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ARTICLE

Assessing the effect of adipose-tissue-derived stem cell conditioned medium on follicles and stromal cells in bovine ovarian tissue culture

BIOGRAPHY

Francisco Vitale earned his medical degree in 2016, and completed his residency in gynaecology and obstetrics in 2021 at Hospital Universitario Austral (Argentina). He is currently a PhD researcher at the Université Catholique de Louvain (Belgium). His work focuses on developing strategies to improve the outcomes of ovarian follicles cultured *in vitro*.

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KEY MESSAGE

Follicular outcomes following in-vitro culture of human ovarian tissue remain suboptimal, mainly showing poor development and viability rates. This study showed that the addition of adipose-tissue-derived stem cell conditioned medium to culture enhances follicle survival, development and oestradiol production, and the viability of stromal cells.

ABSTRACT

Research question: Does adipose-tissue-derived stem cell conditioned medium (ASC-CM) supplementation enhance follicle and stromal cell outcomes *in vitro*?

Design: Bovine ovaries ($n = 8$) were sectioned and cultured *in vitro* for 8 days in two different groups: (i) standard culture (OT Ctrl D8); and (ii) culture with ASC-CM supplementation (OT + CM D8). Half of the culture medium was replaced every other day, and stored to measure the production of oestradiol. Follicle classification was established using haematoxylin and eosin staining. Follicle and stromal cell DNA fragmentation was assessed by TUNEL assays, while growth differentiation factor-9 (GDF-9) staining served as a marker of follicle quality. Additionally, three factors, namely vascular endothelial growth factor (VEGF), interleukin 6 (IL-6) and transforming growth factor beta 1 (TGF-β1), were evaluated in ASC-CM in order to appraise the potential underlying mechanisms of action of ASC.

Results: The OT + CM D8 group showed a significantly higher proportion of secondary follicles ($P = 0.02$) compared with the OT Ctrl D8 group. The OT + CM D8 group also demonstrated significantly lower percentages of TUNEL-positive follicles ($P = 0.014$) and stromal cells ($P = 0.001$) compared with the OT Ctrl D8 group. Furthermore, follicles in the OT + CM D8 group exhibited a significant increase ($P = 0.002$) in expression of GDF-9 compared with those in the OT Ctrl D8 group, and oestradiol production was significantly higher ($P = 0.04$) in the OT + CM D8 group. All studied factors were found to be present in ASC-CM. VEGF and IL-6 were the most widely expressed factors, while TGF-β1 showed the lowest expression.

Conclusions: Addition of ASC-CM to culture medium enhances follicle survival, development and oestradiol production, and promotes the viability of stromal cells. VEGF, IL-6 and TGF-β1 could be paracrine mediators underlying the beneficial effects.

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KEY WORDS

In-vitro development
In-vitro growth
Mesenchymal stem cells
Ovarian tissue culture
Bovine ovarian follicles

INTRODUCTION

In-vitro culture (IVC) of ovarian tissue has emerged as a promising technique to develop mature metaphase II oocytes from the pool of primordial follicles in the ovarian cortex (*Telfer and Andersen, 2021*; *Telfer et al., 2023*). This approach avoids reimplantation of ovarian tissue, which is of great benefit to patients at high risk of ovarian metastases, such as blood-borne malignancies or ovarian cancers (*Dolmans et al., 2010*, *2013*). However, the outcomes of follicles developed in-vitro remain suboptimal, with poor follicle survival and development (*McLaughlin et al., 2018*; *Yang et al., 2020a*). Despite extensive efforts to optimize IVC conditions, studies have shown that only a limited proportion of primordial follicles are capable of progressing to the next growth stage without any significant damage, and the majority face death or growth arrest (*McLaughlin et al., 2018*; *Telfer et al., 2008*).

Intraovarian folliculogenesis encompasses a complex network of cell-to-cell communication and signalling pathways (*Cecconi et al., 2012*; *Craig et al., 2007*), so IVC conditions that best resemble in-vivo milieu are likely to generate less cellular distress and yield better outcomes. In recent years, numerous researchers have attempted to enhance IVC results through the use of different culture additives. It has been documented that cell-based co-culture systems may replicate the intraovarian microenvironment by releasing various paracrine factors (*Tagler et al., 2012*; *Tingen et al., 2011*; *Yang et al., 2020b*). The ability of adipose-tissue-derived stem cells (ASC), a type of mesenchymal stem cell, to promote cell proliferation, survival and tissue regeneration (*Al-Ghadban et al., 2021*; *Tsuji et al., 2014*) via secretion of a wide range of growth factors and cytokines encapsulated within exosomes (i.e. small extracellular vesicles that serve as carriers for cell-to-cell communication) is well known in the field of regenerative medicine (*Cacciottola et al., 2021*; *Kapur and Katz, 2013*; *Manavella et al., 2018*; *Trzyna and Banaś-Ząbczyk, 2021*). Supernatant from ASC culture, known as conditioned medium (CM), contains secreted exosomes and hence exerts the same beneficial effects as the original cells (*Bhang et al., 2014*). Indeed, the use of CM as opposed to cellular components has several advantages (*Gunawardena et al., 2019*; *Pawitan, 2014*). First, it avoids the possibility of two different types of cells competing for nutrients in the culture medium. Moreover, CM can be easily collected, stored and standardized, ensuring consistent and reproducible outcomes. Finally, CM circumvents any concerns associated with unwanted cell differentiation, which may arise when using stem cells in co-culture systems (*Sagaradze et al., 2019*).

In the field of regenerative medicine, it has been widely reported that cytokines and growth factors encapsulated within ASC-derived exosomes, including vascular endothelial growth factor (VEGF), interleukin 6 (IL-6) and transforming growth factor 1 (TGF-β1), promote a proliferative and anti-apoptotic effect on cultured cells (*Al-Ghadban et al., 2021*; *Trzyna and Banaś-Ząbczyk, 2021*). Besides its proangiogenic effects, VEGF has been described as a key factor involved in early follicle activation and development *in vitro*, mainly via the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway (*Irusta et al., 2010*; *Yang and Fortune, 2007*). Moreover, IL-6 has been associated with numerous biological follicle functions including promotion of in-vitro proliferation of granulosa cells and inhibition of cell apoptosis (*Tingen et al., 2011*). The role of TGF-β1 has been widely reported in the regulation of folliculogenesis (*Knight and Glister, 2006*). Indeed, TGF-β1 is known to stimulate proliferation and differentiation of granulosa cells in IVC systems (*Fazzini et al., 2006*).

The main objective of this study was to determine whether the addition of CM from ASC to culture medium could improve the survival and development of ovarian follicles, and the viability of stromal cells in a bovine ovarian tissue IVC system. A secondary objective was to determine the presence of certain paracrine factors (VEGF, IL-6 and TGF-β1) in ASC-CM that may be involved in proliferation and anti-apoptosis cell mechanisms.

MATERIALS AND METHODS

Culture and passage of ASC

Previously characterized human ASC from female donors were acquired commercially (Stempro; Invitrogen, USA). They were subjected to IVC and passaged as described by *Manavella et al. (2018)*. Briefly, ASC were seeded at a density of 5×10^3 cells/cm^2, and cultured at 37°C in 5% CO_2 in MesemPRO RS medium (Gibco, USA) with 2% MesemPRO growth supplement (Gibco) and 1% L-glutamine (Gibco). After achieving 80–90% confluency, the cells were detached and passaged by enzymatic digestion using Accutase (Sigma-Aldrich, USA). All ASC used in the current study were between passages 3 and 4.

Preparation of ASC-CM

Upon reaching 80–90% confluency, the culture medium was replaced completely with serum-free alpha-modified Eagle's medium (α-MEM; Gibco) supplemented with 2 mg/ml glucose (Sigma, USA), and then cultured for a further 72 h at 37°C in 5% CO_2. This period of time was chosen to maximize the obtention of paracrine factors secreted by ASC. The collected supernatant was centrifuged at 1200 *g* for 5 min and filtered twice using a 0.22-μm filter, before being aliquoted and stored at -80°C.

ASC-CM analyses

Concentrations of VEGF (Cat: ab222510; Abcam, UK), IL-6 (Cat: ab178013; Abcam) and TGF-β1 (Cat: BMS249-4; Invitrogen) were measured in ASC-CM by enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions. All experiments were carried out in duplicate. Sensitivity, intra-assay and interassay coefficients of variation are detailed in Supplementary Table 1.

Ovarian tissue processing and IVC

Fresh bovine ovaries (n = 8) were obtained from slaughtered animals (12–18 months) at the time of evisceration, and transported to the laboratory within 30 min in Leibovitz medium (Gibco) tubes covered in ice. Only normal-sized (4 × 2 × 2 cm), oval-shaped ovaries without visible cysts were used in the experiment. Upon arrival, the ovaries were sectioned into thin strips and any surplus medulla was removed using 10 ml pre-warmed dissection medium containing Leibovitz medium (Gibco) supplemented with 2 mM sodium pyruvate (Sigma), 2 mM glutamine (Sigma), 3 mg/ml bovine serum albumin (BSA; Sigma), 50 μg/ml penicillin G (Sigma) and 50 μg/ml streptomycin (Sigma). They were subsequently cut into fragments of 4 × 2 × 1 mm. One cortical fragment per sample served as a non-cultured control (D0) and was fixed immediately, while two fragments were placed in four-well culture dishes filled with 400 μl of pre-warmed culture medium

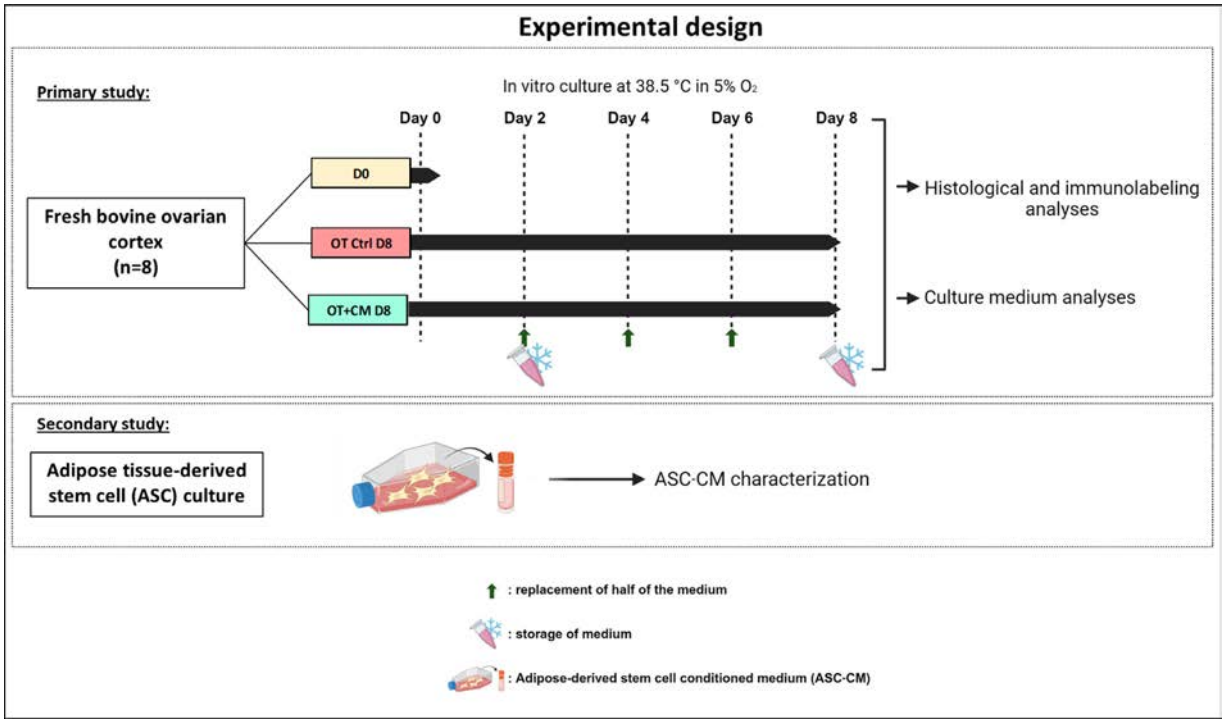

FIGURE 1 Experimental design. Primary study: Fresh bovine ovaries (n = 8) were fragmented into cortical fragments and cultured for 8 days in two different groups: ovarian tissue cultured alone (OT Ctrl D8); and ovarian tissue with conditioned medium (CM) derived from adipose-tissue-derived stem cells (ASC) (OT + CM D8). One cortical fragment per ovary served as the non-cultured control (D0). All cortical fragments were collected for histological and immunolabelling analyses. Half of the culture medium was replaced every other day, and medium from days 2 and 8 was stored at -80°C for future analysis. Secondary study: ASC-CM was analysed to identify and measure paracrine factors associated with proliferative and anti-apoptotic effects.

composed of α-MEM supplemented with 25 mM HEPES (Gibco), 1 mg/ml BSA (Sigma), 3 mM glutamine (Sigma), 30 μg/ml penicillin G (Sigma), 50 μg/ml streptomycin (Sigma), 5 μg/ml transferrin (Fisher Scientific), 5 ng/ml selenium (Sigma), 50 μg/ml ascorbic acid (Sigma), 10 ng/ml insulin (Sigma) and 1 ng/ml human FSH (Gonal F; Merck Serono, UK). IVC was then carried out in two different groups: (i) ovarian tissue cultured alone (OT Ctrl D8); and (ii) ovarian tissue with ASC-CM (OT + CM D8). The OT + CM D8 group contained a combination of standard culture medium and ASC-CM at a 1:1 ratio from the beginning of culture. Every other day, half of the culture medium from both groups was changed. On days 2 and 8, retrieved culture medium was stored at -80°C for further analysis. A schematic design of the experiment is shown in FIGURE 1.

Both IVC groups remained in identical conditions for 8 days at 38.5°C in a low oxygen environment (5% O_2) using a hypoxic subchamber incubator (ProOx C21; BioSpherix, USA). In-vitro culture in physiological oxygen concentrations has previously shown an improvement in follicular viability (*Vitale et al., 2023*).

Sample processing

Ovarian fragments from all groups were fixed in 4% formaldehyde, embedded in paraffin and cut serially into 5-μm sections. Haematoxylin and eosin (H&E; Merck, USA) staining was performed on every eighth section to assess follicle density and classification. Sections in between underwent immunolabelling analyses. All slides were digitized using the Panoramic P250 Flash III scanner (3DHistech, Hungary) and analysed by CaseViewer (v2.3).

Histological evaluation

Follicle density and stage were investigated on five H&E-stained tissue sections per sample. To mitigate the possibility of counting twice, only follicles containing a visible oocyte were examined. Atretic follicles were separated from viable follicles if they showed ooplasm eosinophilia, pyknosis of granulosa cells and/or cytoplasmic contraction (*Gougeon and Chainy, 1987*). Follicle density was determined as the number of viable follicles per mm^3, taking section thickness into account. Follicles were only classified if they were viable according to developmental stage: (i) primordial (oocyte surrounded by a single layer of flattened granulosa cells; (ii) primary (oocyte surrounded by a single layer of cuboidal granulosa cells); and (iii) secondary (oocyte surrounded by two or multiple layers of cuboidal granulosa cells). The proportion of follicles at each stage was then established. Follicle and oocyte diameters of secondary follicles were assessed according to *Kitajima et al. (2014)*. For each measurement, two perpendicular diameters were recorded, and the average of these values was calculated (*Kitajima et al., 2014*).

Immunohistochemistry

Growth differentiation factor-9 (GDF-9) immunolabelling was used to evaluate follicle competence, as this factor has been reported to play a crucial role in promoting the early stages of folliculogenesis (*Hussein et al., 2006*). Four tissue sections per sample were analysed. Briefly, after blocking endogenous peroxidase activity with 3% H_2O_2 and demasking in citrate buffer (pH 6) for 75 min at 98°C, non-specific binding sites were blocked by incubation in 10% fetal bovine serum for

30 min. Sections were first incubated overnight with mouse anti-GDF-9 primary antibodies (1:1000, AB-325-AG012, AnshLab, RRID:AB_2756501), and then with EnVision anti-mouse HRP-conjugated secondary antibodies (1:2, K4001, Dako, RRID:AB_2827819). Human testicular cancer served as the positive control (Supplementary Figure 1), and mouse immunoglobulin G (Dako) served as the negative control. Slides were digitized using the Pannoramic P250 Flash III scanner (3DHistech), and growing follicles (primary and secondary) were examined for GDF-9 analysis. Using ImageJ software (http://rsb.info.nih.gov/ij/), growing follicles were digitally isolated from adjacent stromal cells with the 'clear outside' tool option. A colour threshold was then determined to separate GDF-9 signalling from the rest. After setting the scale of the image, the GDF-9-stained area and total area of each follicle were calculated in μm^2 and expressed as a percentage of GDF-9 staining.

TUNEL immunofluorescence

DNA fragmentation was analysed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assays using the In Situ Cell Death Detection Kit, TMR red (Roche, Switzerland), as described by *Vanacker et al. (2012)*. Four tissue sections per sample were analysed. Briefly, after pretreatment with 20 mg/ml proteinase K working solution (Roche) in 10 mM Tris-HCl for 30 min at 37°C, sections were incubated with a TUNEL reaction mixture: 50 ml enzyme solution (terminal deoxynucleotidyl transferase) and 450 ml label solution (nucleotide mixture in reaction buffer) for 60 min at 37°C. Finally, the slides were incubated in 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories, USA) and covered with a fluorescent mounting medium (Enzo, USA). DNAse was used to damage DNA in positive control sections (Supplementary Figure 1), while negative controls were incubated with a label without enzyme solution. The slides were digitized using the Panoramic P250 Flash III scanner (3DHistech), and analysed with CaseViewer (v2.3). Follicles were classified as TUNEL-positive when the oocyte or ≥50% of granulosa cells were stained by TUNEL (*Amorim et al., 2012*). Results were presented as the percentage of TUNEL-positive follicles out of the total follicles. For stromal cell analysis, three areas showing TUNEL-positive stromal cells were examined at high magnification. The percentage of TUNEL-positive cells over TUNEL-negative (stained by DAPI alone) cells was calculated using ImageJ software (http://rsb.info.nih.gov/ij/).

Oestradiol production

Oestradiol concentrations were measured in culture medium obtained from both IVC groups (OT Ctrl and OT + CM) on days 2 and 8 using an ELISA kit (Cat: ab108667; Abcam) in accordance with the manufacturer's instructions. All experiments were conducted in duplicate.

Statistics

GraphPad Prism software for Windows (v9.2) was used to conduct statistical analyses. One-way analysis of variance (ANOVA) was applied, followed by Fisher's least significant difference (LSD) post-hoc test. The paired *t*-test was used to analyse oestradiol production. Data expressed as a proportion were analysed after logit transformation, and results have been expressed as mean ± SD. A *P*-value <0.05 was considered to indicate significance.

