Suboptimal mid-luteal progesterone levels are associated with aberrant endometrial gene expression potentially resulting in implantation failure

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Abstract

Research Question:

What is the difference of endometrial transcriptomics between women with normal and low mid-luteal progesterone during the implantation window?

Design:

An endometrial biopsy and serum progesterone level were taken from participants during the mid-luteal phase (LH+7 to LH+9). A total of 12 participants were recruited. The participants were categorised into two groups based on their progesterone levels: normal progesterone (>15 ng/ml, n=6) and low progesterone (<15 ng/ml, n=6). Global endometrial gene expression between the two groups was compared by microarray techniques. Principal component analysis (PCA) was used to display the gene's expression pattern. Pathway and gene ontology enrichment analysis were performed to determine the biological mechanism of progesterone on the endometrium.

Results:

Several key genes related to endometrial receptivity were found to be regulated by progesterone. With regard to gene ontology and pathway analysis, progesterone was shown to be mainly involved in structure morphogenesis predominantly during a process of decidualisation, extracellular matrix-receptor interaction, and cell adhesion. Distinct differences in the transcriptomic profiles between the two groups were observed indicating potential impairment of endometrial receptivity in women with suboptimal progesterone levels. There was a relatively similar pattern of gene expression between endometrial samples with progesterone levels approximately 10 ng/ml and >15 ng/ml. Thus, a progesterone level of between 10-15 ng/ml seems to be sufficient to induce endometrial receptivity.

Conclusions:

Abnormally low progesterone below the threshold of 10-15 ng/ml during the implantation window results in aberrant endometrial gene expression that may affect implantation potential.

Trial registration:

This trial was registered at clinicaltrials.gov as NCT04323683

Keywords: Progesterone monitoring: Endometrial gene expression: Endometrial receptivity: Assisted reproductive technology: Microarray

Introduction

The human endometrium undergoes cyclical changes in response to fluctuations in steroid hormone levels during the menstrual cycle. It is essential that the endometrium reaches a receptive stage initiating embryo-endometrial dialogue which results in invasion, placentation, fetal development, and finally parturition (Finn and Martin, 1974, Paria et al., 2002). The time that the endometrium becomes receptive is the window of implantation (WOI) taking place around day 21 of a 28-day menstrual cycle or approximately 7 days following the LH surge (Navot et al., 1991, Prapas et al., 1998, Riesewijk et al., 2003). During this period, the endometrial environment is conducive to blastocyst implantation.

Progesterone is a major contributing factor in transforming the non-receptive to the receptive endometrium. Progesterone concentration physiologically peaks at the mid-luteal phase corresponding to the window of implantation (Reed and Carr, 2015). Progesterone inhibits the proliferative effect of oestrogen on uterine epithelial cells and induces endometrial receptivity by promoting stromal cell proliferation/differentiation and decidual growth. Furthermore, it is involved in the regulation of expression of key cell adhesion molecules, growth factors, and cytokines essential for embryo implantation (Okada et al., 2018).

Endometrial transcriptomics has been shown to be regulated by cyclical hormonal change (Ruiz-Alonso et al., 2012, Haouzi et al., 2012, Mirkin et al., 2005, Díaz-Gimeno et al., 2011). A whole genome association study has yielded insights into endometrial transcriptomic changes during the natural cycle (Talbi et al., 2006) supporting the concept of a receptive gene expression profile (Garrido-Gómez et al., 2013, Bhagwat et al., 2013, Díaz-Gimeno et al., 2011). Suboptimal progesterone level is involved in delayed endometrial maturation and abnormal patterns of gene expression (Young et al., 2017) resulting in implantation failure, therefore sufficient progesterone concentration appear to be important for successful implantation.

