Alterations in vaginal microbiota among pregnant women with COVID-19

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Abstract

The maintenance of vaginal microbiota is an important factor to achieve optimum pregnancy outcomes. The study aims to describe the alterations in the composition of vaginal microbiota in pregnant women with coronavirus disease 2019 (COVID-19). This was a prospective case-control study. Vaginal swabs were collected from uninfected pregnant women (n = 28) and pregnant women with COVID-19 (n = 19) during the active phase of infection and within a month after recovering from infection. The vaginal microbiota on the swabs was examined by 16S rRNA gene sequencing. Shannon index indicates that alpha diversity is significantly higher in women with COVID-19 (p = 0.012). There was a significant decrease in Firmicutes (p = 0.014) with an increase in Bacteroidota (p = 0.018) phyla and a decrease in Lactobacillus (p = 0.007) genus in women with COVID-19 than those of uninfected pregnant women. The relative abundance of L. crispatus, L. iners, L. gasseri, and L. jensenii were lower in the COVID-19 group than in uninfected pregnant women. In subgroup analysis, the amount of Ureaplasma spp. was higher in women with moderate/severe than those of asymptomatic/mild disease (p = 0.036). The study revealed that vaginal dysbiosis with low abundance of Lactobacillus species occurred in pregnant women infected with severe acute respiratory syndrome coronavirus-2. These findings may lead to new studies to elucidate the risk of pregnancy adverse outcomes related to COVID-19.

KEYWORDS
COVID-19, pregnancy adverse outcome, vaginal microbiome, vaginal microbiota

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has emerged worldwide, causing infections that range from asymptomatic to severe disease. COVID-19 has been shown to have potential adverse effects on pregnancy and neonatal outcomes. Pregnancy is a risk factor for the severity of COVID-19 disease, with an increased risk of intensive care unit admission, maternal morbidity, and mortality.1,2 Furthermore, pregnancy complications such as preeclampsia and preterm birth (PTB) are more likely to occur in women diagnosed with COVID-19.3,4
The predominance of Lactobacillus species (spp.) play a key role in inhibiting nondomestic and potentially harmful microorganisms to epithelial cells.\textsuperscript{5–7} Lactobacilli maintain the protective low vaginal pH through secretion of lactic acid.\textsuperscript{8} Pregnant women with decreased amounts of Lactobacillus crispatus, L. gasseri, and L. jensenii in the vaginal microbiota are more likely to deliver preterm.\textsuperscript{9} Likewise, the abundance of Gardnerella vaginalis increases the risk of PTB.\textsuperscript{5,10}

The mechanism of action of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in pregnancy varies and remains unknown. The SARS-CoV-2 genome has been identified in the vaginal mucosa of a pregnant woman.\textsuperscript{11} Indeed, the role of SARS-CoV-2 infection in vaginal microbiome composition in pregnant women with COVID-19 has not yet been investigated. Therefore, we anticipate that COVID-19 may unfavorably affect the composition of the vaginal microbiota, resulting in adverse pregnancy outcomes. We aimed to describe the alterations in the composition of vaginal microbiota in pregnant women with COVID-19.

2 | MATERIALS AND METHODS

2.1 | Study population

A prospective study was conducted at Koc University Hospital between August 2020 and August 2021. Pregnant women (n = 19) with active or recently infected within 1 month with SARS-CoV-2 were included in the study. The SARS-CoV-2 infection was confirmed by a positive nasopharyngeal polymerase chain reaction (PCR) test. The signs and symptoms of COVID-19 were evaluated in all women with positive PCR tests for SARS-CoV-2 infection. COVID-19 was classified according to NIH COVID-19 clinical guidelines.\textsuperscript{12} Patients with symptoms including fever, myalgia, or gastrointestinal system symptoms were categorized as a mild disease and those who required oxygen (O\textsubscript{2}) supplementation as moderate/severe disease (MSD). Recovery was defined as clinical improvement in combination with a negative nasopharyngeal PCR test. The single vaginal swab was collected from each participant at the time of COVID-19 and within 1 month after recovery from COVID-19. In three patients, longitudinal microbiota analysis was performed with a collection of vaginal swabs before, during active infection, and 2 months after recovery. The overview of patients is presented in Figure 1.