RESULTS

Follicle outcomes

Follicle density, classification and morphology

In total, 1197, 807 and 1007 viable follicles were counted in the D0 group, the OT Ctrl D8 group, and the OT + CM D8 group, respectively. A significant decrease in follicle density was observed in both IVC groups (OT Ctrl D8: $P = 0.002$; OT + CM D8: $P = 0.010$) compared with the D0 group. However, the OT + CM D8 group exhibited significantly higher ($P = 0.025$) follicle density compared with the OT Ctrl D8 group (FIGURE 2A). Regarding follicle classification, there was a significant decrease in the proportion of primordial follicles in both IVC groups (OT Ctrl D8: 57.50% ± 5.37%, $P < 0.001$; OT + CM D8: 51.63% ± 4.80%, $P < 0.001$) compared with the D0 group (78.63% ± 3.29%), but a significantly higher proportion were detected in the OT Ctrl D8 group ($P = 0.033$) compared with the OT + CM D8 group. In terms of the proportion of primary follicles, significant increases were encountered in both IVC groups (OT Ctrl D8: 39.13% ± 4.91%, $P < 0.001$; OT + CM D8: 41.50% ± 5.18%, $P<0.001$) compared with the D0 group (18.88% ± 3.87%). The proportion of secondary follicles was significantly higher in the OT + CM D8 group (6.87% ± 3.27%) compared with the OT Ctrl D8 group (3.62% ± 3.20%, $P = 0.028$) and D0 group (2.37% ± 1.40%, $P = 0.003$) (FIGURE 2B). Concerning the proportion of atretic follicles between the two IVC groups, significantly lower values were obtained in the OT + CM D8 group (9.25% ± 3.41%, $P = 0.002$) compared with the OT Ctrl D8 group (23.75% ± 6.54%) (FIGURE 2C). Follicle and oocyte diameters were measured in morphologically normal secondary follicles. Twenty-four follicles were analysed in the D0 group, 32 in the OT Ctrl D8 group, and 60 in the OT + CM D8 group. No significant differences in follicle or oocyte diameters were observed between the groups (TABLE 1).

GDF-9 staining of follicles

In total, 177, 223 and 233 growing follicles were analysed in the D0 group, OT Ctrl D8 group and OT + CM D8 group, respectively. The OT + CM D8 group yielded the highest percentage of GDF-9 staining (22.80% ± 3.38%), and this differed significantly from the OT Ctrl D8 (16.38% ± 2.25%, $P = 0.001$) and the D0 (14.14% ± 2.16%, $P<0.001$) groups. The OT Ctrl D8 group displayed a significantly higher ($P = 0.033$) percentage of GDF-9 staining compared with the D0 group (FIGURE 3A,B).

DNA fragmentation

Follicles. In total, 448, 192 and 259 follicles were evaluated in the D0 group, the OT Ctrl D8 group and the OT + CM D8 group, respectively. The proportion of TUNEL-positive follicles was significantly higher in both IVC groups (OT Ctrl D8: 24.49% ± 6.36%, $P < 0.001$; OT + CM D8: 16.76% ± 7.53%, $P < 0.001$) compared with the D0 group (2.26% ± 2.11%). However, a significantly lower proportion was found in the OT + CM D8 group ($P = 0.014$) compared with the OT Ctrl D8 group (FIGURE 4A).

Stromal cells. The proportion of TUNEL-positive stromal cells was significantly higher in both IVC groups (OT Ctrl D8: 28.98% ± 4.88%, $P < 0.001$; OT + CM D8: 18.05% ± 3.48%, $P < 0.001$) compared with the D0 group (0.27% ± 0.11%). The OT + CM D8 group showed a significantly lower ($P = 0.001$) proportion compared with the OT Ctrl D8 group (FIGURE 4B).

Oestradiol production. In the group cultured with ASC-CM (OT + CM), there was a significant increase in oestradiol

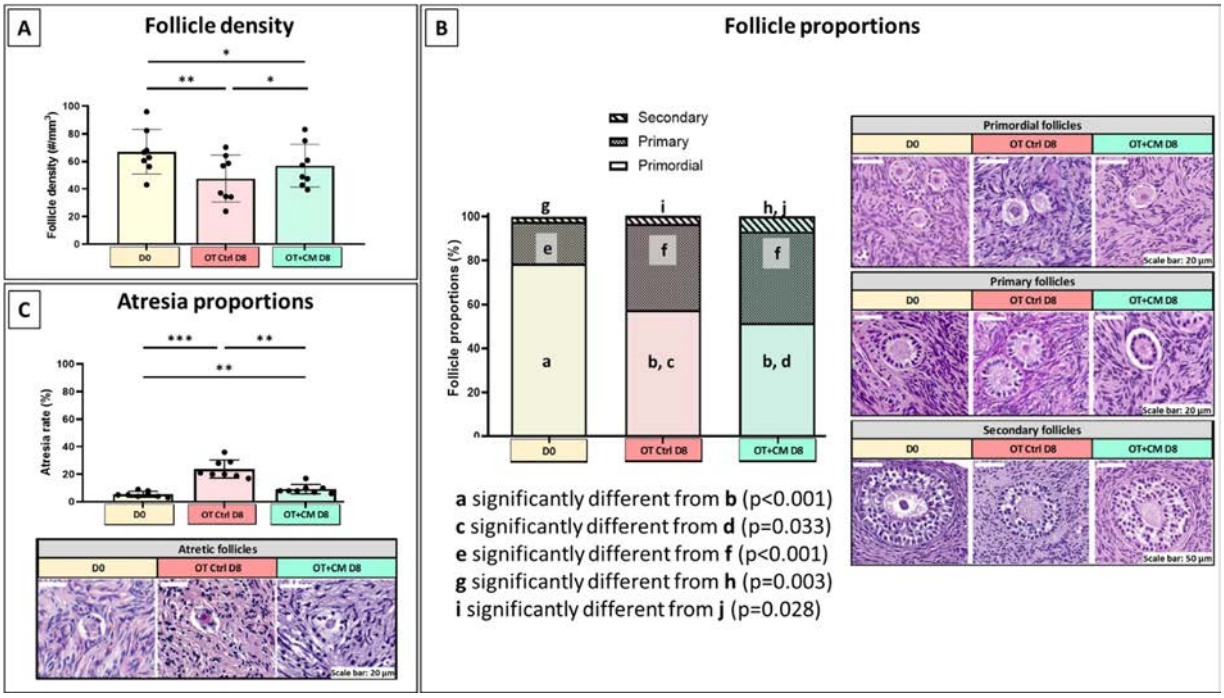

FIGURE 2 Density and proportion of follicles and proportion of atretic follicles. (A) Follicle density (no. of viable follicles/mm^3, mean ± SD) analysed by one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) post-hoc test [**P = 0.002; *P = 0.010 (D0 versus OT + CM D8); P = 0.025 (OT + CM D8 versus OT Ctrl D8)]. (B) Proportion of follicles (percentage, mean ± SD) assessed by one-way ANOVA and Fisher's LSD post-hoc test, and representative photomicrographs of each stage of follicule development, namely primordial (oocyte surrounded by a single layer of flattened granulosa cells), primary (oocyte surrounded by a single layer of cuboidal granulosa cells) and secondary (oocyte surrounded by two or multiple layers of cuboidal granulosa cells). (C) Proportion of atretic follicles (percentage, mean ± SD) evaluated by one-way ANOVA and Fisher's LSD post-hoc test [***P < 0.001; **P = 0.008 (D0 versus OT + CM D8); P = 0.002 (OT + CM D8 versus OT Ctrl D8)], and representative photomicrographs of atretic follicles in each group. n = 8 ovaries per group. D0, non-cultured controls; OT Ctrl, ovarian tissue cultured alone; OT + CM, ovarian tissue with conditioned medium derived from adipose-tissue-derived stem cells.

production by day 8 (84.11 ± 41.76 pg/ml, P = 0.022) compared with day 2 (57.02 ± 43.55 pg/ml). Furthermore, on day 8 of IVC, the OT + CM group yielded a significantly higher concentration of oestradiol compared with the OT Ctrl group (57.30 ± 27.41 pg/ml, P = 0.046) (FIGURE 5). The concentration of oestradiol was also measured in ASC-CM before being added to the OT + CM group, which displayed values below detectable levels (data not shown).

ASC-CM characterization and analyses. Concentrations of VEGF, IL-6 and TGF-β1 were measured in ASC-CM. Among studied factors, VEGF (531.41 ± 37 pg/ml) and IL-6 (545.43 ± 11.31 pg/ml) were the most highly expressed, while TGF-β1 showed values of 63.25 ± 2.10 pg/ml. In standard culture medium, the concentrations of these factors were below detectable levels (data not shown).

TABLE 1 SECONDARY FOLLICLE AND OOCYTE DIAMETERS

	D0[a] (24 follicles)	OT Ctrl D8[b] (32 follicles)	OT + CM D8[c] (60 follicles)	*P*-value
Follicle diameter (μm)	86.28 ± 27.84	88.92 ± 29.69	96.20 ± 34.04	a,b: 0.754 a,c: 0.261 b,c: 0.394
Oocyte diameter (μm)	40.17 ± 14.45	44.64 ± 16.54	47.14 ± 19.28	a,b: 0.252 a,c: 0.087 b,c: 0.635

Data are mean ± SD.

Assessed by one-way analysis of variance and Fisher's least significant difference post-hoc test.

D, day; D0, non-cultured controls; OT Ctrl: ovarian tissue cultured alone; OT + CM, ovarian tissue with conditioned medium derived from adipose-tissue-derived stem cells.

DISCUSSION

This study demonstrates that the addition of ASC-CM to bovine ovarian tissue IVC medium enhances follicle survival, development and oestradiol secretion, and promotes the viability of stromal cells.

Supplementation of ASC-CM to culture medium had a positive influence on follicle stage progression, as shown by the lower proportion of primordial follicles and higher proportion of secondary follicles in the OT + CM D8 group compared with the OT Ctrl D8 group. Early-stage follicle development is a gonadotrophin-independent process, and requires local coordination of activating factors from somatic cell–oocyte bidirectional crosstalk (*Li and Albertini, 2013*). It was reported recently that CM from ASC contains various biomolecules, which could

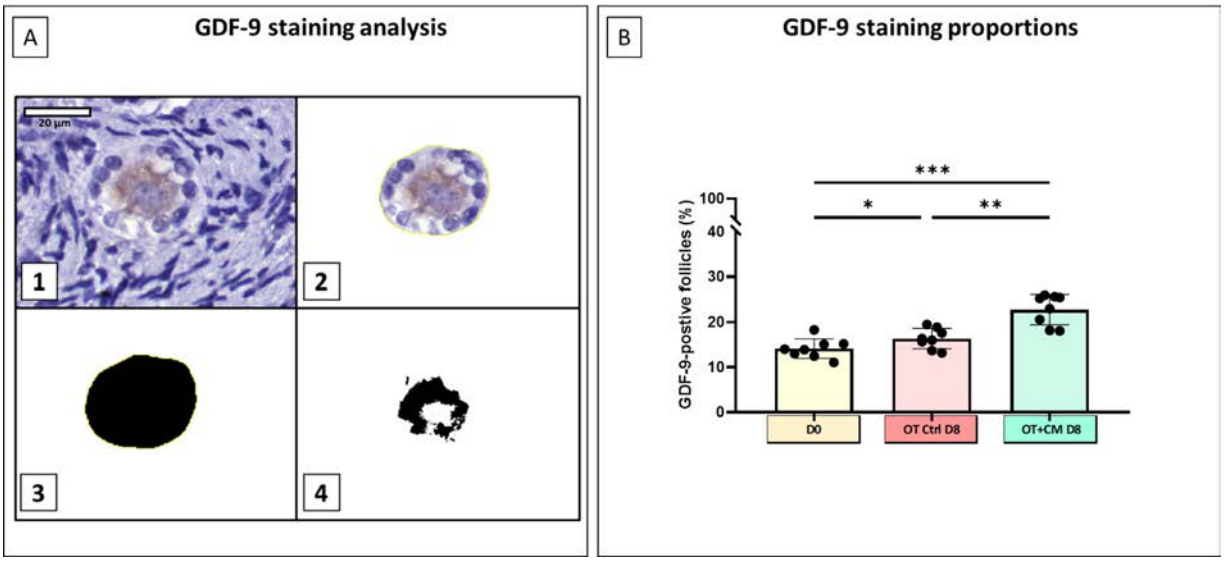

FIGURE 3 Analysis of growth differentiation factor-9 (GDF-9) staining and proportion of follicles stained. (A) GDF-9 staining analysis. (1) Growing (primary) follicle surrounded by stromal cells. Scale bar = 20 μm. (2) Digital follicle isolation from surrounding cells. (3) Measurement of total follicle area. (4) Colour deconvolution and measurement of GDF-9 area within the follicle. (B) Proportion of follicles stained with GDF-9 (percentage, mean ± SD) assessed by one-way analysis of variance and Fisher's least significant difference post-hoc test (***$P < 0.001$; **$P = 0.001$; *$P = 0.033$). $n = 8$ ovaries per group. D0, non-cultured controls; OT Ctrl, ovarian tissue cultured alone; OT + CM, ovarian tissue with conditioned medium derived from adipose-tissue-derived stem cells.

modulate follicle activation signalling pathways (*Cacciottola et al., 2023*; *Jiao et al., 2022*; *Tomaszewski et al., 2019*). In line with the current findings, *Hosseini et al. (2019)* detected an increased rate of follicle development in human ovarian cortical fragments co-cultured with mesenchymal stem cells for 8 days compared with ovarian cortex cultured alone.

Despite the significant increase in the proportion of secondary follicles in the OT + CM D8 group in this study, there were no significant differences in the diameters of secondary follicles or oocytes between any of the groups (**TABLE 1**). Only secondary follicles were considered in this analysis, as they represent the main follicle

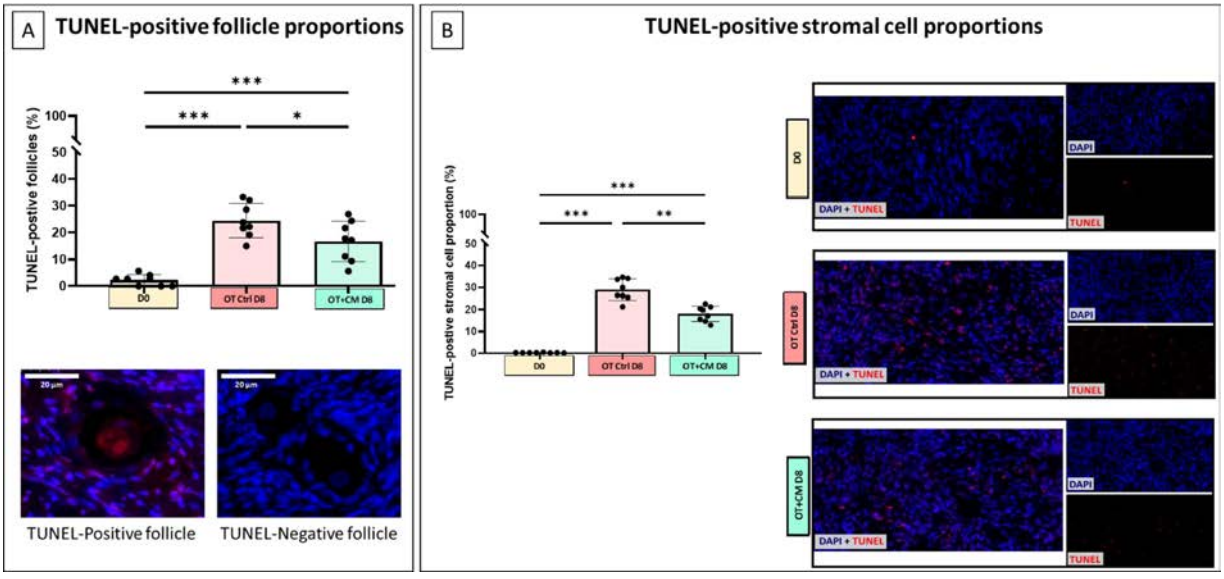

FIGURE 4 Proportions of TUNEL-positive follicles and stromal cells. (A) Proportion of TUNEL-positive follicles (percentage, mean ± SD) analysed by one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) post-hoc test (***$P < 0.001$; *$P = 0.014$). Representative photomicrographs of TUNEL-positive and TUNEL-negative follicles. TUNEL (red), DAPI (blue). Scale bar = 20 μm. (B) Proportion of TUNEL-positive stromal cells (percentage, mean ± SD) assessed by one-way ANOVA and Fisher's LSD post-hoc test (***$P < 0.001$; **$P = 0.001$). Representative photomicrographs of TUNEL-positive and TUNEL-negative (stained by DAPI alone) stromal cells in each group. TUNEL (red), DAPI (blue). $n = 8$ ovaries per group. D0, non-cultured controls; OT Ctrl, ovarian tissue cultured alone; OT + CM, ovarian tissue with conditioned medium derived from adipose-tissue-derived stem cells.

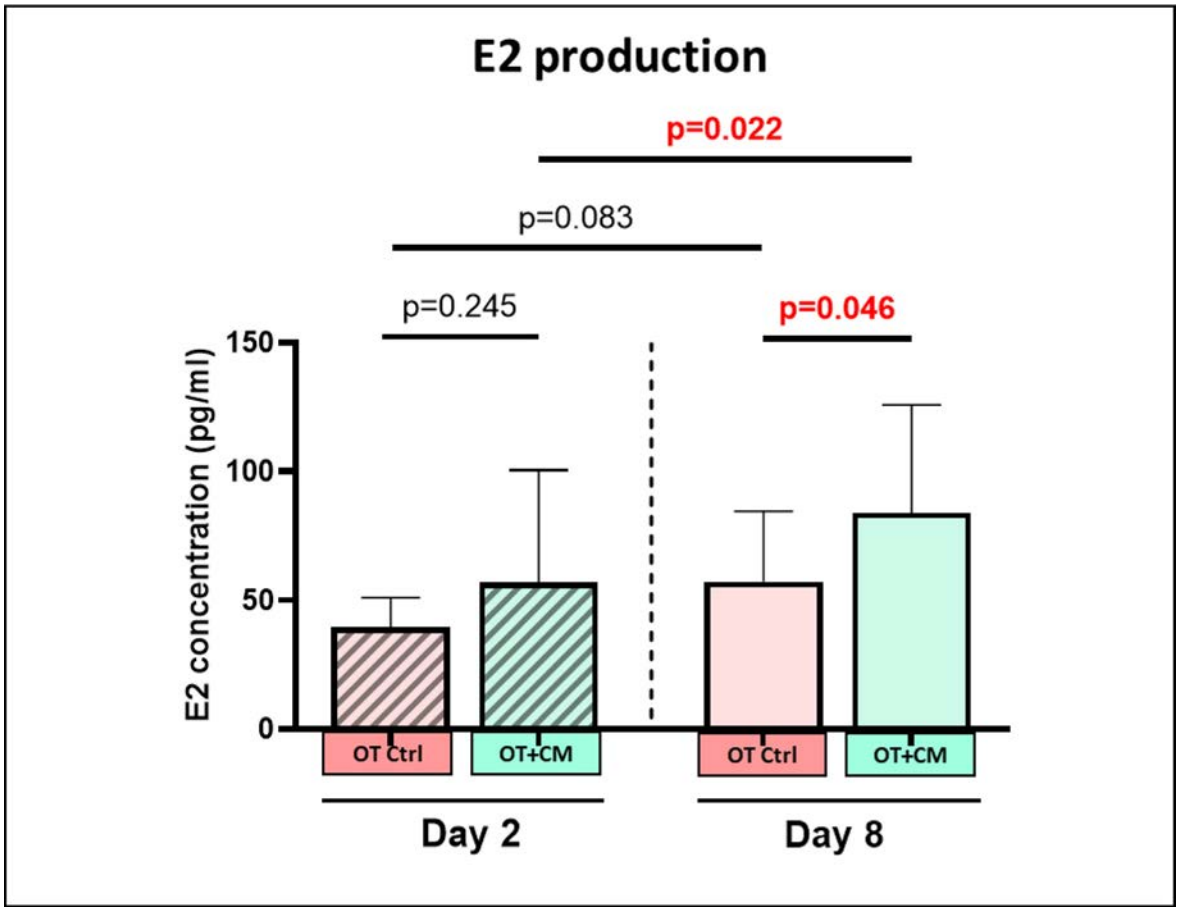

FIGURE 5 Production of oestradiol (E2; pg/ml, mean ± SD) analysed by paired *t*-test. $n = 8$ ovaries per group. OT Ctrl, ovarian tissue cultured alone; OT + CM, ovarian tissue with conditioned medium derived from adipose-tissue-derived stem cells.

outcome following the follicle activation step (after 8 days of IVC). However, it is important to emphasize that follicle growth is a slow process *in vitro*, requiring several cycles of replication of granulosa cells (*Braw-Tal and Yossefi, 1997*). Indeed, previous research from *Itoh and Hoshi (2000)* identified a substantial increase in the diameter of bovine follicles when co-cultured with mesenchymal stem cells alone after 30 days of culture. It is therefore possible that the culture period used in the present study (8 days) may not have been long enough to elucidate discernible differences. Something else to consider is that once follicles reach the secondary stage in the multistep IVC system (*McLaughlin et al., 2018*), they should be isolated from the surrounding ovarian cortex and cultured further to continue their development. It is likely that secondary follicles in the present study had reached their growth limit due to the influence of surrounding ovarian cells.

