Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

ORIGINAL ARTICLE: REPRODUCTIVE SCIENCE

Endometrial gene expression differences in women with coronavirus disease 2019

Lucía de Miguel-Gómez, Ph.D.,[a] Patricia Sebastián-León, Ph.D.,[a] Mónica Romeu, M.D., Ph.D.,[b,c] Nuria Pellicer, M.D., Ph.D.,[b,c] Amparo Faus, B.Sc.,[a] Antonio Pellicer, M.D., Ph.D.,[d,e] Patricia Díaz-Gimeno, Ph.D.,[a] and Irene Cervelló, Ph.D.[a]

[a] IVI Foundation, La Fe Health Research Institute, Valencia, Spain; [b] Reproductive Medicine Research Group, La Fe Health Research Institute, Valencia, Spain; [c] La Fe University Hospital, Valencia, Spain; [d] Women's Health Area, Human Reproduction Unit, La Fe University Hospital, Valencia, Spain; and [e] Pediatrics, Obstetrics, and Gynecology Department, University of Valencia, Spain.

Objective: To study the potential effect of coronavirus disease (COVID-19) on the endometrial transcriptome of affected, symptomatic women for the detection of altered gene expression.
Design: Pilot study of the endometrial transcriptomes of women manifesting COVID-19 compared with those of women without COVID-19 undergoing hysteroscopic procedures for benign gynecologic disorders using RNA sequencing.
Setting: Hospital and university laboratories.
Patient(s): Women with (n = 14) and without a COVID-19 (n = 10) diagnosis based on a nasopharyngeal swab analysis using quantitative reverse-transcription polymerase chain reaction. The endometrium of the patients with COVID-19 had previously been tested for severe acute respiratory syndrome coronavirus 2 infection, revealing the absence of the virus in this tissue.
Intervention(s): Endometrial biopsy sample collection.
Main Outcomes Measure(s): Endometrial gene expression and functional analysis of symptomatic patients with COVID-19 vs. individuals without the infection.
Result(s): The systemic disease COVID-19 altered endometrial gene expression in 75% of the women, with the patients exhibiting a preponderance of 163 up-regulated (e.g., *UTS2*, *IFI6*, *IFIH1*, and *BNIP3*) and 72 down-regulated genes (e.g., *CPZ*, *CDH3*, and *IRF4*) (false discovery rate<0.05). A total of 161 dysregulated functions (36 up-regulated and 125 down-regulated) were typically enriched in the endometria of the patients with COVID-19, including up-regulation in pathways involved in the development of immune responses to viruses and cytokine inflammation, reflecting elicitation of a COVID-19 response pathway.
Conclusion(s): Coronavirus disease 2019 affects endometrial gene expression despite the absence of severe acute respiratory syndrome coronavirus 2 RNA in endometrial tissues. (Fertil Steril® 2022;■:■–■. ©2022 by American Society for Reproductive Medicine.)
Key Words: Endometrium, COVID-19, endometrial gene expression, transcriptomics, women's reproductive health

DIALOG: You can discuss this article with its authors and other readers at https://www.fertstertdialog.com/posts/35284

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causal agent of the coronavirus disease 2019 (COVID-19) pandemic. Between the first unexplained pneumonia cases reported in Wuhan (China) and the time this manuscript was written (July 27, 2022), SARS-CoV-2 infected >568 million people and caused >6 million deaths worldwide (1).

Coronavirus disease 2019 commonly produces pulmonary manifestations, which usually correlate with a severe form of the illness and poor outcomes. However, a myriad of extrapulmonary manifestations have also been reported along with these pulmonary symptoms, mainly in mild

Received May 11, 2022; revised September 5, 2022; accepted September 8, 2022.
L.M.-G. has nothing to disclose. P.S.-L. has nothing to disclose. M.R. has nothing to disclose. N.P. has nothing to disclose. A.F. has nothing to disclose. A.P. has nothing to disclose. P.D.-G. has nothing to disclose. I.C. has nothing to disclose.
P.D.-G. and I.C. should be considered similar in author order.
Supported by grants from Miguel Servet research contract for research into health (CP20/00118) from the Instituto de Salud Carlos III (ISCIII), co-funded by the European Union (Fondo Social Europeo [El FSE invierte en tu futuro]) (to P.D.-G.).
Supported by grants from IVI Foundation (2004-FIVI-035-IC) and FERRING COVID-19 INVESTIGATIONAL GRANT in Reproductive Medicine and Maternal Health for the submitted work (to I.C.).
Supported by a grant from Miguel Servet research contract for research into health (CPI19/00149) from the Instituto de Salud Carlos III (ISCIII), co-funded by the European Union (Fondo Social Europeo [El FSE invierte en tu futuro]) (to I.C.).
Correspondence: Irene Cervelló, Ph.D., IVI Foundation, La Fe Health Research Institute, 106 Fernando Abril Martorell Avenue. La Fe University Hospital, Biopolo, 1st floor, Valencia 46026 (E-mail: Irene.cervello@ivirma.com, irene_cervello@iislafe.es).

Fertility and Sterility® Vol. ■, No. ■, ■ 2022 0015-0282/$36.00

https://doi.org/10.1016/j.fertnstert.2022.09.013

and moderate cases or during severe disease. Such symptoms include dizziness, skin manifestations, arrhythmia, liver injury, hematuria, or even loss of taste and smell (2). Thus, COVID-19 is considered a heterogeneous and multisystem disease (2).

Some complications associated with COVID-19 are not directly caused by the viral infection but, instead, may result from global immune dysregulation triggered by the virus (3). In fact, the most severe cases exhibit increased levels of proinflammatory cytokines, which provoke a cytokine storm, which is directly related to COVID-19 progression (4, 5) and other biologic responses, such as neutrophil extracellular traps (NETs) or production of inflammatory molecules that, when sustained, can result in tissue damage (6, 7). Indeed, cytokine storms are induced by cross-talk between infected cells and host immune cells, affecting multiple organs, even those without active viral replication (4, 8, 9). Accordingly, very low or no viral RNA expression was detected in the affected lungs (3), skin (10), and kidneys (11) of symptomatic patients with COVID-19 and pneumonia, cutaneous manifestations, and acute kidney injury or proteinuria, respectively. These observations prompted us to ask whether such a scenario (i.e., tissue changes in the absence of direct viral infections) might also occur in the female reproductive system.