The uninfected pregnant women (n = 28) were recruited from the prospective study entitled “Vaginal, Placental and Neonatal Buccal Mycobiota, and Microbiome in Preterm Birth.” The study was initiated in April 2020 (ClinicalTrials.gov Identifier: NCT04165252). Maternal age and gestational week-matched pregnant women were selected for the healthy controls. Inclusion criteria for the healthy controls are as follows: age older than 18 years with a singleton pregnancy. The exclusion criteria were multiple pregnancies, major fetal structural defects and/or chromosomal abnormalities, stillbirth, having used antibiotics and/or antifungal medication within 2 weeks at the time of sample collection, the presence of vaginal bleeding at the time of sample collection, and sexual intercourse within 72 h of sample collection.

FIGURE 1  An overview of study design the cohort includes pregnant women with COVID-19 (n = 19) and uninfected pregnant women (n = 28), recruited from ClinicalTrials. NCT04165252 study. Longitudinal study cases are shown in purple. The three preterm birth cases are shown in black circles. COVID-19, coronavirus disease 2019.
Maternal characteristics, medical and obstetrical history were recorded for all participants. Maternal height and weight were measured at the same time with vaginal swabs collection. Gestational age was determined from the last menstrual period and confirmed from the measurement of fetal crown-rump length at the first-trimester scan. Written informed consent was obtained from all participants. Koç University Research Ethics Board approved the study protocol. The study complies with the declaration of Helsinki.

2.2 | Sample collection, processing, and sequencing

Vaginal samples were collected with REMEL ESwabs. Vaginal swabs were placed into the sterile tubes and then stored at −80°C until DNA extraction. Frozen vaginal swabs were immersed in sterile PBS then DNA extraction was performed using the Qiagen DNeasy PowerSoil Kit (Qiagen), as described by the manufacturer’s. DNA concentration was quantified by Qubit (Thermo Fisher Scientific).

Library preparation was performed using QIAseq 16S/ITS Panel Kit (Qiagen) for sequencing the V1–V9 region of the 16S rRNA bacterial gene. Library quantification was done using QIAseq Library Quant Assay (Qiagen) kit following the manufacturer's instructions with Applied Biosystems QuantStudio 7 Flex Real-Time PCR (Applied Biosystems Inc.). Sequencing was performed with the Illumina MiSeq platform using the MiSeq v3 Reagent Kit (Illumina).

2.3 | Bioinformatics

FASTQ files were demultiplexed by the different regions using the module in the GeneGlobe Data Analysis Center. (https://geneglobe.qiagen.com/tr/analyze). The resulting paired-end FASTQ files containing V1–V2 region sequences were used to profile the microbiota of the samples with Mothur (v.1.45.3). High quality sequences were aligned with SILVA bacterial reference database (v.138.1). Chimeric sequences were removed using the VSEARCH program embedded in the Mothur. Then, the sequences were assigned with taxonomic annotation using the Wang approach implemented in the Mothur. Silva (v.138.1) was used as the reference database for the assignment. Finally, sequences with no more than 3% dissimilarity were clustered into one Operational Taxonomic Unit for the analysis of diversity and composition.

2.4 | Statistical analysis

Continuous variables were expressed as a median and interquartile range, whereas categorical variables were expressed as percentages. Mann–Whitney U and Fisher exact tests were applied for comparison of maternal demographic and clinical characteristics between COVID-19 patients with healthy controls. Data were analyzed using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp.).

The alpha diversity, beta diversity, and vaginal microbiota composition of pregnant women with COVID-19 and uninfected pregnant women were assessed. The vaginal microbiota of asymptomatic/mild, moderate, or severe cases were compared with those of the uninfected pregnant women. Alpha diversity indices were calculated by the summary.single command embedded in the Mothur. Beta-diversity was defined using the Bray–Curtis distance and generated using the dis.shared command in the Mothur. The evaluation of differences in the alpha diversity metrics and microbiota composition was performed by Wilcoxon signed-rank test using Python 3.7. The significance of group dissimilarity based on the Bray–Curtis distance matrix was evaluated by the analysis of molecular variance (AMOVA) test using Python. Statistical significance was set as \( p < 0.05 \). Statistical data were visualized with GraphPad Prism 8.0.2.