It is hypothesised that women with suboptimal progesterone levels result in a non-receptive endometrium as this steroid is responsible for endometrial preparation to achieve implantation. Compromised pregnancy rates have been shown when mid-luteal progesterone levels are lower than

the threshold of 10-15 ng/ml in a natural cycle (Jordan et al., 1994, Radwanska et al., 1981, Hull et al., 1982). The gene expression pattern between women with normal and women with low progesterone will be compared in this study. If aberrant gene expression is found in the lower progesterone group compared with the normal group, it would suggest the importance of having sufficient progesterone levels during the mid-luteal phase. We further investigated the key genes associated with implantation and pathways regulated by progesterone for a better understanding of progesterone activities. This is the first study aiming to clarify the effect of low versus normal progesterone on gene expression profile during the human mid-luteal phase.

Objectives

Primary objective

To determine the relationship of mid-luteal serum progesterone and endometrial gene expression by comparing endometrial transcriptomics in women with low progesterone versus women with normal progesterone during the implantation window.

Secondary objective

To investigate genes associated with implantation, gene ontology, and pathway analysis regulated through progesterone activities.

Methods

Participant characteristics

This study was approved by the Nottingham Research Ethics Committee, United Kingdom (NRES reference: 13/EM/0277). This study was conducted at the Nottingham University Research and Treatment Unit in Reproduction (Nurture), Nottingham, UK. Written informed consent was obtained from all participants in accordance with the guidelines in The Declaration of Helsinki. Women who met the following criteria were recruited: aged 18-35 years, regular menstrual cycles with an interval of 25-35 days, a body mass index (BMI) between 18 – 25, no history of taking hormonal medication in the last 3 months. Based on progesterone concentration on the day of endometrial biopsy (LH+7 to LH+9), participants were categorised into two groups based on normal progesterone (≥ 15 ng/ml) and low progesterone levels (< 15 ng/ml). A total of 12 endometrial samples were selected based on progesterone levels to proceed with microarray analysis.

Study protocol

Participants were asked to use barrier methods of contraception or abstain from sexual intercourse**.** Urinary luteinising hormone (LH) kit (Ovuquik One Step; Quidel, SanDiego, CA, USA) was given to detect the LH surge beginning on day 10 of the natural cycle, the day of the urinary LH surge was considered to be LH=0. Women with positive LH test denoting ovulation were arranged to undergo an endometrial biopsy on day LH+7 to LH+9 of the menstrual cycle. A pregnancy test was performed prior to endometrial biopsy. Women who had a negative pregnancy test were eligible to undergo biopsy. An endometrial biopsy was obtained using Pipelle de Cornier endometrial sampler (CCD, Paris). The device was introduced into the uterus until resistance from the fundus was felt. Negative pressure was generated and the device rotated through 360° as it was gradually withdrawn. An endometrial biopsy was undertaken from all four walls of the endometrial cavity under aseptic conditions. Endometrial samples were frozen in liquid nitrogen and stored at -80°C. Blood samples for progesterone were obtained on the day of the endometrial biopsy.

Blood sample analysis

Blood sampling was undertaken in accordance with a standard phlebotomy protocol in the morning (8.00-10.00 AM). The blood tube was centrifuged (4000 rpm for 20 minutes), and the supernatant was used for analysis. Serum progesterone concentrations were measured using automated electro chemiluminescent immunoassays (Abbott Diagnostics, UK). The measurement was performed according to manufacturer's instructions using a commercially available chemiluminescent immunoassay kit. The progesterone assay had a sensitivity of ≤ 0.1 ng/ml. The intra-assay coefficient of variation was 2.9 % and the inter-assay coefficient of variation was 3.5 %.

RNA extraction and quality control

Total RNA was extracted from the endometrium samples by using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The RNA concentration was measured using the Nanodrop RNA quantification. Sample concentration and purity were determined by spectrophotometry, and RNA integrity was confirmed using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano Kit (Agilent Technologies). Only samples surpassing the minimal quality threshold (RIN > 8.0) were used in the subsequent transcriptomic assessment.

Transcriptome profiling with Affymetrix GeneChip

cDNA was prepared from 200ng of RNA as per the GeneChip WT-PLUS Reagents Kit (Affymetrix), followed by in vitro transcription to produce cRNA, end-labelled and hybridized for 16 hours at 45°C to Clariom S array Human (Affymetrix). All steps were performed by Gene Chip Fluidics Station 450 (Affymetrix) according to the manufacturer's instructions. Detection was performed using a GeneChip Scanner 3000 7G (Affymetrix).