Since the beginning of the pandemic, the reproductive medicine community has been intrigued by the potential impact of COVID-19 on the female reproductive tract and, particularly, the endometrium, which is essential for embryo implantation and, thus, the focus of this study. Because collecting the samples of reproductive tissue is quite challenging in these cases (it would involve invasive work on patients with the infection), several in silico studies have sought to predict the consequences of viral infections on reproduction in women. In May 2020, our group examined expression data in silico and found that the endometrium was likely safe from viral entry because of the low levels of *ACE2* and *TMPRSS2* (the respective host-cell receptor and protease described as essential for SARS-CoV-2 infection) (12). Then, another group reported low expression of *ACE2* and *TMPRSS2* throughout the menstrual cycle and subsequently predicted that the risk of uterine infection ranged from 0.1% to 1.2% based on the expression of *ACE2* (13). Consistent with these predictions, similar findings were independently reported in other in vivo human studies in which the virus was not detected in oocytes (14), the ovarian medulla, or vaginal and follicular fluids (15, 16) obtained from patients with COVID-19. In corroboration, we did not detect SARS-CoV-2 in 14 endometrial biopsy samples collected from women with COVID-19, whereas their levels of *ACE2* were very low (17). Taking these findings together, it seems unlikely that endometrial tissue is directly infected by SARS-CoV-2.

However, we hypothesize that in the absence of direct viral infections of the endometrium, cytokine storms (or systemic inflammation) that occur with COVID-19 may indirectly, and unpredictably, affect the female reproductive tract. Although the menstrual cycle has been found to be altered in patients with COVID-19 (18), there are currently no prospective studies on how systemic global gene expression changes that occur during COVID-19 affect women's reproductive health. Therefore, our objective was to describe, for the first time, how systemic COVID-19 alters global gene expression (focusing on key processes for a normal menstrual cycle and related fertility outcomes) in the endometria of symptomatic women using the whole-RNA, next-generation sequencing (RNA-seq), high-throughput technology.

MATERIAL AND METHODS

Study Design

A total of 24 endometrial samples were prospectively collected for this study. Fourteen biopsy samples (COVID-19 group) were collected from patients hospitalized for COVID-19 at Hospital Universitari i Politècnic La Fe (Valencia, Spain) (17); these patients were symptomatic (patients with mild-to-severe symptoms were included, whereas asymptomatic or very severe cases were not recruited) and had a positive test result (cycle threshold<40) for SARS-CoV-2 infection, as indicated by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) of nasopharyngeal swab samples, as previously described (17). The other 10 endometrial samples (control group) were derived from patients admitted to the same hospital who underwent hysteroscopic procedures for benign gynecologic disorders and tested negative for COVID-19 based on qRT-PCR of nasopharyngeal swab samples. Notably, the different gynecologic disorders in these control patients did not change the results between the groups because of normal endometrial tissue diagnoses in all the cases. The patients' characteristics (including age and menstrual cycle stage at the time of endometrial biopsy sample collection, menstrual cycle length, and COVID-19 symptoms) are presented in Supplemental Table 1 (available online).

Samples with good RNA quality (percentage of RNA fragments >200 nucleotides [DV_{200}] >30%) were analyzed using RNA-seq for the detection of changes in gene expression using an untargeted approach that compared both the groups (COVID-19 and control). Raw sequencing data were preprocessed, normalized, and functionally interpreted using bioinformatic pipelines that were used to identify disease-altered genes and their functions in the endometria. The study design is depicted in Figure 1A.

Ethical Approval

The research ethics committee of Hospital Universitari i Politècnic La Fe (Valencia, Spain) approved this study (registration number: 2020-268-1) on May 12, 2020 (addendum: November 18, 2020). All study participants received written and oral information on the characteristics of the study and signed informed consent forms (or consented orally in the presence of at least 1 witness in the case of patients with COVID-19).

Sample Collection

All endometrial biopsy samples (from both the COVID-19 and control groups) were collected via endometrial aspiration using an intrauterine cannula (Cornier Pipelle, ref. 4164,

Gynetics, Lommel, Belgium) and preserved in RNAlater (ref. AM7021; Invitrogen, Vilnius, Lithuania) until cryopreservation. After the removal of RNAlater, the endometrial biopsy samples (median size, 3 mm^2) were frozen at −80°C until their analysis.

