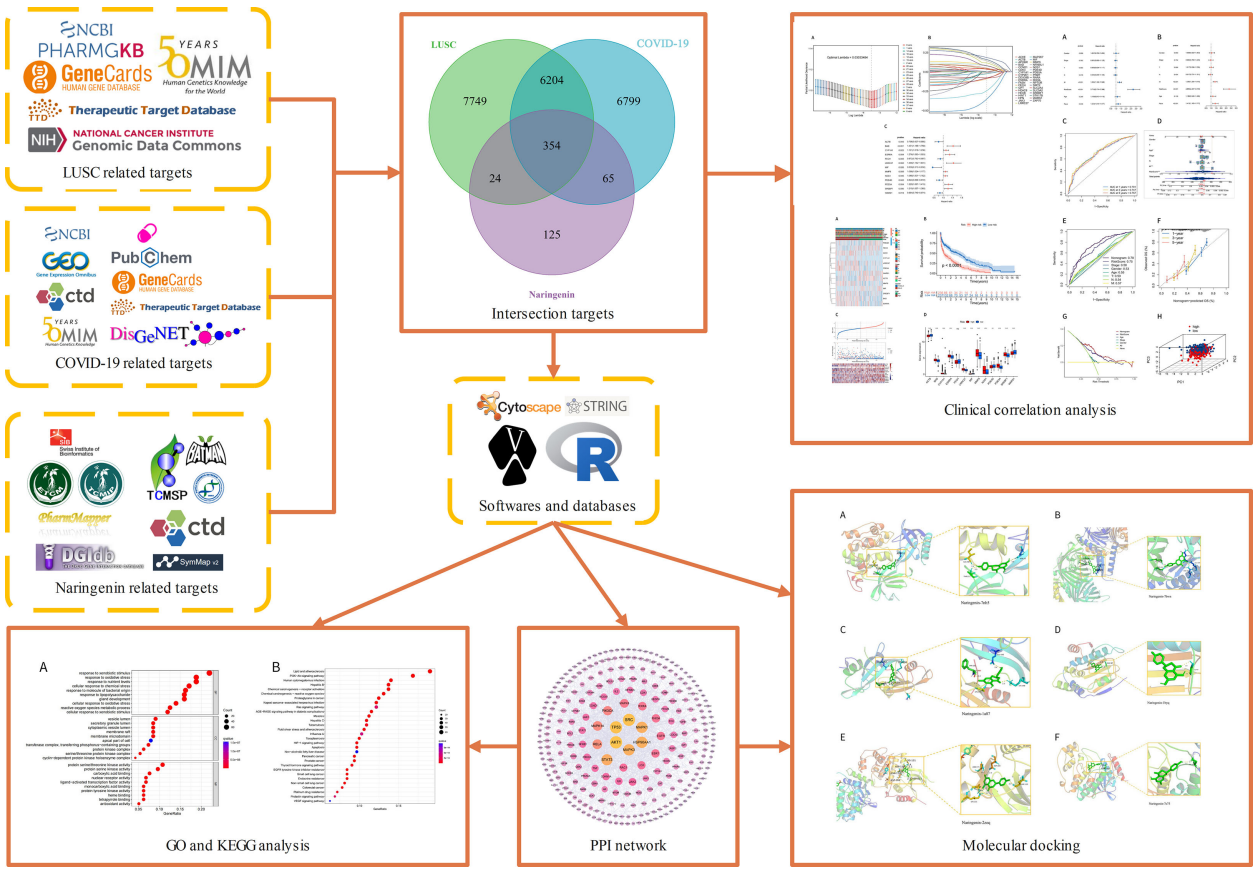

FIGURE 1
Flow of the entire investigation.

targets associated with COVID-19 were obtained by searching the following eight databases: 1) DisGeNET(http://www.disgenet.org/), 2) Comparative Toxicogenomics Database(CTD, http://ctdbase.org/), 3) DrugBank(https://go.drugbank.com/), 4) PubChem(https://pubchem.ncbi.nlm.nih.gov/), 5)Therapeutic Target Database, 6) GeneCards, 7) NCBI Gene, 8)Online Mendelian Inheritance in Man. Targets gathered from public databases and GEO datasets were combined. The targets of LUSC and COVID-19 were then intersected to create a gene set that is connected to COVID-19/LUSC.

2.4 Fishing of *naringenin*-related targets

From the following databases, many pharmacological targets connected to *naringenin* were gathered: 1) CTD (http://ctdbase.org/) (49), 2) Bioinformatics Analysis Tool for Molecular mechANism of Traditional Chinese Medicine(BATMAN, http://bionet.ncpsb.org.cn/batman-tcm/) (50), 3) Integrative Pharmacology-based Research Platform of Traditional Chinese Medicine (TCMIP, http://www.tcmip.cn/TCMIP/) (51), 4) Symptom Mapping (SymMap, https://www.Symmap.org/) (52), 5) Swiss Target Prediction (http://www.swisstargetprediction.ch/) (53), 6) Chemical Association Networks (STITCH, http://stitch.embl.de/) (53), 7) PharmMapper (http://www.lilabecust.cn/pharmmapper/) (54), 8) Encyclopedia of Traditional Chinese Medicine (ECTM, http://www.tcmip.cn/ETCM/) (51), and 9) Drug Gene Interaction Database (DGIdb, https://www.dgidb.org/) (55). The target genes were transformed to standard gene symbols by using the UniProt database (https://www.uniprot.org/) with the limitation of "Human species". After deleting the duplicate data, there were 568 targets of *naringenin*.

2.5 Acquisition of *naringenin* targets in COVID-19/LUSC

Using the Venn diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) and Microsoft Excel, it was possible to further achieve the goal of removing the repeated targets among *naringenin*, LUSC, and COVID-19. Then, the intersection of *naringenin*-related targets and COVID-19/LUSC-related targets was then screened for common targets.

2.6 Construction of LUSC/COVID-19-related prognostic signature for lung squamous cell carcinoma patients

Univariate Cox analysis was performed on 354 common targets, and Lasso Cox regression was performed on significantly expressed genes based on a 1000 ten-fold cross-validation to identify COVID-19-related genes. Optimal prognostic genes were identified based on multivariate Cox regression analysis ($P< 0.05$), and the best model parameters were used for signature construction, followed by the calculation of risk scores.

$$\mathbf{Risk\ score} = \mathbf{Exp\ gene1} \times \beta\ \mathbf{gene1} + \mathbf{Exp\ gene2} \times \beta\ \mathbf{gene2} + \ldots \mathbf{Exp\ gene\ n} \times \beta\ \mathbf{gene\ n}$$

2.7 Analysis of prognosis signature

Patients were divided into high- and low-risk groups based on median values of a risk score to determine the prognosis of the signature. We used the survival package to calculate overall survival (OS) for patients with LUSC in different groups and performed univariate and multivariate independent prognostic analyses to evaluate the independent prognostic value of the risk prediction signature. The pheatmap package was used to plot patient survival status and gene expression heatmap based on the risk scores. The survival ROC package was used to calculate the 1-, 3-, and 5-year area under the receiver operating characteristic curve(ROC) curve (AUC) of signature in the LUSC patients.

2.8 Construction of nomogram and validation of clinical subgroups

Nomograms were constructed for age, gender, T stage, N stage, M stage, and risk score using the survival and rms packages. Calibration curves were plotted to show the difference between the predicted and actual outcomes of the nomogram. Decision curve analyses are used to verify the accuracy of the signature in predicting the survival of patients with LUSC.

2.9 Principal component analysis

PCA analysis was performed using limma and scatterplot3d packages to explore the distribution of patients with the high and low-risk groups.

2.10 Analyses of the protein-protein interaction network and hub targets

PPI networks contribute to a better understanding of target-related pathogenesis at the protein level. Thus, the STRING 11.5b database (https://string-db.org/) was used to fabricate the PPI network and acquire hub targets. The organism was selected as "Homo sapiens" and the minimum required interaction score with a correlation degree ≥0.900 was the cut-off value (56). Subsequently, the PPI network was visualized and analyzed by Cytoscape 3.9.1 software (https://cytoscape.org/). The degree values in the PPI network were calculated by using the NetworkAnalyzer CytoNCA of Cytoscape 3.9.1 software. Then, targets with degree values higher than the median were filtered as hub targets (57).

2.11 Enrichment analyses for common targets

To investigate the related functional and pathway analysis of the common targets of COVID-19/LUSC and *Naringenin*, R packages such as "enrichplot", "clusterProfiler" (58), "org.Hs.eg.db", and "ggplot2" were used to perform GO functional analysis and KEGG pathway enrichment analysis (59).Biological processes (BP), cellular components (CC), and molecular functions(MF) were the three categories included in the GO analysis. For enrichment, q-value cutoff = 0.05 and p-value cutoff = 0.05 were set, and the output was utilized to construct the bubble chart (46, 60).

2.12 Molecular docking

The binding situation and interaction force of proteins and small molecules may be anticipated and acquired *via* molecular docking analysis. *Naringenin* was docked with the PPI network's top six hub targets, which included RAC-alpha serine/threonine-protein kinase(AKT1), TP53-target gene 3 protein(TP53), Proto-oncogene tyrosine-protein kinase Src(SRC), Heat shock protein HSP 90-alpha(HSP90AA1), Mitogen-activated protein kinase 3 (MAPK3), and Mitogen-activated protein kinase 1(MAPK1). *Naringenin*'s two-dimensional molecular structure was retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) (61), and its three-dimensional structure was created and optimized using the MM2 force field in ChemBioOffice software (version 2019) (62). Following that, *Naringenin*'s output ligand file was saved in mol2 format. The protein structures of the hub targets were obtained from the PDB database (https://www.rcsb.org/) (63). All water molecules and original ligands were removed from the structures using PyMOL software (https://pymol.org/2/) and saved as PDB files. AutoDockTools (Vina 1.5.6, http://autodock.scripps.edu/) was used to convert the ligand files and original protein receptor to PDBQT file format that be identified by the Autodock Vina software for further molecular docking. Lastly, PyMOL software was used to analyze and present all docking results (64).

3 Results

3.1 Targets identification of *naringenin* and COVID-19/LUSC

All targets of *naringenin* were obtained from Nine open-source databases, namely, BATMAN (1), CTD (326), DGIdb (6), ETCM (44), PharmMapper (299), STITCH (13), Swiss (65), SymMap (65), and TCMIP (44). And 568 targets related to *naringenin* were acquired after eliminating duplicate targets (Figure 2A). Subsequently, a total of 10649 DEGs (4042 upregulated and 6607 downregulated) of LUSC were identified from the TCGA. DEGs volcano plots for LUSC are displayed in Figure 3A. In addition, LUSC related genes collected from PharmGKB, NCBI Gene, TTD, GeneCards, and OMIM were 83, 2424, 7, 5062, and 438, respectively. By merging DEGs from the TCGA and LUSC-related genes from online platforms and removing duplication, we obtained 22232 LUSC related targets (Figure 2B). In total, 4533 DEGs (4561 upregulated and 17 downregulated) of COVID-19 were identified from the GEO. DEGs volcano plots for COVID-19 are displayed in Figure 3B. In addition, COVID-19 related genes retrieved from DisGeNET, CTD, DrugBank, PubChem, TTD, GeneCards, NCBI Gene, and OMIM were 1632, 9892, 40, 622, 73, 1972, 412, and 3, respectively. By merging DEGs from the GEO and LUSC-related genes from online platforms and removing duplication, we obtained 13422 COVID-19 related targets (Figure 2C). Finally, we acquired 354 common targets between *naringenin*, LUSC, and COVID-19, which were shown using the Venn diagram tool (Figure 2D).

3.2 Construction of LUSC/COVID-19-related prognostic signature

Cox regression analysis of 354 common target expression data and survival information was conducted using the coxph method in the "survival package" of the R language. The filtering standard was $P<0.05$, and 37 single-factor significant genes were obtained. Lasso regression analysis was performed on the above single-factor significant gene expression data, and Lasso regression plots and cross-validation plots were drawn (Figures 4A, B), and there were 20 Lasso regression significant genes based on the λ minimum value of LASSO Cox regression (0.03033484), Finally, 13 prognosis-related genes for constructing the model were identified using multifactorial Cox regression analysis, and forest plots were drawn (Figure 4C). Those involved in the model construction (BAD, CYP1A1, ESRRA, LRRC27, MMP9, NOS1, PDE5A, and SREBF1) with risk coefficients greater than 1 were defined as risk factors in LUSC, and their higher expression correlated with the worst OS of LUSC.

3.3 Analysis of prognosis signature

The patients were separated into high- and low-risk groups in order to better examine the prognostic value of the risk signature. The 13 genes in the constructed model were analyzed differentially for each clinical trait and high and low-risk groups, and heat maps were plotted (Figure 5A), demonstrating that (BAD, CYP1A1, ESRRA, LRRC27, MMP9, NOS1, PDE5A, and SREBF1) were highly expressed in the high-risk group. We discovered that patients in the high-risk group had a considerably shorter overall survival than those in the low-risk group in the Kaplan-Meier survival analysis. (Figure 5B). The risk curves show the relationship between LUSC patients' risk scores and survival rates, and we discovered that mortality was higher in high-risk patients than in low-risk individuals. The heatmap showed high- and low-risk levels for 13 genes. For example, 8 genes (BAD, CYP1A1, ESRRA, LRRC27, MMP9, NOS1, PDE5A, and SREBF1) were highly

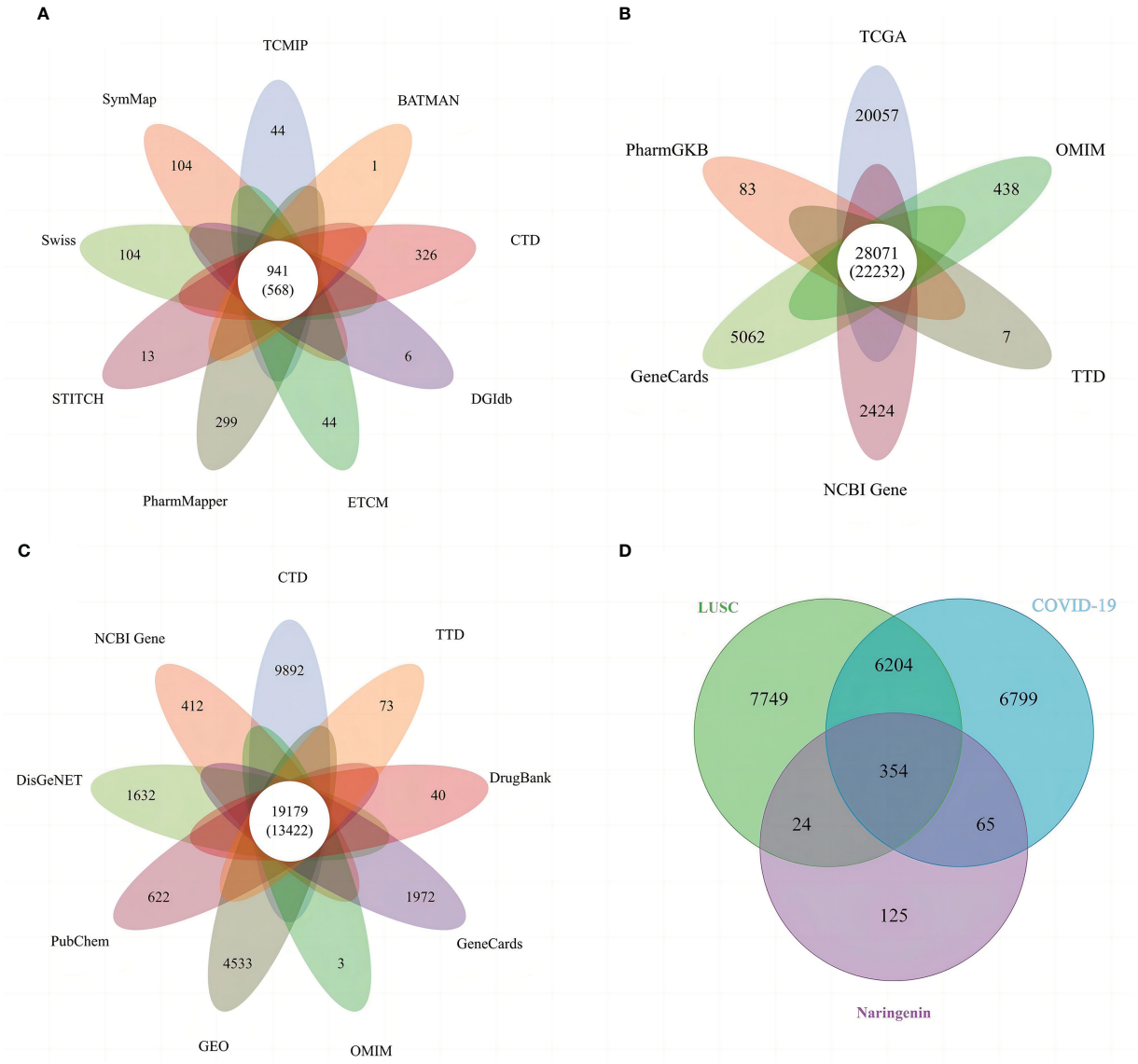

FIGURE 2
(A) Targets connected to naringenin in nine open-source databases. **(B)** Targets connected to LUSC in six open-source databases. **(C)** Targets connected to COVID-19 in nine open-source databases. **(D)** A Venn diagram demonstrating the common targets of naringenin, LUSC, and COVID-19.

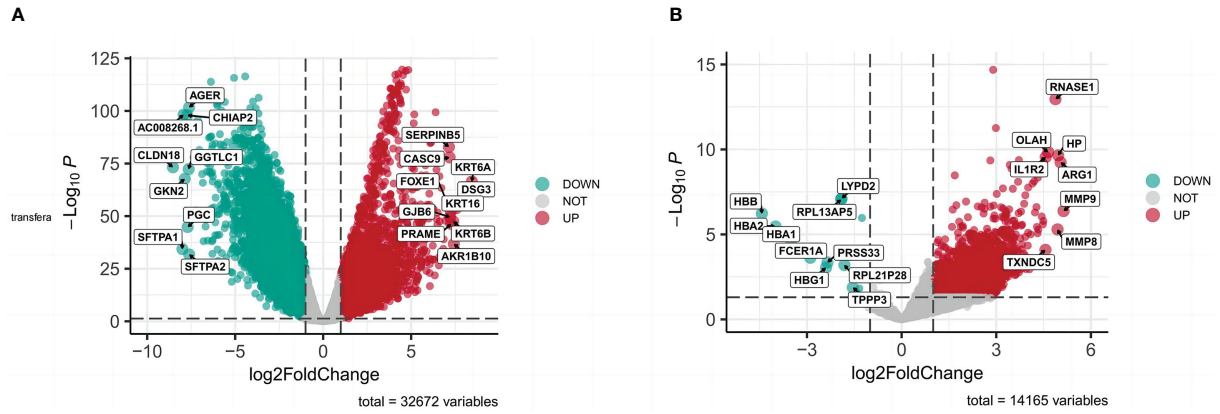

FIGURE 3
(A) DEGs volcano plots for LUSC. **(B)** DEGs volcano plots for COVID-19.