In recent years, it has been acknowledged that oocyte-derived factors from the TGF-β superfamily, particularly GDF-9, regulate follicle stage transition through distinct functions, including growth and expansion of adjacent granulosa cells (*Hreinsson et al., 2002*; *Orisaka et al., 2006*). GDF-9 is currently identified as a key indicator of follicle quality and competence (*Hussein et al., 2006*). Upon activation, the oocyte of the recruited follicle starts secreting GDF-9, which directly triggers the proliferation of granulosa cells, causing follicle stage transition through the developing phases (*Gilchrist et al., 2008*; *Li and Albertini, 2013*). In the present study, the percentage of GDF-9 staining was significantly higher in growing follicles (primary and secondary) in the OT + CM D8 group compared with the OT Ctrl D8 group. Based on these findings, paracrine factors contained within ASC-CM were able to promote follicle progression via stimulation of oocyte-derived factor GDF-9. In line with these results, *Xia et al. (2015)* reported increased expression of GDF-9 in isolated human preantral follicles co-cultured with mesenchymal stem cells.

ASC-CM supplementation boosted the proportion of morphologically viable follicles (non-atretic) compared with IVC without ASC-CM. As stated above, only viable follicles were taken into account to calculate follicle density. It is well known that follicle density decreases during ovarian tissue IVC, which was evidenced in the present study. However, the data demonstrated that follicle density was significantly higher in the OT + CM D8 group compared with the OT Ctrl D8 group. Similar results were obtained by *Sousa et al. (2021)* when investigating the impact of goat ovarian tissue co-cultured with mesenchymal stem cells over 7 days. These differences may be explained by the pro-survival properties of ASC-derived paracrine factors (*Ai et al., 2023*; *Cacciottola et al., 2023*; *Song et al., 2017*). In order to further explore the mechanisms behind cell death, DNA fragmentation was evaluated by TUNEL assays. The OT + CM D8 group yielded a lower proportion of TUNEL-positive follicles compared with the OT Ctrl D8 group. Consistent with the present results,

Green et al. (2019) reported higher viability of mouse follicles co-encapsulated with mouse ASC compared with follicles cultured alone. Similarly, another study demonstrated a significant decrease in the rate of follicle apoptosis when CM from mesenchymal stem cells was added to standard IVC medium (Jia et al., 2017).

The current study also demonstrated a significant improvement in the viability of ovarian stromal cells in the OT + CM D8 group compared with the OT Ctrl D8 group. These specialized cells are known for their role in orchestrating essential functions within the ovarian microenvironment. They provide signalling cues that facilitate proper folliculogenesis (Kinnear et al., 2020; Telfer et al., 2023), offer structural and biochemical support (Ouni et al., 2019), and regulate immune responses and inflammation through release of cytokines (Zhang et al., 2020). Compromised viability of stromal cells could disrupt these intricate signalling networks, potentially leading to impaired follicle development and reduced oocyte quality.

Steroid hormones such as oestradiol have proved vital to the in-vitro development of bovine preantral follicles, enabling oocytes to acquire the necessary competence as they transition through the growth stages (Chauvin et al., 2022; Drummond, 2006). Granulosa cells and theca cells of growing follicles secrete oestradiol, and this production increases proportionally with follicle stage progression and proliferation of granulosa cells (Tajima et al., 2006). As expected, medium collected at the end of IVC from the OT + CM D8 group had a significantly higher concentration of oestradiol compared with the OT Ctrl D8 group, reflecting the higher proportion of secondary follicles in the OT + CM D8 group. These results confirm the findings of Park et al. (2021), who reported that the expression of two key steroidogenic genes, namely aromatase and steroidogenic acute regulatory protein, was significantly higher in isolated mouse granulosa cells after being cultured with CM from mesenchymal stem cells.

In the OT + CM group, tissue was cultured in a medium comprising 50% standard culture medium and 50% ASC-CM (1:1 ratio). This strongly implies that the paracrine factors present in ASC-CM provide a greater benefit than the compounds found in the standard culture medium for cultured ovarian tissue.

Subsequent studies could assess whether varying the proportions of ASC-CM and standard culture medium leads to additional modifications in follicle outcomes. Furthermore, a broader proteomics analysis could contribute to better insight into the factors contained in ASC-CM.

As described in earlier studies (Borgese et al., 2020; de Souza et al., 2020; Tomaszewski et al., 2019), the present study found notable concentrations of VEGF, IL-6 and TGF-β1 in ASC-CM. Besides its proangiogenic effects, VEGF has been shown to be a strong cell survival factor (Byrne et al., 2005; Kosaka et al., 2007). By activating the PI3K/Akt pathway, VEGF has been described as a key factor involved in early follicle activation and development (Irusta et al., 2010; Yang and Fortune, 2007). VEGF receptors have been found to be expressed in bovine follicles (Greenaway et al., 2004), and the concentration of VEGF mRNA increased gradually through the growth stages (Yang and Fortune, 2007). Yang and Fortune (2007) also demonstrated that addition of VEGF to culture medium increased the proportion of secondary follicles in a dose-dependent manner in bovine cortical fragments cultured for 10 days. Studies have also shown that various interleukins, such as IL-6, are involved in ovarian folliculogenesis (Tomaszewski et al., 2019). IL-6 receptors were found mainly in granulosa cells and theca cells of developing bovine follicles (Samir et al., 2017). This multifunctional interleukin has been associated with numerous biological activities, including proliferation of granulosa cells and inhibition of cell apoptosis (Tingen et al., 2011). Previously, it has been proposed that IL-6 induces anti-apoptotic gene expression (B-cell lymphoma 2 gene family) (Karaoz et al., 2010). In addition, the role of TGF-β1 has been widely reported in the regulation of folliculogenesis (Knight and Glister, 2006). Oocytes produce TGF-β1, which is critical for oocyte–granulosa cell communication, formation of transzonal projections and intercellular metabolic cooperation (Chen et al., 2015). Indeed, TGF-β1 is known to stimulate proliferation and differentiation of granulosa cells in IVC systems in cows (Fazzini et al., 2006), and several TGF-β receptors (TGFBR1, TGFBR2 and TGFBR3) have been documented in granulosa cells of bovine follicles (Matiller et al., 2019). In addition, TGF-β1 plays an important role in the formation of extracellular matrix and secretion of its components. TGF-β1 supplementation has been found to promote collagen production and boost proliferation of chicken granulosa cells *in vitro* (Zhou et al., 2021).

The current study has both strengths and limitations. One of its strengths lies in the assessment of follicle development *in vitro* from different aspects, such as the proportion of secondary follicles, GDF-9 expression and oestradiol production, which adds robustness to the results. Additionally, the findings successfully demonstrate the beneficial impact of ASC-CM on both cell compartments, namely ovarian follicles and stromal cells. In recent years, an increasing body of evidence has highlighted the essential role of stromal cells in folliculogenesis. On the other hand, the main limitation of the study is that only three paracrine factors were assessed in ASC-CM. Further proteomics studies on ASC-CM are currently being studied by the authors' team to encompass novel target factors, and improve understanding of the protective effects it exerts on ovarian tissue cultured *in vitro*.

In conclusion, this study demonstrates that the addition of ASC-CM to bovine ovarian tissue IVC medium enhances follicle survival, development and oestradiol secretion, and promotes the viability of stromal cells. ASC-derived paracrine factors (VEGF, IL-6 and TGF-β1) associated with pro-survival and anti-apoptotic effects were identified in CM. Overall, these findings on the beneficial role of ASC-CM on IVC outcomes for ovarian tissue offer a firm foundation for further investigations into optimal culture conditions.

DATA AVAILABILITY

Data will be made available on request.

AUTHOR CONTRIBUTIONS

F.V.: experimental design, experimental procedures, analyses, statistical analysis, interpretation of results and article preparation. L.C.: experimental design, statistical analysis and interpretation of results. A.C: experimental design and interpretation of results. L.H: analyses and interpretation of results. J.D.: interpretation of results, article preparation and revision. M.M.D.: experimental design, interpretation of

results, and article preparation and revision.

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SUPPLEMENTARY MATERIALS

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RBMO

ELSEVIER

ARTICLE

The outcome of tissue cryopreservation on the cellular, molecular and epigenetic characteristics of endometrial tissue and stromal cells

BIOGRAPHY

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KEY MESSAGE

Endometrial tissue cryopreservation led to an up-regulation of some pluripotency and *DNMT* gene expressions, and down-regulation of hsa-miR145-5p. Nevertheless, cells from fresh and cryopreserved tissue exhibit very similar profiles related to cellular properties and histone modifications. In general, this study demonstrates that both fresh and cryopreserved tissues may be suitable for further medical and cell therapy research.

ABSTRACT

Research question: What impact does the cryopreservation of endometrial tissue have on cell characteristics and molecular and epigenetic profile changes in endometrial tissue and stromal cells?

Design: Cellular properties, such as proliferation efficiency, surface marker expression and the differentiation potency of endometrial stromal cells (ESC) isolated from fresh (Native) and cryopreserved (Cryo) tissue were compared. Moreover, changes in the expression of genes associated with pluripotency, endometrial function and epigenetic regulation and microRNA (miRNA, miR) were assessed, as were levels of DNA methylation and histone modifications.

Results: Native and Cryo cells exhibit very similar profiles including cell surface marker expression, differentiation potency and histone modifications, except for a decrease in proliferative potency and cell surface marker SUSD2 expression in Cryo cells. It was demonstrated that endometrial tissue cryopreservation led to an up-regulated expression of genes associated with pluripotency (*NANOG, OCT4* [also known as *POU5F1*]). This confirms that despite being recovered from cryopreserved differentiated tissue, cells retained their stemness properties. In addition, alterations in DNA methyltransferase (*DNMT1, DNMT3A, DNMT3B*) gene regulation were observed, along with a down-regulation of hsa-miR145-5p in Cryo ESC.

Conclusions: These findings contribute to a deeper understanding of the complex effects of endometrial tissue cryopreservation, providing insights for both medical and basic research applications. Since different tissues possess unique characteristics, it is essential to select the most suitable cryopreservation method for each tissue individually. Furthermore, the study findings indicate the potential utility of slow-cooling cryopreservation for both normal and pathological endometrial tissue samples, with the purpose of isolating stromal cell cultures.

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KEYWORDS

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Histone modification
microRNA

INTRODUCTION

Ensuring access to viable human tissues is crucial for many medical applications and advancing research with a focus on personalized care. The long-term preservation of biological samples can be achieved using very low temperatures, with biological and chemical reactions being dramatically reduced during this process, which is called cryopreservation (*Jang et al., 2017*). The cryopreservation of reproductive system material such as gametes, embryos and tissues is especially widely used in reproductive medicine (*Canose et al., 2023*). It can ensure the constant availability of cryopreserved materials that could be used off the shelf as needed, and cryopreserved cells and tissues are also a common part of basic research applications. Therefore, a deeper understanding of the impact of cryopreservation on sample quality, assessing the molecular, genetic and epigenetic changes, needs to be properly taken into account. Therefore, this study aimed to establish the main characteristics at the cellular and molecular/epigenetic levels of cells isolated from either fresh (Native) or cryopreserved (Cryo) tissue.

The two most popular tissue cryopreservation protocols are slow cooling with a lower concentration of the cryoprotectant agent (slow freezing) and fast cooling with a higher concentration of the cryoprotectant agent (vitrification). Both techniques have advantages and disadvantages; however, it has recently been demonstrated that slow freezing is more suitable for mucosal tissues such as cervico-vaginal and colorectal tissue compared with vitrification, which dramatically decreased the cell recovery rate from thawed tissue (*Hughes et al., 2018*). Therefore, in the present study, on the basis of Hughes and colleagues' findings (*Hughes et al., 2018*), the slow cooling method was chosen as the cryopreservation protocol.

To prevent intracellular ice formation, which could be detrimental to cell viability, cell-penetrating cryoprotectant agents, such as dimethyl sulfoxide (DMSO), are used (*Best, 2015*). An analysis of the effect of DMSO on equine endometrial tissue viability after cryopreservation revealed that, compared with a fresh tissue control, tissue viability was decreased in the absence of DMSO, while there was no difference in viability when DMSO was added during cryopreservation. Moreover, it has been shown that cryopreservation using a cryoprotectant did not impact the gene expression of oestrogen and progesterone receptors (*Thompson et al., 2019*).

A previously published study analysing cervical tissue demonstrated that the viability of explants of cryopreserved-thawed samples was not affected compared with native tissue (*Fox et al., 2017*). Moreover, stromal cells isolated from cryopreserved endometrial tissue exhibit a similar response to hormone stimuli as do stromal cells from fresh tissue (*Heidari-Khoei et al., 2022*). Additionally, cells isolated from thawed tissue do not lose their potency to organize into two- and three-dimensional structures (*He et al., 2020*) or organoids that also respond to hormonal stimulation (*Bui et al., 2020*; *Heidari-Khoei et al., 2022*). Recently, the current authors have demonstrated that the cryopreservation of placental tissue leads to slight variations in the epigenetic profile as well as the up-regulation of genes associated with an early-senescence state in isolated mesenchymal stromal cells (MSC) (*Navakauskiene et al., 2023*). A recent study by Baušytė and colleagues, using isolated stromal cells from non-cryopreserved endometrial tissue and their potential clinical application, demonstrated the beneficial therapeutical effects of such cells in an in-vivo model (*Baušytė et al. (2023)*.

When working with biological samples derived from patients, immediate use is not always possible, and cryopreservation is a favourable solution for long-term storage before application. However, despite recent studies demonstrating that cryopreservation does not have a detrimental effect on the properties of thawed tissue, the effect of cryopreservation on endometrial epigenetics has not yet been assessed. Therefore, the aim of this study was to investigate whether the cryopreservation of endometrial tissue could induce alterations on a molecular level in cells isolated from these tissues with the hope of providing recommendations for further cell therapy applications.

This study assessed the endometrial tissue outcomes of a standard cryopreservation protocol in terms of molecular changes, focusing on epigenetic changes such as histone modification, DNA methylation and microRNA (miRNA, miR) levels. It was shown that cryopreservation can lead to several differences in the cellular and molecular aspects of isolated stromal cells, including a slight decrease of proliferation efficiency in later passages (from passages 10 to 15), a reduction in the percentage of SUSD2+ (sushi domain containing 2) cells, an increment of *DNMT* gene expression and suppressed levels of hsa-miR145-5p compared with native tissue cells. Nevertheless, a significance of these changes for the general characteristics of stromal cells could not be observed.

MATERIALS AND METHODS

Tissue sample collection

Endometrial tissue samples were obtained from women undergoing IVF as part of assisted reproductive treatment at Vilnius University Hospital Santaros Klinikos Obstetrics and Gynaecology Center Santaros Fertility Center. All participating patients signed informed consent for their involvement in this research. The endometrial biopsy procedures were conducted between days 17 and 22 of the menstrual cycle in preparation for application of assisted reproductive technology. This study received approval from the Vilnius Regional Biomedical Research Ethics Committee under the reference number 158200–18/7–1049–550, issued 3 July 2018, and approval for this research extension was signed on 5 November 2019.

Tissue cryopreservation, thawing and endometrial stromal cell isolation

Freshly collected tissue samples were employed for two purposes: direct isolation of endometrial stromal cells (ESC) or cryopreservation. For both of these approaches, endometrial tissues were washed with phosphate-buffered saline (Genaxxon bioscience, Germany) and cut into pieces measuring 2–3 mm^2. In the cryopreservation process, the cut pieces were placed into cryogenic vials. A cryopreservation medium, composed of 90% fetal bovine serum (Gibco, USA) and 10% DMSO (Sigma-Aldrich, USA), was added to the vials containing the tissue pieces. Subsequently, the cryogenic vials were incubated overnight at −80°C before being transferred to a liquid nitrogen container, where they were stored at temperatures ranging from −196°C to −210°C. After a period of storage in cryopreservation, the thawing process was initiated by transferring cryogenic vials from a liquid nitrogen container to a 37°C

water bath for 3 min, and then ESC were isolated as described elsewhere (*Bausyte et al., 2023*; *Tavakol et al., 2018*).

Fresh samples for native tissue analysis proceeded straight to ESC isolation. Briefly, washed endometrial samples were cut and incubated with collagenase I for 60 min. After incubation, the sample was filtered through a 70 μm cell strainer and the cells that passed through were collected. Isolated ESC were seeded into culture flasks and left to grow to confluence in Dulbecco's Modified Eagle Medium/F12 media supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Genaxxon bioscience, German) at 37°C under 5% CO_2.

Assessment of proliferation and differentiation

To assess cell proliferation the cells were evaluated by staining with Trypan blue and calculating the number of cells in every passage using a haemacytometer. The cumulative population doubling level (PDL) was determined using the following formula:

$$\begin{aligned} PDL(x) = {} & 3.32(\log [\text{Total viable cells at harvest}]/ \\ & [\text{Total viable cells at seed}]) \\ & + PDL\ (x - 1\,\text{passage}) \end{aligned}$$

For all subsequent analyses, only cells from the early passages (3–6 passages) were used. ESC obtained from both fresh and cryopreserved tissues were subjected to differentiation into adipogenic, osteogenic and chondrogenic lineages following the previously described protocol (*Navakauskiene et al., 2023*). The lineage-specific properties of the differentiated cells were observed using the EVOS XL Cell Imaging System (Thermo Fisher Scientific, USA).

Flow cytometry

To detect the cell surface markers, Native and Cryo cells were analysed using flow cytometry. Cell preparation followed previously established procedures (*Zentelyte et al., 2021*). The anti-human antibodies used in this study were as follows: CD31 (BioLegend catalogue no. 303110 RRID:AB_493074), CD34 (Cell Signaling Technology catalogue no. 79253, RRID:AB_2799925), CD44 (EXBIO Praha catalogue no. 1F-221-T100, RRID: AB_10735534), CD73 (BioLegend catalogue no. 344015, RRID:AB_2561808), CD90 (BioLegend catalogue no. 328108 , RRID:AB_893429), CD105 (BioLegend catalogue no. 323203, RRID:AB_755955), SUSD2 (BioLegend catalogue no. 327408, RRID:AB_2561888), SSEA4 (BioLegend catalogue no. 330420, RRID:AB_2629631), CD140b (BioLegend catalogue no. 323608, RRID:AB_2162787), CD146 (BioLegend catalogue no. 361016, (RRID: AB_2564360), CD166 (BioLegend catalogue no. 343903, RRID:AB_2289303), HLA-ABC (BioLegend catalogue no. 311415, RRID:AB_493134) and HLA-DR (BioLegend catalogue no. 307656, RRID: AB_2564168). Appropriate isotype controls were included in this study.

The analysis was conducted using Guava easyCyte with InCyte 2.2.2 software (Luminex, USA). Ten thousand events were collected per sample. Flowing Software 2 (Turku Bioscience, Finland) was employed for the result analysis. Initially, live cell populations were differentiated from debris based on forward and side scatter properties. Subsequently, live cell populations were chosen for fluorescence analysis. Gates were set for each sample using appropriate isotype controls. Examples are shown in Supplementary Figure 1.