Expression array analysis

Whole transcriptome analysis was performed to compare the gene expression profile between the normal progesterone group (n=6) and the low progesterone group (n=6). Gene expression data were

analysed using Partek Genomics Suite 6.6 software (Partek). The raw CEL files were normalized using the robust multiarray average (RMA) background correction with quantile normalization, log base 2 transformation and mean probe-set summarization with adjustment for guanine-cytosine content. Differentially expressed genes were considered significant if P-value was ≤ 0.05 at a fold change (FC) of 1.5 with false-discovery rate (FDR) < 0.05. Principal component analysis (PCA) was used to analyse large amounts of the dataset from the microarray. It displays a multidimensional dataset in three dimensions allowing clear visualisation of the gene's expression pattern. Hierarchical clustering was used to create clusters that have a predetermined ordering from top to bottom to differentiate gene pattern expression among different groups. Enrichment analyses for Gene Ontology (GO) terms and biological pathways (KEGG) were carried out by using the g: Profiler web tool (https://biit.cs.ut.ee/gprofiler/gost) (Raudvere et al., 2019). A P-value of less than 0.05 was considered significant. Gene expression profiles have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE143620.

Microarray Validation by RT-PCR

Microarray validation was carried out by reverse transcriptase-polymerase chain reaction (RT-PCR) in samples from both groups. The RT-PCR was performed for eight selected differentially expressed genes: CXCL13, DKK1, SPP1, IL15, MMP10, ND6, MMP3, and TRH.

PCR was performed in a total volume containing 1.5 µL of cDNA; 0.75 µL of TaqMan Assays: CXCL13 (Hs00757930_m1), SPP1 (Hs00959010_m1), DKK1 (Hs00183740_m1), IL15 (Hs01003716_m1); 7.5 µL TaqMan fast universal master mix; and 5.25 µL H_20. All reactions were run in triplicate. Real-time PCR was run on the ABI 7500 fast real-time PCR system. The reaction underwent a heating step at 95°C for 20 minutes and then cycled 40 times at 95°C for 3 seconds followed by 60°C for 30 seconds. 18S mRNA expression was used for data normalisation. Student's t-test was performed with Statistical Package for the Social Sciences (SPSS) Statistics 26. A p-value < 0.05 was considered to be statistically significant.

Results

Baseline characteristics

There was no significant difference observed in age, cycle length, and BMI between the two groups. All participants were caucasian and non-smokers. Progesterone levels were statistically different between the two groups (Table 1, Figure 1). The annotation of baseline characteristics for each single microarray data is provided in Supplementary 1.

The pattern of gene expression

Principal component analyses (PCA) reveals a clear distinction in the pattern of gene expression between the normal and low progesterone groups (Figure 2). The pattern of gene expression of each woman with normal progesterone displays similarly, whereas the gene expression of the women with low progesterone scattered based on progesterone levels. In the low progesterone group, samples with the lowest progesterone levels of 3.51 and 4.22 ng/ml displayed their position farthest from the normal progesterone group on the left side ((principal component 1 (PC1): principal component 2 (PC2) = (-260, -10) and (-234, 31)). Two samples with progesterone levels of 4.41, 5.78 ng/ml exist below the normal group ((PC1:PC2) = (24, -101) and (32, -113)). The pattern of endometrial transcriptomics of samples with progesterone levels of 9.5, 9.8 ng/ml ((PC1:PC2) = (67, 53) and (76, 64)) was comparable to the normal progesterone group having progesterone levels of 15.5, 16, 17.2, 18.17, 20.37, and 28.61 ng/ml (Figure 2).

Differentially expressed genes, gene ontology and pathway analysis

Global gene expression profiles were analysed by microarray technology comparing the endometrial expression patterns of participants with low progesterone versus normal progesterone at the threshold of 15 ng/ml. A total of 1279 genes displayed a >1.5-fold significant change in expression between the two groups. 805 genes were down-regulated and 474 genes were up-regulated in the low progesterone group compared with the normal group (Supplementary 2).