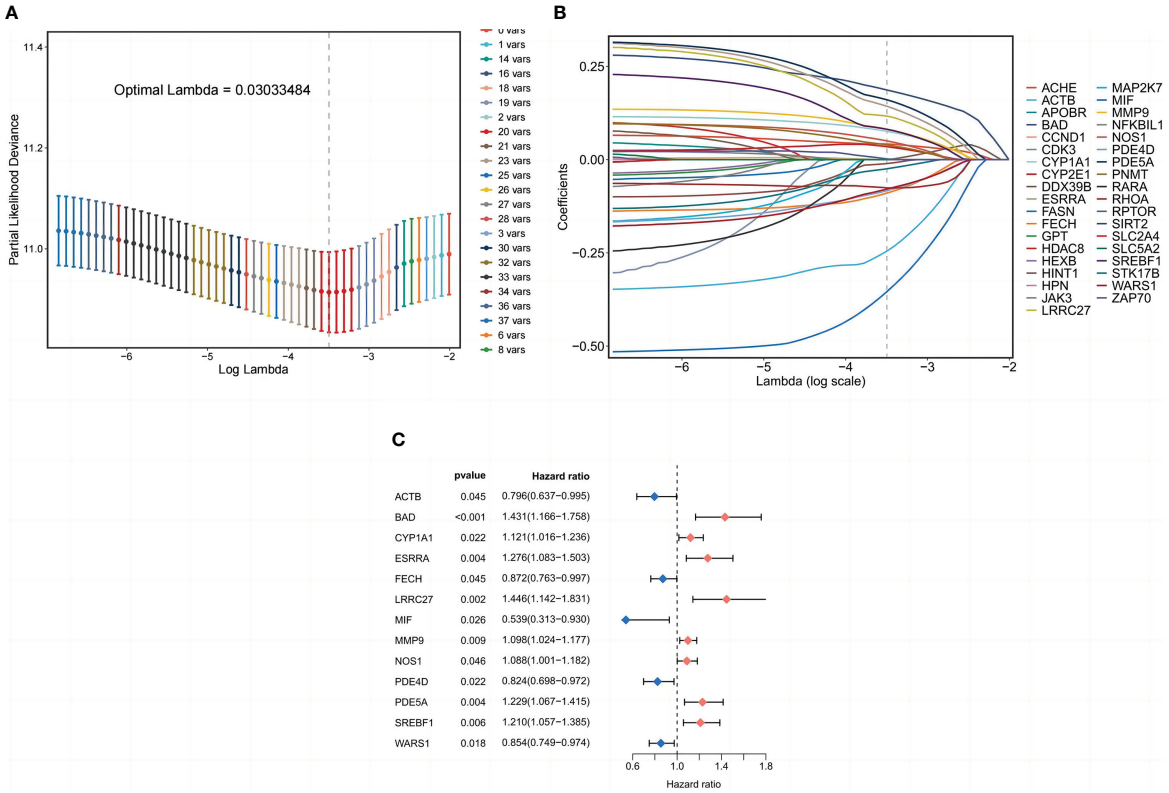

FIGURE 4
Prognostic model of LUSC patients. **(A)** Coefficient profiles plotted for LASSO regression analysis of LUSC. **(B)** Cross-validation error rate plotted for LASSO regression analysis of LUSC. **(C)** Partial presentation of prognostic related genes.

expressed in the high-risk group, which was consistent with the prediction of the model (Figures 5C, D).

3.4 Independent analysis of prognostic factors

Univariate and multivariate Cox regression analyses were performed to determine whether the risk signature has the potential to be a prognostic factor independent of other clinical parameters (Figures 6A, B). The risk score (HR = 2,805, 2.237–3.518; P<0.001) was significantly linked with OS in multivariate Cox regression, demonstrating that the risk signature is an independent prognostic factor for LUSC patients. Additionally, we evaluated the risk score's predictive accuracy using ROC curves. The AUC for the risk score was 0.75, which was higher than those for age (0.55), gender (0.53), and stage (0.58). While in the LUSC cohort, the AUCs for 1-, 3-, and 5-year OS were 0.701, 0.747, and 0.757 (Figure 6C), indicating that the signature has trustworthy diagnostic applicability.

3.5 Construction of nomogram and validation of clinical subgroups and PCA

Age, gender, TNM stage, T stage, M stage, N stage, and the risk score from the signature were used to construct a nomogram (Figure 6D) that could accurately predict the 1-, 3-, and 5-year OS of LUSC patients. Furthermore, ROC curves and decision curves were used to test the effectiveness of nomograms (Figures 6E, F). The nomogram's AUC was 0.780, which was higher than the risk score of 0.75 and indicates that the nomogram has reliable diagnostic significance. Then, decision curve analysis(DCA) demonstrated that the nomogram outperformed the other six molecular categorization techniques in terms of clinical net benefit (Figure 6G). Finally, we used PCA to look at the distribution of risk genes among patients, and the results showed that these genes could be relied upon to generate the signature (Figure 6H).

3.6 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis

GO and KEGG enrichment analyses on common targets were performed to investigate the biological activities and pathways of *naringenin* against COVID-19/LUSC. As a consequence, 3133 GO terms were highlighted (BP: 2793, CC: 92, and MF: 248), as well as 183 KEGG pathways. The top 10 GO terms of each ontology and the top 30 KEGG pathways are presented as bubble charts (Figures 7A, B). Representative BP terms included the response to xenobiotic stimulus, cellular response to chemical stress, response

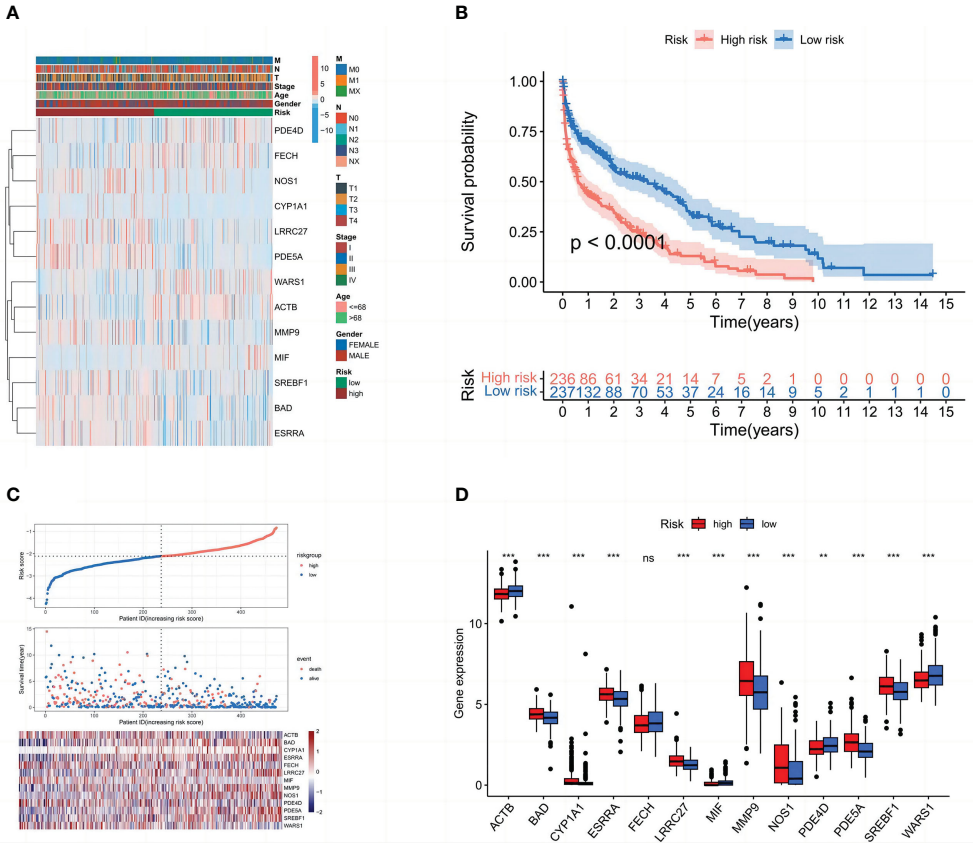

FIGURE 5
Subgroup correlation heat map and survival analysis of high- and low-risk groups. **(A)** Patient clinicopathological characteristics are distributed in different ways. **(B)** Box plot of model gene differential analysis: comparison of model genes differentially expressed in high- and low-risk groups. **(C)** Patient's OS according to Kaplan-Meier curves for high- and low-risk groups. **(D)** The variation in immune checkpoint gene expression between various populations. ns, P=>0.05: **, P<0.01; ***, P<0.001.

to oxidative stress, response to nutrient levels, response to lipopolysaccharide, response to molecule of bacterial origin, cellular response to xenobiotic stimulus, reactive oxygen species metabolic process, cellular response to oxidative stress, gland development, etc. Representative CC terms included protein kinase complex, cyclin-dependent protein kinase holoenzyme complex, vesicle lumen, secretory granule lumen, cytoplasmic vesicle lumen, etc. Representative MF terms included nuclear receptor activity, ligand-activated transcription factor activity, carboxylic acid binding, monocarboxylic acid binding, protein serine/threonine kinase activity, protein tyrosine kinase activity, etc. In addition, representative pathways included the PI3K-Akt signaling pathway, the VEGF signaling pathway, the HIF-1 signaling pathway, Pancreatic cancer, human cytomegalovirus infection, toxoplasmosis, prostate cancer, Chemical carcinogenesis—receptor activation, non-small cell lung cancer, chemical carcinogenesis—reactive oxygen species, Kaposi sarcoma-associated herpesvirus infection, proteoglycans in cancer, small cell lung cancer, endocrine resistance, apoptosis; colorectal cancer, thyroid hormone signaling pathway, tuberculosis, etc. In conclusion, Go and KEGG analysis highlighted that *naringenin*'s anti-inflammatory, antiviral, and anticancer properties are important targets/pathways in COVID-19/LUSC treatment.

3.7 PPI network analysis

The PPI network of common targets contained 268 nodes and 1,358 edges, which represented targets and interactions between targets, respectively. In the fight against COVID-19/LUSC, a node with a darker color and a larger form is more important. According to Figure 8, the top 6 targets with the highest degree values were AKT1 (degree = 55), TP53 (degree = 54), SRC (degree = 54), MAPK1 (degree = 49), MAPK3 (degree = 49), and HSP90AA1 (degree = 49). Consequently, molecular docking with *naringenin* was performed using AKT1, TP53, SRC, MAPK1, MAPK3, and HSP90AA1 as the hub targets for *naringenin* to cure COVID-19/LUSC.

3.8 Molecular docking

In general, stronger binding conformations and higher interaction probabilities result from lower binding energies. Related studies revealed that binding energy< 0 kJ/mol imply spontaneous binding, and -5.0 kJ/mol or lower indicates good binding activity (66). We analyzed the possible binding of *naringenin* with the six COVID-19/LUSC hub targets (AKT1, TP53, SRC, HSP90AA1, MAPK3, and MAPK1) identified

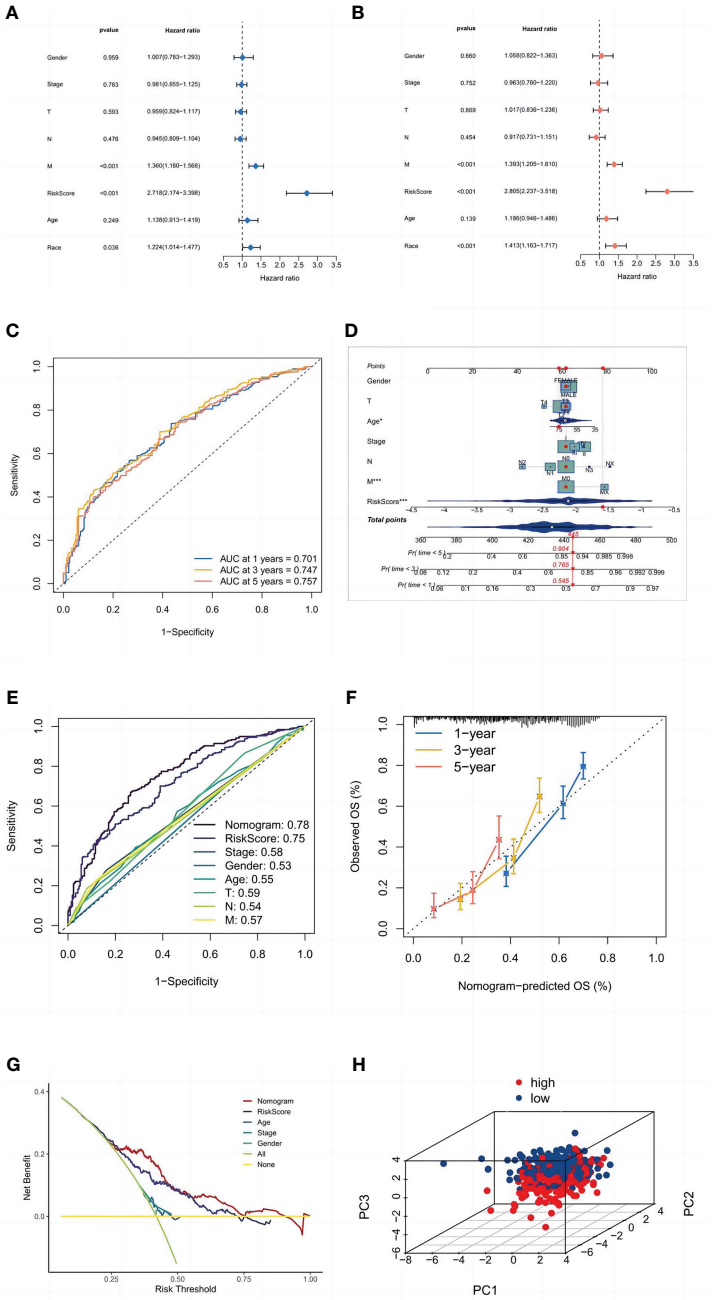

FIGURE 6
Validation of the model. **(A)** Univariate and **(B)** multivariate Cox regression analyses. **(C)** ROC curves of 1, 3, and 5 years. **(D)** The nomogram prediction model. **(E)** ROC curves of risk scores and clinical characteristics. **(F)** A calibration curve. **(G)** A DCA of the nomogram prediction model and the TNM staging system. **(H)** Principal component analysis of high and low-risk groups.

previously and found that all of the docking results showed strong binding activity. Amino acid residues ASN-53, ILE-290, and THR-211 in AKT1, amino acid residues GLN-183, ASN-164, GLN-248, GLU-349, and ASN-345 in TP53, amino acid residues GLU-162, PTR-101, LYS-155, and LYS-198 in SRC, amino acid residues GLY-97 in HSP90AA1, amino acid residues LYS-131, ASP-128, ASN-171, ASP-184, and ASP-123 in MAPK3, amino acid residues ASN-144 in MAPK1, and *naringenin* form hydrogen bonds tightly. Overall, our findings demonstrated the high affinity between *naringenin* with AKT1, TP53, SRC, HSP90AA1, MAPK3, and MAPK1 (Figures 9A–F).

4 Discussion

COVID-19 is a serious, rapidly spreading infectious disease that can be fatal (67). At this time, it is known that older age, male sex, diabetes mellitus, obesity, cardiovascular disease, and cancer are risk

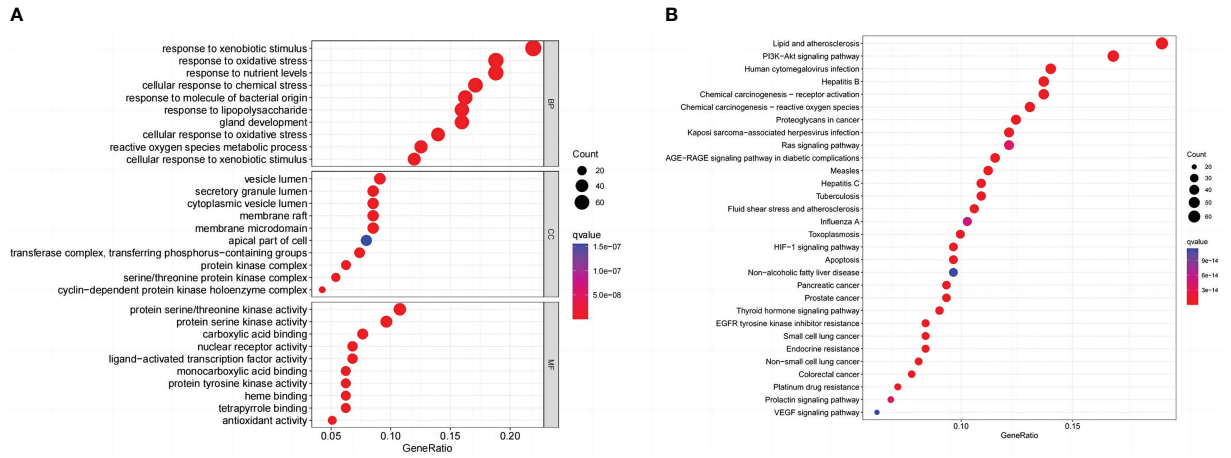

FIGURE 7
Naringenin functional characterization against COVID-19/LUSC. **(A)** GO analysis of naringenin and COVID-19/LUSC intersecting genes. **(B)** KEGG analysis of naringenin and COVID-19/LUSC intersecting genes.

factors for COVID-19 infection and serious outcomes (68, 69). Cancer patients are vulnerable to respiratory viruses due to immunosuppression caused by the disease or therapy. This susceptibility has been demonstrated with influenza, which has been linked to a higher mortality rate in people with solid and hematologic cancer (70). Lung cancer patients from COVID-19 had the highest death risk compared to those with other cancers, which is most likely related to advancing age, a decline in lung reserve, concomitant conditions, and cancer treatment (71). We investigated the potential usefulness and molecular mechanisms of *naringenin* for COVID-19/LUSC using bioinformatics and system pharmacology methodologies based on the extensive biological properties of *naringenin*, including anti-inflammatory, anti-tumor, and antiviral. This might offer a fresh option for raising the survival rate of COVID-19/LUSC patients and stopping the spread of SARS-COV-2 through the digestive system.

In the present study, the potential mechanism and prognostic value of *naringenin* in COVID-19/LUSC were comprehensively investigated through network pharmacology and bioinformatic analyses, and we acquired 354 common targets of *naringenin* against COVID-19/LUSC. According to GO and KEGG enrichment analysis, the mechanisms for *naringenin* treatment of COVID-19/LUSC may be related to oxidative stress, immunoregulation, apoptosis, antiviral, anti-inflammatory, anti-cancer, and associated signaling pathways such as PI3K-Akt, HIF-1, and VEGF. Additionally, molecular docking demonstrated that *naringenin* and the top 6 COVID-19/LUSC related-target proteins had strong binding activity. As a result, the findings illustrate that *naringenin* holds a lot of promise as a treatment for COVID-19/LUSC.