3 | RESULTS

3.1 | Study population

There were no differences in maternal age, body mass index, gestational age at delivery, and birthweight between women in the COVID-19 group with uninfected pregnant women (Table 1). Of 19 women with COVID-19, 13 had asymptomatic/mild disease (68.4%), and 6 had MSD (31.6%). The rate of PTB was 15.3% \(( n = 3)\) in the COVID-19 group; 2 of 6 women (33.3%) with MSD and 1 of 13 women (7.7%) with asymptomatic/mild disease (Table 2). Two patients had COVID-19 in the third trimester and 1 in the second trimester. No other risk factors for PTB were detected.

In asymptomatic/mild disease, 61.5% of those had infection in the second trimester and 38.5% in the third trimester (Table 2). Two women with MSD received both antibiotic and antiviral medications. Seven patients received low molecular weight heparin during the active period of COVID-19 disease (Table 2).

3.2 | The composition of vaginal microbiota in the healthy controls and women with COVID-19

Alpha diversity was evaluated by using the Shannon index. In the COVID-19 group, the Shannon index was significantly elevated, compared to those of the uninfected pregnant women \((0.77 \text{ vs. } 0.40; \ p = 0.012)\) (Figure 2A). In women with asymptomatic/mild disease, there was a significant difference compared to uninfected pregnant women in the Shannon index \((0.77 \text{ vs. } 0.40; \ p = 0.03)\). The Shannon index \((0.76 \text{ vs. } 0.4; \ p = 0.05)\) was higher in women with MSD than the uninfected pregnant women, but it did not reach a statistically significant level (Figure 2A). Beta diversity (Bray–Curtis) analysis indicated that there were no compositional differences between COVID-19 status and uninfected pregnant women \((p = 0.76, \text{ AMOVA})\) (Figure 2B).
### TABLE 1  
Maternal demographic and clinical characteristics of pregnant women with COVID-19 and uninfected pregnant women

| Maternal baseline characteristics and clinical presentations | COVID-19 group (n = 19) | Uninfected pregnant women (n = 28) | p Value |
|-------------------------------------------------------------|-------------------------|------------------------------------|---------|
| Maternal age in years, median (IQR)                         | 33.0 (28.0–36.0)        | 31.0 (29.0–34.0)                   | 0.61    |
| BMI on the admission in kg/m², median (IQR)                 | 26.4 (23.7–28.7)        | 27.0 (24.9–29.1)                   | 0.38    |
| Parity                                                      |                         |                                    |         |
| Nulliparous, n (%)                                         | 8 (42.1)                | 18 (64.3)                          | 0.15    |
| Multiparous, n (%)                                         | 11 (57.8)               | 10 (35.7)                          |         |
| Gestational age at delivery in weeks, median (IQR)         | 38.0 (25.5–40.0)        | 39.0 (37.0–40.0)                   | <0.001* |
| Birthweight in grams, median (IQR)                         | 3200 (725–3990)         | 3265 (2800–4000)                   | 0.25    |
| Mode of delivery                                           |                         |                                    |         |
| Spontaneous vaginal delivery, n (%)                        | 5 (26.3)                | 9 (32.1)                           | 0.75    |
| Cesarean section, n (%)                                     | 14 (73.7)               | 19 (67.9)                          |         |

Abbreviations: BMI, body mass index; COVID-19, coronavirus disease 2019; IQR, interquartile range.

* p < 0.05, statistically significant.