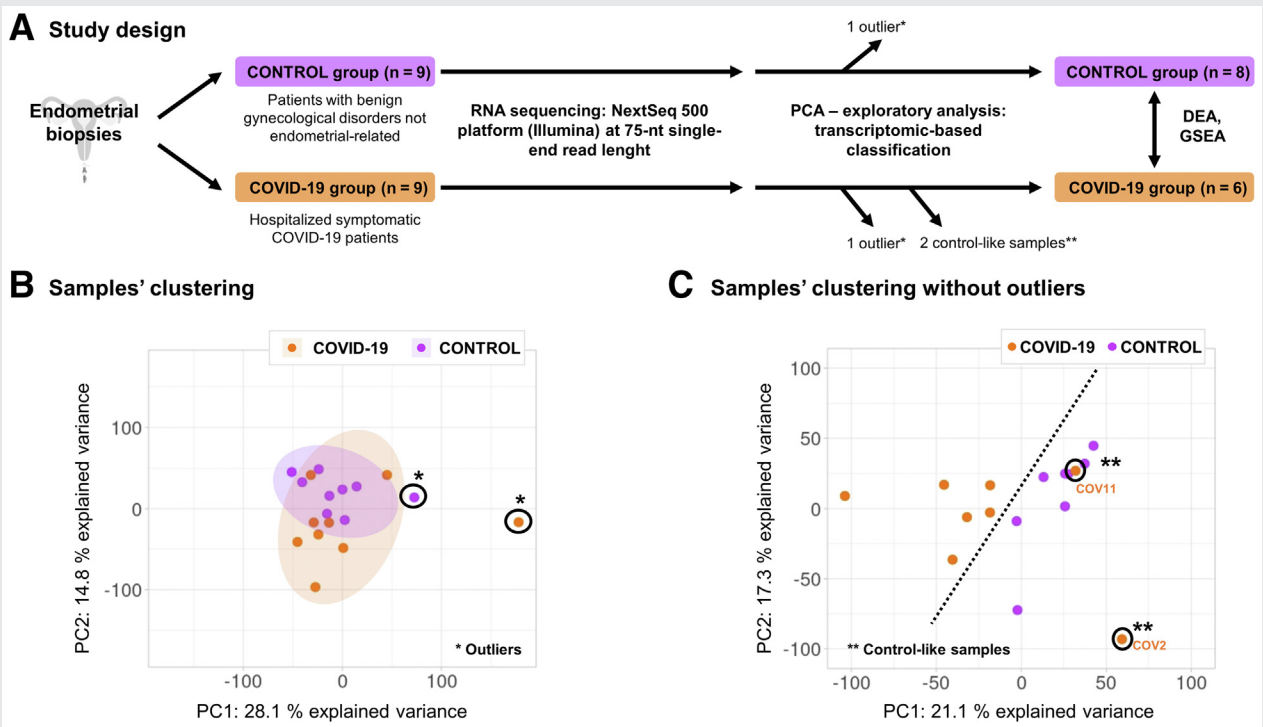
Library Construction and RNA-Seq

Total RNA was extracted from the thawed endometrial biopsy samples using the miRNeasy Mini Kit (ref. 1038703; Qiagen, Hilden, Germany) according to RNeasy FFPE Handbook protocol instructions (Qiagen, Hilden, Germany). The amount of total RNA isolated from each tissue was quantified using the NanoDrop One spectrophotometer (ThermoFisher, Vilnius, Lithuania), and the integrity of the extracted RNA was assessed using the Fragment Analyzer System (Agilent, Santa Clara, CA). Nine endometrial samples from each group (9 from the COVID-19 group and 9 from the control group) with the highest DV_{200} values were selected for RNA-seq analysis. Sequencing libraries were constructed using the TruSeq stranded messenger RNA protocol (Illumina, San Diego, CA), with poly-A selection for ribosomal messenger RNA depletion, followed by chemical fragmentation and complementary DNA synthesis, as per the manufacturer's instructions. The libraries were sequenced in 2 runs using the NextSeq 500 platform (Illumina, San Diego, CA) at a single-end read length of 75 nucleotides using the High Output 75 cycles kit (Illumina, San Diego, CA).

Transcriptomic Data Processing

Raw data were trimmed using fastp (version 0.21) (19) and evaluated for read quality using the FastQC, version 0.11.5, tool (20). STAR algorithm (21) was employed for mapping data quality, using GRCh38 as the reference human genome, and obtaining raw counts. Genes with zero counts in all measured samples were excluded, whereas low-count genes were filtered using the NOISeq R-package (22) in R (version 4.0.5 [March 31, 2021]) (23). The remaining data were normalized using the Remove Unwanted Variation methodology (24) implemented in the RUVseq R-package (25). Briefly, this approach uses general linear model regression to remove unknown sources of data or variation from batch or other variable effects to improve the detection of differentially expressed genes (DEGs). We then corrected for the menstrual cycle phase to remove transcriptomic variability associated with the progression of the menstrual cycle (26). Finally, the principal component analysis (PCA) was performed to evaluate any remaining batch effects, look for outliers, and find transcriptomically related samples in our cohort.

FIGURE 1



Study design. (**A**) The workflow indicates the size of each group throughout the study and the reasons for excluding specific samples. (**B**) An initial principal component analysis showed how the 2 experimental groups overlapped with the presence of 2 outliers. (**C**) After removal of these outliers, the samples were evidently segregated according to the 2 experimental groups: coronavirus disease 2019 and control. This principal component analysis also identified 2 samples from the coronavirus disease 2019 group that behaved similar to the ones from the control group. *Outliers **Control-like samples. COVID-19 = coronavirus disease 2019; DEA = differential expression analysis; GSEA = gene set enrichment analysis; PC = principal component; PCA = principal component analysis.

de Miguel-Gómez. Endometrial changes during COVID-19. Fertil Steril 2022.

Differential Expression Analysis and Functional Enrichment

To identify genes in the endometrium that are most affected by COVID-19, the differential expression analysis (DEA) was performed using the limma R-package (27). DEGs that had an adjusted P value of $<.05$ and a false discovery rate of <0.05 were considered to have been significantly affected by COVID-19 and were subjected to a subsequent functional analysis using the Gene Ontology (version 2021-07-02, experimental and propagated annotation; to elucidate the affected biologic processes, molecular functions, and cellular components) (28) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (release 99.0, August 1, 2021) (29). The genes were arranged in descending order according to their fold change (FC), which was obtained from DEA, and the gene set enrichment analysis was implemented in the clusterProfiler R-package (30). Only significantly enriched functions (false discover rate<0.05) were considered to be affected by COVID-19. All plots were generated using the ggplot2 package in R (31).

Gene Validation

To validate the DEGs identified using the RNA-seq analysis, the complementary DNA from the COVID-19 ($n = 9$) and control ($n = 9$) samples was analyzed using qRT-PCR with PowerUp SYBR Green (ref. A25742; ThermoFisher, Vilnius, Lithuania) on the StepOnePlus system (Applied Biosystems, Carlsbad, CA). Based on their high |FC| values (Supplemental Table 2, available online), we assessed *UTS2* and *IFI6* as up-regulated DEGs and *CPZ* and *CDH3* as down-regulated DEGs. Further, based on their involvement in response to viral infections or inflammatory processes, we assessed *IFIH1*, BNIP3, and *IRF4*. The relative level of gene expression or FC was determined using the $\Delta\Delta$Ct method and normalized to the level of the expression of the housekeeping gene *GAPDH*. The primer sequences (Integrated DNA technologies, Leuven, Belgium) are listed in Supplemental Table 3 (available online).

Statistical Analysis

The qRT-PCR results are expressed as means $\pm$ standard deviations. We used the unpaired t test and Wilcoxon test for normally and nonnormally distributed data, respectively, to identify significant DEGs between the COVID-19 and control groups. GraphPad Prism 8.3 (San Diego, CA) was used for statistical analysis and visualization. In all the analyses, $P < .05$ was considered statistically significant.

RESULTS

The profiling of global endometrial gene expression revealed altered endometrial gene expression in most of the patients with COVID-19.

Among the 24 initial endometrial biopsy samples, 5 out of the 14 samples from the COVID-19 group had low RNA integrity ($DV_{200}<30\%$) and were excluded from the RNA-seq analysis, whereas 1 out of the 10 samples from the control group was excluded because of low quality (Supplemental Table 1). Thus, 18 endometria samples were sequenced as detailed in Figure 1A.

The endometrial biopsy samples from the patients with COVID-19 and controls had similar numbers of good-quality reads per sample (Supplemental Fig. 1, available online). The subsequent analysis of read quality in all the samples revealed 16,546 good-quality measured transcripts.

Exploratory transcriptomic analyses of the 18 endometrial biopsy samples revealed that age, corrected menstrual phase, and RNA integrity did not affect endometrial gene expression. After removing 2 outliers identified using PCA (Fig. 1B), the samples were segregated into 2 groups based on gene expression related to COVID-19 (Fig. 1C). Two patients with COVID-19 (named COV2 and COV11), exceptionally clustered with the controls, were considered to have a "control-like" transcriptomic behavior and were removed from subsequent analysis because their endometria appeared transcriptomically unaffected by COVID-19 despite the other symptoms that they presented with. These findings led us to assume that COVID-19 variably affected this cohort (in terms of affected organs and manifested symptoms), and we estimated that 75% of our recruited symptomatic patients with COVID-19 had altered endometrial gene expression profiles.