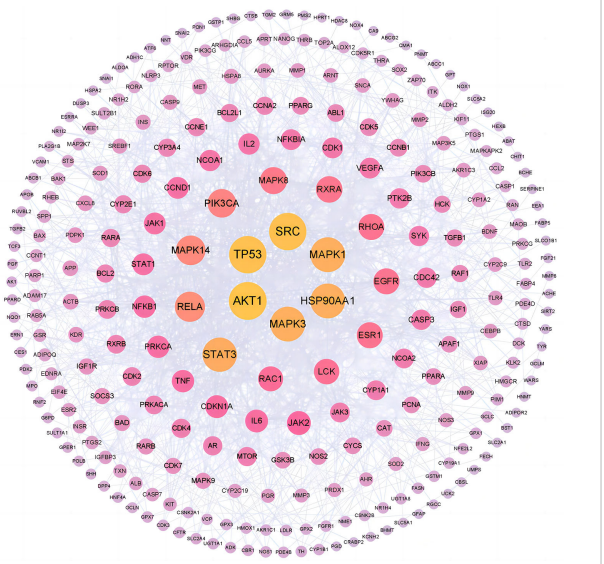

FIGURE 8
PPI network for hub targets of naringenin against COVID-19/LUSC.

4.1 Excellent prognostic analysis of LUSC patients with COVID-19

We first collected 13422 COVID-19 targets, 568 *naringenin* targets, and 22232 LUSC targets, and then further screened out 354 common targets, then selected 472 patient samples for follow-up prognostic analysis. Subsequently, a thirteen-gene signature containing ACTB, BAD, CYP1A1, ESRRA, FECH, LRRC27, MIF, MMP9, NOS1, PDE4D, PDE5A, SREBF1,and WARS1 was developed through univariate and multivariate Cox analyses, LUSC patients with COVID-19 may benefit from independent prognostic variables. Additionally, multivariate ROC analysis demonstrated that risk ratings were significantly more accurate than conventional pathological prognostic variables in predicting OS. Analysis of the nomogram revealed that the prognostic signature may be utilized to predict the outcomes of LUSC patients who have the COVID-19 mutation.

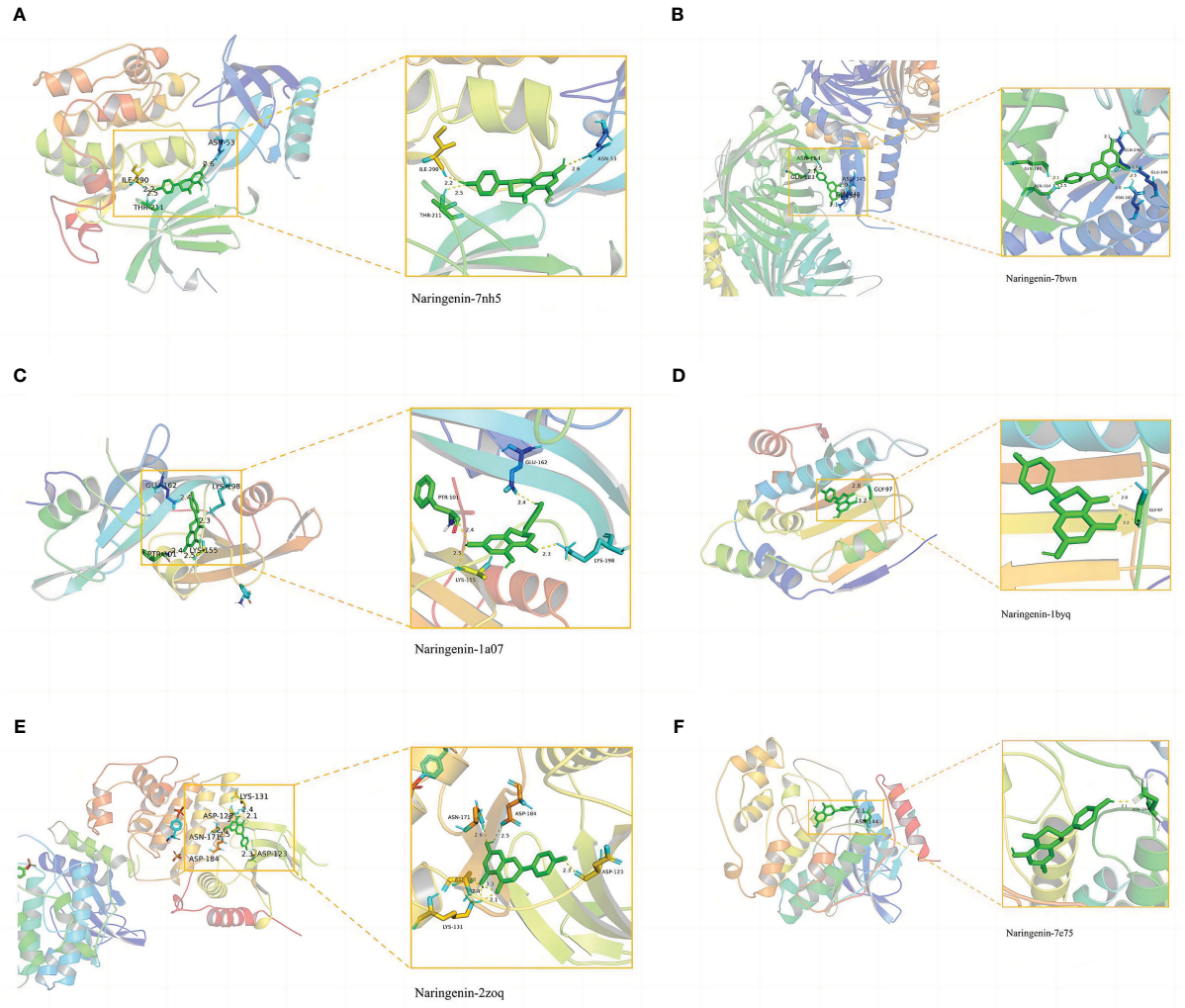

FIGURE 9
Molecular docking of naringenin and the targets AKT1, TP53, SRC, HSP90AA1, MAPK3, and MAPK1. **(A)** The binding of naringenin with the 7nh5 protein of AKT1. **(B)** The binding of naringenin with the 7bwn protein of TP53. **(C)** The binding of naringenin with the 1a07 protein of SRC. **(D)** The binding of naringenin with the 1byq protein of HSP90AA1. **(E)** The binding of naringenin with the 2zoq protein of MAPK3. **(F)** The binding of naringenin with the 7e75 protein of MAPK1.

4.2 Hub targets could be set off by *Naringenin* to fight COVID-19/LUSC

In the PPI network of shared targets, which we first obtained, there were 268 nodes and 1,358 edges for the 354 common targets of *naringenin* and COVID-19/LUSC. In addition, we chose the top six targets in the PPI network as well as essential proteins connected to COVID-19/LUSC for molecular docking with *naringenin*. There were significant variations in the expression of the top six target genes, which included AKT1, TP53, SRC, MAPK1, MAPK3, and HSP90AA1. A member of the serine/threonine protein kinase subfamily known as Akt1 (72), viral protein synthesis is facilitated by overexpressed AKT1, and silencing of AKT1 results in reduced viral RNA expression, suppression of viral capsid protein synthesis, and virus release (73, 74). According to a study, which supports our hypothesis, AKT1 inhibition lowers viral yields in Huh7 cells infected with SARS-CoV-2 (75). An important aspect of the development of cancer is cell migration and motility, in different malignancies, Akt1 activation and expression leads to the advancement of carcinogenesis and metastasis (76). By controlling the expression of certain genes, such as those in the Akt signaling pathway, Akt1 plays a significant role in the development of tumors. Furthermore, AKT1 regulates innate immunity, which affects macrophage immunological function and the activated phenotype. AKT1 activation increases inflammatory and metabolic responses, making it a suitable target for COVID-19 therapy (57, 77, 78). The p53 protein, expressed by the TP53 gene, has been dubbed the "guardian of the genome" due to its involvement in responding to DNA damage by inducing cell cycle arrest, apoptosis, and/or senescence (79). The potential for TP53 gene therapy through SGT-53 to suppress viral infections against the many SARS-CoV-2 variants that have evolved or may develop throughout the COVID-19 pandemic (80). The gene that suppresses tumors One of the most frequently altered genes in human lung cancer is TP53 (81). In addition to causing tumor development, defects in TP53 function impair the response of

malignant cells to anticancer drugs, especially those that induce DNA damage (82) and TP53 mutations are more common than average in LUSC (83). Therefore, it is anticipated that TP53 would be crucial in the fight against LUSC (84). When MAPK family members and the MAPK-STAT3 axis are activated, inflammatory factors such as IL-1, TNF-, and IL-6 are overexpressed (85). The MAPK1 signaling pathway has been associated to ALI/ARDS inflammation (86). Many cytokines, including IL-1, TNF-, and IL-6, play important roles in ALI/ARDS, primarily *via* the MPAK1 signal transduction pathway (87), The study discovered that an inhibitor of MAPK3/MAPK1 following carrageenan induced a reduction in all inflammation parameters assessed, which could be effective in the treatment of numerous inflammatory illnesses (88),Therefore, we have reason to believe that regulating MAPK pathway has positive significance for improving the expression of inflammatory factors in COVID-19. Meanwhile, MAPK1 restoration inhibited the proliferation, migration, and invasion of NSCLC cells (89). HSP90AA1 is a gene that promotes squamous cell lung cancer progression (90) and has an important regulatory role in non-small cell lung cancer (91). Simultaneously, the study discovered that decreasing HSP90AA1 expression can lower inflammatory factors, ROS generation, cell apoptosis rate, and autophagy-related proteins (92). These results further suggested that *naringenin* could be an effective pharmaceutical target for these intersecting genes against LUSC and COVID-19.

4.3 The critical mechanisms for *naringenin* to combat COVID-19/LUSC

The mechanism of *naringenin* against COVID-19/LUSC is strongly linked to oxidative stress, immunoregulation, apoptosis, antiviral, anti-inflammatory, anti-cancer, and related pathways including PI3K-Akt, HIF-1, and VEGF signaling pathway, according to the findings of GO and KEGG enrichment study. Excessive oxidative stress impairs immune system performance, increasing the risk of SARS-CoV-2 viral invasion in the body (93). Cancer, neurodegeneration, cardiovascular conditions, diabetes, and other illnesses also can be brought on by abnormal oxidative stress (94). The major characteristic of cancer cells is reduced apoptosis, which is accomplished by modifying important signaling molecules or pathways. The proto-oncogene Akt, whose expression and activation are increased in a variety of cancers, including LUSC, contributes to the resistance of cancer cells to chemotherapy and radiation therapy (95, 96). High basal levels of PI3K-Akt activation in clinical samples suggested an aggressive type of LUSC (97). The PI3K-Akt signaling pathway is specifically activated by CD147, which is crucial in the entry of SARS-CoV-2 into cells, and its shutting down will prevent some viruses from entering cells (98). Furthermore, by inhibiting the PI3K/AKT/mTOR signaling pathway, SARS-CoV-2 spike pseudovirions promote the appearance of autophagy, which in turn initiates apoptosis (99). Another hallmark of COVID-19 is tissue hypoxia, which is related to overexpression of the HIF-1along with their immunometabolic and immune-response implications (100). HIF-1, as one of the hypoxia signal transcription factors, regulates the expression of genes involved in metabolism in macrophages and T cells, promoting an inflammatory response (101, 102). The HIF-1 pathway regulates oxidative stress, hypoxia, and inflammation, and its activity may promote SARS-CoV-2 infection and affect a variety of physiological processes (103). HIF-1 is a fibroblast master regulator of lipid metabolism that contributes to a tumor-promoting phenotype in lung fibroblasts (104). Therapy for lung cancer may benefit from focusing on the HIF-1/SCD1 axis in CAFs (65). HIF-1 is thus a potential target in research against COVID-19/LUSC due to the importance of HIF-1 stabilization in tumor progression. As is generally known, VEGF is the best-characterized mediator of angiogenesis at the molecular level (105). Cancer cells that overexpress VEGF exhibit enhanced tumorigenicity, invasiveness, proliferation, and EMT features (106). Therefore, VEGF coordinates non-angiogenic events that are crucial for the early spread of tumors (107). When VEGF levels are elevated, it results in high permeability, edema, and tissue injury, which are the pathophysiologic causes of acute lung injury in COVID-19 patients (108, 109). The VEGF signaling pathway, on the other hand, raises angiotensin II(Ang II) levels to promote inflammation, while Ang II can also raise VEGF to promote the release of inflammatory cytokines (110). In conclusion, *Naringenin* may have an antiviral, anti-inflammatory, and anti-cancer effect by regulating oxidative stress, apoptosis, HIF-1, PI3K-Akt, and VEGF signaling pathways in order to alleviate the clinical symptoms of COVID-19/LUSC patients.

Last but not least, molecular docking data showed that *naringenin* has strong binding capabilities with the six COVID-19/LUSC targets, demonstrating that *naringenin* can effectively bind to specific proteins connected to COVID-19/LUSC. According to network pharmacology, *naringenin* can be used to treat SARS-CoV-2-infected patients with LUSC. More experimental investigation, however, is required to confirm and investigate the expected targets and their regulatory processes.

To sum up, We created a trustworthy predictive model for patients with LUSC and COVID-19 as well as many possible treatment targets of COVID-19/LUSC. Further, through the pharmacological actions and possible targets that have been identified, *naringenin* may be used to treat COVID-19/LUSC, including immunomodulation, antivirals, anti-inflammation, etc. Additionally, we were able to find direct binding sites that had a strong affinity for *naringenin* against COVID-19/LUSC, which gave the justification for further clinical trials as well as the proof for its use in clinical settings.

4.4 Strengths and limitations

Notably, our study offered some novel insights into *naringenin* in the therapy of COVID-19/LUSC and suggested plausible molecular pathways and prospective pharmacological targets of *naringenin* for the first time. However, a few remaining issues with our study's limitations must be resolved. Since the results of this study were not verified in actual LUSC patients with COVID-19, future confirmation of these findings will need the recruitment

of actual LUSC patients with COVID-19. Second, additional *in vivo* and *in vitro* studies are necessary to confirm the hypothesized mechanisms and pharmacological targets in order to confirm the potential therapeutic application of *naringenin* for COVID-19/LUSC.

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.

Author contributions

S-fZ, KW, and X-fH conceived and designed this research. W-yW and XJ wrote the manuscript and participated in the design of the study. W-yW and XJ were responsible for the bioinformatics analysis and network construction. P-qX and W-xS carried out the data analysis and data interpretation. PW helped to modify the manuscript. 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1187882/full#supplementary-material

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EDITED BY
Jeff M. P. Holly,
University of Bristol, United Kingdom

REVIEWED BY
Pietro Locantore,
Catholic University of the Sacred Heart, Rome, Italy
Roberto Novizio,
Agostino Gemelli University Polyclinic (IRCCS), Italy

*CORRESPONDENCE
Yimin Yan
yanyimin180@163.com
Zhe Dai
daizhe@znhospital.cn

†These authors have contributed equally to the work

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Transient low T3 syndrome in patients with COVID-19: a new window for prediction of disease severity

Mingyao Zhong[1,2†], Yue Gao[2†], Hongling Hu[3†], Xuan Zhu[2], Lulu Gan[2], Ling Li[1], Cheng Xiang[1], Yimin Yan[1,2*] and Zhe Dai[3*]

[1]Department of Endocrinology, Xiaogan Hospital Affiliated to Wuhan University of Science and Technology, The Central Hospital of Xiaogan, Xiaogan, Hubei, China, [2]Department of Internal Medicine, Medical College of Wuhan University of Science and Technology, Wuhan, China, [3]Department of Endocrinology, Zhongnan Hospital of Wuhan University, Wuhan, China

Objective: To investigate the relationship of low T3 syndrome with disease severity in patients with COVID-19.

Methods: The clinical data of 145 patients with COVID-19 were retrospectively collected, and patients were divided into a low T3 group and a normal T3 group. Logistic regression models were used to assess predictive performance of FT3. Receiver operating characteristic (ROC) analysis was used to evaluate the use of low T3 syndrome in predicting critical disease. Kaplan-Meier analysis was used to analyze the impact of low T3 syndrome on mortality.

Results: The prevalence of low T3 level among COVID-19 patients was 34.48%. The low T3 group was older, and had lower levels of hemoglobin, lymphocytes, prealbumin, and albumin, but higher levels of white blood cells, neutrophils, CRP, ESR, and D-dimer (all $p<0.05$). The low T3 group had greater prevalences of critical disease and mortality (all $p<0.05$). Multivariate logistic regression analysis showed that the Lymphocytes, free T3 (FT3), and D-dimer were independent risk factors for disease severity in patients with COVID-19. ROC analysis showed that FT3, lymphocyte count, and D-dimer, and all three parameters together provided reliable predictions of critical disease. Kaplan-Meier analysis showed the low T3 group had increased mortality ($p<0.001$). Six patients in the low T3 group and one patient in the normal T3 group died. All 42 patients whose T3 levels were measured after recovery had normal levels after discharge.

Conclusion: Patients with COVID-19 may have transient low T3 syndrome at admission, and this may be useful for predicting critical illness.

KEYWORDS

low T3 syndrome, thyroid, COVID-19, critical illness, prediction

1 Introduction

Many studies have demonstrated that COVID-19 is associated with functional abnormalities of the thyroid (1–3), including low T3 syndrome, subclinical hypothyroidism, and subacute thyroiditis (4, 5). The most common of these is low T3 syndrome (6), and this condition had a prevalence of 64% in one population of COVID-19 patients (7). Low T3 syndrome, also referred to as non-thyroidal illness syndrome (NTIS), is a metabolic disorder of thyroid gland caused by a non-thyroidal illness that can occur upon exposure to a variety of stressful conditions that manifest as a decreased level of T3, but normal levels of T4 and TSH. Previous studies showed that a low free T3 (FT3) level was a reliable prognostic indicator for patients with a variety of diseases. In particular, a low FT3 level is a reliable predictor for death in ICU patients (8, 9).

In November 2021, researchers first discovered the Omicron variant of SARS-CoV-2 in Botswana (10). This strain is highly transmissible (11), and 3 weeks after its discovery it replaced Delta as the predominant SARS-CoV-2 variant (12). Although Omicron is generally associated with reduced disease severity and mortality (13), it is also associated with a higher incidence of critical illness and poor prognosis in unvaccinated older adults (14). Therefore, early prediction of critical illness is particularly important. The COVID-19 diagnostic and treatment guidelines clearly indicate that Lymphopenia is an early warning indicator for severe COVID-19 (15). Many studies have shown that elevated D-dimer is associated with severe COVID-19 (16). The value of low T3 syndrome in predicting the prognosis of COVID-19 patients remains unclear. Therefore, we retrospectively examined the predictive value of low T3 syndrome in patients who were critically ill with COVID-19.

2 Patients and methods

2.1 Patients

The clinical data of 145 patients diagnosed with COVID-19 from January to March 2020 in Xiaogan Central Hospital (Hubei Province) were retrospectively analyzed and divided into a low T3 group (FT3 < 3.1 pmol/L, n = 50) and a normal T3 group (FT3 ≥ 3.1 pmol/L, n = 95) (Figure 1). All clinical parameters were recorded within 24 h after admission. Disease severity was classified according to clinical symptoms and chest imaging results as: mild (mild symptoms, with no imaging features of pneumonia), ordinary (symptoms of fever and cough, with imaging features of pneumonia), severe (dyspnea with respiratory rate of 30/min or more, blood oxygen saturation of 93% or less, partial pressure ratio of arterial oxygen to inspired oxygen [PaO_2/FiO_2] below 300 mmHg, and/or pulmonary infiltration greater than 50% within 24 to 48 h after admission) or critically ill (respiratory failure, infectious shock, and/or multi-organ dysfunction or failure). All patients signed informed consent documents. The study protocol was approved by the Ethics Committee of the Xiaogan Central Hospital and followed the ethical principles of the Declaration of Helsinki, as amended in 2013.