### TABLE 2  
Clinical features of pregnant women with COVID-19

| Maternal clinical presentations | Total COVID-19 population (n = 19) | Asymptomatic or mild disease (n = 13) | Moderate or severe disease (n = 6) | p Value |
|--------------------------------|------------------------------------|---------------------------------------|-----------------------------------|---------|
| Gestational age at COVID-19 in weeks, median (min–max) | 26 (19.0–35.0)                     | 22.0 (18.0–35.0)                      | 30.0 (25.0–35.0)                   | 0.32    |
| Second trimester, n (%)        | 11 (57.9)                          | 8 (61.5)                              | 3 (50)                            | 1.0     |
| Third trimester, n (%)          | 8 (42.1)                           | 5 (38.5)                              | 3 (50)                            | 1.0     |
| Presenting signs and symptoms  |                                    |                                       |                                   |         |
| Fever, n (%)                   | 9 (52.6)                           | 4 (30.8)                              | 6 (100)                           | 0.01*   |
| Cough, n (%)                   | 9 (47.4)                           | 3 (23.1)                              | 6 (100)                           | 0.003*  |
| Dyspnea, n (%)                 | 7 (36.8)                           | 1 (7.7)                               | 6 (100)                           | <0.001* |
| Myalgia and fatigue, n (%)     | 14 (73.7)                          | 9 (69.2)                              | 5 (83.3)                          | 1.0     |
| Diarrhea/GI symptoms           | 5 (26.3)                           | 3 (23.1)                              | 2 (33.3)                          | 1.0     |
| Headache, n (%)                | 10 (52.6)                          | 6 (46.2)                              | 4 (66.7)                          | 0.33    |
| Antepartum therapy             |                                    |                                       |                                   |         |
| Antibiotics, n (%)             | 2 (10.5)                           | -                                     | 2 (33.3)                          | N/A     |
| Antiviral, n (%)               | 3 (15.8)                           | 1 (7.7)                               | 2 (33.3)                          | 0.22    |
| Prednisolone, n (%)            | 2 (10.5)                           | -                                     | 2 (33.3)                          | N/A     |
| Low molecular weight heparin, n (%) | 7 (36.8)                     | 1 (7.7)                               | 6 (100)                           | <0.001* |
| Oxygen support without ICU admission, n (%) | 4 (21.1)               | -                                     | 4 (66.7)                          | N/A     |
| Admission to ICU, n (%)        | 2 (10.5)                           | -                                     | 2 (33.3)                          | N/A     |
| Preterm birth, n (%)           | 3 (15.8)                           | 1 (7.7)                               | 2 (33.3)                          | 0.22    |
| Admission to NICU, n (%)       | 3 (15.8)                           | 1 (7.7)                               | 2 (33.3)                          | 0.22    |

Note: p Value is given for the comparison of asymptomatic/mild disease with moderate/severe disease groups.

Abbreviations: COVID-19, coronavirus disease 2019; GI, gastrointestinal symptoms; ICU, intensive care unit; NICU, neonatal intensive care unit.

*p refers to a statistically significant value (<0.05).
There were no statistically significant differences in the alpha diversity of vaginal microbiota between the second and third trimesters of uninfected pregnant women according to the Shannon index (0.41 vs. 0.5; \( p = 0.22 \), respectively).

The vaginal microbiota composition and relative abundances of the bacterial phylum, genera, and species for two groups are summarized in Figure 3. The three phyla; Firmicutes (86.12%), Actinobacteria (11.74%), and Bacteriodata (0.57%) accounted for 99.94% of the bacterial species in the COVID-19 group. In the COVID-19 group, Firmicutes was significantly lower compared to uninfected pregnant women (86.12% vs. 96.07%; \( p = 0.014 \)) while the amount of Bacteriodota was significantly higher than uninfected pregnant women (0.57% vs. 0.45%; \( p = 0.018 \)) (Figure 3A). At the genus level, the amount of Lactobacillus sp. was found to be significantly lower in the COVID-19 group than in uninfected pregnant women (80.1% vs. 93.8%; \( p = 0.007 \)) (Figure 3B).

L. crispatus, Lactobacillus iners, Lactobacillus gasseri, and L. jensenii showed trends toward a decline in COVID-19 group (30.6%, 29.1%, 15.8%, and 7.6%, respectively) compared to the uninfected pregnant women (32.6%, 36.9%, 16.9%, and 8%, respectively) but the differences were not statistically significant (\( p > 0.05 \)) (Figure 3C).

In the COVID-19 group, among anaerobe taxa, Prevotella timonensis was significantly more abundant compared to uninfected pregnant women (0.12% vs. 0.08%; \( p = 0.03 \)). In addition to these, pregnant women with COVID-19 disease had a higher proportion of G. vaginalis than uninfected pregnant women (2.1% vs. 2%; \( p = 0.2 \)).

In pregnant women with COVID-19 disease, L. iners decreased the most among Lactobacillus species while the relative abundances of G. vaginalis, Mycoplasma hominis, and Ureaplasma spp. increased in this group of women (Figure 4).