Gene ontology and pathway analysis were carried out to identify critical progesterone activities on the endometrium during the receptive phase. Enrichment analysis is arranged according to biological processes, molecular functions and cellular components (Figure 3A, 3B, and 3C). The enriched biological processes were mainly involved in anatomical structure morphogenesis, tissue development, cell adhesion and biological adhesion (Figure 3A). With respect to molecular function, differentially expressed genes were mainly associated with glycosaminoglycan binding, extracellular matrix structural constituent, sulfur compound binding, ion binding, and heparin binding (Figure 2B). For cellular component annotation classification, significant genes were mainly localized in the extracellular matrix, endomembrane system, collagen-containing extracellular matrix, endoplasmic reticulum and cytoplasm (Figure 3C). Full lists of genes were provided in Supplementary 3.

Pathway analysis of the differentially expressed genes was carried out using the Kyoto Encyclopedia of Genes and Genomes (KEGG). Enriched KEGG pathways are shown in Figure 3D. Among these differentially regulated pathways, ECM-receptor interaction, focal adhesion, and PI3K-Akt signalling pathway were the most enriched pathways. Extracellular matrix and adhesion appear to be dominant in both pathway and gene ontology analysis. Full lists of genes were provided in Supplementary 3.

Differentially expressed genes associated with decidualisation and implantation

The functional analysis in our study shows that 254 genes (Supplementary 2) are associated with anatomical structure morphogenesis which is characteristic of the decidualisation process (Okada et al., 2018). An in vitro cell culture model by Lucas et al. showed 898 differentially expressed decidualisation-associated genes between undifferentiated endometrial stromal cells and the cells exposed to progesterone for two days (Lucas et al., 2020). A total of 33 overlapping genes were extracted indicating highly potential genes regulating the process of decidualisation (Figure 4A, Supplementary 2).

Endometrial receptivity-related genes that regulated by progesterone were examined by comparing the genes identified by our study with the previous evidence reporting gene associated endometrial receptivity, we found 11% of the 238 ERA genes (27/238) (Díaz-Gimeno et al., 2011) and 14% of the 57 genes in the meta-analysis of genes-related endometrial receptivity (9/57) by Altmäe et al. (Altmäe et al., 2017) were differentially expressed between the low and normal progesterone groups (Table 2, Figure 4B). Pathway analysis revealed that those 27 overlapping genes with ERA were associated with mineral absorption (Figure 5, Supplementary 4). Seven genes were present in all three studies: SPP1, IL15, DKK1, CLDN4, BCL6, MT1H, and MT1G. Progesterone-dependent genes that are over-representative during implantation window are also potential genes involved in the process of implantation. We compared our gene list with a study by Chi et al. that reported 653 differentially expressed genes with altered progesterone receptor (PGR) binding between proliferative and mid-luteal phase (Chi et al., 2020) and study by Young et al. that showed 182 differentially expressed genes between women receiving adequate and inadequate progesterone supplementation during the luteal phase (Young et al., 2017)(Figure 4C). Seven overlapping genes were found among three studies: CILP, CRYAB, CYP4B1, DKK1, KLF4, MAMDC2, and SLIT3. Full lists of genes were provided in Supplementary 2.

Bar charts were generated to present the directionality between this study and other studies. The fold change was adjusted to make comparable to this study, the original fold change was multiply by -1. It was found that the majority of the overlapping genes were up- or down-regulated in the same direction between our study and four other studies for all comparisons (Figure 6): 66% ERA (18/27 genes) (Díaz-Gimeno et al., 2011), 82% Lucus et al. (27/33 genes) (Lucas et al., 2020), 87% Chi et al. (103/118 genes) (Chi et al., 2020), 100% Young et al. (21/21 genes) (Young et al., 2017). A total of 21/37 overlapping genes were analysed between our study and young et al. as fold changes of some genes were not provided in the original publication.