The inclusion criteria were receipt of thyroid function testing within 24 h after admission; age of 18 years or more; and presence of the clinical diagnostic criteria for COVID-19 based on the National Health Board COVID-19 Guidelines (version 8). The exclusion criteria were previous primary thyroid disease or another endocrine disease; recent use of medications that could affect thyroid hormone secretion and/or metabolism (including amiodarone, interferon, and glucocorticoids); exposure to iodine-containing contrast media prior to thyroid testing; and presence of a chronic disease, such as hepatic insufficiency, renal insufficiency, or heart failure; high level (≥10.0 μIU/mL) or low level (<0.1 μIU/mL) of thyroid-stimulating hormone (TSH).

2.2 Data collection

Demographic and clinical data of patients were extracted from the hospital records system. All data regarding clinical manifestations, disease severity, laboratory results, imaging results, and clinical outcomes were recorded. Blood samples were collected within 24 h after admission. The Roche Cobas e602

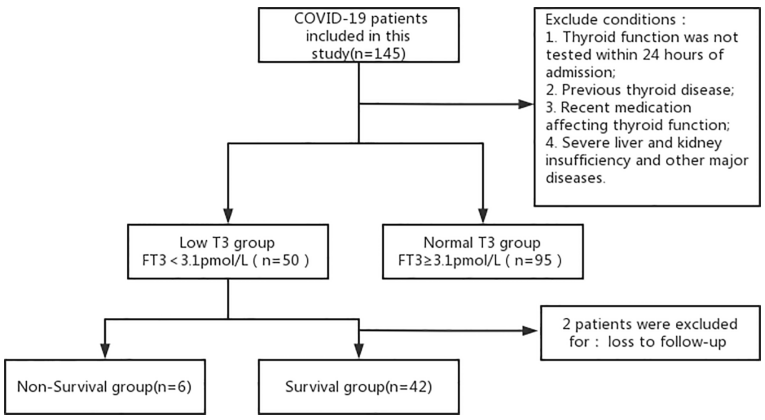

FIGURE 1
Disposition of patients who were admitted for COVID-19 and received thyroid hormone testing.

electrochemiluminescence analyzer (Roche, Germany) was used to determine the levels of FT3, FT4, and TSH. RT-PCR was used to confirm SAS-CoV-2 infection from a throat swab.

During the collection of lung CT imaging data (soon after admission), two experienced physicians read the films and performed quantitative analysis of the distribution, size, and number of lesions. All 50 patients in the low T3 group were followed up; 6 of them died and 44 survived, 42 of whom returned to the outpatient clinic for rechecking of thyroid function at 1 month after discharge. One of the 95 patients in the normal T3 group died.

2.3 Statistical analysis

Data were analyzed using SPSS version 22.0. A difference was considered statistically significant when the P value was below 0.05. Non-normally distributed data were expressed as medians and interquartile ranges (IQRs), and the significance of differences was determined using the Mann-Whitney U test. Categorical data were expressed as numbers and percentages, and the significance of differences was determined using the Chi-squared test or Fisher's exact test.

To assess predictive performance of FT3 in this small sample size, we firstly performed univariable logistic regressions to select potential laboratory factors associated with COVID-19 severity. Statistically significant factors were further included into multivariable logistic regression. Area under Receiver operating characteristic curve (AUC) and 95% confidence interval (CI) were reported for each selected factor and final model.

To further explore prognostic effect of FT3, Kaplan-Meier survival curves were used to determine the relationship of low FT3 with different endpoints, and survival times and 95% confidence intervals (CIs) were reported.

3 Results

3.1 Clinical characteristics of patients with low T3 and normal T3

We retrospectively examined the records of 145 patients who had COVID-19 and received thyroid hormone testing (Table 1). Overall, 34.50% of the patients had low T3, 52.40% were female, and the median age was 50 years (IQR 39, 60). Comparison of the two groups indicated the low T3 group was older (median age: 57 years (48, 67) vs 45 years (35, 54), $p < 0.001$) and low SpO2 (28.00% vs 6.30%, $p < 0.001$).

The two groups also had differences in many laboratory findings. In particular, the low T3 group had significantly lower levels of FT3, TSH, lymphocytes, hemoglobin, pre-albumin, total plasma protein, and albumin, and significantly higher levels of white blood cells, neutrophils, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and D-dimer (all $P < 0.05$). Thus, the low T3 group had higher levels of inflammatory indicators and lower levels of nutritional parameters.

Analysis of disease severity showed that the low T3 group had a smaller proportion of patients with ordinary disease (58% vs 80%, $p < 0.05$), but greater proportions of patients with severe disease (26.00% vs 13.68%, $p < 0.05$) and critical disease (16.00% s 5.26%, $p < 0.05$). The low T3 group also had a lower rate of discharge (88.00% vs 98.95%, $p < 0.05$), and a greater rate of death (12.00% vs 1.05%, $p < 0.05$).

3.2 Univariate and multivariate logistic regression analysis on disease severity

Univariate logistic regression analysis showed that FT3, White blood cells, Neutrophils, Lymphocytes, Pre-albumin, Albumin, CRP, ESR, D-dimer were associated with disease severity. Multivariate logistic regression analysis showed that the Lymphocytes 0.87(95% CI: 0.82–0.93, P<0.001), FT3 0.80(95% CI: 0.51–0.93, P=0.043), and D-dimer 5.78(95% CI: 1.48–22.54, P=0.011) were independent risk factors for disease severity in patients with COVID-19. (Table 2).

3.3 Predictive value of lymphocytes, D-dimer, and FT3 with disease severity

Analysis of all 145 patients showed that as disease severity increased, the levels of FT3 and lymphocytes decreased, and the level of D-dimer increased (all $P < 0.05$; Table 3). Pairwise comparisons indicated the serum FT3 level was significantly lower in patients with severe disease and critical disease than in those with ordinary disease (both $p < 0.05$), but the FT3 level was not significantly different in patients with severe disease and critical illness (Figure 2).

We used ROC analysis to determine the predictive performance of FT3 level, lymphocyte count, and D-dimer level on critical illness (Table 4, Figure 3). The area under the curve (AUC) was 0.724 (95% CI: 0.622–0.825, $P < 0.001$) for FT3, 0.698 (95% CI: 0.591–0.804, $P < 0.001$) for lymphocytes, and 0.783 (95% CI: 0.684–0.881, $P < 0.001$) for D-dimer. Thus, a lower FT3 level and lymphocyte count and a higher D-dimer level were significantly associated with critical illness. The combined use of all three parameters had an AUC of 0.802 (95% CI: 0.706–0.898, $P < 0.001$).

3.4 Clinical characteristics and cumulative morality of patients with low T3

To explore the potential value of low T3 for clinical outcome assessment, we analyzed the low T3 group by comparison of survivors (n = 42) and non-survivors (n = 6; Table 5). The non-survivors had significantly lower levels of FT3 and lymphocytes, and a significantly higher level of D-dimer (all $p < 0.05$). Kaplan-Meier analysis confirmed that patients with low T3 had greater cumulative mortality (p < 0.001; Figure 4).

3.5 Thyroid hormone levels before and after discharge of patients with low T3

42 patients with low T3 were measured after recovery had normal levels after discharge. Analysis of the 42 patients with low T3 levels indicated these patients had significantly greater post-discharge levels of FT3 and FT4 (both $p < 0.05$; Table 6). The post-discharge level of TSH was also greater, but the difference was not statistically significant ($P > 0.05$).

4 Discussion

Our assessment of the prognostic value of FT3 in patients who had COVID-19 indicated that patients with low FT3 syndrome were older, had more severe clinical symptoms, and had worse clinical outcomes. We also found lower levels of FT3 and lymphocytes and a higher level of D-dimer in patients with critical illness and in non-survivors, suggesting that a low T3 level is related to COVID-19 severity. Our ROC analysis demonstrated

TABLE 1 Clinical characteristics and outcomes of all patients, the low T3 group, and the normal T3 group.

Variable	All patients (n=145)	Low T3 (n=50)	Normal T3 (n=95)	p value
Age (years), median (IQR)	50 (39,60)	57 (48,67)	45 (35,54)	<0.001
Gender, n (%)				
Male	69 (47.59)	18 (36.00)	51 (53.68)	0.043
Female	76 (52.41)	32 (64.00)	44 (46.32)	0.043
Clinical parameters on presentation, n (%)				
Temperature ≥ 37.3°C	47 (32.41)	15 (30.00)	32 (33.68)	0.363
SpO_2 ≤ 93%	20 (13.79)	14 (28.00)	6 (6.32)	<0.001
Heart rate > 100 bpm	26 (17.93)	14 (28.00)	12 (12.63)	0.022
Mean arterial pressure, mmHg	94 (87,99)	91 (83,100)	95 (89,99)	0.182
Laboratory results				
FT3 (2.43-6.01pmol/L)	3.43 (2.80-4.00)	2.64 (2.41-2.80)	3.76 (3.44-4.26)	<0.001
FT4 (9.01-19.05pmol/L)	15.37 (13.45-17.19)	14.73 (13.31-16.32)	15.67 (13.76-17.24)	0.274
TSH (0.35-4.94uIU/mL)	1.65 (1.00-2.84)	1.10 (0.62-2.66)	1.90 (1.33-2.87)	0.002
White blood cells (3.5-9.5×10^9/L)	4.56 (3.41-5.68)	5.33 (3.58-6.81)	4.43 (3.34-5.40)	0.005
Hemoglobin (130-175g/L)	131.00 (120.00-144.00)	124 (117-134.25)	138.00 (126.00-149.00)	<0.001
Neutrophils (1.8-6.3×10^9/L)	2.84 (2.04-4.29)	4.27 (2.48-6.51)	2.47 (1.84-3.50)	<0.001
Lymphocytes (1.1-3.2×10^9/L)	0.94 (0.66-1.42)	0.84 (0.57-1.00)	1.11 (0.72-1.54)	<0.001
Pre-albumin (200-400m g/L)	143.20 (109.00-190.90)	109.10 (75.30-143.58)	164.40 (132.30-206.70	<0.001
Total plasma protein (65-85g/L)	66.60 (63.80-73.00)	65.50 (63.03-69.85)	67.60 (64.30-73.85)	0.042
Albumin (40-55g/L)	38.10 (35.70-40.90)	35.75 (33.48-37.93)	39.80 (37.40-41.90)	<0.001
Procalcitonin (0-0.5ng/mL)	0.20 (0.15-0.34)	0.18 (0.16-0.35)	0.21 (0.14-0.34)	0.829
C-reactive protein (0-3mg/L)	17.91 (6.91-37.05)	36.91 (17.81-74.00)	14.90 (5.13-25.72)	<0.001
Erythrocyte sedimentation rate (0-15mm/h)	41.00 (22.00-57.00)	53.50 (30.50-84.75)	31.50 (21.00-47.25)	0.004
D-dimer (0-1mg/L)	0.27 (0.23-0.37)	0.36 (0.26-0.65)	0.26 (0.23-0.30)	<0.001
Disease severity, n (%)				
Ordinary	105 (72.41)	29 (58.00)	76 (80.00)	0.005
Severe	26 (17.93)	13 (26.00)	13 (13.68)	0.066
Critical	13 (8.97)	8 (16.00)	5 (5.26)	0.031
Clinical outcome, n (%)				
Discharge	138 (95.17)	44 (88.00)	94 (98.95)	0.012
Death	7 (4.83)	6 (12.00)	1 (1.05)	0.012

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TABLE 2 Univariate and multivariate logistic regression analysis on disease severity.

Variables	Univariate analysis		Multivariate analysis	
	OR (95%CI)	P Value	OR (95%CI)	P Value
FT3	0.42 (0.25-0.71)	0.001	0.80 (0.51-0.93)	0.043
TSH	0.98 (0.80-1.19)	0.817		
White blood cells	1.65 (1.33-2.03)	<0.001	1.45 (0.85-2.46)	0.171
Hemoglobin	0.99 (0.97-1.00)	0.125		
Neutrophils	1.13 (1.08-1.18)	<0.001	1.01 (0.80-1.28)	0.952
Lymphocytes	0.87 (0.82-0.91)	<0.001	0.87 (0.82-0.93)	<0.001
Pre-albumin	0.99 (0.98-1.00)	0.005	1.00 (0.98-1.01)	0.577
Total plasma protein	1.01 (0.96-1.07)	0.626		
Albumin	0.72 (0.63-0.83)	<0.001	0.91 (0.70-0.95)	0.040
CRP	1.02 (1.01-1.03)	<0.001	1.02 (1.01-1.04)	0.042
ESR	1.03 (1.01-1.05)	0.004	1.00 (0.96-1.04)	0.952
D-dimer	11.17 (2.14-58.28)	0.004	5.78 (1.48-22.54)	0.011

that a low FT3 level was a reliable and independent risk factor for critical disease (AUC = 0.724), and the addition of lymphocyte count and D-dimer further improved the predictive performance (AUC = 0.802). Our analysis of the 42 patients who had low T3 syndrome and were survivors showed that thyroid function increased after recovery.

We can suggest several possible general mechanisms that explain the association between NTIS and COVID-19-related adverse outcomes. Importantly, these different mechanisms are not mutually exclusive. One possible mechanism is that the virus causes direct damage of cells in thyroid tissues. This seems plausible because angiotensin converting enzyme 2 (ACE2) functions as a receptor for the SARS-CoV-2 spike protein (17), and has high expression in thyroid tissues (18).

Second, the increased levels of cytokines induced by severe COVID-19 could explain the relationship of low T3 syndrome with poor outcome. A cytokine storm affects the course and severity of disease (19). Our low T3 group was sicker and had a stronger inflammatory response, with significantly higher levels of leukocytes, CRP, and ESR, important indicators of inflammation. This systemic inflammatory response can cause an increase in inflammatory cytokines such as IL-1, IL-6 and TNF-α (20), leading to suppression of the hypothalamic-pituitary-thyroid axis (3) and reduced 5'-monodeiodinase activity (21), resulting in decreased TSH secretion, and decreased conversion of T4 into rT3. Severe inflammation is also considered a major cause of disseminated intravascular coagulation (DIC) (22), and many COVID-19 patients have thrombosis and DIC, consistent with our finding of an elevated D-dimer level in patients with low T3.

Third, the association of a low T3 level with more severe COVID-19 disease may be because normal levels of thyroid hormones are important in protecting the lungs from injury (23). In particular, serum T3 can increase the synthesis of lung surface-active substances, reduce alveolar surface tension, and increase lung compliance, thereby improving lung function (24). In contrast, a low T3 level may lead to lower levels of lung surface-active substance and aggravate lung function in patients with COVID-19. Consistent with our findings, previous studies of patients with low FT3 and sepsis had reduced oxygen saturation, greater involvement of lung lesions, greater use of oxygen therapy, and were more likely to experience respiratory failure.

Fourth, the relationship of low T3 level with anemia with poor nutritional status may be responsible for the association of low T3 level with disease severity. Previous research reported that a low serum albumin level alone was a sufficient indicator of malnutrition in patients hospitalized with COVID-19 (25).A negative nitrogen balance and organismal depletion associated with the disease can also lead to a decrease in serum thyroid hormone transporter protein levels, inhibiting T3 production as well as T4 transport in tissues (26). Another study showed that NTIS was related to a significantly decreased level of T3 and a significantly increased level of reverse T3 (rT3, an inactive form of T3) during the acute phase of

TABLE 3 Levels of FT3, lymphocytes, and D-dimer in COVID-19 patients with different disease severity.

	Normal range	All (n=145)	Ordinary (n=106)	Severe (n=26)	Critically ill (n=13)	p value
FT3 (pg/mL)	3.1–6.8	3.43 (2.80,4.00)	3.59 (2.96,4.08)	3.07 (2.55,3.35)	2.89 (2.45,3.25)	0.001
Lymphocytes ($\times10^9$/L)	1.1–3.2	0.94 (0.66,1.42)	1.07 (0.76,1.49)	0.69 (0.52,1.17)	0.68 (0.46,0.88)	0.002
D-dimer (mg/L)	0–1	0.27 (0.23,0.37)	0.026 (0.23,0.30)	0.39 (0.25,2.21)	0.40 (0.31,1.20)	<0.001

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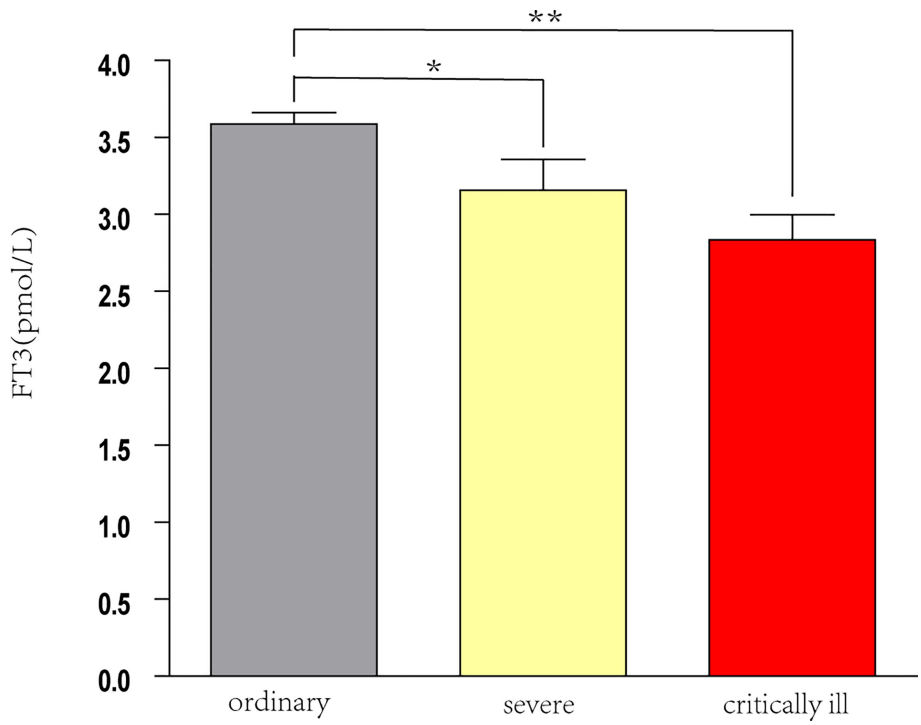

FIGURE 2
FT3 levels in patients with different severity of COVID-19. *$P < 0.05$, **$P < 0.001$.

disease. This may be related to the decreased level of thyroid hormone binding protein and albumin, as well as reduced binding activity (27).