3.3 | The variations of vaginal microbiota composition in relation to the severity of COVID-19

The amount of L. iners was higher in the moderate/severe group (49.3%) than in asymptomatic/mild (28.4%) and uninfected pregnant women (36.2%). The proportions of L. gasseri and L. jensenii were lower in moderate/severe cases when compared to uninfected pregnant women (12% vs. 16.6% and 0.01% vs. 7.8%, respectively) (Figure 5).

The abundance of P. timonensis was higher in asymptomatic/mild disease compared to uninfected pregnant women (0.15% vs. 0.08%; \( p = 0.042 \)). In addition, the amount of G. vaginalis increased in the asymptomatic/mild group when compared to the uninfected pregnant women (1.9% vs. 5%; \( p = 0.47 \)). Ureaplasma spp. was significantly higher in the moderate/severe group than those in the asymptomatic/mild group (2.05% vs. 0.1%, \( p = 0.036 \)) (Figure 5).

It was not possible to deeply analyze the data at the species level in the longitudinal group due to the low sample size. During the active phase of the disease, there was an increase in the proportion of Actinobacteria and Bacteriodata, which returned to similar levels seen before the active disease. The level of Firmicutes remained stable during the active phase of the disease (Figure 6).
cavity by ascending from the vagina and cervix, resulting in the development of intrauterine infection, and subsequent inflammatory response in fetoplacental tissues that eventuates PTB.\textsuperscript{16,17} Since evidence of the relationship between dysbiosis in vaginal microbiota and PTB is accumulating, we can speculate that one of the mechanisms for the explanation of the increased rate of COVID-19-associated PTB may be vaginal dysbiosis.\textsuperscript{5,18,19}

Vaginal dysbiosis is defined as an increase of alpha diversity in vaginal microbiome communities.\textsuperscript{20} Our study indicated that the Shannon index was remarkably high (0.77) in the COVID-19 group compared to the healthy controls (0.4). Recent studies have revealed that vaginal dysbiosis has a negative impact on vaginal protective mechanisms via increasing local proinflammatory effectors.\textsuperscript{21,22}

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**FIGURE 3** The vaginal microbiome composition of UPW and women with COVID-19 UPW (n = 28) and women with COVID-19 (n = 19). (A) The most abundant bacteria at the phylum level are represented in two groups. (B) The most abundant bacteria at the genus level are represented in two groups. (C) The relative abundance of different bacteria species in two groups. Only phyla and genera present at relative abundances >0.01% are reported. COVID-19, coronavirus disease 2019; UPW, uninfected pregnant women.
We identified diminished Lactobacillus communities in women with COVID-19, which was markedly low those with MSD when compared to the uninfected pregnant women (81.5% vs. 94.27%; \(p = 0.07\)). Within subgroup analysis, we found that L. gasseri and L. jensenii were less in patients with MSD compared to the uninfected pregnant women (12% vs. 16.6% and 0.01% vs. 7.8%, respectively).

Several studies have shown that pregnant women with low amounts of L. crispatus, L. gasseri, or L. jensenii in their vaginal microbiota are more likely to deliver before term. In a case-control study, the increased abundance of L. gasseri was found to be associated with decreased risk of early spontaneous PTB. Disruption of the balance of vaginal microbiota leads to invasion of several facultative or strict anaerobes, including G. vaginalis, M.
hominis, Prevotella spp., Fusobacterium spp., Ureaplasm spp., and Porphyromonas spp., as well as displacement of Lactobacilli by these other species.23–25 Aligning with the aforementioned results, we identified a significantly higher abundance of Bacteroidota in pregnant women with COVID-19. In particular, P. timonensis was only identified in women with COVID-19 (0.12%). Notably, we determined an increase in anaerobic species such as P. timonensis abundance in women with severe disease (p = 0.042) and Veillonella ratti in the asymptomatic/mild group (p = 0.037). SARS-CoV-2 infection has been shown to trigger the production of prostaglandins and proinflammatory mediators resulting in widespread tissue ischemia,26,27 and based on our results, we postulate that ischemia in genitourinary compartments could be a predisposing factor for the overgrowth of anaerobes in vaginal microbiota. In our previous article published by our group, placental ischemia related to disease severity was presented.28