Differential Gene Expression and Validation of the RNA-Seq Results

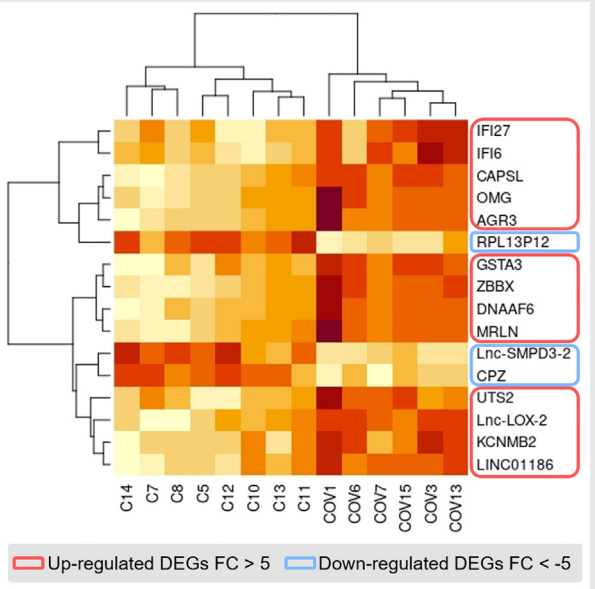
Our DEA revealed 235 DEGs (163 up- and 72 down-regulated) in the altered endometria of the patients with COVID-19 (Supplemental Table 2). The top 10 up-regulated DEGs exhibited over fivefold increases (e.g., *UTS2* exhibited a 16.126-fold increase), whereas the changes in the down-regulated genes ranged from -3- to -5.165-fold (Supplemental Table 2). Notably, only 3 down-regulated DEGs passed our |FC| cutoff of ≥ 5, whereas 13 up-regulated genes met this criterion (Fig. 2), suggesting that COVID-19-associated dysregulation of endometrial genes is mediated through increased rather than decreased expression.

Next, to validate the expression of DEGs in the RNA-seq results, we analyzed the gene expression of 2 up-regulated (*UTS2*, FC $= 8.871$; *IFI6*, FC $= 3.740$) (Fig. 3A) and 2 down-regulated (*CPZ*, FC $= -1.866$; *CDH3*, FC $= -1.511$) (Fig. 3B) DEGs using qRT-PCR. We additionally validated the expression of *IFIH1* (FC $= 1.601$), involved in defense response to symbionts, positive regulation of type I interferon (IFN-I) production, and the COVID-19 KEGG pathway (Fig. 3A and Supplemental Fig. 2, available online); *BNIP3* (FC $= 1.601$), implicated in response to viral infections (Fig. 3A); and *IRF4* (FC $= -1.786$), described in the regulation of anti-inflammatory interleukin 4 production (Fig. 3B). Comparison with the RNA-seq values is presented in Figure 3C.

Functional Analysis

The functional enrichment analysis was performed to identify underlying biologic functions associated with the significantly dysregulated DEGs in the endometria of the patients with COVID-19. We identified 161 enriched, dysregulated functions (36 up- and 125 down-regulated) in the endometria of the

FIGURE 2



Results of the gene set enrichment analysis. A heatmap depicts 13 out of 235 significantly differentially expressed genes with a false discovery rate of <0.05 and fold change of >5. DEG = differentially expressed gene; FC = fold change.

de Miguel-Gómez. Endometrial changes during COVID-19. Fertil Steril 2022.

patients with COVID-19 (Fig. 4 and Supplemental Fig. 3, available online), involving a myriad of biologic processes (15 up- and 52 down-regulated), molecular functions (12 down-regulated), cellular components (12 up- and 7 down-regulated), and/or signaling pathways (9 up- and 54 down-regulated). Specifically, the endometrial transcriptomes obtained from the patients with COVID-19 were enriched in biologic processes associated with response to viral infections, positive regulation of IFN-I production, the COVID-19 KEGG pathway (Supplemental Fig. 2, available online), NET formation (Fig. 4), and ribosome-guided messenger translation. Further, the DEGs were mainly enriched in the mitochondria and cilia (Fig. 4 and Supplemental Fig. 3). Remarkably, the molecular functions related with the immune system (i.e., T helper 17 cell differentiation as well as T-cell and platelet activation), anti-inflammatory cytokine production (i.e., regulation of interleukin 4 production), and gene transcription (i.e., DNA-binding transcription activator activity and transcription regulatory region nucleic acid binding) were down-regulated (Fig. 4 and Supplemental Fig. 3).

DISCUSSION

Although our sample size was limited, this was the first study to analyze changes in global gene expression in the endometrium of women with COVID-19 during the first 10 months of the global pandemic (2020).