Subgroup analysis

According to PCA result, the gene expression pattern of samples with progesterone levels of approximately 10 ng/ml had a relatively similar expression to samples with progesterone levels of ≥15 ng/ml, thus we further performed subgroup analysis by comparing gene expression profile between normal progesterone group and the group with progesterone levels of approximately 10 ng/ml (the first low progesterone subgroup); and between normal progesterone group and the group with lower progesterone levels of 3-6 ng/ml (the second low progesterone subgroup). Only 8 differentially expressed genes were found in the comparison between normal progesterone group and the first low progesterone subgroup, whereas 1309 genes were differentially expressed between normal progesterone group and the second low progesterone subgroup. The hierarchical clustering of both comparisons was shown in Figures 7A and 7B. According to pathway analysis, no pathway involved in implantation was significantly enriched between normal progesterone group and the first low progesterone subgroup (Figure 8, Supplementary 5) whereas the pathways involved with ECM-receptor interaction, focal adhesion, mineral absorption were significantly enriched between normal progesterone group and the second lower progesterone subgroup (Figure 9, Supplementary 6).

Microarray Validation by RT-PCR

RT–PCR was used to verify mRNA expression levels indicated by microarray analysis. Eight genes were selected for this purpose. According to Figure 10, four genes were significantly up-regulated, and four genes were significantly down-regulated, which is consistent with the microarray results ($P < 0.05$ for all comparisons between low and normal progesterone group)

Discussion

This is the first study comparing the transcriptomic profile of the endometrium in women with normal mid-luteal progesterone versus women with low mid-luteal progesterone during the receptive phase of the natural cycle. Distinct differences in the transcriptomic profiles between women with low and normal progesterone at the threshold of 15 ng/ml were observed indicating potential impairment of endometrial receptivity in women with suboptimal progesterone levels. There was a relatively similar

pattern of gene expression between endometrial samples with progesterone levels approximately 10 ng/ml and >15 ng/ml. Thus, a progesterone concentration of between 10-15 ng/ml appears to be sufficient to induce endometrial receptivity. In this study, participants in both groups had regular cycle approximately 28 days suggesting the progesterone levels in this study tends to be measured in an ovulated cycle, although some women had low progesterone levels (Table 1).

A difference in transcriptomic patterns between the two groups according to the PCA plot was observed suggesting that abnormally low progesterone may induce inappropriate endometrial transcriptomic profiles in which may result in implantation failure. Once the progesterone level was higher than the threshold of 15 ng/ml the pattern of endometrial expression of all samples in normal progesterone group becomes relatively similar to each other, however, the pattern of endometrial transcriptomics at 9.5 and 9.8 ng/ml is comparable to that observed in the normal progesterone group. Subgroup analysis with hierarchical clustering revealed that a large number of genes were differentially expressed between samples with normal progesterone and samples with very low progesterone (3-6 ng/ml), whereas only 8 differentially expressed genes were found in the comparison between samples with normal progesterone and samples with progesterone levels $\approx$ 10 ng/ml. It would suggest that aberrant endometrial gene expression is caused by suboptimal progesterone levels and mid-luteal progesterone of approximately 10 ng/ml appears to be adequate for successful implantation. There was no significant enriched biological process, molecular functions, and cellular component between normal progesterone group and group with progesterone levels of approximately 10 ng/ml. The pathway involved in implantation was also not significantly enriched between the two groups suggesting a relatively similar effect on the endometrium between the two groups. A pathway involved in ECM-receptor interaction and focal adhesion should be highlighted as progesterone-related pathways, as they were significantly enriched in both main analysis and subgroup analysis between normal progesterone group and the group with progesterone levels of 3-6 ng/ml.