Pregnant women are more likely to have more severe SARS-CoV-2 infection than nonpregnant women due to physiological, mechanical, and immunological changes during pregnancy.2,29 Data supported that pregnancy itself is a risk factor for severe disease related to COVID-19.4,30 Recently, in a large population-based cohort study, fetal death and PTB occurred more frequently in women with SARS-CoV-2 infection than noninfected women (adjusted odds ratio [aOR]: 2.21; 95% confidence interval [CI]: 1.58–3.11; p < 0.001 and OR: 2.17; 95% CI: 1.96–2.4; p < 0.001, respectively).1 In that study, the prevalence of PTB (15.3%) was high, especially in the severe COVID-19 (2 out of 3 PTB), as compared to the prevalence reported before COVID-19 (9.6%).31 We didn't find an association between the time of infection during pregnancy and PTB. Even with the small sample size of our study, the rate of PTB increased in women with severe COVID-19 disease compared to those with asymptomatic/mild disease, aligning with a recent meta-analysis.32 The abundance of Ureaplasma and Mycoplasma species increases the risk of preterm delivery through chorioamnionitis, salpingitis, bacterial vaginosis, and postpartum endometritis.20,23 We found that the abundance of Ureaplasma spp. was significantly higher in women with MSD than in those of asymptomatic/mild disease (2.05% vs. 0.1%, p = 0.036). Our findings combined with previous evidence from microbiota studies indicate that PTB in women with severe COVID-19 disease could be a consequence of impaired vaginal composition.

In our longitudinal study of three patients, we were unable to perform analysis at the species level because of the small sample size. We observed that, during the active phase of the disease, there was an increasing trend in the proportion of Actinobacteria and Bacteroidota that decreased to predisease levels. Ceccarani et al.35 revealed that the vaginal flora of healthy women consisted of mainly Firmicutes and Bacteroidota, albeit with a low abundance of Actinobacteria.

There are several limitations to our study. First, our cohort sample size was too small to detect a statistically significant difference between women with severe COVID-19 and women with asymptomatic/mild disease, although we were able to show some significant differences between the groups of pregnant women. Second, a potential confounding factor that may differ among the groups was the use of antibiotics in severe cases during the active stage of infection at the time of sample collection.

Overall, COVID-19 disease in pregnant women causes dysbiosis in vaginal microbiota with a significant reduction in the abundance of Lactobacillus species including L. crispatus, L. iners, L. gasseri, and L. jenseni in conjunction with an increase in the amount of P. timonensis, V. ratti, and Ureaplasm spp.. Based on these findings, we suggest that COVID-19 promotes an unfavorable vaginal microenvironment, which may be the underlying cause of the increased risk of adverse pregnancy outcomes such as PTB. These results raise clinically relevant questions regarding the use of microbiome-associated biomarkers as a risk assessment tool for PTB in pregnant women during COVID-19. New studies should be undertaken to look at potential methods to modify the vaginal microbiota in pregnant women with COVID-19 infection to minimize the risk of adverse birth outcomes.

AUTHOR CONTRIBUTIONS

Ebru Celik: conceptualization, investigation, data curation, methodology, project administration, writing – original draft preparation, and writing – review and editing. Gulin Ozcan: conceptualization, investigation, data curation, methodology, formal analysis, visualization, writing – original draft preparation and editing. Cansel Vatansever: investigation, methodology, visualization, and writing – original draft preparation. Enriati Paerhati: data curation, methodology, formal analysis, visualization, and writing – original draft preparation. Mert Ahmet Kuskucu: conceptualization, methodology, and writing – original draft preparation. Sebile Guler: conceptualization, methodology, supervision, and writing – review and editing. Ozlem Dogan: conceptualization and writing – original draft preparation. Attila Gursoy: conceptualization, methodology, supervision, and writing – review and editing. Ozlem Keskin: conceptualization, methodology, supervision, and writing – review and editing. Fusun Can: conceptualization, investigation, data curation, methodology, project administration, supervision, writing – original draft preparation, and writing – review and editing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author upon reasonable request. The data are not publicly available due to privacy or ethical restrictions.
ETHICS STATEMENT
The ethical approval was obtained for the healthy controls and pregnant women with COVID-19 from Koc University Research Ethics Board (No:2019.093IRB.030 and No: 2020.138.IRB1.028). The written consent forms were obtained from all participants.

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