Several previous studies showed that the level of D-dimer (28) and the lymphocyte count (29) were associated with poor outcome in COVID-19 patients. Another study found that a D-dimer level of 2.0 μg/mL or more upon admission was the optimal cut-off for predicting in-hospital mortality from COVID-19 (30). Other studies reported that low T3 syndrome was strongly associated with the severity and prognosis of critical illnesses. For example, a prospective trial of 480 patients in intensive care units reported that the FT3 level was an independent and robust predictor of mortality (31). These previous studies led us to speculate that a low T3 level could be useful as a predictor of critical COVID-19, because early identification of patients who have a risk of progression to severe COVID-19 is essential for providing timely treatments. Our analysis of the low T3 group demonstrated that the level of T3 was lower in non-survivors and in those with more severe disease, and our ROC analysis demonstrated that the FT3 level was a acceptable predictor of critical COVID-19. Recent studies of COVID-19 patients also demonstrated an association between lymphopenia and thyroid function (32), indicating a potential interaction between the hypothalamic-pituitary-thyroid axis and the immune system (33). The levels of lymphocytes and D-dimer affect the relationship between COVID-19 and the thyroid, because a strong inflammatory response and coagulation dysfunction predict worse clinical outcome. In agreement, our ROC analysis indicated that using the combination of the levels of T3, lymphocytes, and D-dimer led to better prediction of critical disease.

TABLE 4 Diagnostic performance of lymphocytes, D-dimer, and FT3 in predicting critical COVID-19.

Parameter	AUC	95%CI	P
FT3	0.724	0.622,0.825	<0.001
Lymphocyte count	0.698	0.591,0.804	<0.001
D-dimer	0.783	0.684,0.881	<0.001
All 3 parameters	0.802	0.706,0.898	<0.001

AUC: area under the curve.

It is possible that COVID-19 could have a long-term impact on thyroid function. However, our follow-up of 42 survivors in the low T3 group demonstrated that recovery from COVID-19 was related to recovery of thyroid function. In particular, the COVID-19 patients in the low T3 group had normalization of the levels of T3 and T4 after discharge, and all of these patients had serum T3 levels in the normal range. Some researchers suggested the use of thyroid hormone supplementation to restore the normal serum levels of patients with NTIS. However, this idea remains highly controversial and there is still no clear evidence that this supplementation provide a benefit (7, 34). Our patients experienced restoration of normal serum T3 levels after recovery from COVID-19, and none of them received thyroid hormone therapy.

There are several possible reasons why the serum T3 levels of our patients normalized after recovery from COVID-19. After

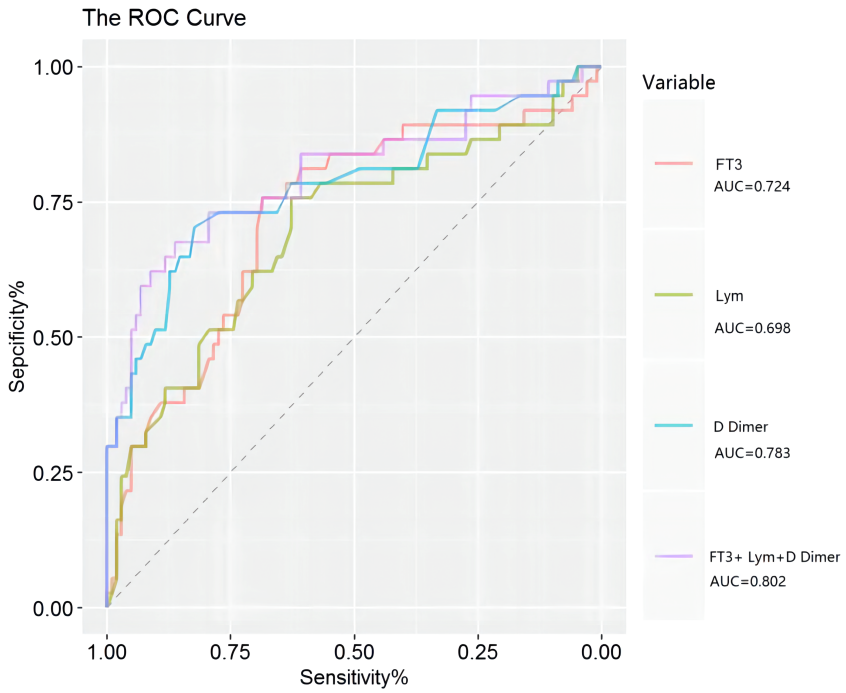

FIGURE 3
ROC curves for FT3, lymphocytes, and D-dimer in predicting critical COVID-19.

disease recovery, the viral load decreases, and this could reduce the direct damage of the thyroid gland caused by SARS-CoV2. At the same time, the clearance of inflammatory cytokines from the body after recovery reduces their effect on deiodinase, thus promoting the production of T3. In addition, the negative nitrogen balance (caused by fever and inadequate nutrition during the course of the disease) normalized after recovery, and the increased serum level of protein enables the increased synthesis of thyroid hormone. The

TABLE 5 Clinical data of patients in the low T3 group who were survivors and non-survivors.

Characteristic	Survivors (n=42)	Non-survivors (n=6)	p value
Clinical symptoms, n (%)			
Fever	40 (95.24)	5 (83.33)	0.318
Cough	28 (66.67)	6 (100.00)	0.109
Dyspnea	13 (30.95)	5 (83.33)	0.028
Fatigue	16 (38.10)	2 (33.33)	0.608
Laboratory tests, mean (IQR)			
FT3 (pmol/L)	2.68 (2.53,2.80)	2.47 (1.91,2.77)	0.034
Lymphocyte count ($\times10^9$/L)	0.98 (0.89,1.14)	0.68 (0.47,0.92)	0.008
Albumin (g/L)	38.70 (36.35,40.35)	33.90 (32.40,35.10)	0.073
D-dimer (mg/L)	0.25 (0.24,0.35)	0.81 (0.37,3.51)	0.035
C-reactive protein (mg/dL)	10.90 (7.79,34.45)	73.25 (39.83,137.49)	0.073
Treatments, n (%)			
Oxygen therapy	8 (19.05)	4 (66.67)	0.043
Invasive/Noninvasive mechanical ventilation	0	3 (50.00)	0.003

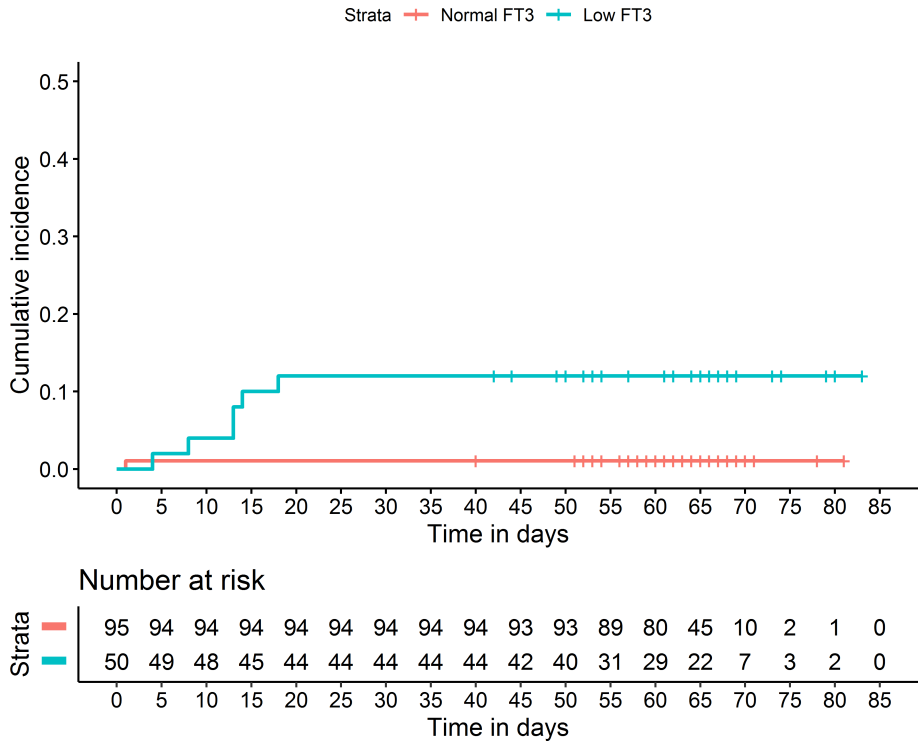

FIGURE 4
Kaplan-Meier analysis of the effect of FT3 on mortality of COVID-19 patients.

TABLE 6 Levels of FT3, FT4, and TSH of patients in the low T3 group (n = 42) at admission and after discharge.

Measurement time	FT3 (pmol/L)	FT4 (pmol/L)	TSH (μIU/mL)
Admission	2.62 ± 0.10	17.10 ± 5.81	1.81 ± 0.18
Post-discharge	4.05 ± 0.98	17.65 ± 1.29	1.93 ± 0.21
p value	<0.001	0.003	0.299

mechanism responsible for the effect of low T3 level on COVID-19 severity and the mechanism responsible for the normalization of the T3 level after recovery from COVID-19 are still unclear and need further study.

This study was limited in that it was a retrospective study of patients with COVID-19 whose thyroid function was assessed upon admission. A second limitation is that post-discharge follow-up was only performed for patients in the low T3 group, and this could have biased the results. A third limitation is that the sample size was small and all patients were from a single center. A large prospective study is needed to further examine the relationship of low T3 level with COVID-19 severity.

In conclusion, our results suggest that low T3 level is a transient injury caused by COVID-19, and is closely related to disease severity. A low T3 level was also a good predictor for critical illness, and may be useful for the early evaluation of COVID-19 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 Xiaogan Central Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MZ and YG wrote the manuscript. YY and ZD conducted the design of the study and reviewed/edited the drafts, and is guarantor. XZ, LG, LL and CX collected and analyzed the data. HH revised the manuscript. All authors contributed to the article and approved the submitted article.

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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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EDITED BY
Jeff M.P. Holly,
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Tsvetelina Velikova,
Sofia University, Bulgaria

*CORRESPONDENCE
Mario Rotondi
mario.rotondi@unipv.it

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Thyroid hormones modifications among COVID-19 patients undergoing pulmonary rehabilitation

Laura Croce[1,2], Elisabetta Zampogna[3], Francesca Coperchini[1], Pietro Costa[1], Patrizia Pignatti[4], Dina Visca[3,5], Antonio Spanevello[3,5] and Mario Rotondi[1,2]*

[1]Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy, [2]Unit of Endocrinology and Metabolism, Laboratory for Endocrine Disruptors, Istituti Clinici Scientifici Maugeri, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Pavia, Italy, [3]Respiratory Rehabilitation of the Institute of Tradate, Istituti Clinici Scientifici Maugeri, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Tradate, Italy, [4]Allergy and Immunology Unit, Istituti Clinici Scientifici Maugeri, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Pavia, Italy, [5]Department of Medicine and Surgery, Respiratory Diseases, University of Insubria, Varese, Italy

Introduction: Patients with severe COVID-19 often experience long-lasting disabilities that can improve after pulmonary rehabilitation. Moreover patients with severe COVID-19 display thyroid function alterations due to a non-thyroidal illness syndrome (NTIS). The aim of our study was to evaluate thyroid function parameters among patients hospitalized for COVID-19 who were eligible or not to respiratory rehabilitation and their modifications during follow-up.

Materials and methods: Post-COVID-19 patients referred to a Respiratory Rehabilitation Unit were evaluated. Outpatients, not candidate for rehabilitation, were enrolled as Control group. Patients who had completed a 4-week-rehabilitation program were enrolled as Rehabilitation Group. All patients were evaluated at T0 (4 weeks after the discharge home in Control Group and after completion of rehabilitation in Rehabilitation Group) and at T1 (3 months after T0).

Results: The final study group included 39 patients (20 in the Rehabilitation group and 19 in the Control group). Patients in the Rehabilitation Group had more frequently received invasive or non-invasive ventilation, had a longer length-of-stay in referring hospitals, had a higher number of comorbidities and displayed a worse performance at 6-minute-walking-test (6MWT) and Short-Physical-Performance-Battery-test (SPPB). FT3 values were lower at T0 in the Rehabilitation Group, while TSH and FT4 values were similar in the two groups. While no significant modifications in thyroid-function-parameters were observed in the Control Group, a significant increase in FT3 value was observed in the Rehabilitation Group at T1. Participants of both groups had improved the results of 6MWT at T1, while SPPB values improved only in the Rehabilitation Group.

Conclusions: COVID-19 patients after pulmonary rehabilitation experience an increase in FT3 values during follow-up, paralleled with an amelioration of functional capabilities.

KEYWORDS

thyroid, COVID-19, pulmonary rehabilitation, non-thyroidal-illness, low-T3 syndrome

Introduction

Coronavirus-Disease-19 (COVID-19) is the disease caused by SARS-CoV-2, characterized by various clinical manifestations, going from mild/asymptomatic forms (in about 81% of infected people), to severe or critical (1). The most typical and frequent symptoms at onset are fever, cough, and shortness of breath. Additional symptoms can include weakness, fatigue, nausea, vomiting, diarrhea and anosmia. A significant percentage of cases requires admission to intensive-care-units (ICU) due to acute respiratory distress syndrome that requires mechanical ventilation support (2). In a subset of patients a severe and life threatening complication, the "Cytokine storm", can occur, characterized by a fulminant and fatal hyper-cytokinemia associated with multi-organ failure (3, 4). Patients who survive the acute phase of COVID-19 often experience long lasting symptoms and disabilities, including fatigue, dyspnea, muscle weakness and impaired mobility, with a consequent decrease in quality of life (5). In particular, in hospitalized patients without any prior motor limitation recovering from COVID-19, a high prevalence of muscle weakness and physical performance impairment has been observed, especially among those requiring mechanical ventilation, sedation, and prolonged intensive care unit (ICU) stay (6, 7). Rehabilitation intervention following the acute phase of COVID-19 (including positioning and respiratory management, medicine, physiotherapy, and psychological support) can help reduce hospital length of stay and improve patient status and quality of life (8, 9).

Among the several organs possibly affected by COVID-19, also the thyroid was object of extensive study during this pandemic (10). The most agreed upon finding is that patients with COVID-19 can experience a "non-thyroidal illness syndrome", especially in severe cases (11–16). This syndrome is characterized by a wide spectrum of thyroid function alterations, most commonly a reduction in free tri-iodothyronine (FT3) and thyroxine (FT4) circulating levels, and can have a prognostic significance in critically ill patients (17). In particular, the "cytokine storm" that characterizes the most severe COVID-19 cases can significantly impact of thyroid function and cause a severe non-thyroidal illness (18).

The aim of our study was to evaluate thyroid function parameters among patients hospitalized for COVID-19 who were eligible or not to respiratory rehabilitation and their modifications during follow-up.

Materials and methods

Study participants

The study included post-COVID-19 inpatients and outpatients referred to Istituti Clinici Scientifici (ICS) Maugeri, Tradate, Italy between March 13, 2020 and July 31, 2021. The study was approved by the Central Ethics Committee of ICS Maugeri (CEC 2279; March 12, 2020), and patients signed the consent form. Inpatients were transferred from intensive and sub-intensive care units, pneumology units or general wards after SARS-CoV 2 negative test, to perform respiratory rehabilitation. Outpatients, not candidate for rehabilitation, (Control group), were discharged home after hospitalization for acute illness and SARS-CoV 2 negative test and were enrolled for the study at the follow-up visit 4 weeks from the discharge at home (T0). Patients of the Rehabilitation Group were enrolled after completion of the 4-week-rehabilitation program (T0). All patients were re-evaluated 3 months after T0 (T1).

The inclusion criteria were:1) availability of thyroid function parameters measurement (including TSH, FT3, FT4) at baseline (T0) and at the 3 months follow-up visit (T1); 2) availability Short Physical Performance Battery (SPPB) at baseline (T0) and at the 3 months follow-up visit (T1). The exclusion criteria were: 1) presence of pre-existing thyroid diseases 2) ongoing therapy with any drug potentially interfering with thyroid function 3) the presence of any thyroid function parameter outside the normal range at baseline.

The following evaluations were performed: clinical examination and anthropometric assessment. Data regarding length of stay (LoS) before admission for pulmonary rehabilitation, previous treatment for acute respiratory failure (ARF) such as Invasive Mechanical Ventilation (IMV), Non-Invasive mechanical Ventilation (NIV), steroid therapy or oxygen use, presence of pulmonary fibrosis at chest CT and arterial blood gases were collected. In individuals under long-term oxygen therapy, assessment had been performed under oxygen at the usual oxygen inspiratory fraction (FiO2).

The burden of comorbidities was estimated through the Cumulative Illness Rating Scale Comorbidities Index (CIRS-CI) and the Cumulative Illness Rating Scale Severity Index (CIRS-SI) (19). CIRS-CI was calculated assigning to each item a score between 0 (none) and 4 (extremely severe), total score reflecting the mean value of the first 13 items. CIRS-CI was obtained by the sum of the

items with score ≥3. Data regarding inflammatory markers, including C-reactive protein (CRP), platelet count and D-dimer were collected.

Rehabilitation program and functional outcome measures

A multidisciplinary program was applied. Endurance exercise training, strength training involving upper and lower peripheral muscle, individual educational sessions and when necessary, tailored diet and psychological support were included in the 4-weeks inpatient program. Intensity, timing and modality of training were tailored to the individual patient according to age, clinical severity, length of immobilization, comorbidities, starting from a minimum of one, 20 minute daily session up to two/three, 30 minute daily sessions.

The following outcome measures were assessed when allowed by patients' clinical conditions and safety or organizational issues:

i) The lower extremity function was assessed by means of the Short Physical Performance Battery test (SPPB) (20, 21) with the predicted normal values of Bergland et al. (22). The total SPPB score ranges from 0 to 12: 1–2: severe; 3–8 moderate disability; 9–12 normal.

ii) Exercise tolerance was assessed by the Six minutes Walking Test (6MWT) (23) using the predicted values of Enright et al. (24). The baseline value of patients unable to perform the test was considered as 0 for analysis.

Serum thyroid function assays

TSH, FT3 and FT4 were measured with the Alinity I system (Abbott Laboratories, IL, USA) which is an automated analyzer that utilizes chemiluminescent microparticle immunoassay (CMIA) principle, by using anti-analyte coated paramagnetic microparticles and anti-analyte acridinium-labeled conjugates. The reaction is measured as relative light units, which have a direct or inverse relationship with the amounts of analyte in the sample.

The intra-assay coefficient of variation (CV) values ranged from 2.7 to 3.8% for FT3, from 2.6 to 3.1% for FT4, from 1.5 to 2.1% for TSH, from 2.3 to 2.8%.

The analytical sensitivities were 1.25 pg/ml for FT3, 0.42 ng/dl for FT4, 0.0083 mIU/l for TSH (third-generation TSH assay). Normal ranges were for TSH 0.35-4.94 μUI/ml, for FT3 1.71-3.71 pg/ml, for FT4 0.70-1.48 ng/dl. Quality control pools at low, normal, and high concentrations for all parameters were present in each assay.

Statistical analysis

Statistical analysis was performed using the SPSS Software (SPSS, Inc.). Between-groups comparisons were performed using the Student's t-test for unpaired data and the Mann–Whitney U-test according to a normal or a non-parametric distribution; comparisons were performed using the Student's t-test for paired data and the Wilcoxon's test according to a normal or a non-parametric distribution. Frequencies among groups were compared using the χ2-test with Fisher's correction when appropriate. A p value of <0.05 was considered statistically significant. Results are expressed as mean ± SD for normally distributed variables and median and interquartile range (IQR) for non-parametric variables.