A cut-off value of mid-luteal progesterone remains uncertain due to limited evidence. The threshold of 15 ng/ml was taken from a study by Basnayake et al. (2018) that reported compromised pregnancy rate in women with mid-luteal progesterone lower than 15.7 ng/ml (Basnayake et al., 2018). Although it is a large study recruiting a total of 4582 participants, we acknowledge that the reliability of the exact threshold for a natural cycle is limited due to inclusion of women undergoing both natural and artificial frozen-thawed embryo transfer cycles. However, Hull et al. (1982) showed a mid-luteal progesterone threshold of approximately 10 ng/ml in pregnant patients following unstimulated cycles (Hull et al., 1982). Due to the uncertainty of the mid-luteal progesterone threshold, subgroup analysis will provide more detail for transcriptomic analysis. In this study, progesterone samples were taken in the morning, according to clinical practice, when concentrations have been reported to reached a peak (Filicori et al., 1984, Syrop and Hammond, 1987). Therefore, we considered that the threshold of 10 ng/ml and the higher cut-off value of 15 ng/ml is reasonable to carry out the differential gene expression analysis.

After comparison our gene list to other studies, we found differentially expressed genes that are strongly associated with decidualisation and implantation. Our result suggests that SPP1, IL15, DKK1, CLDN4, BCL6, MT1H, and MT1G are endometrial receptivity-related genes that were regulated under the progesterone effect. CILP, CRYAB, CYP4B1, DKK1, KLF4, MAMDC2, and SLIT3 are progesterone-dependent genes that typically over-representative during the implantation window. Noticeably, DKK1 was present in both two comparisons and also a decidualisation-related gene (Lucas et al., 2020) highlighting the importance of DKK1 during implantation. DKK1 is normally upregulated during the implantation window (Díaz-Gimeno et al., 2011), low progesterone group in our study allows significantly downregulation of DKK1 expression possibly resulting in a negative effect on implantation. CXCL13, which is the most highly expressed gene in this study, it is progesterone-dependent genes (Young et al., 2017) and also involved in endometrial receptivity (Díaz-Gimeno et al., 2011). CXCL13 is associated with regulation of immune response (Hannan and Salamonsen, 2007, Salamonsen et al., 2007) and tissue invasion (Franasiak et al., 2015, Dominguez et al., 2008). The

expression of CXCL13 was significantly down-regulated in women with implantation failure in comparison to controls (Li et al., 2017). CXCL13 is also down-regulated in the low progesterone group in our study suggesting the potential impairment of endometrial receptivity in this group.

Our results showed that optimal endometrial transcriptomics is achieved under a sufficient amount of progesterone. One-fourth of recurrent implantation failure (RIF) is caused by the displacement of the window of implantation, plausibly resulting from suboptimal progesterone levels (Ruiz-Alonso et al., 2013). The endometrial biopsy was obtained to perform the ERA test on the receptive period of cycle (day LH+7 in a natural cycle or day P+5 in an artificial cycle) and the results were grouped into pre-receptive, receptive and post-receptive (Díaz-Gimeno et al., 2013). Based on the ERA test that showed non-receptive results, it is divided into pre-receptive (85.0%) or post-receptive (12.6%) for endometrial tissue assessed during the receptive phase (Katzorke et al., 2016). It has been shown that a suboptimal progesterone level is associated with delayed endometrial maturation and abnormal patterns of gene expression (Young et al., 2017). On the other hand, excess progesterone results in the advancement of endometrial maturation (Van Vaerenbergh et al., 2011). Consequently, abnormally low progesterone levels inadequate to achieve the receptive stage of endometrium would be expressed as a pre-receptive stage which is the majority of the non-receptive group and excess progesterone could result in post-receptive expression. The personalised embryo transfer according to the ERA result has a potential to improve pregnancy rates in women with RIF who have non-receptive results (Hashimoto et al., 2017, Ruiz-Alonso et al., 2013), suggesting the receptive state of endometrium could be achieved by abnormally low or high progesterone however the timing of implantation is delayed or advanced. Collectively, abnormal progesterone levels are associated with the displacement of the window of implantation, therefore embryo transfer typically performed at the presumed receptive phase will not be successful. Progesterone monitoring appears to be useful and it might also integrate with a genomic diagnostic tool to assess endometrial receptivity in the future. However, there was a lack of evidence for a certain level of upper limit of progesterone concentration during the mid-luteal phase. Too high

progesterone levels could have a detrimental effect on endometrial receptivity, further study is required to figure out the upper limit of progesterone level during the receptive period of menstrual cycle.