Immunoblot analysis

Proteins were extracted directly from Native or Cryo tissues that were used for ESC isolation or from isolated stromal cells cultured *in vitro* for a few passages, following the methodology outlined in a previous study (*Savickiene et al., 2014*). Protein amounts in samples were determined using a Bradford Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The samples underwent fractionation in a 7.5–15% gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane and the membrane was then blocked with 5% milk solution by incubation for 1 h with agitation. After blocking, the membrane was placed in a specific primary antibody solution and incubated overnight at 4°C.

The primary antibodies used in this analysis, with dilutions performed according to the manufacturer's suggestions, were: GAPDH (Abcam catalogue no. ab8245, RRID:AB_2107448), H3 (Millipore catalogue no. 06-755, RRID: AB_11211742), H4 (Millipore catalogue no. 07-108, RRID:AB_2279758), EZH2 (Thermo Fisher Scientific catalogue no. PA5-29129, RRID:AB_2546605), H3K4me3 (Millipore catalogue no. 05-745R, RRID:AB_1587134), H3K9me3 (Millipore catalogue no. 07-442, RRID:AB_310620), H3K27me3 (Millipore catalogue no. 07-449, RRID:AB_310624), H4hyperAc (Millipore catalogue no. 06-946, RRID:AB_310310), H3K9Ac (Cell Signaling Technology catalogue no. 9649, RRID:AB_823528), HDAC1 (Santa Cruz Biotechnology catalogue no. sc-81598, RRID:AB_2118083) and HDAC2 (Santa Cruz Biotechnology catalogue no. sc-9959, RRID:AB_627704). HRP-conjugated secondary antibodies against mouse and rabbit (Advansta, USA) immunoglobulins were applied.

Chemiluminescence was detected using the WesternBright Quantum detection kit (Advansta, USA), and signal detection was carried out on a ChemiDocXRS+ System (BIO-RAD, USA). GAPDH and histones H3 and H4 served as the loading control, and a quantitative assessment of the detected protein band density was conducted using ImageJ software (National Institutes of Health, USA).

Total RNA extraction and quantitative reverse transcription PCR

Total RNA was isolated from either endometrial tissues or cultured cells employing the Quick-DNA/RNA Miniprep Kit (Zymo Research, USA) following the manufacturer's guidelines, and the RNA purity and concentration were measured using the NanoPhotometer (Implen, Germany). Subsequently, up to 1 μg of RNA was subjected to reverse transcription, generating complementary DNA using the LunaScript RT SuperMix Kit (New England Biolabs, USA). Quantitative PCR (qPCR) was conducted with the Luna Universal qPCR Master Mix (New England Biolabs, USA) on a Rotor-Gene 6000 thermocycler using Rotor-Gene 6000 series software (Corbett Life Science, QIAGEN, Germany). The geometric mean of the cycle threshold (C_T) values of *GAPDH* and *RPL13A* were used for normalizing the mRNA levels. Primer sequences are detailed in Supplementary Table 1.

Methylated DNA immunoprecipitation

The extraction of genomic DNA from tissues and cell culture samples was performed using a Quick-DNA/RNA Miniprep Kit (Zymo Research, USA). To achieve DNA fragments ranging from 200 to 500 bp, genomic DNA diluted with TE buffer to reach a concentration of 10–20 ng/μl underwent fragmentation

using a Bioruptor Pico sonication device (Diagenode, Belgium), performing seven cycles of 15 s on and 90 s off.

An amount of 10% of the DNA used for immunoprecipitation was set aside as the total DNA amount used for the immunoprecipitation, denoted as Input DNA. Before immunoprecipitation, DNA samples were denatured by incubating at 95°C for 10 min. A mixture consisting of 320 ng DNA, 0.8 μl of 1 mg/ml anti-5-methylcytosine antibody (DNA:antibody ratio 1:2.5; AnaSpec, EGT Group catalogue no. BI-MECY-0100, RRID: AB_11232821, Eurogentec, Belgium) and 15 μl of ZymoMag Protein A magnetic beads (Zymo Research, USA) was incubated at 37°C for 1 h on a rotator. Immunoprecipitated DNA was magnetically extracted using a magnetic tube rack. The qPCR analysis employed Luna Universal qPCR Master Mix (New England BioLabs, United States) with the Rotor-Gene 6000 system (Corbett Life Science, QIAGEN, Germany). The sequences of the gene promoter and exon-specific primers used for the qPCR analysis are provided in Supplementary Table 2. The percentage of DNA input values for immunoprecipitated DNA samples was calculated according to the following formulas:

$$\Delta C_T = \{(C_T[\text{Input DNA}] - \text{Log}_2 10) - C_T[\text{MeDIP DNA}]\}$$

$$\%\ \text{of Input} = 100\% \times 2^{\Delta C_T}$$

where MeDIP is methylated DNA immunoprecipitation.

miRNA assay

For miRNA expression analysis, RNA underwent reverse transcription using the TaqMan MicroRNA Reverse Transcription Kit and TaqMan MicroRNA Assay (Applied Biosystems, USA). The miRNA assays employed for analysis were hsa-miR29b-3p, hsa-miR145-5p, hsa-miR125b-5p and hsa-miR21-5p. Quantification of miRNA expression levels was performed using the TaqMan™ MicroRNA Assay and TaqMan™ Universal PCR Master Mix II (Applied Biosystems, USA) with the Rotor-Gene 6000 system (Corbett Life Science, QIAGEN, Germany). RNU48 was used as the normalization reference for miRNA levels.

Statistical analysis

GraphPad Prism 8 (GraphPad Software, USA) was employed for statistical analysis. The results are presented as medians with interquartile ranges. To evaluate significant differences between the tested groups, a non-parametric Mann–Whitney *U*-test was applied, with a significance level (α value) set at 0.05.

RESULTS

The results of the analysis demonstrated that both ESC isolated from fresh (Native) and from cryopreserved (Cryo) tissue were able to proliferate during long-term cultivation (evaluated up to 20 passages); however, the proliferation efficiency of the Cryo cells slightly decreased compared with Native cells after the 10th passage (FIGURE 1A). However, for all subsequent analyses, cells from only the early passages (3–6 passages) were used.

Native and Cryo ESC were also characterized by the surface marker expression of typical mesenchymal stem/stromal cell markers (CD44, CD73, CD90, CD105, CD166, SSEA4, HLA-ABC) and markers used for characterizing ESC (CD140b, CD146, SUSD2). Native and Cryo cells exhibited a very similar expression of the evaluated cell surface markers, the only significant change observed being in the SUSD2 level, which significantly decreased in Cryo cells ($P = 0.0012$) (FIGURE 1B). Also, cells from both sources had low or no expression of endothelial and haematopoietic cell markers (CD31, CD34, HLA-DR).

The potential of cell differentiation toward mesenchymal lineages (adipogenic, osteogenic, chondrogenic) was also tested, and it was demonstrated that both cell types (Native and Cryo) were capable of tri-lineage differentiation (FIGURE 1C). Nevertheless, in the chondrogenic differentiation assay, Cryo cells formed a few smaller structures instead of one larger structure. Despite a decrease in proliferation potential during the later cultivation phase of Cryo cells, Native and Cryo cells exhibit a very similar profile of cell surface marker expression and differentiation potential.

After the characterization of Native and Cryo cells, gene expression analysis was undertaken to identify potential changes that could be the result of the cryopreservation process. This involved comparing mRNA directly isolated from Native and Cryo tissues, or from cultivated ESC isolated from these tissues (Native and Cryo cells, respectively) (FIGURE 2).

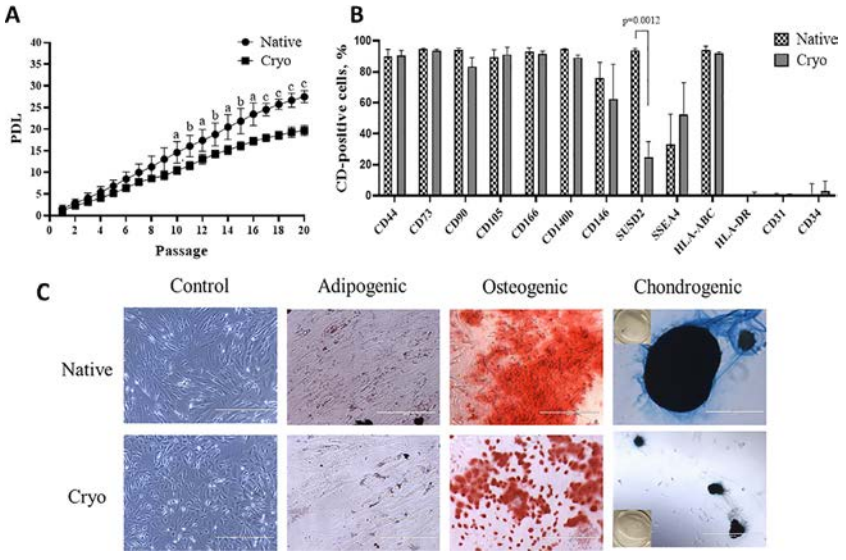

FIGURE 1 Comparison of the characteristics of endometrial stromal cells isolated from non-cryopreserved (Native) and cryopreserved (Cryo) tissues. (A) Proliferation potential of Native and Cryo cells during cultivation *in vitro*. The graph represents the population doubling level (PDL). Results are presented as medians with interquartile ranges ($n = 3$). (B) Analysis of cell surface marker expression of Native and Cryo cells using flow cytometry. Results are presented as medians with interquartile ranges ($n = 3$). (C) Efficiency of differentiation into adipogenic, osteogenic and chondrogenic lineages of Native and Cryo cells. The results are visualized after staining with Oil Red O, Alizarin Red S and Alcian Blue, and representative images are provided (scale bars for control and osteogenic differentiation = 400 μm, adipogenic differentiation = 100 μm, and chondrogenic differentiation = 1000 μm). Statistical significance was determined using the Mann–Whitney *U*-test: (a)– $P = 0.0357$; (b) $P = 0.0179$; (c) $P = 0.0286$.

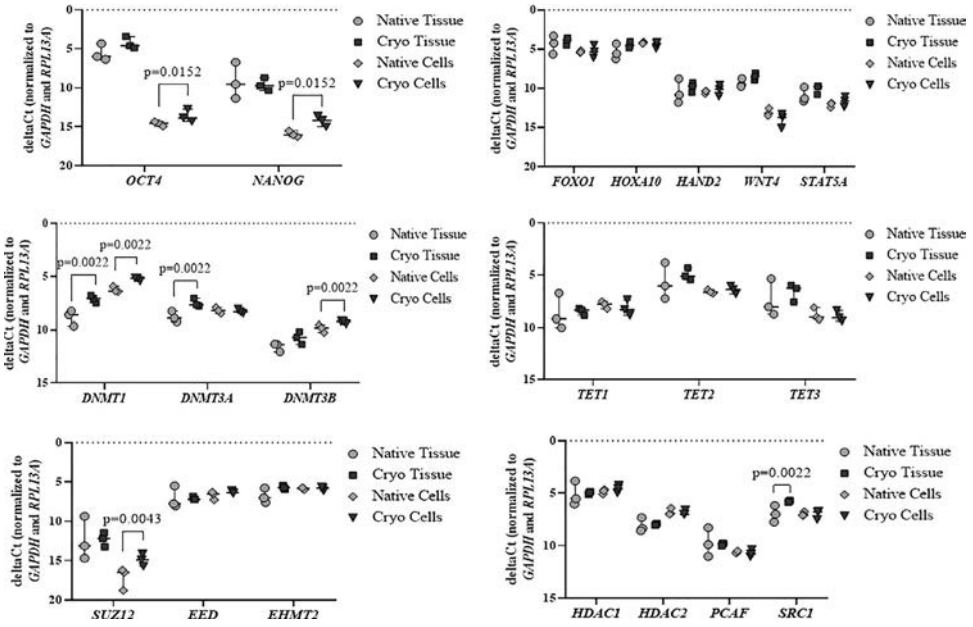

FIGURE 2 Comparison of gene expression levels in non-cryopreserved (Native) and cryopreserved (Cryo) tissues, as well as Native and Cryo cells. The gene expression levels were normalized using the geometric mean of *GAPDH* and *RPL13A*. Graphs are presented with the *y*-axis inverted for better visualization, and higher delta cycle threshold (C_t) values correspond to lower gene expression. The middle line in the graphs represents the median, ($n = 3$). Statistical significance was determined using the Mann–Whitney *U*-test.

Gene expression analysis revealed that the expression of the pluripotency markers *OCT4* and *NANOG* was significantly increased in Cryo cells compared with Native cells (in both cases $P = 0.0152$).

Moreover, an increase in the gene expression levels of DNA methyltransferases was detected in the Cryo samples. Elevated *DNMT1* expression was observed in both cases when comparing tissues and cells ($P = 0.0022$). Meanwhile, a significant increase of *DNMT3A* was detected only when comparing Cryo and Native tissues ($P = 0.0022$), and in the case of *DNMT3B*, a significant elevation was detected in Cryo cells compared with Native cells ($P = 0.0022$). Furthermore, a significant elevation of *SUZ12* mRNA levels in Cryo cells compared with Native cells ($P = 0.0043$), and a significant elevation of *SRC1* in Cryo tissue compared with Native tissue ($P = 0.0022$), was determined.

The analysis did not reveal significant changes in the expression of genes related to endometrial function (*FOXO1, HOXA10, HAND2, WNT4, STAT5A*), DNA demethylation (*TET1, TET2, TET3*) and other genes associated with histone modifications (*EED, EHMT2, HDAC1, HDAC2, PCAF*) that could be the result of tissue cryopreservation. In summary, these findings demonstrated the gene expression changes induced by cryopreservation, emphasizing its impact on key pluripotency markers and DNA methyltransferases, while highlighting the stability of the expression of certain genes related to endometrial function and other epigenetic regulatory mechanisms such as DNA demethylation and histone modifications.

In the next stage of evaluating the impact of endometrial tissue cryopreservation, the levels of histone modifications associated both with active and repressed chromatin were analysed, as were the levels of epigenetic factors catalysing these modifications (FIGURE 3). The results revealed that the cryopreservation of endometrial tissue did not have a significant impact on either the selected histone modifications (H3K4me3, H3K9Ac, H4hyperAc, H3K9me3, H3K27me3), on histone deacetylases (HDAC1, HDAC2) or on isoforms of the Polycomb suppressive complex 2 subunit (EZH2α, EZH2p). These findings suggest that histone modifications associated both with active and repressed chromatin states remain unaffected during the endometrial tissue cryopreservation process, as well as after the isolation of cells from cryopreserved tissue.

As the gene expression analysis revealed that cryopreservation impacts the expression of genes encoding DNA methyltransferases, a subsequent evaluation of DNA methylation levels in the promoter and exon regions of genes associated with endometrial function was conducted (FIGURE 4). It was seen that the DNA methylation level was significantly down-regulated in the *GAPDH* promoter site in Cryo cells compared with Native cells ($P = 0.0348$). A similar tendency of down-regulation in Cryo cells compared with Native cells was also observed in the *FOXO1* and *STAT5A* promoters, and in the positive DNA methylation control *H19* site, although this was not statistically significant ($P = 0.1$). Moreover, the DNA methylation level of the *HAND2* promoter site was down-regulated in Cryo tissue compared with Native tissue ($P = 0.0143$), while in both the *HAND2* and *HOXA10* exon regions a decrease in methylation was detected when comparing Cryo and Native cells ($P = 0.0143$ in both cases). These findings suggest that the cryopreservation of endometrial tissue induces a down-regulation of DNA methylation levels in specific genomic regions in cryopreserved tissues and isolated cells, which may be associated with changes in the expression of these genes, but do not fundamentally change the potential functioning of the cells.

The expression levels of miRNA that are important in post-translational regulation in reproduction tissues were analysed to

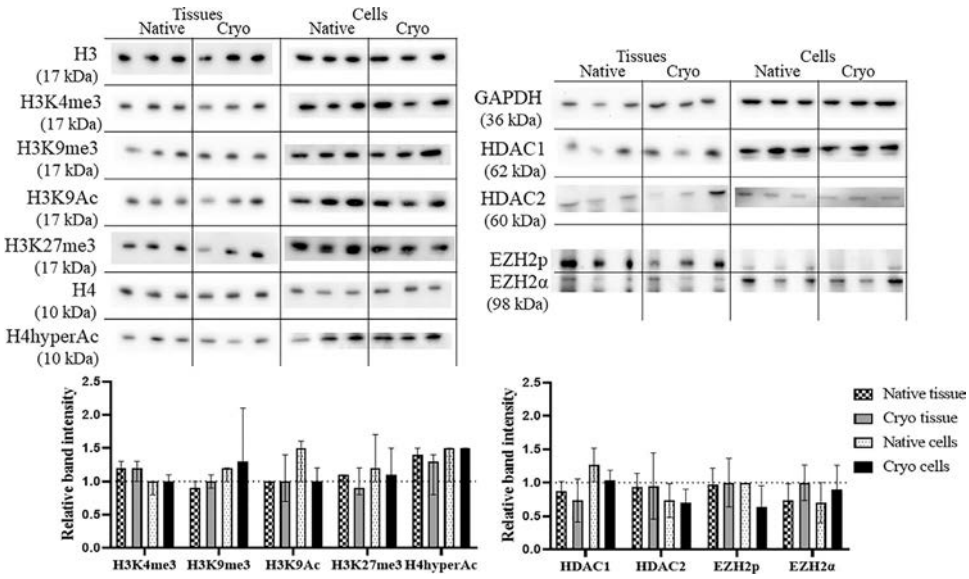

FIGURE 3 Assessment of the levels of histone modifications and their regulators in non-cryopreserved (Native) and cryopreserved (Cryo) tissues, as well as isolated cells. The analysis was conducted by performing an immunoblot analysis. Protein band intensity was measured using ImageJ software. The levels of histone modifications were normalized to histones H3 and H4, while epigenetic regulators were normalized to GAPDH. The results are presented as medians and ranges (n = 3). Statistical significance was determined using the Mann–Whitney U-test.

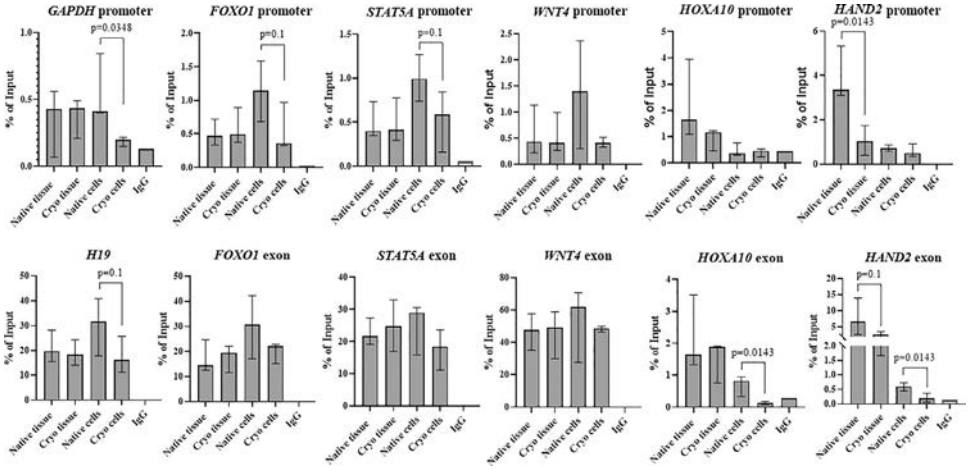

FIGURE 4 DNA methylation levels in the promoter and exon regions of genes associated with endometrial tissue function in non-cryopreserved (Native) and cryopreserved (Cryo) tissues, as well as isolated cells. The results were obtained by methylated DNA immunoprecipitation (MeDIP) followed by quantitative PCR analysis. To assess the efficiency of immunoprecipitation, the study analysed the promoter region of *GAPDH*, which is expected to exhibit a low level of methylation. In contrast, *H19*, a region recognized for its methylated state, served as validation for the successful capture of methylated DNA regions. IgG represents the negative MeDIP test control. Columns show the medians and the error bars represent the ranges (n = 3). Statistical significance was determined using the Mann–Whitney U-test.

assess the impact of cryopreservation (FIGURE 5). The results revealed a significant decrease in the levels of hsa-miR145-5p in both Cryo tissue and Cryo cells compared with their respective Native counterparts (P = 0.0357 and P = 0.0286, respectively). Additionally, the expression changes in hsa-miR29b-3p did not reach statistical significance (P = 0.1). Meanwhile, no changes were observed in the levels of hsa-miR125b-5p and hsa-miR21-5p between the analysed groups. To sum up, the cryopreservation process significantly influences the decrease in hsa-miR145-5p levels. Nevertheless, the functional outcome of this miRNA alteration warrants further investigation.