Results

Out of 185 individuals (in and outpatients) post-COVID-19 screened during the study period, 39 patients (20 in the Rehabilitation group and 19 in the Control group) were included in the study. As shown in Table 1, the patients in the two groups were similar in terms of age, sex and BMI. Apart from a slightly lower PaO_2/FiO_2 ratio among the Rehabilitation Group, no difference in baseline blood gas parameters were observed. The levels of three different markers of inflammation (PCR, D-dimer and platelet count) were similar in the two groups. While a similar percentage of patients had needed oxygen therapy during the acute phase of the infection, a higher percentage of patients in the rehabilitation group had received invasive or non-invasive ventilation. The two Groups were similar in terms of number of patients who had needed steroid therapy in the acute phase of the disease, with a similar cumulative steroid dose. A higher number of patients was still receiving low-dose steroid therapy at T0 in the Rehabilitation Group. In details, the seven patients in the rehabilitation Group were receiving a median prednisone dose of 37.5 mg (IQR 25-37.5) per day, while the only patient in the Control Group receiving steroid therapy was taking 25 mg of prednisone per day. The LoS in referring hospitals for acute COVID-19 was longer in the rehabilitation group. Moreover, patients in the rehabilitation group had a more severe condition and higher number of comorbidities as assessed by the CIRS index. Baseline thyroid function evaluation showed significantly lower FT3 values in the Rehabilitation Group, while TSH and FT4 values were similar in the two groups.

As shown in Table 2, no significant variations between T0 and T1 could be observed in the two groups in the levels of inflammation markers and in blood gas parameters.

Figure 1 shows the comparison between thyroid function parameters evaluated at baseline and those evaluated at the 3 months follow-up in the two groups. While no significant modifications in thyroid function parameters were observed in the Control Group, a significant increase in FT3 value was observed in the Rehabilitation Group.

Figure 2 shows the baseline and follow-up values of SPPB and 6MWT. The results show that at baseline both SPPB and 6MWT were significantly higher in the Control Group, suggesting a better performance among these patients. After 3 months, participants of both group had improved the results of the 6MWT, but remained significantly lower in the Rehabilitation Group, while SPPB values improved only in the Rehabilitation Group. No significant variations in terms of

10.3389/fendo.2023.1192561

TABLE 1 comparison of clinical and biochemical characteristics of patients included in the Rehabilitation or in the Control Group.

	Rehabilitation (N 20)	Control (N 19)	*P* value
Age (years)	67.2 ± 8.0	60.5 ± 12.3	0.050
Male, n (%)	14 (70.0%)	12 (63.2%)	0.651
BMI	27.5 ± 5.8	29.2 ± 4.4	0.323
Previous IV, n (%)	8 (40.0%)	0 (0%)	0.002
Previous NIV, n (%)	15 (75.0%)	4 (21.1%)	<0.001
Previous O_2 need, n (%)	17 (85.0%)	13 (68.4%)	0.219
Previous need of steroid therapy during acute COVID-19, n (%)	14 (70.0%)	8 (42.1%)	0.079
Previous cumulative dose of steroid therapy, prednisone equivalents, mg	195.7± 62.4	165.0± 62.1	0.279
Patients requiring low-dose steroid therapy at T0 n (%)	7 (35.0%)	1 (5.3%)	0.022
Pulmonary Fibrosis at Chest CT, n (%)	7 (35.0%)	6 (31.6%)	0.821
LoS in acute hospitals, days	46.4 ± 18.1	29.2 ± 15.2	0.019
PaO_2/FiO_2	375.5 ± 57.8 (n=16)	411.2 ± 39.7 (n=17)	0.046
PaO_2, mm Hg	79.4 ± 11.4	86.3 ± 8.3	0.053
$PaCO_2$, mm Hg	36.7 ± 3.6	36.1 ± 2.1	0.508
pH	7.41 ± 0.04	7.41 ± 0.02	0.761
CRP mg/dl	0.47(0.14-0.84)	0.23 (0.16-0.40)	0.262
D-dimer, ng/ml	500 (330–870)	480 (315–620)	0.752
Platelet count (n x 10^9/L)	243 (190–336)	223 (196-286)	0.820
CIRS-SI, score	1.60 ± 0.24	1.34 ± 0.20	<0.001
CIRS-CI, score	3.40 ± 1.31	1.84 ± 1.46	0.001
TSH*	1.476 (1.120-2.992)	1.346 (0.986-1.687)	0.187
FT3	2.54 ± 0.35	2.83 ± 0.33	0.015
FT4	0.88 ± 0.11	0.95 ± 0.14	0.053

Data are expressed as n (%) or mean ± sd. *data expressed as median (IQR). BMI, body mass index; IV, invasive ventilation; NIV, non-invasive ventilation; O_2, oxygen; LoS, length of stay; PaO2, arterial oxygen tension; PaCO2, arterial carbon dioxide tension; FiO2, inspired oxygen fraction; CIRS-SI, Cumulative Illness Rating Score Severity Index; CIRS-CI, Cumulative Illness Rating Score Comorbidities Index, TSH, thyrotropin; FT3, free tri-iodothyronine; FT4, free-thyroxine; CRP, C-Reactive Protein.

Discussion

The results of the present study show that patients with post-critical COVID-19 experience an increase in FT3 levels at a 3 months follow-up time after completing a respiratory rehabilitation program. This improvement is paralleled by an increase in lower extremity strength and exercise tolerance, as testified by an increase in 6MWT and SPPB. We observed no significant modifications in thyroid function parameters in the group of COVID-19 who were not eligible for rehabilitation. It should be noted that the patients included in the rehabilitation group were characterized by lower FT3 at the beginning of the study. This is not surprising, since these patients were characterized by a worse respiratory performance (as testified by the lower PaO_2/FiO_2 ratio), by a higher percentage of patients who had required both invasive and non-invasive ventilation and by a higher burden of comorbidities. These findings are in line with previous results on post COVID-19 subjects (11–16). Several studies have showed that the alterations in thyroid function typical of the Non-thyroidal illness syndrome occur frequently among COVID-19 patients and are more pronounced among those with a more severe illness and requiring mechanical ventilation (11–16). Our results show that the improvement of the general conditions after pulmonary rehabilitation of COVID-19 is reflected also in an increase in FT3 levels and a reverting of the Non-Thyroidal-Illness Syndrome. Since this improvement is observed at the 3 months visit after the conclusion of rehabilitation program, we cannot exclude that part of this phenomenon may be due to a general health recovery not directly linked with the rehabilitation intervention. This concept would be supported by the finding that an increase in 6MWT

10.3389/fendo.2023.1192561

TABLE 2 Comparison of blood gases and inflammatory parameters at T0 and T1 in the Rehabilitation and Control Groups.

Rehabilitation Group	T0	T1	*p* value
Blood gases			
PaO_2/FiO_2	382.0 ± 56.2	386.0 ± 43.3	0.856
PaO_2, mm Hg	80.2 ± 11.8	81.8 ± 8.6	0.725
$PaCO_2$, mm Hg	36.5 ± 3.6	37.5 ± 3.7	0.327
pH	7.42 ± 0.03	7.41 ± 0.03	0.708
Inflammatory parameters			
CRP mg/dl	0.47 (0.14-0.84)	0.24 (0.10-0.59)	0.110
D-dimer, ng/ml	500 (330-870)	505 (295-638)	0.306
Platelet count (n x 10^9/L)	243 (190-336)	233 (196-278)	0.446
Control Group	**T0**	**T1**	***p* value**
Blood gases			
PaO_2/FiO_2	411.2 ± 39.7	399.2 ± 33.9	0.304
PaO_2, mm Hg	86.3 ± 8.3	83.8 ± 7.1	0.303
$PaCO_2$, mm Hg	36.1 ± 2.1	37.5 ± 4.1	0.166
pH	7.41 ± 0.02	7.40 ± .02	0.122
Inflammatory parameters			
CRP mg/dl	0.23 (0.16-0.40)	0.15 (0.16-0.26)	0.074
D-dimer, ng/ml	480 (315-620)	355 (315-468)	0.109
Platelet count (n x 10^9/L)	223 (196-286)	220 (193-267)	0.538

Data are expressed as mean ± SD or median (IQR). PaO2, arterial oxygen tension; PaCO2, arterial carbon dioxide tension; FiO2, inspired oxygen fraction; CRP, C-Reactive Protein.

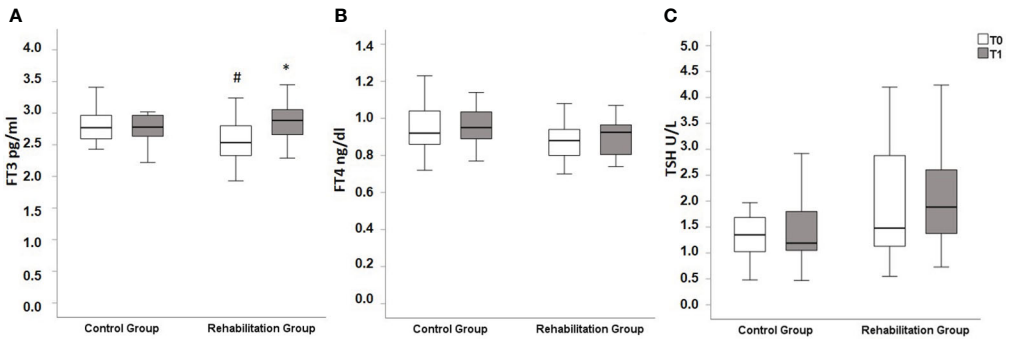

FIGURE 1
Box plot representing thyroid function parameters at baseline (T0, white bars) and at the 3 months follow-up (T1, grey bars). **(A)**: at T0, FT3 values were significantly higher in Control Group when compared with Rehabilitation Group [2.89 (2.65-3.07) pg/ml in Control Group vs 2.54 (2.32-2.82) pg/ml in Rehabilitation Group (p=0.021)]. At T1 FT3 values significantly increased in Rehabilitation Group, reaching a median of 2.89 (2.65-3.07) pg/ml (p=0.007 vs T0), while no differences between T0 and T1 could be observed in Control Group [at T1 2.78 (2.62-2.98) pg/ml, p=0.672 vs T0]. There were no significant differences between the two groups at T1 (p=0.627) **(B)** similar levels of FT4 were observed between the two groups both at T1 [0.92 (0.86-1.07) ng/dl in Control Group vs 0.86 (0.74-0.94) ng/dl in Rehabilitation group, p=0.134] and at T2 [0.95 (0.89-1.04) ng/dl in Control Group vs 0.93 (0.75-0.97) ng/dl in Rehabilitation Group, p=0.113]. No significant variations between T0 and T1 could be observed either in the Control group (p=0.286) nor in the Rehabilitation Group (p=0.243) **(C)** similar levels of TSH were observed between the two groups both at T1 [1.35 (0.99-1.69) U/L in Control Group vs 1.48 (1.12-2.99) U/L in Rehabilitation group, p=0.189] and at T2 [1.19 (1.02-1.81) U/L in Control Group vs 1.88 (1.34-2.63) U/L, p=0.079]. No significant variations between T0 and T1 could be observed neither in the Control group (p=0.601) nor in the Rehabilitation Group (p=0.502). Values reported as median (IQR). TSH, thyrotropin; FT3, free tri-iodothyronine; FT4, free-thyroxine.*p<0.05 vs T0 (Wilcoxon's test). #p<0.05 vs Control group at the same time point (Mann–Whitney test).

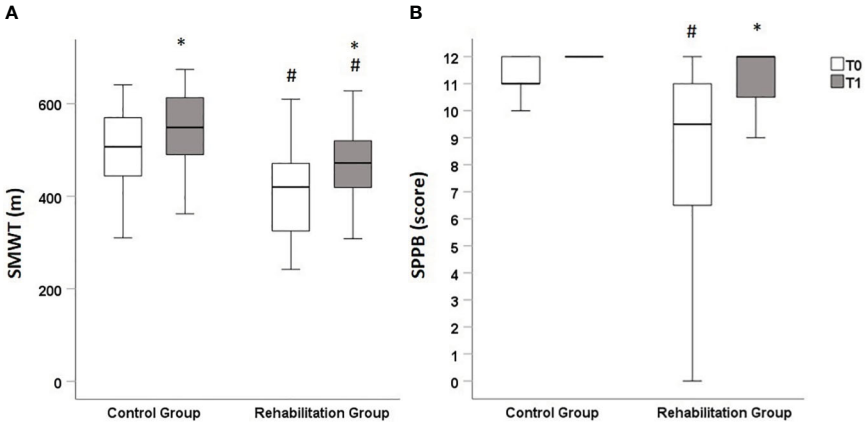

FIGURE 2
Box plot representing results of functional evaluations at baseline (T0, white bars) and at the 3 months follow-up (T1, grey bars). Panel **(A)**: at T0, 6MWT values were significantly higher in Control Group when compared with Rehabilitation Group [522 (444-570) meters in Control Group vs 405 (303-476) meters in Rehabilitation Group, p=0.014]. At T1, 6MWT values significantly increased both in Control Group, reaching a median 549 (480-614) meters (p=0.004 vs T0), and in Rehabilitation Group, reaching a median of 485 (419-520) meters (p=0.001 vs T0). At T2, 6MWT values were still significantly higher in the Control group than in the Rehabilitation Group (p=0.036) Panel **(B)** at T0, SPPB scores were significantly higher in Control Group when compared with Rehabilitation Group [11 (11, 12) points in Control Group vs 10 (6–11) in Rehabilitation Group, p=0.014]. At T1, SPPB values significantly increased in Rehabilitation Group, reaching a median of 12 (10–12) points (p=0.001 vs T0). No significant modifications in SPPB score were observed in Control group (p=0.164), in which all patients had a full score (12) at T2. At T2, SPPB values were similar between the Control group and the Rehabilitation Group (p=0.214). Values reported as median (IQR). 6MWT: six-minute-walking test. SPPB: Short Physical Performance Battery test.*p<0.05 vs T0 (Wilcoxon's test). #p<0.05 vs Control group at the same time point (Mann–Whitney test).

occurred also in patients not undergoing rehabilitation. Indeed, a previous similar study performed among patients undergoing rehabilitation for critical neurological conditions showed an increase in FT3 values in the early stages of rehabilitation (25). In our case, the lack of an evaluation of thyroid function parameters at the moment of acute illness does not allow us to evaluate the effect of early rehabilitation. Nevertheless, the fact that an improvement in FT3 values is observed several months after the acute phases of COVID-19 suggests that these patients can still experience a clinical improvement even in the chronic phases of the disease.

This study has several limitations, mainly due to the limited number of patients included. Nevertheless, the availability of a complete thyroid function evaluation and the exclusion of patients with pre-existing thyroid condition strengthens our results.

In conclusion, COVID-19 patients who have undergone pulmonary rehabilitation experience an increase in FT3 values during follow-up, paralleled with an amelioration of functional capabilities. Prospective studies including a higher number of patients are needed to confirm these promising preliminary results.

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 Central Ethics Committee of ICS Maugeri (CEC 2279; March 12, 2020). The patients/participants provided their written informed consent to participate in this study.

Author contributions

LC, EZ, AS and MR contributed to conception and design of the study. EZ and LC organized the database. LC performed the statistical analysis. LC wrote the first draft of the manuscript. EZ, PC and PP wrote sections of the manuscript. 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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EDITED BY
Jeff M P Holly,
University of Bristol, United Kingdom

REVIEWED BY
Arup Acharjee,
Allahabad University, India
Gianmaria Salvio,
University Hospital Ospedali Riuniti Di Ancona, Italy

*CORRESPONDENCE
Kun Li
likun@hmc.edu.cn

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Impact of SARS-CoV-2 infection on clinical outcomes of *in vitro* fertilization treatments: a systematic review and meta-analysis

Yamei Xue[1], Yuping Xiong[2,3], Xiaohong Cheng[2,3] and Kun Li[2,4]*

[1]Assisted Reproduction Unit, Department of Obstetrics and Gynecology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China, [2]School of Pharmacy, Hangzhou Medical College, Hangzhou, Zhejiang, China, [3]School of Basic Medical Sciences and Forensic Medicine, Hangzhou Medical College, Hangzhou, Zhejiang, China, [4]Zhejiang Provincial Laboratory of Experimental Animal's & Nonclinical Laboratory Studies, Hangzhou Medical College, Hangzhou, Zhejiang, China

The influence of SARS-CoV-2 infection on clinical outcomes in patients undergoing *in vitro* fertilization has been uncertain. Therefore, this systematic review and meta-analysis aimed to evaluate the impact of past SARS-CoV-2 infection on IVF outcomes. A comprehensive search of PubMed, EMBASE, and Cochrane Library databases was conducted from December 2019 to January 2023. Included studies comparing IVF outcomes between patients with prior SARS-CoV-2 infection and controls without previous infection were analyzed. Study quality was assessed using the Newcastle-Ottawa Quality Assessment Scale. Sensitivity analysis, publication bias, and heterogeneity were also examined. The review protocol was registered with PROSPERO (CRD42023392007). A total of eight studies, involving 317 patients with past SARS-CoV-2 infection and 904 controls, met the inclusion criteria. The meta-analysis revealed no significant differences between the infection group and controls in terms of clinical pregnancy rate (OR 0.97, 95% CI 0.73-1.29; P = 0.82), implantation rate (OR 0.99, 95% CI 0.67-1.46; P = 0.96), or miscarriage rate (OR 0.64, 95% CI 0.15-2.65; P = 0.53). Subgroup analyses based on transfer type demonstrated comparable clinical pregnancy rates between the two groups in both fresh embryo transfer (OR 0.97, 95% CI 0.69-1.36; P = 0.86) and frozen embryo transfer (OR 0.96, 95% CI 0.38-2.44; P = 0.94). In conclusion, this meta-analysis suggests that previous SARS-CoV-2 infection does not have a detrimental impact on clinical outcomes in IVF patients. These findings provide valuable insights into assessing the influence of prior SARS-CoV-2 infection on successful pregnancy outcomes in IVF treatment. The systematic review was performed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement. This review was prospectively registered with the International Prospective Register of Systematic Reviews (ID CRD42023392007) on January 16, 2023.

KEYWORDS

coronavirus disease 2019 (COVID-19), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), *In vitro* fertilization (IVF), clinical outcome, meta-analysis, infertility, male, female

Introduction

Coronavirus disease 19 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection, has led to a serious and expanding pandemic around the world. The entry of coronavirus into host cells depends on angiotensin-converting enzyme 2 (ACE2), a cellular receptor, and transmembrane protease serine-2 (TMPRSS2), a cellular protease (1–3). This has raised concerns about the potential impact of SARS-CoV-2 infection on organs with high ACE2 or TMPRSS2 expression that may be more vulnerable to adverse sequelae due to infection (4).