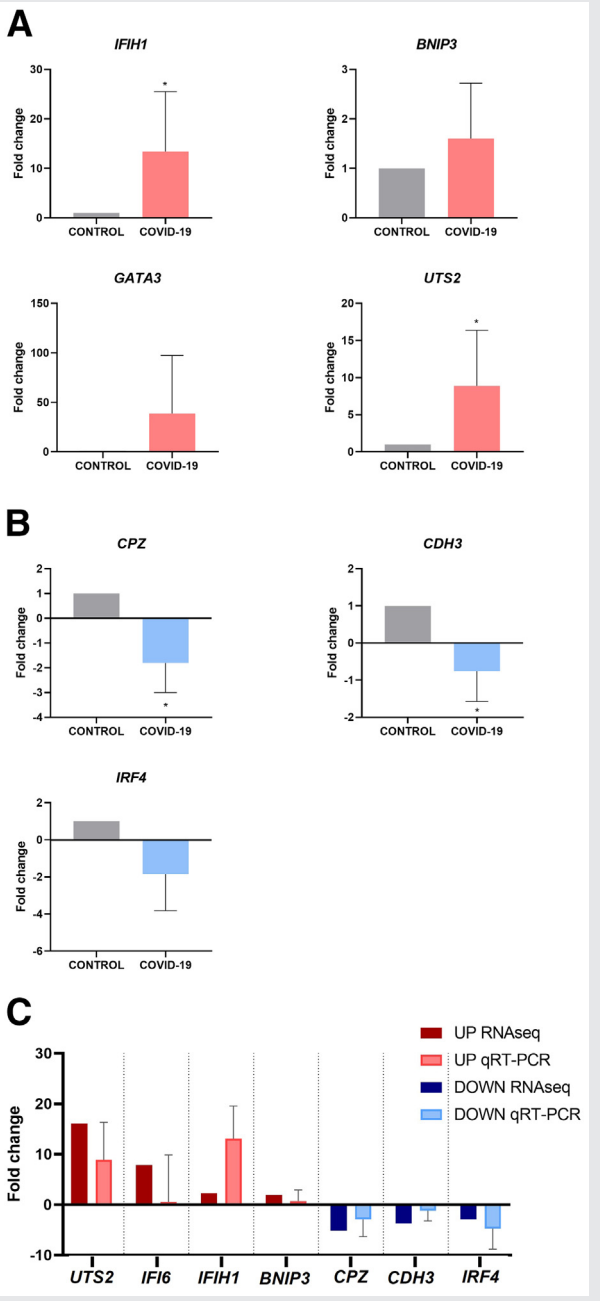
It has been widely reported that COVID-19 produces heterogenic clinical phenotypes in terms of severity and complications (involving only pulmonary or additional renal, hepatic, and/or even cardiac pathophysiologies). The presentation of the disease also varies with age, with older patients having a higher prevalence of severe disease and risk of complications (32, 33). Consistent with COVID-19 being a complex, multiorgan disease, this study highlighted that this disease can also impact the endometrium, at least at the level of gene expression. Indeed, we found that most (75%) of the patients with COVID-19 included in this study presented with an altered endometrial gene expression profile, revealing that the endometrium is commonly affected by this disease. In addition, using a nontargeted approach, we identified key components of the pathogenesis of COVID-19 (Supplemental Fig. 2) among the most altered DEGs in the affected endometria. Overall, this study showcased that this systemic disease affects the normal endometrial gene expression profile in the absence of direct viral infections (17). By correcting for menstrual cycle biases, we overcame the limitation of unequal representation of the different menstrual phases in our COVID-19 and control groups and ensured that the gene dysregulations that we found were truly related to COVID-19 (empowering the results of our low-sample-size study). The latter was verified using PCA, which additionally corroborated that age did not result in a bias in our results.

Other groups have observed some of the same genes and patterns during SARS-CoV-2–mediated disease. For example, NET formation is enhanced in various tissues of patients with COVID-19 because of severe inflammatory reactions (6, 34, 35) and is associated with collateral tissue injury during respiratory viral infections. In this study, we found that the endometrium of the women with COVID-19 exhibited remarkable up-regulation of genes associated with NET formation, suggesting that COVID-19 causes collateral damage to the endometrium as well.

In terms of other host immune responses, we also found dysregulated IFN-I production in the endometria of the patients with COVID-19. This is consistent with previous studies that reported that IFN-I production is impaired in such patients (36). Interestingly, specific interferon-stimulated genes appear to be up-regulated in the peripheral blood of patients with COVID-19 (36). These implicated genes fall into 2 subgroups: those dysregulated in different viral infections (including SARS-CoV-2, severe acute respiratory syndrome coronavirus, or influenza) and those uniquely dysregulated in COVID-19. The second subgroup includes *IFI2*, *IFI6*, and *IFI44* (36), which were also up-regulated in the endometria of our patients, indicating an altered inflammatory environment. Notably, both the *IFI27* gene and the IFN-I signaling pathway are up-regulated in the endometria of women with recurrent pregnancy loss (37), and *IFI6* and *IFI44* are overexpressed in patients with implantation failure (38). Indeed, the entire reproductive system can be altered in response to an inflammatory environment, as has been reported for premature ovarian insufficiency (39).

The other observed changes that we can associate with inflammatory processes include the remarkable 16.126-fold up-regulation of *UTS2* in the endometria of patients with COVID-19. This gene encodes a peptide that acts as a vasoconstrictor and is involved in the pathogenesis of inflammatory diseases such as atherosclerosis and hypertension (40).

FIGURE 3

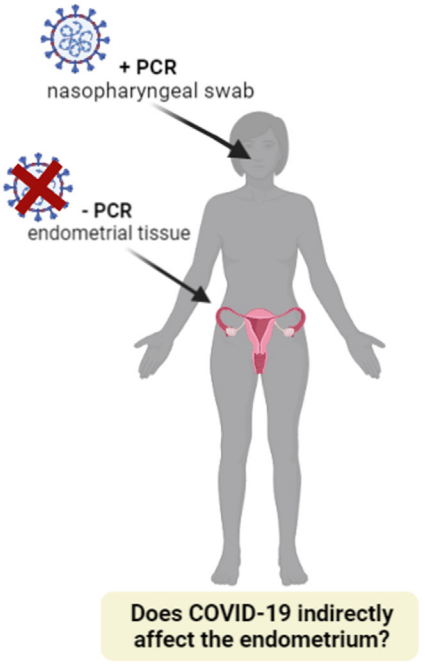


RNA sequencing validation using quantitative reverse-transcription polymerase chain reaction. The graphs show gene expression, presented as mean fold changes ± standard deviations, between endometria of the coronavirus disease 2019 and control groups for 4 up-regulated (**A**) and 3 down-regulated (**B**) differentially expressed genes. (**C**) A comparison between the RNA sequencing and quantitative reverse-transcription polymerase chain reaction results is also shown. *P<.050. COVID-19 = coronavirus disease 2019; DOWN = down-regulated; qRT-PCR = quantitative reverse-transcription polymerase chain reaction; RNAseq = RNA sequencing; UP = up-regulated.

de Miguel-Gómez. Endometrial changes during COVID-19. Fertil Steril 2022.

Interestingly, a recent meta-analysis revealed that several preeclampsia-related pathways were affected in pregnant women with COVID-19, corresponding with an imbalance of vasoactive peptides resulting from the up-regulation of various genes, including *UTS2* (41). Further, studies of other systemic diseases, such as lupus erythematosus (42), several rheumatic diseases (43), irritable bowel syndrome (44), and diabetes (45), revealed dysregulated function of the female reproductive system. Interestingly, these studies also support the fact that the endometrium is influenced by cytokine storms (triggered by viral infections) rather than by the virus itself.