DISCUSSION

In order to evaluate the suitability of fresh and cryopreserved endometrial tissue as well as isolated stromal cells in further medical and cell therapy research and potential applications, this study investigated and presented findings on the possible influence of Native and Cryo states at the cellular, molecular and epigenetic levels. The findings revealed that the two types of cell exhibited similar characteristics, except for a slight decrease in proliferation efficiency observed in cryopreserved tissue-derived cells in later passages.

There are conflicting results on the proliferation efficiency of cryopreserved MSC or mesenchymal stem cells: some

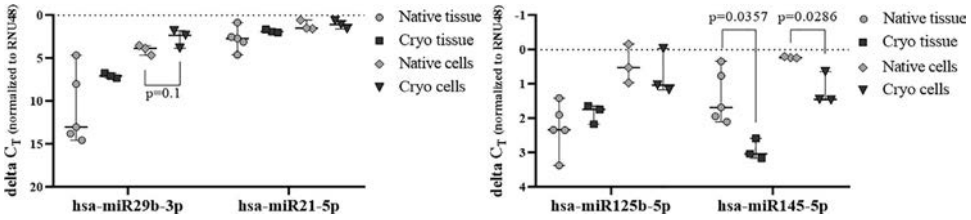

FIGURE 5 miRNA expression evaluation in non-cryopreserved (Native) and cryopreserved (Cryo) tissues, as well as isolated cells. RNU48 was used as an internal control for normalizing the miRNA levels. Graphs are presented with the *y*-axis inverted for better visualization, and higher delta cycle threshold (C_T) values correspond to lower miRNA expression. The middle line in the graphs represents the median ($n = 3$). The native tissue group comprises a sample size of $n = 5$, reflecting the wider distribution of results. Statistical significance was determined using the Mann–Whitney *U*-test.

studies suggest that there is no difference in the pre-and post-cryopreservation proliferation rate (*Bahsoun et al., 2020*; *Li et al., 2017*; *Sugimoto et al., 2021*), while another study confirmed a decrease in the proliferation abilities of cryopreserved MSCs (*Antebi et al., 2019*). Cryopreservation also did not affect the proliferation rates of cells derived from tissues of the reproductive system; the current group has previously shown the absence of changes in the proliferation of placenta tissue cells (*Navakauskienė et al., 2023*), and Gastal and colleagues found no changes in the proliferation of ovarian tissue cells (*Gastal et al., 2019*).

Most of the above-discussed studies assessed only short-term (up to 10 passages) cultivation proliferation rates, and the current results correspond to this, as this study also showed that there were no changes until the 10th passage. However, it was demonstrated that differences between Native and Cryo tissue isolated cells appeared in later passages. It is known that the proliferation rate decreases in later passages due to cellular senescence induced by reactive oxygen species (*Gu et al., 2016*) or to ion transportation alterations (*Marakhova et al., 2019*).

The only detected difference in surface marker expression between Native and Cryo cells includes SUSD2, which is a transmembrane protein participating in cell–cell and cell–matrix adhesion. SUSD2+ ESC are perivascular endometrial stem cells (*Sparovec et al., 2022*), and it has been demonstrated that the down-regulation of SUSD2 leads to cellular senescence (*Zhang et al., 2017*). The current results suggest that endometrial tissue cryopreservation could induce the earlier onset of cellular senescence processes in isolated stromal cells earlier, which, in turn, can influence the decrease of the proliferation capacity in the late passages.

The gene expression analysis in this study confirmed that endometrial tissue cryopreservation led to the up-regulation of pluripotency-associated genes (*NANOG, OCT4*) in isolated and further cultivated ESC. While the long-term (up to 19 passages) cultivation of endometrial mesenchymal stem cells and amniotic fluid mesenchymal stem cells (AF-MSC) did not have an effect on pluripotency genes (*Gasiūnienė et al., 2020*; *Valatkaitė et al., 2021*), the increase of *NANOG* and *OCT4* expression was confirmed in cryopreserved AF-MSC (*Gholizadeh-Ghaleh Aziz et al., 2019*), as well as human Wharton's jelly-derived mesenchymal stem cells (*Shivakumar et al., 2015*) and adipose-derived MSC (*Duan and Lopez, 2018*). Meanwhile, a study analysing the cryopreservation of human dental follicle tissue did not observe any changes in these pluripotency markers (*Park et al., 2014*).

Pluripotency genes that impact numerous downstream targets are intricately regulated in both embryonic and adult stem cell contexts to coordinate normal development and function (*Seymour et al., 2015*). The expression of pluripotency genes usually decreases during differentiation, and this change is irreversible during normal development; however, the overexpression of these genes can be used for generating induced pluripotent stem cells (*Miyamoto et al., 2015*). These results underscore the potential of cryopreservation as a tool for manipulating cellular pluripotency.

Furthermore, the study focused on epigenetic changes that could occur during endometrial tissue cryopreservation. The freeze–thaw process of replicating cells causes DNA damage by disturbing the DNA replication fork and leading to DNA double-strand breaks and changes in epigenetic modifications, while cryoprotectant agents such as DMSO increase chromatin condensation during the freeze–thaw process, which correlates with higher cell viability (*Falk et al., 2018*). This study did not confirm any changes in selected histone modifications, but it was demonstrated that endometrial tissue cryopreservation is accompanied by an increase of DNA methyltransferase (*DNMT1, DNMT3A, DNMT3B*) gene expression. Moreover, a tendency towards a decrease in DNA methylation in specific genome regions in cryopreserved tissue-derived cells was detected, although these changes were not statistically significant and did not reflect a functional role as the gene expression of the analysed regions was not affected.

Another study examining the effect of DMSO during cryopreservation found that even a very small DMSO concentration (0.1%) caused changes in miRNA levels and whole-genome DNA methylation profiles in cardiac tissue model, as well as an up-regulation of *DNMT1* and *DNMT3A* gene expression. However, these changes led to an increase of hypermethylation levels only in regions mostly without regulatory properties (*Verheijen et al., 2019*). Meanwhile, the cytotoxic effect of DMSO is reduced by conducting procedures at low temperatures and limiting the contact time (*Traversari et al., 2022*). Therefore, it is suggested that brief exposure and low-temperature conditions during sample preparation with DMSO during cryopreservation procedures has no significant effect on cellular and molecular alterations.

Moreover, cryopreservation may result in an imbalance of reactive oxygen species and oxidative stress, potentially associated with abnormal promoter hypermethylation and overall hypomethylation (*Reyes Palomares et al., 2022*). The observed

changes in *DNMT* gene expression and the tendencies seen in DNA methylation patterns underscore the need for further research to elucidate the functional implications and long-term effects of endometrial tissue cryopreservation on cellular properties.

In the current study, miRNA assay revealed that hsa-miR145-5p was down-regulated in cryopreserved tissue and remained inhibited in cells isolated from Cryo tissues. An increase in hsa-miR-145 was determined in patients with repeated implantation failure and infertile women with endometriosis (*Jahanbakhsh et al., 2023*; *Saare et al., 2017*), and elevated levels of hsa-miR-145, which directly targets type-1 insulin-like growth factor receptor (*IGF1*), led to impaired attachment of the embryo to the endometrium (*Kang et al., 2015*). However, the down-regulation of miR-145-5p is proposed as a prognostic biomarker for endometrial cancer (*Wu et al., 2020*). miR-145 suppresses the proliferation, invasiveness and stemness of endometriotic cells by targeting various pluripotency factors, cytoskeletal elements and protease inhibitors (*Adammek et al., 2013*). Moreover, miR-145 directly targets *DNMT3A* mRNA (*Zhang et al., 2018*) and *DNMT3B* mRNA (*Xue et al., 2015*). The determined decrease of hsa-miR-145-5p corresponds to the up-regulation of *DNMT* gene expression after endometrial tissue cryopreservation.

It is interesting to consider whether the findings from endometrial tissue slow cooling cryopreservation could be applied to other types of tissue. For example, reviews by Kometas and colleagues (*Kometas et al., 2021*) and Bahroudi and co-workers (*Bahroudi et al., 2022*) on ovarian tissue cryopreservation suggest that while vitrification appears more favourable, there is no significant clinical or biochemical difference between cryopreservation methods. Some of the studies even suggest the superiority of slow cooling in ovarian tissue cryopreservation. Another review by Behl and collaborators (*Behl et al., 2023*), comparing vitrification and slow freezing, revealed that vitrified ovarian tissue exhibits a greater proportion of intact stromal cells without significant differences in follicular qualities. However, compared with endometrial tissue, which is mucosal, ovarian tissue has a more complex structure that could lead to a lower recovery of stromal cells after cryopreservation. As different tissues exhibit different characteristics, optimal cryopreservation should be selected for each individually.

In summary, this study enhances the understanding of the diverse effects of endometrial tissue cryopreservation on cellular characteristics, gene expression and epigenetic modifications such as histone modifications, DNA methylation and miRNA levels. The observed decrease of proliferation efficiency in later passages, which is associated with a reduction in the percentage of SUSD2+ cells, elevated *DNMT* gene expression and suppressed levels of hsa-miR145-5p, underscores the complexity of the changes induced by cryopreservation. This knowledge is crucial for optimizing cryopreservation protocols and advancing the application of cryopreserved tissues across various fields, including regenerative medicine, cell therapy and reproductive health. Moreover, the findings of this study suggest the potential application of slow cooling cryopreservation not only for normal endometrial tissue samples, but also for pathological tissue samples with the purpose of isolating stromal cell cultures.

DATA AVAILABILITY

Data will be made available on request.

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AUTHOR CONTRIBUTIONS

D.Ž., A.Z. and R.N. conceptualized the study; D.Ž., A.Z. and E.G. performed the experiments and data analysis; and D.Ž. wrote the manuscript. All authors critically revised the manuscript for important intellectual content and edited it. All authors approved the final manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.103990.

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RBMO

ELSEVIER

ARTICLE

Pigment epithelium-derived factor expression and role in follicular development

BIOGRAPHY

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KEY MESSAGE

This study presents pigment epithelium-derived factor, expressed in granulosa cells, as a pro-folliculogenesis participant. It interacts with key players, namely FSH and anti-Müllerian hormone, during this process, and monitors the angiogenic and oxidative balance in the growing follicle. This study could be relevant to human ovarian physiology and pathophysiology.

ABSTRACT

Research question: What is the involvement of pigment epithelium-derived factor (PEDF), expressed in granulosa cells, in folliculogenesis?

Design: mRNA expression of *PEDF* and other key factors [*Cyp19*, anti-Müllerian hormone receptor (*AMHR*) and vascular endothelial growth factor (*VEGF*)] in mice follicles was examined in order to typify the expression of *PEDF* in growing follicles and in human primary granulosa cells (hpGC), and to follow the interplay between PEDF and the other main players in folliculogenesis: FSH and AMH.

Results: mRNA expression of *PEDF* increased through folliculogenesis, although the pattern differed from that of the other examined genes, affecting the follicular angiogenic and oxidative balance. In hpGC, prolonged exposure to FSH stimulated the up-regulation of *PEDF* mRNA. Furthermore, a negative correlation between AMH and PEDF was observed: AMH stimulation reduced the expression of *PEDF* mRNA and PEDF stimulation reduced the expression of *AMHR* mRNA.

Conclusions: Folliculogenesis, an intricate process that requires close dialogue between the oocyte and its supporting granulosa cells, is mediated by various endocrine and paracrine factors. The current findings suggest that PEDF, expressed in granulosa cells, is a pro-folliculogenesis player that interacts with FSH and AMH in the process of follicular growth. However, the mechanism of this process is yet to be determined.

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KEY WORDS
Granulosa cells
Folliculogenesis
FSH
AMH
PEDF

INTRODUCTION

Folliculogenesis, the process of follicular formation and development, begins during fetal life with the assembly of primordial follicles, each comprised of an oocyte and surrounding somatic supporting cells (granulosa cells) (*Cortvrindt and Smitz, 2001*), and ends with the formation of fully-grown pre-ovulatory follicles. Folliculogenesis is composed of a complex series of highly coordinated events that require close dialogue between the oocyte and its surrounding granulosa cells. It includes activation of primordial follicles (exit from the dormant stage of primordial follicle and transit to primary follicle), proliferation of granulosa cells leading to follicular growth, oocyte growth and maturation, acquisition of steroidogenic activity, and, finally, ovulation (*Schultz et al., 2018*); otherwise, the follicle will undergo atresia (follicular death) at one of the stages towards ovulation (*Rimon-Dahari et al., 2016*). The whole process, from activation of a primordial follicle to the formation of a pre-ovulatory follicle, occurs along several cycles and is mediated by endocrine and paracrine factors (*Oktem and Urman, 2010*). Follicles are commonly categorized by size, reflecting their developmental stage and their corresponding activities (*Dewailly et al., 2016*). They can also be categorized by their dependence on FSH. Small follicles, from the primordial stage to the small antral stage are FSH independent (*Lebbe and Woodruff, 2013*; *McGee and Hsueh, 2000*); although these follicles can respond to FSH, they are mainly controlled by locally produced growth factors through autocrine/paracrine mechanisms. Larger follicles, from the small antral stage to the ovulatory stage, are FSH dependent, as FSH is the main regulator of their final growth and maturation towards ovulation (*Orisaka et al., 2021*). FSH acts via FSH receptors (FSHR) expressed on granulosa cells (*Yamoto et al., 1992*) that become more abundant as the follicle grows. Once the follicle reaches the pre-antral stage, a minimal quantity of FSHR is required for its further development, or it will undergo atresia (*Zeleznik, 2004*).

Anti-Müllerian hormone (AMH), another central player in folliculogenesis, is an ovarian hormone, expressed and secreted exclusively by granulosa cells, mainly from small growing follicles up to small antral follicles (*Tal et al., 2014*). AMH acts via its type 2 receptors, expressed on granulosa cells. AMH not only regulates the activation of primordial follicles but also the FSH-dependent growth of pre-antral follicles by reducing FSHR expression, thus down-regulating their sensitivity to FSH (*Dewailly et al., 2014*; *Sacchi et al., 2016*; *Silva and Giacobini, 2021*). Hence, FSH and AMH act inversely on pre-antral follicles; the balance between their opposing activities, along with other variables, is critical for female fertility.

Along the cycle, the dynamic expression of various effectors is linked to coordinated changes associated with ovarian angiogenic and inflammatory processes (*Fraser and Lunn, 2000*; *Richards et al., 2008*), as well as hypoxia (*Duncan et al., 2008*; *Hernández-Morales et al., 2021*). During folliculogenesis, blood vessels do not infiltrate the follicle but remain at its periphery, accommodated in the theca cell layer (*Fraser, 2006*). To maintain the avascular state, there is a tight balance between anti- and pro-angiogenic effectors. Until recently, most research focused on the contribution of the pro-angiogenic factor – vascular endothelial growth factor (VEGF) – that is essential to the physiologic growth of the follicle, starting from the secondary stage (*Hernández-Morales et al., 2021*). Moreover, granulosa cells express and respond to inflammatory cytokines and chemokines that promote angiogenesis during the cycle. Thus, an endocrine–angiogenic–inflammatory network is essential for proper folliculogenesis; any disturbance to these processes may contribute to infertility-related syndromes.

Pigment epithelium-derived factor (PEDF) is a 50-kDa secreted glycoprotein (coded by the *SERPINF1* gene) that belongs to the serine protease inhibitor superfamily (*Becerra et al., 2012*; *Filleur et al., 2009*). It is a multi-functional protein that constitutes the most potent anti-angiogenic agent by negating VEGF activity. In addition, PEDF has anti-inflammatory and anti-oxidative properties, as reported by the authors' group and others (*Bar-Joseph et al., 2014*; *Filleur et al., 2009*; *Miller et al., 2016*; *Nemerovsky et al., 2021*; *Wang et al., 2008*; *Yamagishi et al., 2005*). PEDF was found to be widely expressed in a variety of human body tissues, with high expression in the reproductive tissues, including the ovaries. In the ovaries, it is expressed in various components, including follicles (*Chuderland et al., 2013a*), and is important for maintaining proper function of the ovary (*Becerra et al., 2012*).

Previously, the authors demonstrated that both rodent and human granulosa cells produce and secrete PEDF in a hormone-dependent manner (*Chuderland et al., 2013a*,b), and showed a low ovarian concentration of PEDF in several gynaecological pathologies which are correlated with impaired function of the endocrine–angiogenic–inflammatory axis, such as ovarian hyperstimulation syndrome (*Chuderland et al., 2013b*; *Miller et al., 2016*), endometriosis and polycystic ovary syndrome (PCOS) (*Miller et al., 2020*). Administration of recombinant PEDF (rPEDF) negated the symptoms of these pathologies by restoring the proper ovarian balance between *PEDF* and *VEGF* and inflammatory status (*Chuderland et al., 2013b*; *Miller et al., 2016*).

However, to date, the interplay of PEDF with the known key players in folliculogenesis – FSH and AMH – and its role in folliculogenesis have not been characterized. As such, the authors postulated that PEDF participates in the regulation of folliculogenesis by interacting with key factors involved in folliculogenesis.

MATERIALS AND METHODS

Mice

ICR female mice (6–8 weeks old) (Envigo, Israel) were housed in a temperature- and humidity-controlled room at the animal facility of the Sackler Faculty of Medicine, Tel-Aviv University, under artificial illumination for 12 h daily. Animal care was in accordance with institutional guidelines, and was approved by the Institutional Animal Care and Use Committee (Permit No. 01-18-053).

Isolation and classification of mouse follicles

Forty-eight hours after administration of PMSG (5 IU), ovaries from 12 euthanized female mice were excised and put in L-15 medium (01-115-1A; Biological Industries, Israel), supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 mg/ml; Biological Industries). Follicles were mechanically isolated and classified into three groups according to size: secondary stage (100–170 μm), small antral stage (280–380 μm), and large antral stage (500–660 μm). Secondary stage follicles, pooled between mice, were washed and collected in groups of five (to collect enough cells for RNA analysis) in 10 μl of nuclease-free water (Biological Industries); small and

large antral follicles were collected individually. The authors were aware of the limitations that arise from pooling five secondary follicles into one sample and averaging the outcome, but even using a high-sensitivity RNA extraction kit, the follicles had to be pooled for detection.

Immunofluorescence staining and confocal microscopy

For immunofluorescence staining freshly isolated follicles were incubated with a DNA-specific blue fluorochrome (Hoechst 33342; 1μg/ml; 14533; Sigma-Aldrich; St. Louis, MI USA). Images were visualized and acquired by a Leica laser confocal microscope (SP8; Leica Microsystems, Wetzlar, Germany) with standard pinhole (1AU=95 μm) using the same imaging settings for all samples.