SARS-CoV-2 infection has been implicated in various aspects of human fertility. In the male, ACE2 or TMPRSS2 expressed in spermatogonia, peritubular myoid cells, and testicular somatic cells in the testis tissue (5–7); and in some studies, semen parameters were significantly decreased in mildly and moderately infected patients after coronavirus infection, compared to before infection (8, 9). In females, ACE2 and TMPRSS2 are co-expressed in the ovarian cortex, medulla, oocytes (10, 11), endometrium (12, 13), the membrane of trophectoderm, hypoblast, and epiblast cells in blastocysts (14); ACE2 and TMPRSS2 co-expression increased with oocyte maturity (15); and ACE2 is expressed in all stages of follicular maturation in the human ovary (16). Furthermore, SARS-CoV-2 infection has been associated with ovarian dysfunction, disturbs the follicular microenvironment, potentially affects reproductive outcomes in the study (17), and potentially interferes with embryo implantation and pregnancy. Besides, medium, or high SARS-CoV-2 IgG levels in follicular fluid are associated with a lower number of retrieved oocytes (17).

Therefore, it is necessary to evaluate the potential risks of SARS-CoV-2 infection *in vitro* fertilization (IVF). Although the effect of SARS-CoV-2 infection on clinical outcomes of *in vitro* fertilization has been reported, a small sample size was employed in most studies. Meanwhile, the evidence on the effect of SARS-CoV-2 infection on the clinical outcomes and fertility of patients undergoing IVF treatment has not been systematically reviewed. In this study, we aimed to perform a systematic review and meta-analysis to present a comprehensive summary of the available evidence of the effect of SARS-CoV-2 infection on the clinical outcomes of patients undergoing IVF treatment. This study provides valuable insights to evaluate the potential impact of SARS-CoV-2 infection on reproductive outcomes in patients undergoing IVF treatment.

Methods

The systematic review was performed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement. This review was prospectively registered with the International Prospective Register of Systematic Reviews (ID CRD42023392007) on January 16, 2023.

Search strategy

PubMed, EMBASE and Cochrane Library were searched from December 1, 2019, to January 15, 2023, using a search strategy that combined Medical Subject Heading (MeSH) and EMTREE terms. The target terms included "fertilization *in vitro*", "IVF", "*in vitro* fertilization", "intracytoplasmic sperm injection", "ICSI", "coronavirus disease 2019", "COVID-19", "severe acute respiratory syndrome coronavirus 2", "SARS-CoV-2", "infection", "outcome", "pregnancy". The search terms were combined using Boolean operators AND, OR, and NOT. We applied filters to exclude irrelevant articles and ensure the search's reproducibility.

Eligibility criteria

1. population: this review focused on patients who had a history of SARS-CoV-2 infection and underwent IVF treatment. Studies included in this review were required to report clinical outcomes after embryo transfer for both the infection population and non-infection population.
2. Exposure: patients underwent routine serum SARS-CoV-2 antibody tests and/or reverse transcription-polymerase chain reaction (RT-PCR) tests for detecting SARS-CoV-2 RNA at least one time. The COVID group included patients with a positive test before IVF treatment and the control group referred to those patients who have no history of COVID infection.
3. Outcomes: the primary outcome was the clinical pregnancy rate. The secondary outcomes included early miscarriage rate and implantation rate. Studies that reported any of the outcomes above were included in this review.
4. Setting and language: this review did not restrict settings and languages.
5. Study design: all observation studies (case-control studies, cohort studies, and cross-sectional studies) will be included.
6. Exclusion: this review excluded case reports, case series, reviews without original data presented, commentaries, and editor letters. Studies involving preimplantation genetic testing (PGT), oocyte or sperm donation cycles were excluded from this review. Studies that only provided the outcome percentages, rather than the absolute values of each group were excluded as well.

Study selection

Two reviewers (YMX and YPX) independently assessed the titles and abstracts of all records. Full-text studies of selected citations were used to assess the eligibility. Each study was included or excluded according to the inclusion and exclusion criteria. Any discrepancies were resolved through discussion with a third reviewer (KL).

Data extraction

Data were extracted independently by two reviewers (YMX and YPX), and controversial data were discussed and agreed on. The

information collected included publication date, authors, study period, location, study design, setting, sample size, time of COVID-19 diagnosis, methods of COVID-19 detection, the severity of COVID-19, transfer type, and clinical outcomes. When data were analyzed by subgroups (e.g., fresh and frozen embryo transfer) in the studies, the extracted data were pooled for the overall meta-analysis.

Outcome measures

The primary outcome was the clinical pregnancy rate, which was defined as the observation of a gestational sac with fetal heartbeat on ultrasound imaging divided by the number of transfers. The secondary outcomes included early miscarriage rate and implantation rate. The early miscarriage rate was defined as the loss of pregnancy within the first three months divided by the number of clinical pregnancies. The implantation rate was defined as the number of gestational sacs observed divided by the number of embryos transferred.

Quality assessment

The Newcastle-Ottawa Scale (NOS) was used to assess the methodological quality of included studies (18, 19). Two reviewers (YMX and YPX) independently accessed the quality of included comparative cohort studies. The major three domains (eight items) of bias to be assessed consist of selection (items: representativeness of the exposed cohort, selection of non-exposed cohort, and ascertainment of the exposure), comparability (item: comparability based on the study design or analysis), and ascertainment of outcome (items: assessment of the outcome and statistical test). A maximum of one star could be assigned for every item under the selection, ascertainment of outcome, and exposure domain. A maximum of two stars could be assigned for the items under the section of comparability. NOS quality assessment scored more than or equal to 7 as high quality, 4-6 as medium quality, and <4 as low quality. Any unresolved disagreements were evaluated by a third reviewer (KL).

Statistical analysis

Meta-analyses were performed by RevMan ver. 5.4.1 software (Cochrane Collaboration, Copenhagen, Denmark). The Mantel-Haenszel method was used for dichotomous variable data (clinical pregnancy rate, implantation rate, and early miscarriage rate), which were presented as odds ratios (OR) with a two-sided 95% confidence interval (CI). Statistical heterogeneity was assessed by the value of I^2 index and Q test. I^2 values <50% and P value of $\chi 2$ test >0.10 were considered to have low heterogeneity and fixed effects models were used. When I^2 values >50% and a P value of χ^2 test < 0.10 were considered to indicate moderate to high heterogeneity, a random effects model was used to analyze the data (20, 21). $P < 0.05$ was considered statistically significant. Subgroup analyses were performed according to the transfer type: fresh embryo transfer and frozen embryo transfer.

Furthermore, to evaluate the robustness of the effect size, we conducted sensitivity analyses by excluding each study (Leave-one-out meta-analysis) to explore the impact of individual studies on the pooled effect size. Potential publication bias was examined using the symmetry of the funnel plot and the Egger regression test (22). Statistical analyses were performed with Stata version 17.0 (StataCorp LLC, College Station, TX, USA).

Results

Literature screening process

A flowchart of the literature screening process is shown in Figure 1. Our search identified 97 PubMed, 228 EMBASE, and 7 Cochrane Library records. A total of 332 reports were searched; 159 were duplicates, leaving 173 reports. Based on eligibility criteria, after being screened for titles and abstracts, 145 articles were excluded for the following reasons: patients without a history of SARS-CoV-2 infection (n = 87), patients without IVF treatment (n = 32), and no clinical pregnancy outcomes (n = 26). Twenty-eight full articles were obtained to assess its eligibility. Twenty of them were excluded: 4 were case studies, 11 were reviews, 3 were commentaries and editorials, one contained inappropriate study design, and one lacked extract data. Therefore, eight studies were finally included in this review.

Study characteristics

There was a total of 317 patients with past COVID-19 infection and 904 controls, included in 8 studies (23–30). The characteristics of the included studies are presented in Table 1. The publication date of included studies varied from 2021-2023. Of them, one was a prospective observational study, and the remaining 7 were retrospective cohort studies. Two of the 8 included studies were multicenter studies, and the rest were single-center studies. Five studies transferred fresh embryos, 2 transferred frozen-thawed embryos, and 1 transferred fresh and frozen embryos. All included studies have reported the primary outcomes. Regarding secondary outcomes, 4 and 3 studies reported implantation rate and early miscarriage rate, respectively.

Quality of included studies

Table 2 shows the NOS quality scores of the included studies. Overall, 6 of the 8 cohort studies (23, 25–27, 29, 30) were of high quality (NOS score ≥ 7 stars), whereas the remaining two studies (24, 28) scored 6 and were considered medium quality (Figures 2A, B). In the selection domain of NOS, 6 of the 8 included studies scored 4 stars (23, 25–27, 29, 30). The study by Albeitawi et al. (24) and the study by Aizer et al. (28) scored 3 stars because the two studies did not provide the time of COVID-19 diagnosis and the methods of COVID-19 detection.

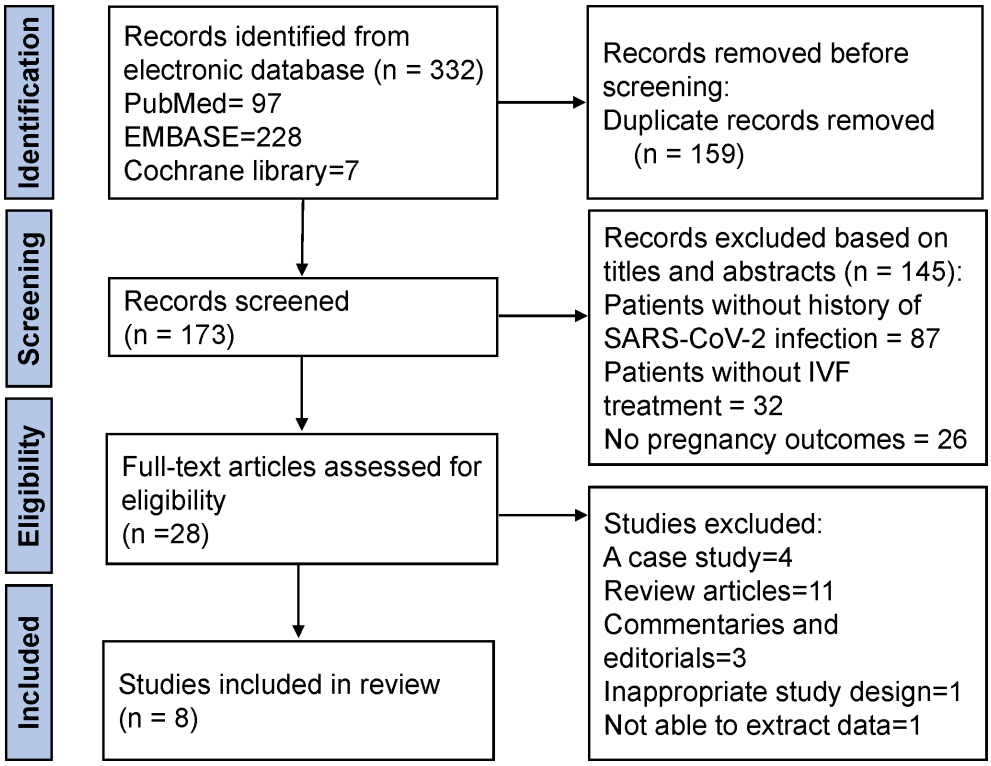

FIGURE 1
Flowchart of the study selection process.

In the comparability item of NOS, 4 studies (23, 26, 27, 30) scored 2 stars, respectively because the studies provided the controls matched with females' age and other parameters. The study by Youngster et al. (25) and the study by Braga et al. (29) scored one star, respectively because the controls were exclusively matched with females' age. The study by Albeitawi et al. (24) and the study by Aizer et al. (28) did not obtain a star because the control groups reported in these two studies did not match the age of the females.

Sensitivity analysis

The results of sensitivity analyses are shown in Figure 3: the overall clinical pregnancy rate (Figure 3A), clinical pregnancy rate in fresh embryo transfer (Figure 3B), clinical pregnancy rate in frozen embryo transfer (Figure 3C), implantation rate (Figure 3D), and early miscarriage rate (Figure 3E). The results indicated that excluding any single study had no significant effect on the total effect size (All P value > 0.05).

Publication bias

As indicated in Figure 4, the funnel plots of the A to E are not asymmetrical and were evenly vertically distributed, demonstrating no or limited publication bias. The results of the Egger test (Figure 4F) showed that there was no publication bias in the overall clinical pregnancy rate, clinical pregnancy rate in fresh embryo transfer, clinical pregnancy rate in frozen embryo transfer, implantation rate, and early miscarriage rate, with Egger values of 0.775, 0.489, 0.626, 0.299, and 0.084, respectively.

Primary outcomes

Clinical pregnancy rate

A total of eight studies involving 317 patients with COVID-19 infection and 904 controls undergoing IVF treatment reported the clinical pregnancy rate. Overall, the Q test and I^2 index showed low heterogeneity between the two groups ($P = 0.40$, $I^2 = 3\%$), and fixed-effects model analysis was used. The meta-analysis results showed that there was no difference between the two groups in the clinical pregnancy rate (OR = 0.97, 95% CI: 0.73-1.29, $P = 0.82$; Figure 5A).

Subgroup analysis

Five studies provided comparison data of clinical pregnancy rate in the fresh embryo transfer. There was low heterogeneity between the two groups ($P = 0.65$, $I^2 = 0\%$). The results of the meta-analysis showed that there was no difference between the two groups in the clinical pregnancy rate (OR = 0.97, 95% CI: 0.69-1.36, $P = 0.86$; Figure 5B).

Three studies provided data regarding frozen embryo transfer. There was moderate heterogeneity between the two groups ($P = 0.09$, $I^2 = 58\%$), and a random effects model analysis was used. The OR was 0.96 (95% CI, 0.38-2.44, $P = 0.94$; Figure 5C). These findings suggest that the type of embryo transfer did not

TABLE 1 Characteristics of included studies.

Author, Y (study period)	City, country	Study design	Setting	Time of diagnosis	Diagnosis COVID-19*	Severity of COVID-19	The number of patients included		Type of transfer	Reference
							Infection	Control		
Wang M et al., 2021 (2020.5-2021.1)	Wuhan, China	Retrospective cohort study	Single-center	At least 4 months between the first diagnosis and IVF treatment	RT-PCR and antibody	Asymptomatic or mild	65	195	Fresh embryo transfer	(23)
Albeitawi S et al., 2022 (2021.9-2021.11)	Irbid, Jordan	Retrospective study	Multicenter	NA	NA	NA	52	98	Fresh embryo transfer	(24)
Youngster M et al., 2022 a (2021.1-2012.6)	Zerifin and Herzliya, Israel	Retrospective cohort study	Multicenter	The time interval from infection to oocyte retrieval: 8-348 days	NA	Asymptomatic or mild	121	121	Fresh embryo transfer	(25)
Wang M et al., 2022 (2020.5-2021.1)	Wuhan, China	Retrospective cohort study	Single-center	Three diagnostic times: the first visit, before the COH procedure, and before oocyte retrieval	RT-PCR and antibody	Asymptomatic, mild, or moderate	50	148	Fresh embryo transfer	(26)
Youngster M et al., 2022 b (2021.1-2021.6)	Zerifin, Israel	Retrospective cohort study	Single-center	Within 1 year before embryo transfer	RT-PCR	NA	41	41	Frozen embryo transfer	(27)
Aizer A et al., 2022 (2021.1-2021.8)	Tel Aviv, Israel	Retrospective cohort study	Single-center	NA	NA	NA	26	234	Frozen embryo transfer	(28)
Braga DPAF et al., 2022 (2019.3-2021.6)	Sao Paulo, Brazil	Historical cohort study	Single-center	Within 6 months before IVF treatment	Antibody	NA	22	66	Fresh embryo transfer	(29)
Adler Lazarovits C et al., 2023(2021.10-2021.11)	Jerusalem, Israel	Prospective observational study	Single-center	NA	RT-PCR	NA	21	13	Fresh and frozen embryo transfer	(30)

*Three methods for diagnosis of COVID-19: RT-PCR, antibody, and other.
COVID-19, coronavirus disease 19; IVF, in vitro fertilization; RT-PCR, reverse transcription-polymerase chain reaction; NA, not available.

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TABLE 2 Quality assessment of included studies based on the Newcastle-Ottawa Scale.

Study (author, y)	Selection				Comparability	Outcome			Quality score
	Representativeness of the exposed cohort	Selection of Non-exposed cohort	Ascertainment of exposure	Demonstration that the outcome of interest was not present at the start of the study	Comparability of cohorts based on the design or analysis [#]	Assessment of outcome	Was follow-up long enough for outcomes to occur?	Adequacy of the follow-up of cohorts	
Wang M et al., 2021 (23)	a)*	a)*	a)*	a)*	c)**	a)*	a)*	a)*	9
Albeitawi S et al., 2022 (24)	a)*	a)*	d)	a)*	d	a)*	a)*	a)*	6
Youngster M et al., 2022 a (25)	a)*	a)*	b)*	a)*	a)*	a)*	a)*	a)*	8
Wang M et al., 2022 (26)	a)*	a)*	a)*	a)*	c)**	a)*	a)*	a)*	9
Youngster M et al., 2022 b (27)	a)*	a)*	a)*	a)*	c)**	a)*	a)*	a)*	9
Aizer A et al., 2022 (28)	a)*	a)*	d)	a)*	d	a)*	a)*	a)*	6
Braga DPAF et al., 2022 (29)	a)*	a)*	a)*	a)*	a)*	a)*	a)*	a)*	8
Adler Lazarovits C et al., 2023 (30)	a)*	a)*	b)*	a)*	c)**	a)*	a)*	a)*	9

Representativeness of the exposed cohort: a) truly representative of the average in vitro fertilization therapy patients in the community, b) somewhat representative of the average in vitro fertilization therapy patients in the community, c) selected group of users, d) no description of the derivation of the cohort. Selection of non-exposed cohort: a) drawn from the same community as the exposed cohort, b) drawn from a different source, c) no description of the derivation of the non-exposed cohort. Ascertainment of exposure: a) secure record, b) structured interview, c) written self-report, d) no description. Demonstration that outcome of interest was not present at the start of the study: a) yes, b) no. Comparability of cohorts based on the design or analysis: a) study controls for female's age, b) study controls for any additional factors, c) study controls for female's age and other factors, d) no study controls for female's age or any other factors. Assessment of outcome: a) independent blind assessment, b) record linkage, c) self-report, d) no description. Was follow-up long enough for outcomes to occur: a) yes, b) no. Adequacy of follow-up of cohorts: a) complete follow-up, b) subjects lost to follow-up unlikely to introduce bias - small number lost ≥ 90% follow-up, c) follow-up rate <90% and no description of those lost, d) no statement.
[#] A maximum of 2 stars can be allotted in this category, one for female age, the other for other controlled factors. *, a maximum of one star could be assigned; **, a maximum of two stars could be assigned.