FIGURE 4



Up-regulated biological processes and metabolic pathways		NES
Subcellular component movement	Microtubule-based movement	3,608
	Cilium movement involved in cell motility	2,702
Response to stimulus	Defense response to symbiont	3,198
	Response to virus	2,372
	Coronavirus disease - COVID-19	2,053
Cellular component organization	Axoneme assembly	2,851
	Mitochondrial respiratory chain assembly	2,344
Developmental process	Determination of bilateral symmetry	2,804
Metabolism	Oxidative phosphorylation	2,376
	Valine, leucine and isoleucine degradation	2,156
	Fatty acid catabolic process	2,123
	Butanoate metabolism	2,085
Translation	Ribosome	2,284
Cytokine production	Positive regulation of IFN I production	2,055
Immune system	Neutrophil extracellular trap formation	1,818

Down-regulated biological processes and metabolic pathways		NES
Digestive and endocrine system	Protein digestion and absorption	-2,801
	Thyroid hormone signaling pathway	-2,142
Developmental process	Ossification	-2,695
Cellular component organization	Neuron projection development	-2,329
	Gap junction	-2,096
Cell motility	Cell adhesion	-2,194
	Cell migration	-2,107
Human diseases	Inflammatory bowel disease	-2,219
	Asthma	-2,094
Cell death	Regulation of endothelial cell apoptotic process	-2,074
Metabolism	Positive regulation of phosphate metabolic process	-2,247
Genetic information processing	Positive regulation of nucleic acid transcription	-2,508
Signal transduction	Wnt signaling pathway	-2,367
	Regulation of BMP signaling pathway	-2,188
	PI3K-Akt signaling pathway	-2,142
	Ras signaling pathway	-2,011
Immune system	Th1 and Th2 cell differentiation	-2,321
	Platelet activation	-2,252
	Hematopoietic cell lineage	-2,168
Cytokine production	Regulation of IL-4 production	-2,276
Response to stimulus	Negative regulation of response to GF stimulus	-2,182
	Regulation of inflammatory response	-2,134
Sensory system	Taste transduction	-2,029

Functional enrichment analysis. The figure shows the main functions that were differentially up- and down-regulated (false discovery rate<0.05) in the endometria of patients with COVID-19 (compared with those in the control group; COV11 and COV2 were considered "control-like" samples). Both the Kyoto Encyclopedia of Genes and Genomes pathways and Gene Ontology biologic processes are included. BMP = bone morphogenetic protein; COVID-19 = coronavirus disease 2019; GF = growth factor; IFN = interferon; IL = interleukin; NES = normalized enrichment score; P13k-Akt = phosphatidylinositol 3-kinase and protein kinase B; PCR = polymerase chain reaction; Ras = rat sarcoma virus; Th = T helper.

de Miguel-Gómez. Endometrial changes during COVID-19. Fertil Steril 2022.

Changes in lipid metabolism are also consistent with SARS-CoV-2 infection. We found that the endometrial expression of genes involved in this process was altered in the patients with COVID-19. Despite not being directly linked to inflammation, this correlates with the differential lipid profile observed in the serum of patients with COVID-19, which, in turn, seems to be associated with the severity of the disease (46, 47).

We found the expression of genes with direct roles in endometrial function to be altered by COVID-19. In particular, *AGR3* was among the top 10 up-regulated DEGs in the endometria of the patients with COVID-19, and the protein it codes for has been shown to be overexpressed in the endometrial ciliated luminal epithelium of women with recurrent implantation failure and recurrent pregnancy loss (48). Alterations in ciliated cells also occur in other reproductive disorders such as endometriosis (49, 50). Further, these reports describe the alteration of ribosome and phosphorylation processes during an endometrial pathology (49), supporting our findings of these 2 mechanisms being up-regulated in our group of patients with COVID-19.

The up-regulation of genes associated with reproductive disorders indicates that COVID-19 could negatively affect endometrial function, thereby potentially disrupting menstrual cycles and consequently compromising embryo implantation and fertility. However, to address this hypothesis, it would be necessary to investigate whether proinflammatory reactions (that can alter the normal function of the female reproductive system) (51) subside after the infection or persist for a short or even long term (52). Nevertheless, a more detailed dataset from a larger cohort that takes into account other factors that cause irregular menstrual cycles (i.e., baseline ovarian reserve and hormone measurements for the given cycle as well as follow-up of the menstrual cycle) is required to refine the correlation. Because alterations in the menstrual cycle have previously been described in other systemic

disorders (42–45), it could be worthwhile to conduct an in silico analysis that compares our data with their transcriptomes to elucidate whether COVID-19 directly produces unique genetic signatures or indirectly induces molecular changes with the general inflammation it elicits.

Although this study emphasized the up-regulation of genes and their pathways because of their high |FC| values, the down-regulated processes were also noteworthy. For example, smell and taste disorders are common in patients with COVID-19 (53), indicating the down-regulation of the taste transduction pathway, as shown in our analysis. Further, CPZ, a carboxypeptidase involved in the Wnt signaling pathway (54), was among the most down-regulated DEGs and pathways in the COVID-19–affected endometria. The Wnt pathway has well described roles in multiple endometrial processes and is critical for implantation (55). Two other down-regulated DEGs, *CDH3* and *IRF4*, are involved in cell adhesion during embryo implantation (56) and decidualization (57), respectively. Taken together, the down-regulated DEGs we identified in this study also suggest that COVID-19 could alter endometrial function.

This study provides novel transcriptomic findings elucidating the potential impact of COVID-19 on the endometrium. Our bioinformatic approach for processing the global endometrial transcriptomes was concise and powerful, highlighting our findings despite the small sample size. Nevertheless, our small cohort (resulting from the risk of collecting high-quality samples from patients with the infection) implied several limitations, such as interpatient variability in terms of menstrual cycle parameters, fertility status, day of biopsy sample collection, and age, among others. By eliminating the effect of 2 of these confounding variables (menstrual phase and gynecologic disorders), our endometrial COVID-19 signature became more reliable. In addition, because of health measures and the risk of infection, we only collected biopsy samples from symptomatic, hospitalized patients (asymptomatic women were not recruited). Finally, regarding the 2 endometria with control-like expression profiles from the COVID-19 group, we carefully reviewed the medical records of COV11 and found that she might have had a false-positive result in her SARS-CoV-2 diagnostic test. Interestingly, the 2 patients with control-like expression profiles were the youngest in the cohort. Because of our small sample size, we cannot draw conclusions based on age at this point, especially considering that all the patients reported respiratory symptoms. However, age has been shown to be correlated with the severity of COVID-19 (58, 59), and in this regard, it might be possible that the endometrium of younger patients is not affected to the same degree. Overall, additional research with a larger sample size would be needed to further validate the results of our RNA-seq and functional analyses and advance the understanding of the implications of COVID-19 on the human endometrium.

CONCLUSION

In conclusion, our transcriptomic results revealed that human endometrial gene expression and, possibly, endometrial function are affected by COVID-19. The changes in the endometria of patients with COVID-19 seem to occur in the absence of direct viral infection, along with the down-regulation of other vital functions and host immune responses. Still, more studies are needed to elucidate whether these molecular changes also occur in patients with aggressive COVID-19 symptoms and/or asymptomatic women. Finally, evaluating whether these changes persist after disease remission or resolve in the following menstrual cycle(s) as the endometrium cyclically regenerates itself would be of high clinical interest.