Humans

Isolation and culture of human primary granulosa cells

Cells were obtained from women (age 20−45 years) undergoing IVF treatment due to male factor infertility [Helsinki IRB Ref. No. 0240-19-SZMC, approval date 15 August 2019 with extensions: S. Zedek, 'The role of pigment epithelium derived factor (PEDF) in the human ovary']. They were isolated from aspirated follicular fluid after oocyte retrieval, as described previously (*Breckwoldt et al., 1996*; *Chuderland et al., 2013b*). Follicular fluid was centrifuged, and the resulting pellets were resuspended in haemolysis buffer [10 mM Tris, 0.84% NH_4Cl (pH 7.4)] and then washed several times in Dulbecco's phosphate-buffered saline (PBS; Biological Industries). The pellets were resuspended in fresh culture medium [Dulbecco's Modified Eagle's Medium F-12 (DMEM-F12); Biological Industries], supplemented with L-glutamine (2mM), penicillin (10,000 IU/ml), streptomycin (100 mg/ml) and 10% FBS (all Biological Industries), counted and then seeded on to six-well plates (Thermo Fisher Scientific, USA) to allow cell adherence, confirming the presence of human primary granulosa cells (hpGC) alone. hpGC were incubated for 5 days at 37°C in a controlled atmosphere of 5% CO_2 in air, as described previously (*Chuderland et al., 2013a*), to reduce the effect of the previous IVF hormone stimulatory treatment, and subjected to daily washes with fresh culture medium. Cells were cultured in pools (two to three women per pool) to collect enough cells for RNA analysis. In a preliminary analysis, the samples were categorized into two groups: age ≤35 years and age ≥36 years. Upon analysis, however, no discernible difference was found between the two groups, so all analyses continued without age categorization.

hpGC stimulation

Following the incubation period, hpGC were serum-starved (DMEM F-12, 0.1%) for 17 h and stimulated for either 4 or 24 h with several stimulators: recombinant human FSH α/β (rFSH, derived from Chinese hamster ovary cell line; 5925-FS-01; R&D Systems, USA; 50 ng/ml) for 4 and 24 h; rPEDF (derived from *Escherichia coli*; ab56289; Abcam, UK; 5 nM); or recombinant human Müllerian inhibiting substance/AMH (rAMH, derived from *E. coli*; 1737-MS-010; R&D Systems; 60 ng/ml) for 4 h. Cells were then rinsed in PBS and harvested for mRNA analysis.

RNA isolation, reverse transcription, polymerase chain reaction and real-time polymerase chain reaction

Follicles were lysed using the lysis buffer supplied in the TaqMan MicroRNA Cells-to-CT Kit (4391848, Ambion; Thermo Fisher Scientific) and subjected to real-time polymerase chain reaction (PCR) as described in Section 3.5. Total RNA was reverse transcribed using 9 μl of the Cells-to-C_T sample lysates and the High-Capacity cDNA Reverse Transcription Kit (4368814; Applied Biosystems, USA). Data were analysed in two distinct ways: (1) per follicle, representing the follicle as a functioning unit. Analysis of a full follicle, filtered by size (i.e. developmental stage), by expression of the selected genes of all the granulosa cells within the follicle (un-normalized data of the PCR test; secondary follicles were collected and analysed in groups of five, then the outcome was divided by 5 to represent a single follicle); and (2) per cell, representing the expression of the selected genes in a single granulosa cell. An endogenous normalizing control (*HPRT1*; Applied Biosystems) was used, thus disregarding the size of the follicle and the number of granulosa cells. hpGC RNA was extracted using Trizol reagent (Bio-Tri; Bio-lab Chemicals, Israel), in accordance with the manufacturer's instructions, and quantified with the Nano-Drop spectrophotometer (ND-1000; Thermo Fisher Scientific). Total RNA was reverse transcribed using a high-capacity cDNA Reverse Transcription Kit (4368814; Applied Biosystems), and used for relative gene expression analysis (20 ng of cDNA per reaction was used as an amplification template). *HPRT1* or *RPLP2* served as the endogenous normalizing control.

Changes in mRNA expression were detected and analysed by quantitative PCR on the StepOnePlus Real-Time PCR System (Applied Biosystems), using SYBR green reagent (Power SYBR Green PCR Master Mix; Applied Biosystems) and SYBR primers (Applied Biosystems; TABLE 1), or TaqMan and TaqMan primers (Applied Biosystems; TABLE 2). Relative expression was calculated using the comparative $_{\Delta}$Ct.

Statistics

All statistical analyses were performed using SPSS software (IBM Corp., USA) or GraphPad Prism 9.0.0. (GraphPad Software, Inc., USA). Data normality was assessed using Kolmogorov Smirnov test. For multiple comparisons, the data were evaluated by One-Way ANOVA analysis, followed by Tukey's post-hoc test (figures

TABLE 1 LIST OF QUANTITATIVE POLYMERASE CHAIN REACTION SYBR PRIMERS

Primer name	Species	Forward (5'to3')	Reverse (5'to3')
PEDF	Mouse	CCAAGTCTCTGCAGGACATGAAG	GGTTTGCCAGTAATCTTGCTG
HPRT1	Mouse	CTCATGGACTGATTATGGACAGGA	GCAGGTCAGCAAAGAACTTATAGCC
FSHR	Human	GGTGCATTTTCAGGATTTGG	CTGCCTCTATCACCTCCAAGA
HPRT1	Human	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
AMHR	Human	CCCTGCTACAGCGAAAGAAC	ATGGCAACCAGTTTTCCTTG
VEGF	Human	AAGGAGGAGGGCAGAATCAT	CTGCATGGTGATGTTGGACT
Cyp19	Human	GACTCTAAATTGCCCCCTCTG	CAGAGATCCAGACTCGCATG

TABLE 2 LIST OF QUANTITATIVE POLYMERASE CHAIN REACTION TAQMAN PRIMERS

Primer name	Species	TaqMan Gene Expression Assay ID
PEDF	Mouse	Mm00441270_m1
VEGF	Mouse	Mm00437306_m1
AMH	Mouse	Mm004310795_g1
FSHR	Mouse	Mm00442819_m1
RPLP2	Mouse	Mm00782638_s1
BCl2	Mouse	Mm00437796_m1
Foxo1	Mouse	Mm00490672_m1
ASPN	Mouse	Mm00445945_m1
Cyp19	Mouse	Mm00484049_m1
Cyp17A	Mouse	Mm00484040_m1
PEDF	Human	Hs01106937_m1
VEGF	Human	Hs00900055_m1

2, 3,4). To specify the significance of differences between two experimental groups (figures 5,6,7), we applied a Wann-Whitney test followed by Welch's correction. A P-value < 0.05 was considered to indicate significance.

RESULTS

Gene expression during mouse folliculogenesis

mRNA expression of key genes in mice follicles was examined at three developmental stages of folliculogenesis (FIGURE 1A): secondary stage follicles (100-170 μm; FSH-independent follicles), and small and large antral stage follicles (280–380 μm and 500–660 μm, respectively; FSH-dependent follicles). The relative expression of each mRNA in the whole follicle was

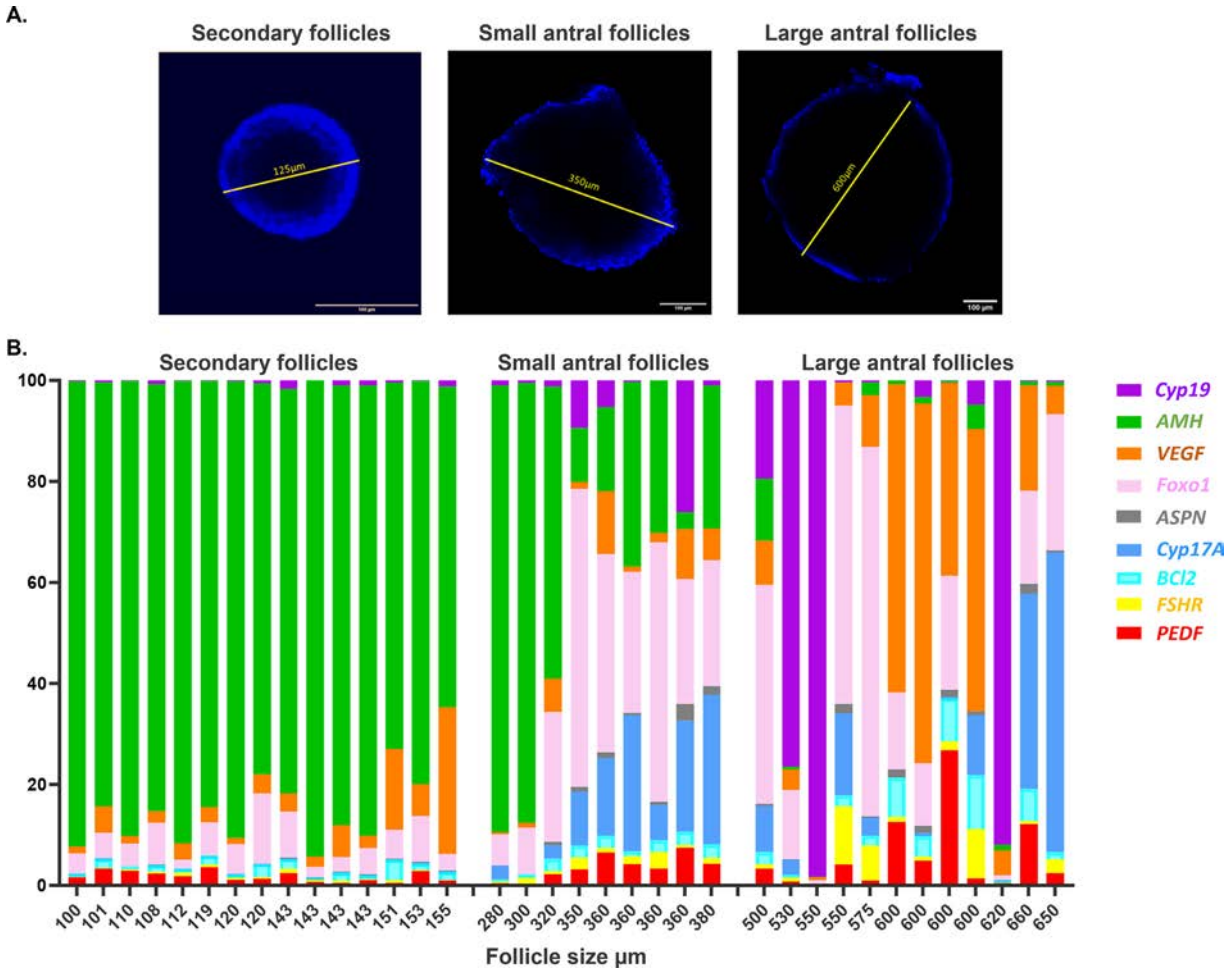

FIGURE 1 Expression panel of selected genes during follicular growth. Three developmental stages (100–170 μm, secondary stage; 280–380 μm, small antral stage; 500–660 μm, large antral stage) of mouse ovarian follicles were isolated and analysed individually. (A) Representative images of follicles in each group size, stained with Hoechst 33342. All scale bars 100 μm. (B) Follicle mRNA was extracted and subjected to quantitative polymerase chain reaction (relative expression), and calibrated by *HPRT1*. Secondary follicles were collected and analysed in groups of five; to represent a single follicle, the average mRNA expression levels and follicular size are presented. Each bar represents the expression panel of the examined genes for an individual follicle of a specific size, expressed as 100%.

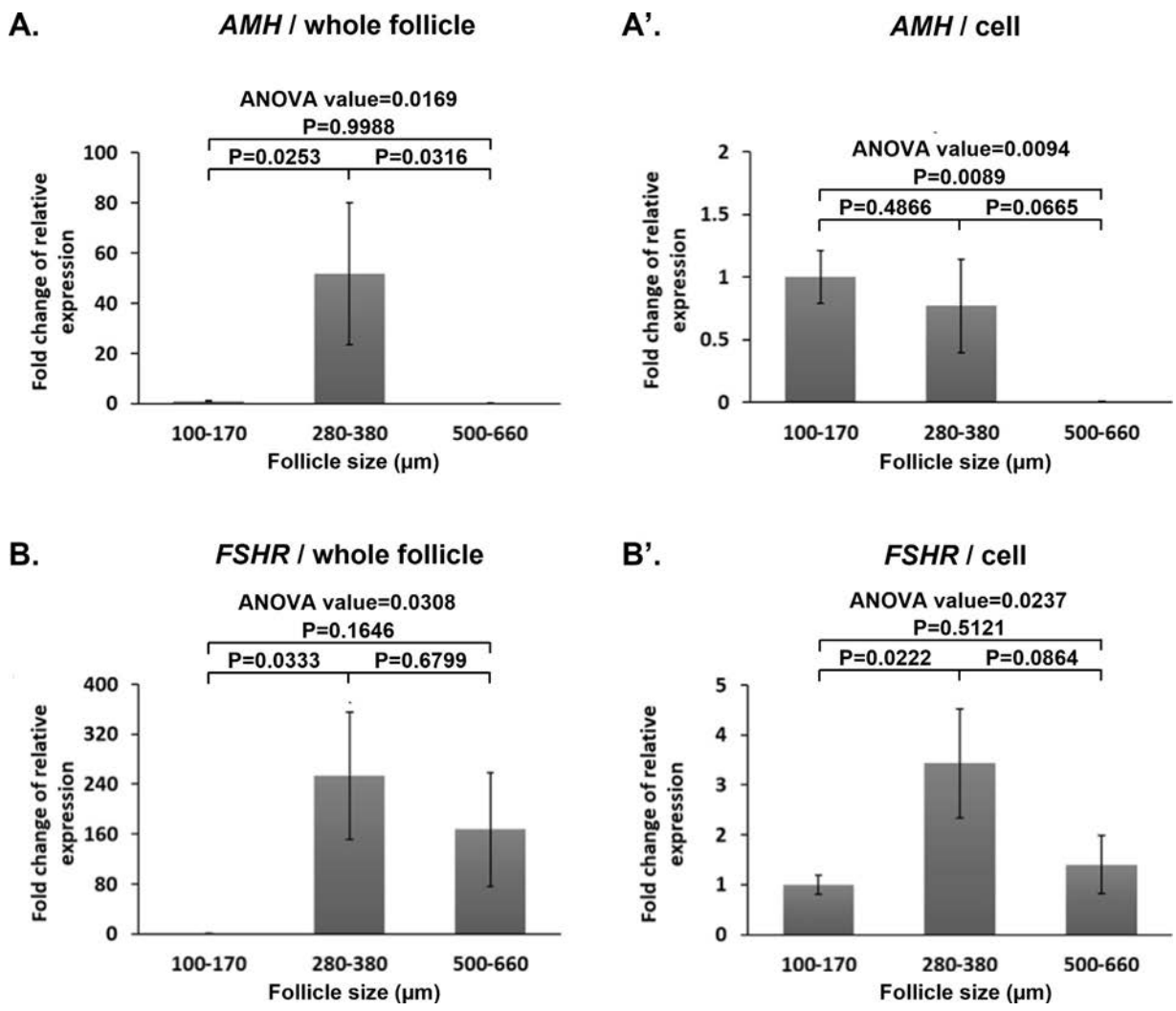

FIGURE 2 mRNA expression of anti-Müllerian hormone (*AMH*) and FSH receptor (*FSHR*) in mouse follicles. Ovarian follicles were collected individually from mice primed with PMSG (FSH analogue). Collected follicles were divided into three groups based on their size (100–170 μm, secondary stage; 280–380 μm, small antral stage; 500–660 μm, large antral stage). Each follicle was subjected to RNA extraction using TaqMan MicroRNA Cells-to-CT Kit unique protocol. Corresponding cDNA was subjected to quantitative polymerase chain reaction analysis for specific gene detection. (A, A') *AMH* mRNA. (B, B') *FSHR* mRNA. The analysis was performed for the absolute gene values of whole follicles (A, B), and for relative expression of genes in a single follicular cell (A', B'), normalized to the geometric mean of *HPRT1* and *RPLP2* housekeeping genes. Each group size consisted of at least nine follicles. Data are presented as fold change ratio relative to expression of secondary follicles (100–170 μm), as mean ± SEM. Data were analysed by one-way analysis of variance, followed by Tukey's post-hoc test. ANOVA, analysis of variance.

examined (FIGURE 1B), and differing levels of expression of the examined genes were detected.

As such, the expression of each gene of interest was followed, and analysed at the whole follicle level, considering the increased number of granulosa cells as follicles grow and develop; and at the cellular level (normalized to a reference gene; indicating the activity of a single cell; FIGURE 2). Changes in genes (*AMH* and *FSHR*) with a well-described pattern of expression during folliculogenesis were followed in order to establish the reliability of the system. At the whole follicle level, *AMH* expression was found to resemble a Gauss pattern: it was barely detected in secondary stage follicles, its expression was highly up-regulated in small antral follicles, and there was virtually no expression in large antral follicles ($P = 0.0253$ and 0.0316, respectively, versus secondary follicles; FIGURE 2A). *FSHR* expression increased as the follicle grew ($P = 0.0333$ secondary follicles versus small antral follicles; FIGURE 2B), and remained elevated in large antral follicles. When examining the expression of these genes at the cellular level, *AMH* expression was found to be down-regulated as the follicle grew ($P = 0.0089$ for large antral follicles versus secondary follicles; FIGURE 2A'), whereas the *FSHR* expression pattern was similar to that of a whole follicle ($P = 0.0222$ for large antral follicles versus secondary follicles; FIGURE 2B'). These data are consistent with the literature.