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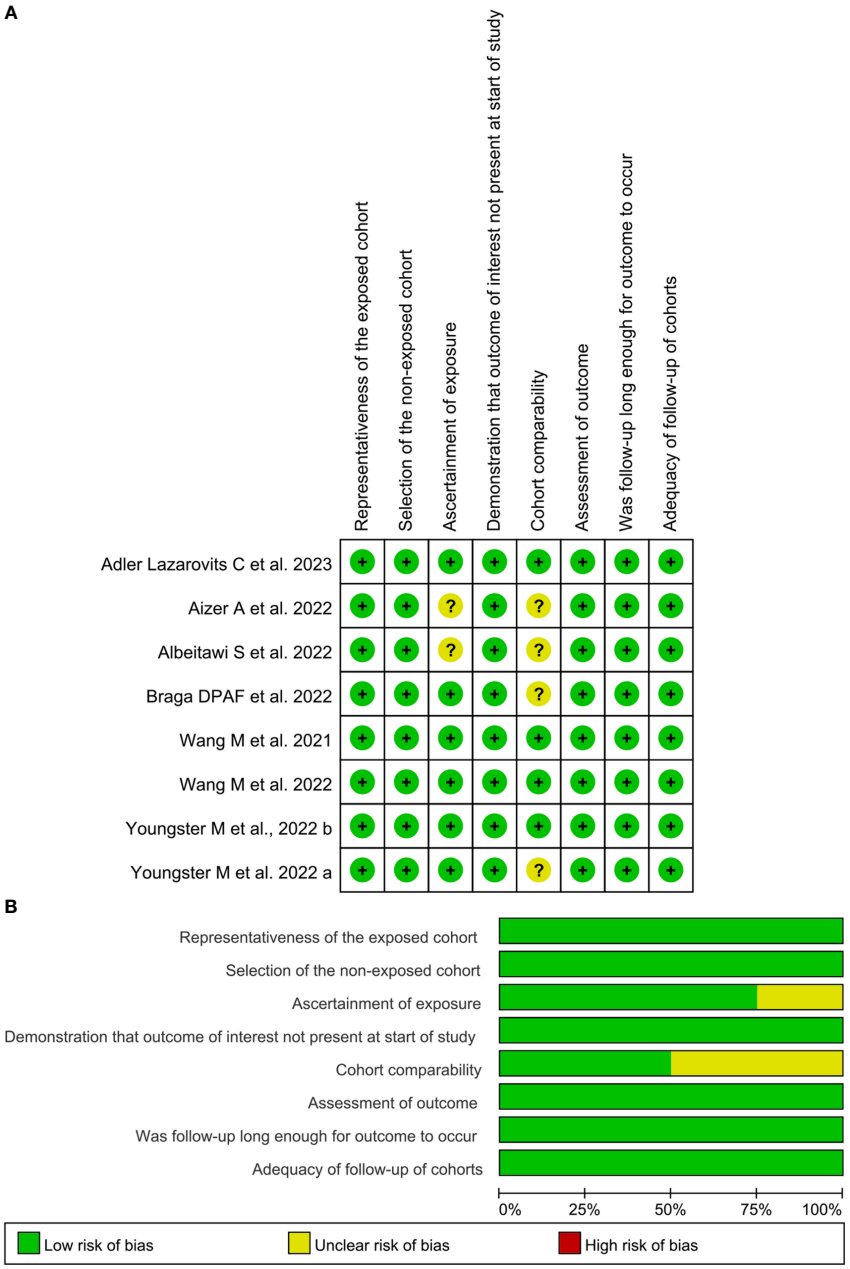

FIGURE 2
Risk of bias of included studies based on the Newcastle-Ottawa Scale. **(A)**, the Newcastle-Ottawa Scale rating per study; **(B)**, Summative Newcastle-Ottawa Scale rating. Images **(A, B)** were generated with RevMan Version 5.4.

significantly affect the clinical pregnancy rate in patients with prior SARS-CoV-2 infection.

Secondary outcomes

Implantation rate

Four studies including 904 embryos transferred (158 embryos transferred from patients with COVID-19 infection and 746 embryos from controls) reported implantation rate. There was low heterogeneity between the two groups (P = 0.17, I^2 = 41%), and a fixed effects model analysis was used. Meta-analyses of these studies showed no significant difference between the COVID-19 infection and control groups (OR 0.99, 95% CI 0.67-1.46, P = 0.96; Figure 5D) suggesting that COVID-19 infection does not affect the implantation rate in IVF treatment.

Early miscarriage rate

Three studies including 99 transfer cycles in the infection group and 223 transfer cycles in the control group have investigated the early miscarriage rate. There was low heterogeneity between the two groups (P = 0.19, I^2 = 39%), and a fixed effects model analysis was used. No difference was found in the early miscarriage rate between the COVID-19 infection and control groups (OR 0.64, 95% CI 0.15-

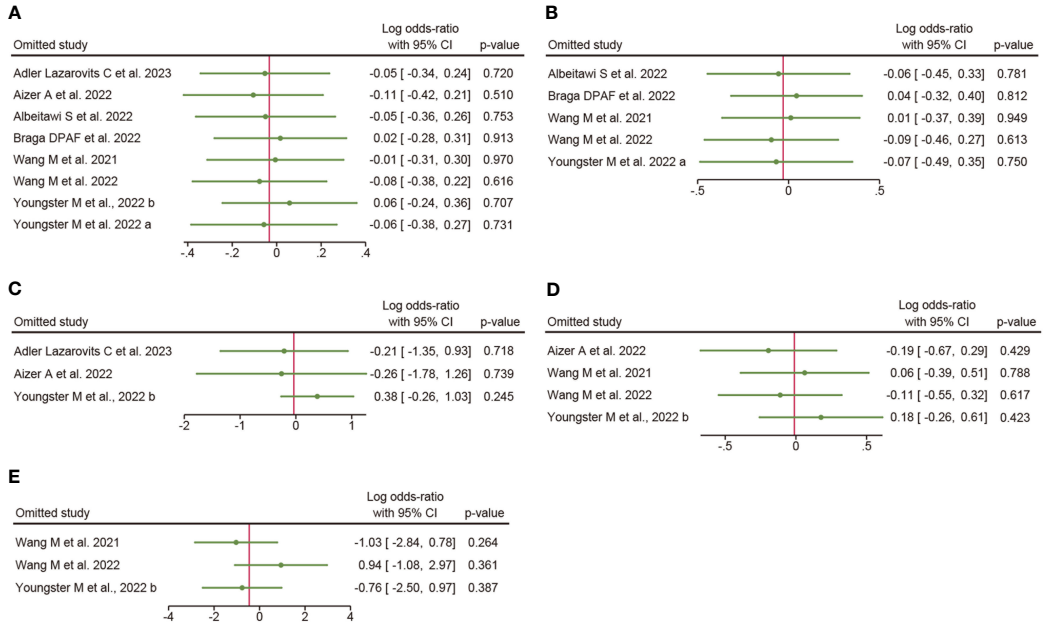

FIGURE 3
Sensitivity analysis. **(A)**, overall clinical pregnancy rate; **(B)**, clinical pregnancy rate in fresh embryo transfer; **(C)**, clinical pregnancy rate in frozen embryo transfer; **(D)**, implantation rate; **(E)**, early miscarriage rate.

2.65, $P = 0.53$; Figure 5E), suggesting that COVID-19 infection does not affect the early miscarriage rate in IVF treatment.

Discussion

The SARS-CoV-2 virus remains a significant global public health concern. In the early stage of the pandemic, the American Society for Reproductive Medicine (ASRM) and the European Society of Human Reproduction and Embryology (ESHRE), independently recommended suspending fertility services except for the most urgent cases (31, 32). More recently, with increased knowledge of SARS-CoV-2 and its transmission, reproductive care has gradually resumed within certain restrictions (33). However, there are insufficient data to show that SARS-CoV-2 infection negatively influences clinical outcomes in patients undergoing IVF treatments.

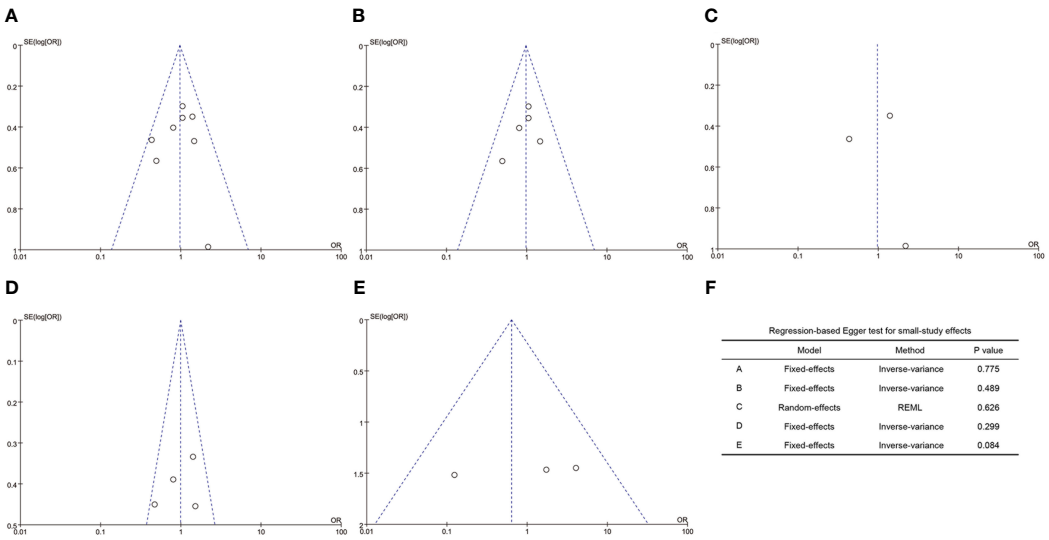

FIGURE 4
Funnel plots and Egger's test. **(A)**, overall clinical pregnancy rate; **(B)**, clinical pregnancy rate in fresh embryo transfer; **(C)**, clinical pregnancy rate in frozen embryo transfer; **(D)**, implantation rate; **(E)**, early miscarriage rate; **(F)**, values of the Egger test.

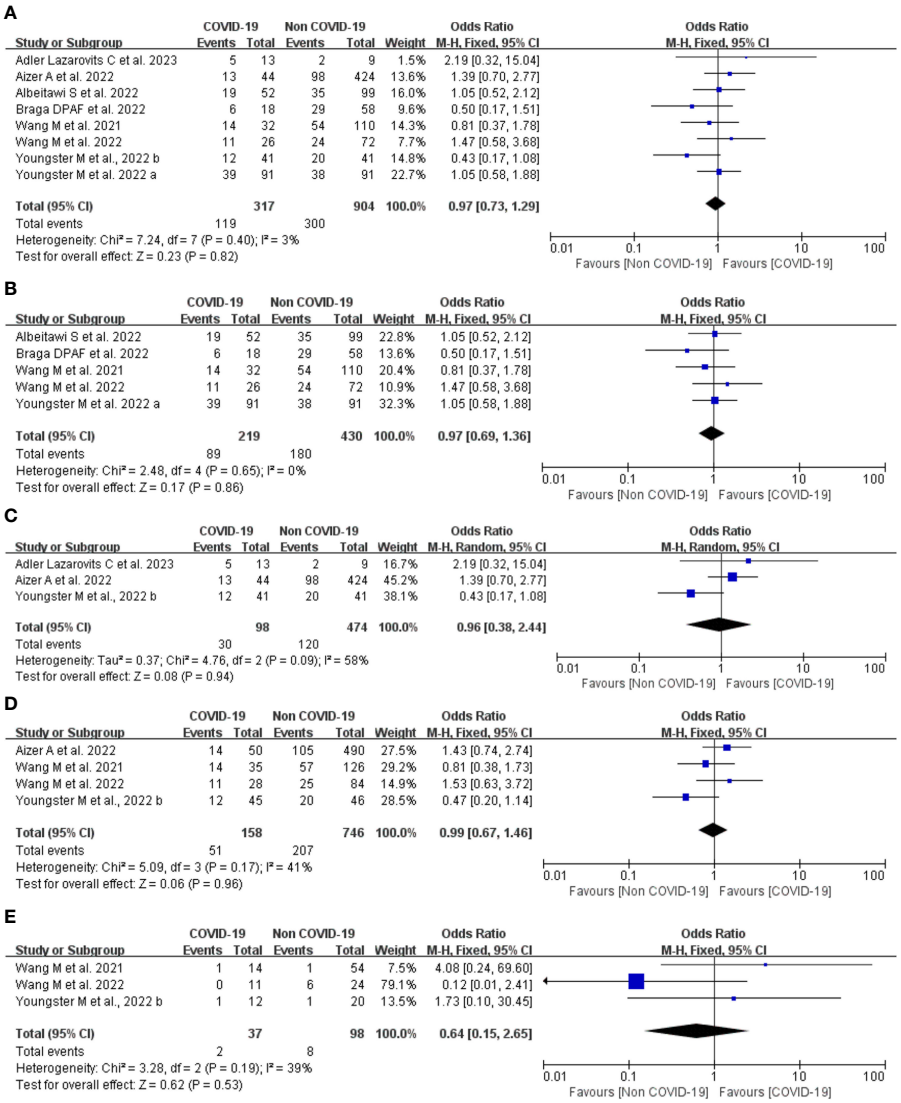

FIGURE 5
Forest plot of studies of COVID-19 vs. non-COVID-19 for the clinical outcomes. **(A)**, overall clinical pregnancy rate; **(B)**, clinical pregnancy rate in fresh embryo transfer; **(C)**, clinical pregnancy rate in frozen embryo transfer; **(D)**, implantation rate; **(E)**, early miscarriage rate; M-H = Mantel-Haenszel method.

The results of this study indicate that past infection with SARS-CoV-2 had no impact on IVF treatment outcomes in terms of clinical pregnancy rate, implantation rate, and miscarriage rate. No significant difference was found in the subgroup analysis of clinical pregnancy rate for fresh and frozen embryo transfers.

Due to the outbreak of a new virus, little is known about the pathophysiology of SARS-CoV-2 and its potential impact on human endometrial and early embryo attachment. Significant progress has been made in understanding the molecular machinery of virus entry into host cells (1–3). However, contradictory data are available on the expression, interaction, and function of ACE together with another TMPRSS2 in human endometrial receptivity and early embryo implantation. A recent transcriptomic analysis indicated that the expression of ACE2 was significantly higher in the implantation window and TMPRSS2 increased during embryo implantation (12). On the contrary, another study suggested a low level of ACE1, ACE2, and TMPRSS2 in human endometrial cells at the transcripts level (13). Especially, the co-expression of ACE2 and TMPRSS2 proteins in human mature oocytes and preimplantation embryos (11, 34), and the expression of genes required for SARS-CoV-2 infection in trophectoderm cells (35), further increase the potential risk of SARS-CoV-2 infection on embryo survival and implantation. It has also been supposed that couples infected with SARS-CoV-2 may have poor reproductive outcomes after IVF treatment.

Several studies have examined the impact of SARS-CoV-2 on ovarian function during stimulation. In a small study including nine women with past infection undergoing oocyte retrieval, no difference

in the levels of serum estradiol on the day of ovulation trigger, and serum progesterone on the day of oocyte retrieval, the ratios of serum estradiol/oocyte, and oocytes/follicles aspirated was reported when compared to the non-exposed group (36). However, another study reported a negative effect on oocyte yield in women who had a past SARS-CoV-2 infection more than 180 days before oocyte retrieval, compared to those had, who had a past infection 90-180 days and ≤90 days (25). The authors pointed out that their results need to be considered with caution as the sample size of the study was small (25).

As for the risk of vertical transmission of virus infection through gametes or IVF, a recently published study examined the viral RNA of SARS-CoV-2 in oocytes from women who were positive on the day of oocyte collection and found that the viral RNA was not detected in all oocytes (37). Up until now, several studies have reported that no viral RNA was found in follicular fluid (38–40), cumulus cells (39), ovarian medulla (40), vaginal secretions (40–42), and endometrial tissue (39) in SARS-CoV-2 positive women. Based on the above research results, the ovary, uterus, and genital tract are considered to be at low risk of SARS-CoV-2 infection.

To our knowledge, this systematic review is the first study to examine the effect of a history of SARS-CoV-2 infection on the clinical outcomes of fresh and frozen ET cycles. The study was conducted using a prospectively registered protocol and a comprehensive search strategy. The main strengths of the present study included a large number of patients, 317 patients with COVID-19 infection, and 904 controls undergoing IVF treatment, including 8 studies. The comparisons were performed not only for the main outcomes but also according to transfer type. Furthermore, in this review, we strictly followed the reporting guidelines while searching databases, selecting eligible articles, assessing quality, and analyzing the data.

There are still several limitations in the current study. First, the number of included studies was relatively small and the quality of included data was medium because most of the included studies are retrospective designs. Therefore, we conducted sensitivity analyses by excluding one study to evaluate the robustness of the effect size. The results indicated that excluding any single study had no significant effect on the total effect size. Second, the included patients exhibited heterogeneity in the baseline characteristics, such as time of COVID-19 diagnosis, detection methods, and severity of COVID-19, which could represent confounding factors and affect the outcomes. Our meta-analysis showed that a major of the results had low heterogeneity, and only the groups of frozen embryo transfers had moderate heterogeneity. To avoid the effect of moderate heterogeneity, a random effect model analysis was employed. A further limitation was that the time intervals between SARS-CoV-2 infection and IVF treatment exist differences in participants, which may not reflect the true effect of past SARS-CoV-2 infection on IVF outcomes. Finally, the potential limitation of this meta-analysis was the absence of data on live birth outcomes. Further detailed research is needed to investigate the long-term effects of SARS-CoV-2 infection on infertility treatment outcomes.

Conclusion

In this systematic review and meta-analysis, past SARS-CoV-2 infection did not appear to harm the clinical outcomes of patients with a history of SARS-CoV-2 infection undergoing IVF treatment. The results can provide evidence for healthcare professionals who suggest treatment interventions and for couples who contemplate pregnancy through IVF. Further studies are warranted to further confirm these findings.

Author contributions

YXu and KL conceived the concept and drafted the original manuscript. YXu, YXi, XC, and KL assessed the literature and discussed the manuscript. YXu and KL revised and edited the work. 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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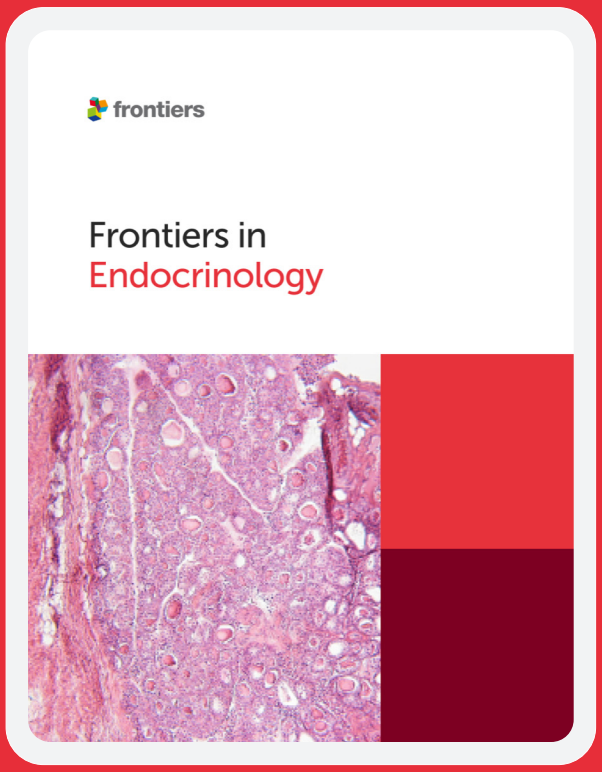

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