Acknowledgments: The authors express their sincere thanks to Hospital Universitari i Politècnic La Fe Biobank, especially to Raquel Amigo, M.Sc., and to all the medical care staff of this hospital for their assistance in obtaining the endometrial biopsy samples. In addition, the authors thank Amparo Martínez, Ph.D., and Vicente Sentandreu, M.Sc., from the Genomic Core of the Universitat de València (Central Service for Experimental Research [SCSIE], Burjassot, València).

DIALOG: You can discuss this article with its authors and other readers at https://www.fertstertdialog.com/posts/35284

REFERENCES

1. World Health Organization. WHO coronavirus (COVID-19) dashboard with vaccination data. Available at: https://covid19.who.int. Accessed July 27, 2022.
2. Elrobaa IH, New KJ. COVID-19: pulmonary and extra pulmonary manifestations. Front Public Health 2021;9:711616.
3. El Jamal SM, Pujadas E, Ramos I, Bryce C, Grimes ZM, Amanat F, et al. Tissue-based SARS-CoV-2 detection in fatal COVID-19 infections: sustained direct viral-induced damage is not necessary to drive disease progression. Hum Pathol 2021;114:110–9.
4. Coperchini F, Chiovato L, Rotondi M. Interleukin-6, CXCL10 and infiltrating macrophages in COVID-19-related cytokine storm: not one for all but all for one. Front Immunol 2021;12:668507.
5. Hu B, Huang S, Yin L. The cytokine storm and COVID-19. J Med Virol 2021;93:250–6.
6. Zuo Y, Yalavarthi S, Shi H, Gockman K, Zuo M, Madison JA, et al. Neutrophil extracellular traps in COVID-19. JCI Insight 2020;5:e138999.
7. Hadjadj J, Yatim N, Barnabei L, Corneau A, Boussier J, Smith N, et al. Impaired type I interferon activity and inflammatory responses in severe COVID-19 patients. Science 2020;369:718–24.
8. Delorey TM, Ziegler CG, Heimberg G, Normand R, Yang Y, Segerstolpe A, et al. COVID-19 tissue atlases reveal SARS-CoV-2 pathology and cellular targets. Nature 2021;59:107–13.
9. Yang L, Xie X, Tu Z, Fu J, Xu D, Zhou Y. The signal pathways and treatment of cytokine storm in COVID-19. Signal Transduct Target Ther 2021;6:1–20.
10. García-Gil MF, Monte-Serrano J, García MG, Prieto-Torres L, Pascual-del-Riquelme AJ, Flecha IC, et al. Absence of SARS-CoV-2 RNA detection in tissue samples of COVID-19-related cutaneous lesions analyzed by real-time RT-PCR. J Eur Acad Dermatol Venereol 2021;35:e318–21.
11. Akilesh S, Nast CC, Yamashita M, Henriksen K, Charu V, Troxell ML, et al. Multicenter clinicopathologic correlation of kidney biopsies performed in COVID-19 patients presenting with acute kidney injury or proteinuria. Am J Kidney Dis 2021;77:82–93.
12. Henarejos-Castillo I, Sebastian-Leon P, Devesa-Peiro A, Pellicer A, Diaz-Gimeno P. SARS-CoV-2 infection risk assessment in the endometrium: viral infection-related gene expression across the menstrual cycle. Fertil Steril 2020;114:223–32.
13. Vilella F, Wang W, Moreno I, Roson B, Quake SR, Simon C. Single-cell RNA sequencing of SARS–CoV-2 cell entry factors in the preconceptional human endometrium. Hum Reprod 2021;36:2709–19.