Next, the expression levels of *PEDF* and *VEGF* mRNA were followed at the whole follicle level (FIGURE 3A,B); both were found to be elevated during follicular growth, with a significant difference in large antral follicles compared with secondary follicles ($P = 0.0391$ and 0.0061, respectively). At the cellular level (FIGURE 3A',B'), *PEDF* mRNA expression remained stable during follicle development, but *VEGF* expression was significantly elevated in large antral follicles ($P = 0.0131$ compared with small

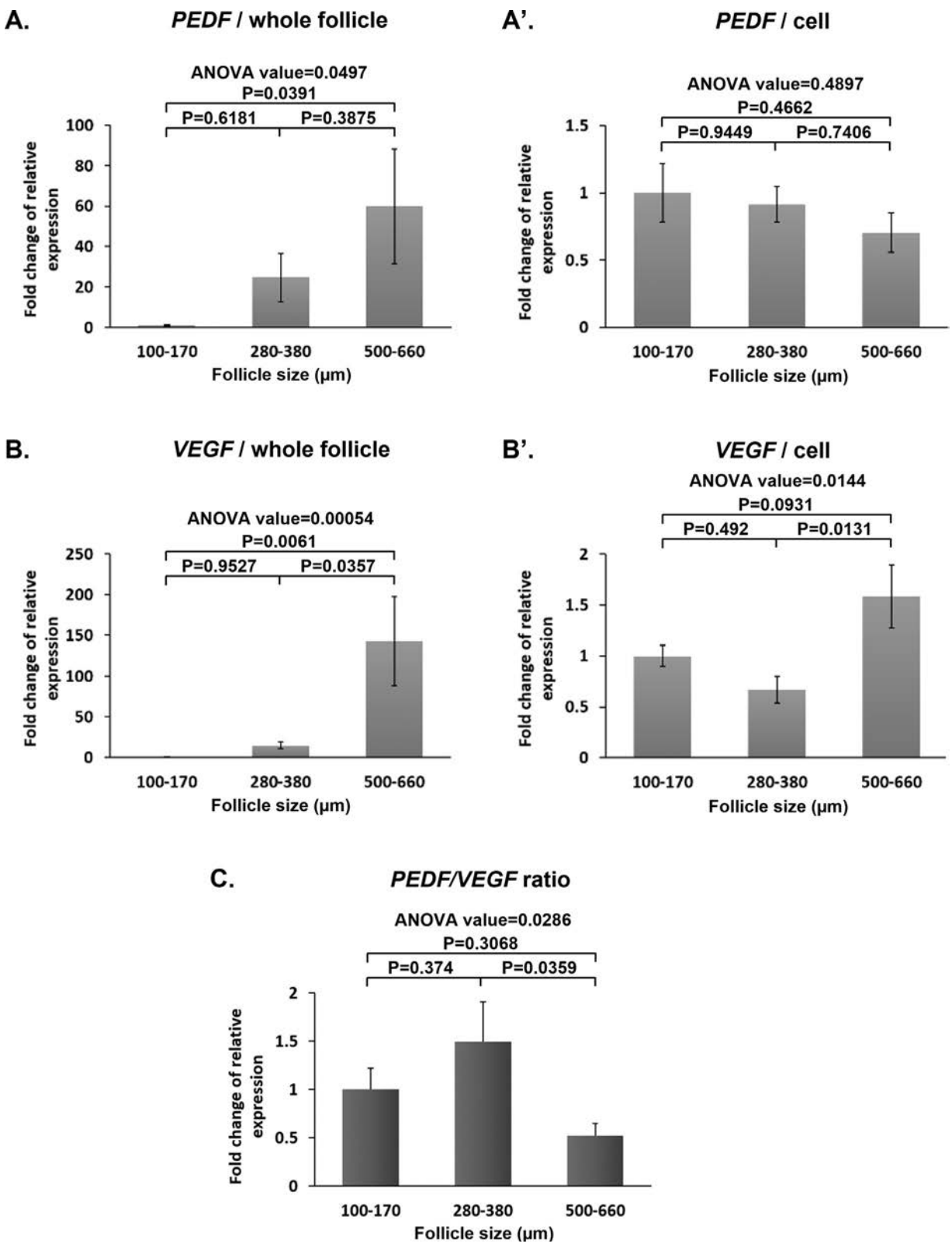

FIGURE 3 mRNA expression of pigment epithelium-derived factor (*PEDF*) and vascular endothelial growth factor (*VEGF*) in mouse follicles. Ovarian follicles were collected individually from mice primed with PMSG (FSH analogue). Collected follicles were divided into three groups based on their size. Each follicle was subjected to RNA extraction using TaqMan MicroRNA Cells-to-CT Kit unique protocol. Corresponding cDNA was subjected to quantitative polymerase chain reaction for specific gene detection. (A, A') *PEDF* mRNA. (B, B') *VEGF* mRNA. The analysis was performed for the absolute gene values of whole follicles (A, B), and for relative expression of genes in a single follicular cell (A', B'), normalized to the geometric mean of *HPRT1* and *RPLP2* housekeeping genes. (C) *PEDF/VEGF* ratio in experimental groups. Each group consisted of at least nine follicles. Data are presented as fold change ratio relative to expression of the smallest follicle group, shown as mean ± SEM. Data were analysed by one-way analysis of variance, followed by Tukey's post-hoc test. ANOVA, analysis of variance.

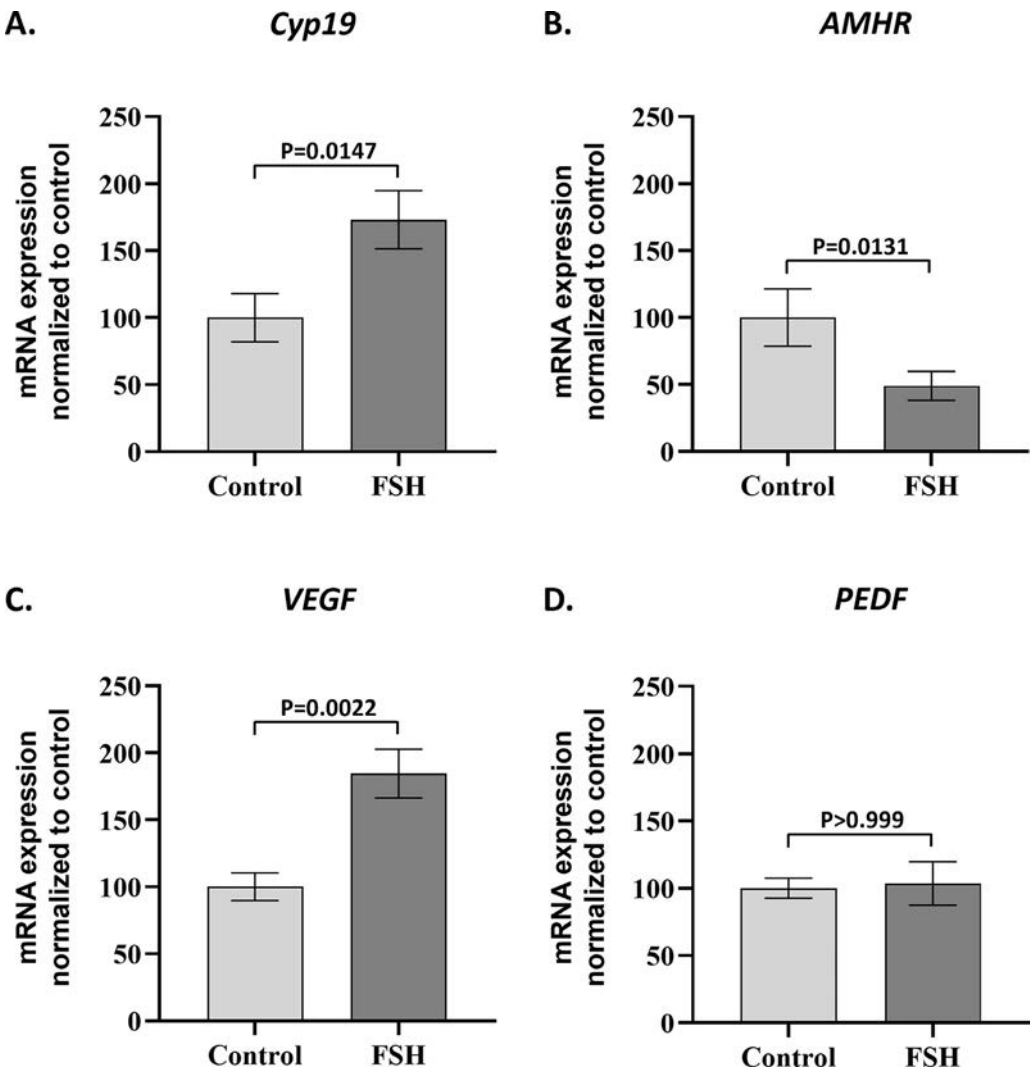

FIGURE 4 mRNA expression of selected genes in human primary granulosa cells (hpGC) after a short period of stimulation with recombinant FSH (rFSH). Serum-starved hpGC were treated with rFSH 50 ng/ml for 4 h. Untreated cells served as the control. mRNA was subjected to quantitative polymerase chain reaction analysis with specific primers for *Cyp19*, anti-Müllerian hormone receptor (*AMHR*), vascular endothelial growth factor (*VEGF*) and pigment epithelium-derived factor (*PEDF*), and calibrated by *HPRT1*. Data are presented as mean ± SEM of relative expression. Data were analysed by Mann–Whitney test, followed by Welch's correction. Experiments were conducted at least three times, and each sample was assayed in duplicate. Cells were cultured in pools (two to three women per pool).

antral follicles; FIGURE 3B'). As the *PEDF/VEGF* ratio is crucial for activation of the angiogenic switch, this was analysed and found to decrease in pre-ovulatory follicles ($P = 0.0359$ versus secondary follicles), favouring angiogenesis. These findings point to PEDF as an important effector contributing to the maintenance of folliculogenesis.

Stimulation of hpGC

Data from the mouse model studies led the authors to investigate the potential role of PEDF during the final stages of folliculogenesis, and its interplay with FSH and AMH, using hpGC. The cells were stimulated for a short period (4 h); as *PEDF* was the main gene of interest, another stimulation period (24 h) was used to follow changes in its expression.

FSH stimulation

The effect of FSH on the expression of genes that are known to be involved in folliculogenesis was tested (FIGURE 4). After a short period of stimulation, changes that have been reported previously in the literature (*Convissar et al., 2017*; *Fraser, 2006*; *Shoham and Schachter, 1996*) were noted [i.e. a significant increase in the expression levels of *Cyp19* and *VEGF* mRNA ($P = 0.0147$ and 0.0022, respectively), accompanied by a decrease in the expression of *AMHR* mRNA ($P = 0.031$)]. No change in the expression of *PEDF* mRNA was noted (FIGURE 4D).

To investigate any effect of FSH on *PEDF* mRNA, the cells were stimulated for longer periods (8 and 24 h). After 8 h of stimulation, the effect was similar to that obtained after 4 h of stimulation (data not presented). After 24 h of stimulation (FIGURE 5), significant increases in the expression levels of *PEDF* ($P = 0.036$), *Cyp19* ($P = 0.0043$) and *VEGF* mRNA ($P = 0.0066$) were noted (FIGURE 5A,C). There was no significant change in the expression of *AMHR* mRNA (FIGURE 5B).

AMH stimulation

The effect of a short period of AMH stimulation on the mRNA expression of *Cyp19*, *FSHR*, *VEGF* and *PEDF* was investigated (FIGURE 6). The expression

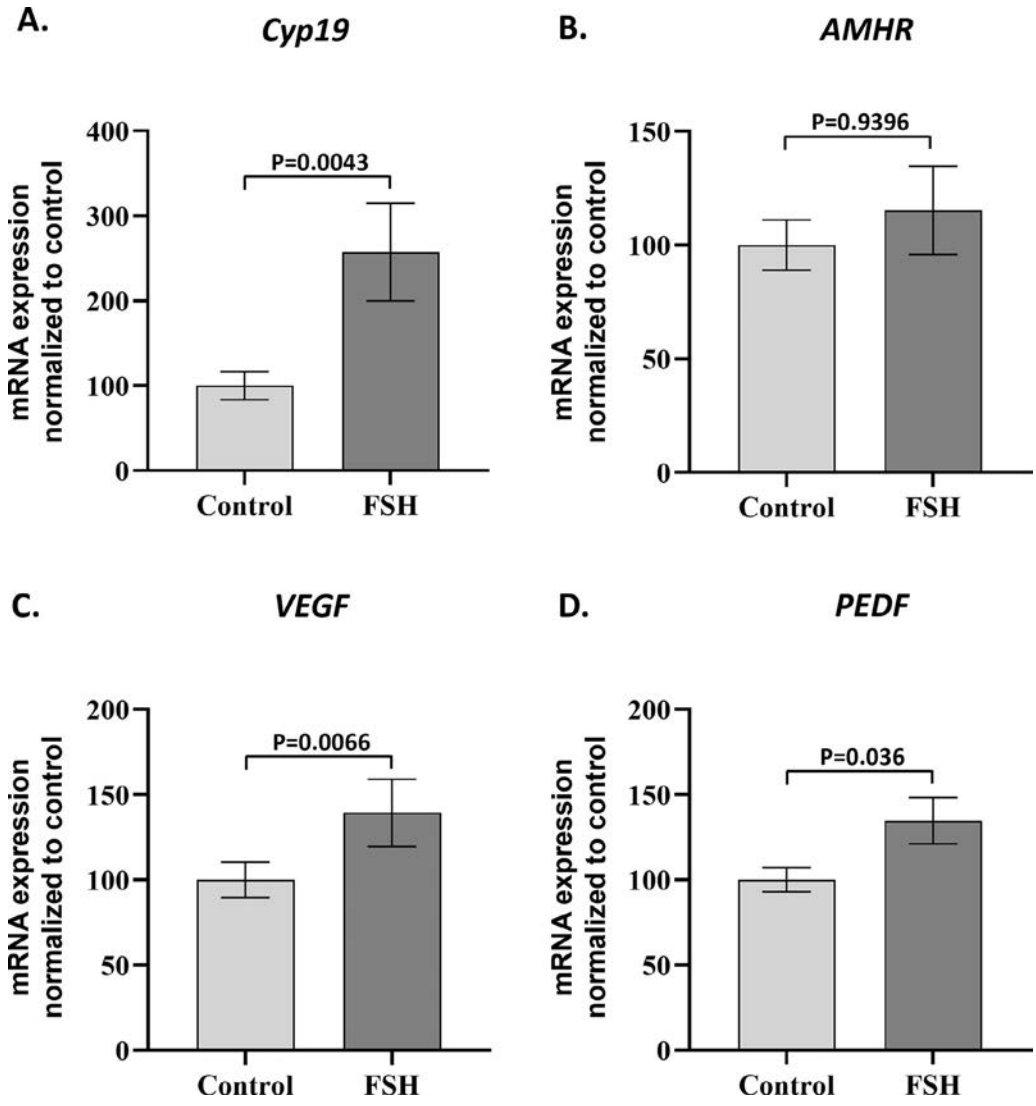

FIGURE 5 mRNA expression of selected genes in human primary granulosa cells (hpGC) after a long period of stimulation with recombinant FSH (rFSH). Serum-starved hpGC were treated with rFSH 50 ng/ml for 24 h. Untreated cells served as the control. mRNA was subjected to quantitative polymerase chain reaction analysis with specific primers for *Cyp19*, anti-Müllerian hormone receptor (*AMHR*), vascular endothelial growth factor (*VEGF*) and pigment epithelium-derived factor (*PEDF*), and calibrated by *HPRT1*. Data are presented as mean ± SEM of relative expression. Data were analysed by Mann–Whitney test, followed by Welch's correction. Experiments were conducted at least three times, and each sample was assayed in duplicate. Cells were cultured in pools (two to three women per pool).

levels of *Cyp19* and *FSHR* mRNA were not affected (FIGURE 6A,B), whereas the expression levels of *PEDF* and *VEGF* mRNA decreased significantly ($P = 0.0148$ and 0.0234, respectively; FIGURE 6C,D).

PEDF stimulation

Finally, the involvement of PEDF in folliculogenesis and its effect on the mRNA expression of selected genes were assessed. No significant changes in the expression levels of *Cyp19* or *VEGF* mRNA were detected following a short period of stimulation with rPEDF (FIGURE 7A,C, respectively); however, a significant decrease in the expression of *AMHR* mRNA was noted ($P = 0.012$, FIGURE 7B).

DISCUSSION

Ovarian folliculogenesis has long been of interest to scientists and clinicians. It is a phenomenon of extraordinary complexity, involving many paracrine and endocrine factors. The main objective of this study was to elucidate the involvement of PEDF in folliculogenesis, focusing on the inter-relationships between PEDF and the well-described factors FSH and AMH. The growing follicle is confronted with two opposing forces: FSH, which promotes follicular development; and AMH, which represses the follicle's progress through two of its developmental stages.

PEDF is a multifunctional protein that possesses anti-angiogenic (*Simonovic et al., 2001*), anti-inflammatory (*Wang et al., 2008*) and anti-oxidative properties (*Yamagishi et al., 2005*). PEDF is a multifunctional protein that possesses anti-angiogenic (*Simonovic et al., 2001*), anti-inflammatory (*Wang et al., 2008*), and anti-oxidative properties (*Yamagishi et al., 2005*). Previous data from our lab (*Bar-Joseph et al., 2020*, *2016*, *2014*;

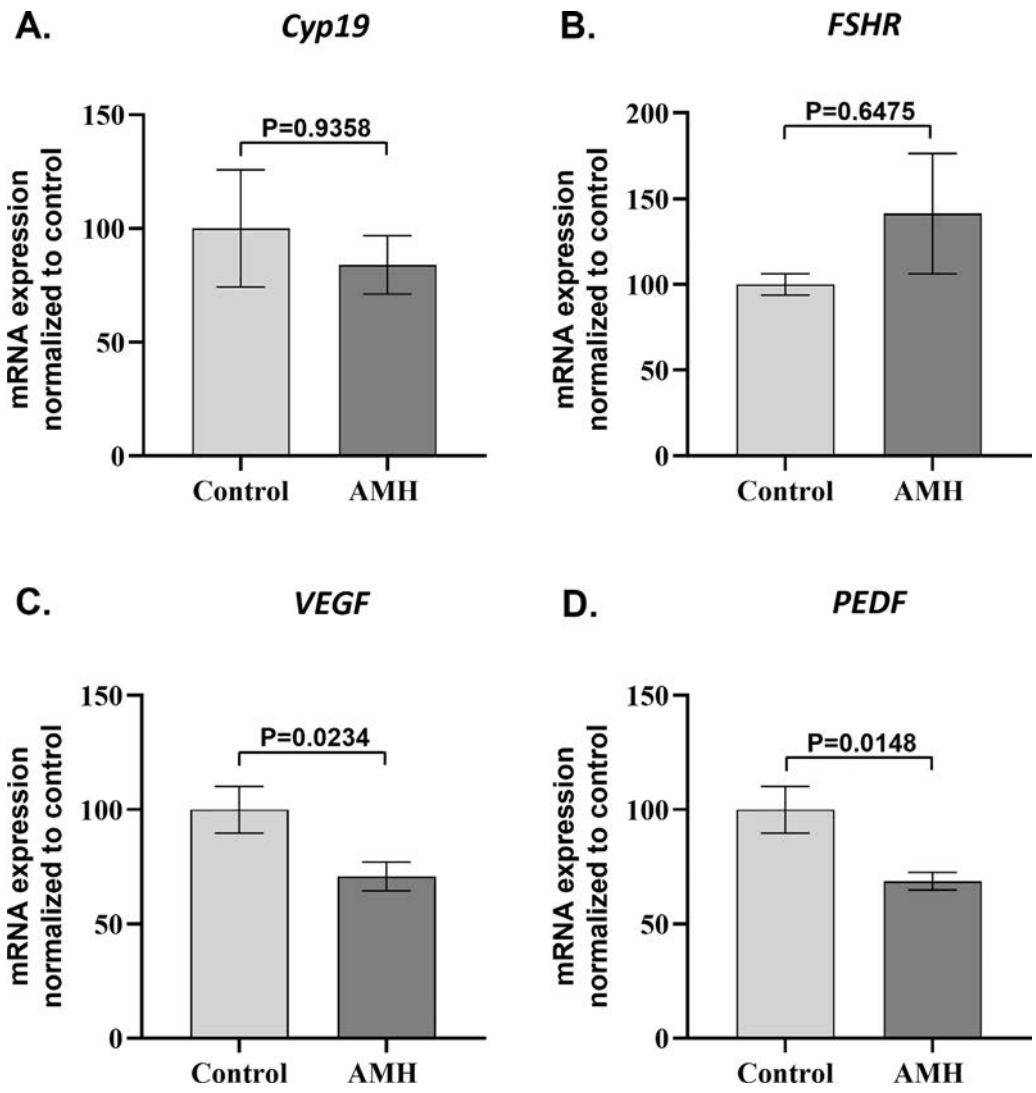

FIGURE 6 mRNA expression of selected genes in human primary granulosa cells (hpGC) after a short period of stimulation with recombinant anti-Müllerian hormone (rAMH). Serum-starved hpGC were treated with rAMH 60 ng/ml for 4 h. Untreated cells served as the control. The corresponding mRNA was subjected to quantitative polymerase chain reaction analysis with specific primers for *Cyp19,* FSH receptor (*FSHR*), vascular endothelial growth factor *(VEGF*) and pigment epithelium-derived factor (*PEDF*), and calibrated by *HPRT1*. Data are presented as mean ± SEM of relative expression (. Data were analysed by Mann–Whitney test, followed by Welch's correction. Experiments were conducted at least three times, and each sample was assayed in duplicate. Cells were cultured in pools (two to three women per pool).