Variation in progesterone production could be expected among different menstrual cycles of the same woman and among different women. Intercycle variation (within-women) is considerably lower than interindividual variation as intercycle variance accounted for approximately 20-30% of the total variance (intercycle variation and interindividual variation) in luteal phase progesterone (Lenton et al., 1983, Sukalich, 1994, Gann et al., 2001). Due to low intercycle variation, progesterone measurement is highly predictable and reproducible between cycles (Chatterton et al., 2005). With regard to the daytime variation in a cycle, we acknowledge the limitation in our study and the single point progesterone measurement as it was previously reported that progesterone is secreted in pulses and fluctuate depending on LH pulsatile release over the course of a day, with highest concentrations in the morning (0800-1000hrs) with little fluctuation thereafter (Filicori et al., 1984); (Syrop and Hammond, 1987). The authors also conclude that the effect of clinically significant fluctuations is minimised by the use of timed sampling (Syrop and Hammond, 1987). In this work, we limit the mid-luteal progesterone timed sampling to 8.00 -10.00 AM to minimise the variation and to allow a reasonable comparison between the 12 samples reported.

Our results suggest that the cut-off value of mid-luteal progesterone at the threshold of 15 ng/ml, could be lowered to 10 ng/ml in the natural cycle. Frozen-thawed embryo transfer could be performed in either a natural cycle or artificial hormonally-controlled cycles. It is challenging whether this threshold is acceptable in an artificial cycle. In the artificial cycle, exogenous oestrogen inhibits ovulation and progesterone supplementation is given to replace progesterone physiologically produced by the corpus luteum in the natural cycle. The recent and largest retrospective study in 2018 (Basnayake et al., 2018) recruiting a total of 4582 participants undergoing either artificial or natural frozen-thawed embryo transfer cycles proposed the cut-off value at 15.7 ng/ml and another prospective study by Labarta et al., which recruited only artificial cycle, showed the thresholds at 9.2 ng/ml (Labarta et al., 2017). The most

recent retrospective study in 2019 also showed improved pregnancy rates in women who have a relatively similar progesterone level to the previous study at 10 ng/ml (Cédrin-Durnerin et al., 2019). Those studies demonstrated comparable results with our study ranging from approximately 10-15 ng/ml. The route of progesterone should be taken into consideration as the different routes of administration allow different serum concentrations of progesterone, for example, vaginal progesterone administration yields lower serum progesterone levels in comparison to intramuscular progesterone (Cicinelli and De Ziegler, 1999). Vaginal progesterone was given in all three studies suggesting that vaginal progesterone is able to replace endogenous progesterone from the corpus luteum at the levels of approximately 10-15 ng/ml in the artificial cycle. In cases of suboptimal progesterone level, the decision of the day of embryo transfer would be changed to either the different day in the same cycle or defer to another cycle where the optimal progesterone levels are obtained. However, due to small sample size in our study, further larger studies investigating the effect of progesterone levels on both endometrial transcriptomics and pregnancy outcomes in multiple cycles for both natural and frozen-thawed cycles are needed to confirm the predictive value of progesterone monitoring.

Conclusion

Suboptimal mid-luteal progesterone concentrations below 10-15 ng/ml are associated with aberrant expression of endometrial genes regulating processes such as extracellular matrix remodelling, decidualisation, and embryo-endometrial adhesion during the implantation window. Therefore, mid-luteal progesterone monitoring might be useful to predict implantation potential, however, future clinical studies are needed to ensure its clinical benefit.

Declaration of Competing Interest

The authors declare no conflicts of interest related to this manuscript.

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Authors' contributions

WEM designed the study. LP, NR collected and processed the endometrial biopsies. SS, MC, and SM performed the experiments, processed the experimental data and performed the analysis. SS, WM, JHM, KW, JT and KJ assisted in the final interpretation of the results and writing the manuscript.

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