14. Barragan M, Guillén JJ, Martin-Palomino N, Rodriguez A, Vassena R. Undetectable viral RNA in oocytes from SARS-CoV-2 positive women. Hum Reprod 2021;36:390–4.
15. Kteily K, Pening D, Diaz Vidal P, Devos M, Dechene J, Op de Beeck A, et al. Risk of contamination of semen, vaginal secretions, follicular fluid and ovarian medulla with SARS-CoV-2 in patients undergoing ART. Hum Reprod 2022;37:235–41.
16. Boudry L, Essahib W, Mateizel I, Van de Velde H, De Geyter D, Piérard D, et al. Undetectable viral RNA in follicular fluid, cumulus cells, and endometrial tissue samples in SARS-CoV-2–positive women. Fertil Steril 2022;117:771–80.
17. de Miguel-Gómez L, Romeu M, Castells-Ballester J, Pellicer N, Faus A, Mullor JL, et al. Undetectable viral RNA from SARS-CoV-2 in endometrial biopsies from women with COVID-19: a preliminary study. Am J Obstet Gynecol 2022;226:434–7.
18. Lebar V, Laganà AS, Chiantera V, Kunič T, Lukanović D. The Effect of COVID-19 on the menstrual cycle: a systematic review. J Clin Med 2022;11:3800.
19. Chen S, Zhou Y, Chen Y, Gu J. Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 2018;34:i884–90.
20. Andrew S. FastQC a quality control tool for high throughput sequence data. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/. Accessed April 10, 2022.
21. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15–21.
22. Tarazona S, Furió-Tarí P, Turrà D, Di Pietro AD, Nueda MJ, Ferrer A, et al. Data quality aware analysis of differential expression in RNA-seq with NOISeq R/Bioc package. Nucleic Acids Res 2015;43:e140.
23. R: the R project for statistical computing. https://www.r-project.org/. Accessed April 10, 2022.
24. Gagnon-Bartsch JA, Jacob L, Speed TP. Removing unwanted variation from high dimensional data with negative controls. 2013. Technical report. http://statistics.berkeley.edu/techreports/820. Accessed April 12, 2022.
25. Risso D, Ngai J, Speed TP, Dudoit S. Normalization of RNA-seq data using factor analysis of control genes or samples. Nat Biotechnol 2014;32:896–902.
26. Devesa-Peiro A, Sebastian-Leon P, Pellicer A, Diaz-Gimeno P. Guidelines for biomarker discovery in endometrium: correcting for menstrual cycle bias reveals new genes associated with uterine disorders. Mol Hum Reprod 2021;27:gaab011.
27. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 2015;43:e47.
28. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. Nat Genet 2000;25:25–9.
29. Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 2000;28:27–30.
30. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. Omics 2012;16:284–7.
31. Wickham H. ggplot2: elegant graphics for data analysis. 2nd ed. NY: Springer-Verlag; 2016.
32. Lusczek ER, Ingraham NE, Karam BS, Proper J, Siegel L, Helgeson ES, et al. Characterizing COVID-19 clinical phenotypes and associated comorbidities and complication profiles. PLoS One 2021;16:e0248956.
33. Davis HE, Assaf GS, McCorkell L, Wei H, Low RJ, Re'em Y, et al. Characterizing long COVID in an international cohort: 7 months of symptoms and their impact. EClinicalMedicine 2021;38:101019.
34. Veras FP, Pontelli MC, Silva CM, Toller-Kawahisa JE, de Lima M, Nascimento DC, et al. SARS-CoV-2–triggered neutrophil extracellular traps mediate COVID-19 pathology. J Exp Med 2020;217:e20201129.
35. Fuentes Baldarrago AL, Patel A, Masso Silva JA, Moshensky A, Perera S, Ma LJ, et al. Neutrophil extracellular trap formation (NETOSIS) increases with severity of disease in COVID-19 patients. American Thoracic Society International Conference; 2021 May 14–19;Virtual Conference.
36. McClain MT, Constantine FJ, Henao R, Liu Y, Tsalik EL, Burke TW, et al. Dysregulated transcriptional responses to SARS-CoV-2 in the periphery. Nat Commun 2021;12:1–8.
37. Li Y, Wang R, Wang M, Huang W, Liu C, Fang Z, et al. RNA sequencing of decidua reveals differentially expressed genes in recurrent pregnancy loss. Reprod Sci 2021;28:2261–9.
38. Bersinger NA, Wunder DM, Birkhäuser MH, Mueller MD. Gene expression in cultured endometrium from women with different outcomes following IVF. Mol Hum Reprod 2008;14:475–84.
39. Huang Y, Hu C, Ye H, Luo R, Fu X, Li X, et al. Inflamm-aging: a new mechanism affecting premature ovarian insufficiency. J Immunol Res 2019;2019:8069898.
40. Sun SL, Liu LM. Urotensin II: an inflammatory cytokine. J Endocrinol 2019;240:R107–17.
41. Beys-da-Silva WO, da Rosa RL, Santi L, Tureta EF, Terraciano PB, Guimarães JA, et al. The risk of COVID-19 for pregnant women: Evidences of molecular alterations associated with preeclampsia in SARS-CoV-2 infection. Biochim Biophys Acta Mol Basis Dis 2021;1867:165999.
42. Medeiros PB, Febrônio MV, Bonfá E, Borba EF, Takiuti AD, Silva CA. Menstrual and hormonal alterations in juvenile systemic lupus erythematosus. Lupus 2009;18:38–43.
43. Orlandi M, Vannuccini S, El Aoufy K, Melis MR, Lepri G, Sambataro G, et al. Menstruation-related disorders-dysmenorrhea and heavy bleeding-as significant epiphenomena in women with rheumatic diseases. Front Pharmacol 2022;13:807880.
44. Viganò D, Zara F, Usai P. Irritable bowel syndrome and endometriosis: new insights for old diseases. Dig Liver Dis 2018;50:213–9.
45. Kiconco S, Teede HJ, Earnest A, Loxton D, Joham AE. Menstrual cycle regularity as a predictor for heart disease and diabetes: findings from a large population-based longitudinal cohort study. Clin Endocrinol (Oxf) 2022;96:605–16.
46. Liu Y, Pan Y, Yin Y, Chen W, Li X. Association of dyslipidemia with the severity and mortality of coronavirus disease 2019 (COVID-19): a meta-analysis. Virol J 2021;18:1–11.
47. Caterino M, Costanzo M, Fedele R, Cevenini A, Gelzo M, Di Minno A, et al. The serum metabolome of moderate and severe COVID-19 patients reflects possible liver alterations involving carbon and nitrogen metabolism. Int J Mol Sci 2021;22:9548.
48. Tempest N, Batchelor E, Hill CJ, Al-lamee H, Drury J, Drakeley AJ, et al. Anterior gradient protein 3 and S100 calcium-binding protein P levels in different endometrial epithelial compartments may play an important role in recurrent pregnancy failure. Int J Mol Sci 2021;22:3835.
49. Devesa-Peiro A, Sebastian-Leon P, Garcia-Garcia F, Arnau V, Aleman A, Pellicer A, et al. Uterine disorders affecting female fertility: what are the molecular functions altered in endometrium? Fertil Steril 2020;113:1261–74.
50. Devesa-Peiro A, Sebastian-Leon P, Parraga-Leo A, Pellicer A, Diaz-Gimeno P. Breaking the ageing paradigm in endometrium: endometrial gene expression related to cilia and ageing hallmarks in women over 35 years. Hum Reprod 2022;37:762–76.
51. Weiss G, Goldsmith LT, Taylor RN. Inflammation in reproductive disorders. Reprod Sci 2009;16:216–29.
52. Carp-Veliscu A, Mehedintu C, Frincu F, Bratila E, Rasu S, Iordache I, et al. The effects of SARS-CoV-2 infection on female fertility: a review of the literature. Int J Environ Res Public Health 2022;19:984.
53. Mastrangelo A, Bonato M, Cinque P. Smell and taste disorders in COVID-19: from pathogenesis to clinical features and outcomes. Neurosci Lett 2021;748:135694.
54. Moeller C, Swindell EC, Kispert A, Eichele G. Carboxypeptidase Z (CPZ) modulates Wnt signaling and regulates the development of skeletal elements in the chicken. Development 2003;130:5103–11.
55. Tepekoy F, Akkoyunlu G, Demir R. The role of Wnt signaling members in the uterus and embryo during pre-implantation and implantation. J Assist Reprod Genet 2015;32:337–46.
56. Pathare AD, Hinduja I. Endometrial expression of cell adhesion genes in recurrent implantation failure patients in ongoing IVF cycle. Reprod Sci 2022;29:513–23.
57. Vasquez YM, Mazur EC, Li X, Kommagani R, Jiang L, Chen R, et al. FOXO1 is required for binding of PR on IRF4, novel transcriptional regulator of endometrial stromal decidualization. Mol Endocrinol 2015;29:421–33.

58. Casas-Deza D, Bernal-Monterde V, Aranda-Alonso AN, Montil-Miguel E, Julián-Gomara AB, Letona-Giménez L, et al. Age-related mortality in 61,993 confirmed COVID-19 cases over three epidemic waves in Aragon, Spain. Implications for vaccination programmes. PLoS One 2021;16:e0261061.

59. Ho FK, Petermann-Rocha F, Gray SR, Jani BD, Katikireddi SV, Niedzwiedz CL, et al. Is older age associated with COVID-19 mortality in the absence of other risk factors? General population cohort study of 470,034 participants. PLoS One 2020;15:e0241824.