Chuderland et al., 2014, *2013a*, *2013b*, *2013c*; *Miller et al., 2020*, *2016*; *Nemerovsky et al., 2021*, *2022*; *Silber et al., 2020*), described PEDF in the ovaries, specifically in GCs of healthy women and mice. We characterized a strong link between PEDF expression and ovarian function: (i) in physiological cycles, stimulation of GCs with LH/hCG induces a reduction in PEDF level concomitantly to VEGF elevation, thus promoting an angiogenic balance that favors blood vessel formation towards corpus luteum formation (*Bar-Joseph et al., 2016*; *Chuderland et al., 2013a*); (ii) in pathophysiological conditions, PEDF level is reduced and the angiogenic balance is compromised. For example, in OHSS (caused by excessive permeability of blood vessels in the ovary during assisted reproduction treatments) PEDF level is low; treatment of OHSS-induced mice with recombinant PEDF restored the level of the angiogenic balance in their ovaries to the physiological level and alleviated OHSS symptoms, putting PEDF as part of the pathogenesis and treatment of OHSS, and of other pathologies (as PCOS and endometriosis). Altogether, data points to PEDF as an essential factor in the ovary; however, neither the role of PEDF in folliculogenesis, nor the interplay among PEDF, FSH and AMH have been investigated yet.

In order to characterize the expression of PEDF during folliculogenesis, an 'individual fingerprint' was constructed, presenting the relative mRNA expression of key genes (proportion of 100% of examined genes) during three developmental stages of mouse folliculogenesis. This study showed that different genes present different patterns of expression during these three stages, namely a profound increase in *Cyp19* mRNA expression (coding for aromatase protein, producing oestrogen) in large antral follicles, and a decrease in *AMH* mRNA expression. The large antral group is very heterogenous, and includes large antral follicles that will not continue to develop properly and will undergo

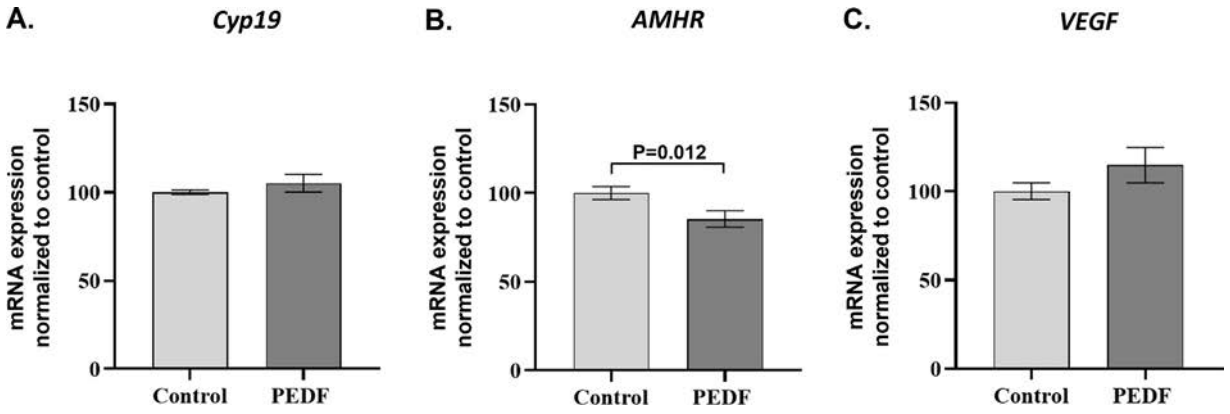

FIGURE 7 mRNA expression of selected genes in human primary granulosa cells (hpGC) after a short period of stimulation with recombinant pigment epithelium-derived factor (rPEDF). Serum-starved human primary granulosa cells were treated with rPEDF 5 nM for 4 h. Untreated cells served as the control. Corresponding mRNA was subjected to quantitative polymerase chain reaction analysis with specific primers for *Cyp19*, anti-Müllerian hormone receptor (*AMHR*) and vascular endothelial growth factor (*VEGF*), and calibrated by *HPRT1*. Data are presented as mean ± SEM of relative expression. Data were analysed by Mann–Whitney test, followed by Welch's correction. Experiments were conducted at least three times, and each sample was assayed in duplicate. Cells were cultured in pools (two to three women per pool).

atresia. As such, these follicles do not express a physiological pattern of the examined genes (only three follicles had a high proportion of *Cyp19* mRNA expression).

Once it had been established that the study protocol of follicular classification aligned with the literature, the changes in individual genes at various stages of follicular development were followed. Data were analysed at two levels: whole follicle level and cellular level. The findings have been presented through these two methods in order to emphasize the significance of the results that compare *PEDF* mRNA expression in follicles at different developmental stages. As the follicle is an independently functioning unit, the authors aimed to distinguish between the function of the whole unit (not normalized; considering the size, number of granulosa cells, and developmental stage of the follicle) and the function of a single granulosa cell (normalized to control gene; evaluating the dynamic activity of the cells during folliculogenesis).

The findings demonstrate a similar trend in *AMH, FSHR* and *VEGF* mRNA expression patterns, which were used as reference genes, for both levels (whole follicle and cellular) relating to their known physiological activities within the growing follicle. It should be noted that, in the whole follicle setting, mRNA expression of secondary follicles (used as reference) was very low compared with that of antral follicles, reflecting the small number of cells in secondary follicles, regardless of the examined gene. Thus, the cellular analysis is very important to emphasize the expression pattern of individual genes, and *PEDF* specifically, suggesting its role during folliculogenesis. Furthermore, as indicated by the 'fingerprint' analysis, the population of large antral follicles is very heterogeneous. In the mouse model, an FSH analogue (PMSG) was administered to the females before follicle isolation in order to recruit more antral follicles. Allegedly, this should have protected the follicles from atresia; however, a single PMSG dose is evidently not sufficient to prevent atresia in all follicles from atresia, and it is likely that atresia occurred in those with low FSHR [non-atretic follicles should have elevated FSHR (*Chun et al., 1996*)]. Thus, only a few of the large antral follicles continued towards ovulation, with atresia of the others. Therefore, mean mRNA expression of the examined genes in both analysis methods of this group was less significant.

The expression of *VEGF* mRNA in both analysis methods was low in secondary follicles and small antral follicles, but high in antral follicles. VEGF is a key player in folliculogenesis; it is expressed alongside *VEGFR* in pre-antral follicles and their surrounding stroma (*Abir et al., 2010*), promoting follicular growth and oocyte maturation (*Duncan et al., 2008*; *Hernández-Morales et al., 2021*) by stimulating the formation of new blood vessels surrounding the follicle (*Fraser, 2006*; *Losordo and Isner, 2001*). Under the increasing hypoxic conditions within the follicle, hormones such as oestrogen and FSH regulate *VEGF* mRNA expression in granulosa cells, mediated by Hif1-α. Thus, the elevation detected in this study corresponds with the physiological needs of proper oogenesis, folliculogenesis and ovulation. Finally, this study showed that, unlike other examined genes, the expression of *PEDF* mRNA differed between the two analysis methods. *PEDF* mRNA expression was elevated as the follicle grew, yet its cellular expression was stable throughout follicular development. Although both *VEGF* and *PEDF* mRNA expression were elevated during follicular growth, the elevation in *VEGF* mRNA expression in the large antral follicles was much higher than that of *PEDF* mRNA expression (150% versus 60%, respectively), resulting in an anti-angiogenic balance during the avascular early stages of folliculogenesis, and a pro-angiogenic balance in later stages.

Next, this study focused on the direct interplay between PEDF, FSH and AMH during the final stages of folliculogenesis, using an established hpGC system (*Bussenot et al., 1993*). These cells had been exposed to ovulation triggering as part of the IVF protocol. Thus, a well-described protocol (*Chuderland et al., 2013a*; *Ophir et al., 2019*; *Sasson and Amsterdam, 2002*) that reverts the hpGC response to hormonal stimulation, specifically LH/human chorionic gonadotrophin (*Ophir et al., 2019*), was used, subjecting the cells to a daily exchange of fresh media before the stimulation. The reference genes (*Cyp19, AMHR* and *VEGF*) responded to a short period of stimulation with FSH, as expected from the literature, indicating

that hpGC are a suitable research model; however, this study was not able to detect any change in expression of *PEDF* mRNA. The authors' previous work showed that hormonal stimulation leads to a reduction in *PEDF* mRNA expression, both *in vitro* and *in vivo* (*Chuderland et al., 2013b*; *Miller et al., 2016*). In a search for an effect of FSH on *PEDF* mRNA expression, the cells were stimulated for a longer period. An increase in *PEDF* mRNA expression was detected, corresponding with reports that granulosa cells, derived from follicles in the follicular phase, secrete anti-angiogenic factors that shield against premature vascularization (*Fraser, 2006*). This increase in *PEDF* mRNA expression, only detected after 24 h of stimulation, could also imply an indirect effect of FSH on *PEDF* mRNA expression.

As detected in mouse follicles, the *PEDF/VEGF* ratio tends to favour *VEGF* after FSH stimulation, to encourage angiogenesis (*Fraser, 2006*; *Kuo et al., 2011*). However, an increase in *PEDF* mRNA expression at this stage could also be important to maintain a coordinated compartmentalization of blood vessels around the follicle that should not penetrate the follicle prematurely, facilitating both its anti-angiogenic and anti-oxidative traits. Previously, the authors showed that PEDF exerts an anti-oxidative effect in granulosa cells (*Bar-Joseph et al., 2014*) and oocytes (*Nemerovsky et al., 2021*). Thus, it is proposed that PEDF facilitates both its anti-angiogenic and anti-oxidative traits during folliculogenesis.

Following AMH stimulation, a decrease in *PEDF* mRNA expression was detected. In addition, PEDF stimulation led to a significant decrease in *AMHR* mRNA expression, pointing to a reciprocal relationship between their activities. Furthermore, AMH stimulation led to a decrease in *VEGF* mRNA expression that could reflect the preventive effect of AMH on the developmental progress of secondary follicles.

FSH and AMH push follicular growth in opposite directions. These data suggest that PEDF reinforces the pro-developmental effect of FSH by reducing the expression of *AMHR* mRNA, thus decreasing the influence of AMH on the growing follicle; the reciprocal regulation of *PEDF* mRNA expression by AMH supports this conclusion. This counter effect can be demonstrated in PCOS, where there are many pre-antral and small antral AMH-secreting follicles (*Rudnicka et al., 2021*), correlating with relatively low FSH and PEDF concentrations, and significantly high concentrations of AMH and ovarian vascularization (*Chuderland et al., 2014*; *Karakas, 2017*; *Sathyapalan et al., 2018*). In addition, FSH was found to be the only stimulator that up-regulated *PEDF* mRNA expression; all other examined effectors down-regulated *PEDF* mRNA expression. This elevation contributes to a strict angiogenic balance, negating VEGF both directly and indirectly via Hif1α (*Mao et al., 2020*); and to the regulation of oxidative stress in granulosa cells within the hypoxic follicle. The dual effect of PEDF during folliculogenesis allows follicular growth and health. When considering the contribution of PEDF to folliculogenesis, the many regulators that affect the ovary in general, and PEDF in particular, should be taken into account.

CONCLUSIONS

PEDF is a major pro-folliculogenesis player that interacts with the main participants in follicular growth and development. It appears to contribute to the regulation of angiogenesis and oxidative stress during folliculogenesis, allowing successful ovulation. Although the mechanism of action is not yet known, this study could be relevant to human ovarian physiology and pathophysiology, as explored in previous papers.

DATA AVAILABILITY

No data was used for the research described in the article.

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AUTHOR CONTRIBUTIONS

Conceptualization: RS, HB-J, LN and IB-A. Data curation: HB-J, AE-B, RT and IB-A. Formal analysis: RT, LN and CE. Funding acquisition: RS and IB-A. Investigation: RT, LN and CE. Methodology: HB-J, RS and AE-B. Supervision: RS and HB-J. Validation: HB-J. Writing – original draft: RT and LN. Writing – review and editing: RT, LN, HB-J, AE-B, CE, IB-A and RS. All authors reviewed the drafts and edited the manuscript, participating in analysis and interpretation of the results, fulfilling the four criteria recommended by the International Committee of Medical Journal Editors.

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RBMO

ARTICLE

Use of artificial intelligence embryo selection based on static images to predict first-trimester pregnancy loss

BIOGRAPHY

Alejandro Chavez-Badiola, a leader in fertility medicine, combines clinical expertise with technological innovation. Having founded NHFC Mexico and IVF 2.0, he is now focusing on Conceivable Life Sciences, a company at the forefront of applying cutting-edge automation technology to IVF.

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KEY MESSAGE

Correlation was found between the risk of spontaneous abortion and embryo rank as determined by artificial intelligence using the ERICA algorithm, with classification accuracy of 67.4%. This preliminary study suggests that ERICA may provide useful information for couples regarding their risk of spontaneous abortion.

ABSTRACT

Research question: Can an artificial intelligence embryo selection assistant predict the incidence of first-trimester spontaneous abortion using static images of IVF embryos?

Design: In a blind, retrospective study, a cohort of 172 blastocysts from IVF cases with single embryo transfer and a positive biochemical pregnancy test was ranked retrospectively by the artificial intelligence morphometric algorithm ERICA. Making use of static embryo images from a light microscope, each blastocyst was assigned to one of four possible groups (optimal, good, fair or poor), and linear regression was used to correlate the results with the presence or absence of a normal fetal heart beat as an indicator of ongoing pregnancy or spontaneous abortion, respectively. Additional analyses included modelling for recipient age and chromosomal status established by preimplantation genetic testing for aneuploidy (PGT-A).

Results: Embryos classified as optimal/good had a lower incidence of spontaneous abortion (16.1%) compared with embryos classified as fair/poor (25%; OR = 0.46, $P = 0.005$). The incidence of spontaneous abortion in chromosomally normal embryos (determined by PGT-A) was 13.3% for optimal/good embryos and 20.0% for fair/poor embryos, although the difference was not significant ($P = 0.531$). There was a significant association between embryo rank and recipient age ($P = 0.018$), in that the incidence of spontaneous abortion was unexpectedly lower in older recipients (21.3% for age ≤35 years, 17.9% for age 36–38 years, 16.4% for age ≥39 years; OR = 0.354, $P = 0.0181$). Overall, these results support correlation between risk of

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Declaration: JC holds stock in Conceivable Life Sciences, IVF 2.0 and Althea Science. ACB has received fees and/or meeting support from Conceivable Life Sciences and IVF 2.0, holds stock in Conceivable Life Sciences and IVF 2.0, and holds a Mexican patent for ERICA. ACB and JC have patents for Conceivable Life Sciences submitted for adjudication. AFSF, GMR, GS and RVM have received consulting fees from Conceivable Life Sciences and IVF 2.0, and hold stock options in Conceivable Life Sciences. AJD holds stock in IVF 2.0. DG has no conflicts of interest to declare.

KEY WORDS
Artificial intelligence
Embryo morphology
Miscarriage
Assisted conception
Embryo ranking

spontaneous abortion and embryo rank as determined by artificial intelligence; classification accuracy was calculated to be 67.4%.

Conclusions: This preliminary study suggests that artificial intelligence (ERICA), which was designed as a ranking system to assist with embryo transfer decisions and ploidy prediction, may also be useful to provide information for couples on the risk of spontaneous abortion. Future work will include a larger sample size and karyotyping of miscarried pregnancy tissue.

INTRODUCTION

Spontaneous abortion, otherwise known as early miscarriage or pregnancy loss in the first trimester, is a common complication of pregnancy (*Kolte et al., 2015*). The miscarriage rate is reported as 10–20% (with the lower figure pertaining to late pregnancy recognition) among naturally conceived pregnancies (*Cohain et al., 2017*). It has been suggested that pregnancies conceived through IVF have a slightly higher risk of miscarriage, although numbers vary (*Bashiri et al., 2018*). Spontaneous abortion can be a devastating experience for patients, leading to a range of stressful emotions and grief, with many couples giving up on further treatment before they become parents (*Domar et al., 2018*; *Rooney et al., 2022*). The management of spontaneous abortion also increases the time until the next opportunity for pregnancy, thus prolonging the infertility journey (*Munné et al., 2019*). The risk of miscarriage increases with maternal age, with the most common cause being chromosomal abnormality, principally aneuploidy (*Cimadomo et al., 2018*; *Gruhn et al., 2019*). With the exception of donor egg cycles, oocyte age is closely related to recipient age, with most patients receiving their own eggs from fresh or frozen cycles. Currently, the most common intervention for poor-prognosis patients with a high likelihood of spontaneous abortion is preimplantation genetic testing for aneuploidy (PGT-A). PGT-A is perhaps the most controversial procedure in assisted reproductive technology (ART) because of its invasive nature (embryo biopsy is required), its expense, and mixed messages from randomized controlled trials regarding its ability to improve (cumulative) pregnancy rates. As such, the Human Fertilization and Embryology Authority (HFEA), the UK regulator, has variously assigned PGT-A a red/amber light, several red lights and (currently) a green light for reducing the likelihood of miscarriage for most patients with infertility (https://www.hfea.gov.uk/treatments/treatment-add-ons/pre-implantation-genetic-testing-for-aneuploidy-pgt-a/). The HFEA system for rating 'add-ons' is no longer referred to as 'traffic lights', but uses a five-category rating system.

Nowadays, artificial intelligence applications are widely used in many areas of clinical medicine and medical research (*Curchoe et al., 2020*; *Yu et al., 2018*). ART lends itself particularly well to artificial intelligence applications, as it usually generates treatment-associated data in a variety of formats including images (from light microscope and camera), video, natural language and time. All of these parameters can potentially be used to train machine-learning algorithms to predict treatment success at different stages. Examples of these include artificial-intelligence-enhanced embryo selection (*Bori et al., 2021*; *Chavez-Badiola et al., 2020*; *Tran et al., 2019*; *VerMilyea et al., 2020*), sperm selection (*Zhang et al., 2021*), semen analysis (*Hicks et al., 2019*; *Mahdavi et al., 2011*) and many others (*Curchoe et al., 2020a*,*b*; *Kragh et al., 2021*; *Riegler et al., 2021*). Artificial intelligence is attractive because it facilitates the interrogation of large amounts of data, far more than the human eye can see or the mind is able to process, in a very rapid time frame. It thereby creates the possibility of identifying patterns and correlations amongst features not previously recognized as being important (*Bori et al., 2020*; *Gardner et al., 2000*).

ERICA (IVF 2.0 Ltd, UK) is an artificial intelligence system initially trained to anticipate the ploidy potential of blastocysts based on an assessment of either single static images or single images it extracts from time-lapse videos and metadata (*Chavez-Badiola et al., 2020*). ERICA first extracts texture patterns from the static images (light microscope and standard camera). This comprises a pre-processing step in which the images are adjusted to standardize the pixel to micrometre ratio, and each image is then convoluted with kernel filters, and segmented into four regions of interest (i.e. background, zona pellucida, trophectoderm and inner cell mass). A feature extractor designed to quantify predictors of embryo viability (e.g. size, shape of inner cell mass, and total energy and entropy measurements) is used to generate a feature vector. Next, ERICA ranks embryos based on the identification and scoring of blastocysts using extracted image-based features, and combining them with the metadata for each embryo using a binary classification model generated by a deep neural network. A total of 94 features have been extracted successfully by ERICA using these deep neural networks and artificial vision. The resulting model classification and confidence defines a score for each blastocyst in a given cycle, and ranks them according to their prognosis. In a previous study (*Chavez-Badiola et al., 2020*), these features were introduced into the training software, achieving accuracy of 0.70 in the validation set, and a positive predictive value for euploidy (as determined by PGT-A) of 0.79 in the test set. The ERICA algorithm assigns each embryo a value between 0 and 1, based on its putative anticipated euploidy potential, categorizing it as either optimal, good, fair or poor. The purpose is to assist embryologists in ranking the order in which blastocysts from a given cohort are to be transferred (*Chavez-Badiola et al., 2020*).

Based on the known association between aneuploidy and spontaneous abortion, the objective of this research was to perform an initial feasibility study to determine whether embryo ploidy ranking using ERICA could potentially be used to predict the occurrence of spontaneous abortion. Specifically, this study tested the hypothesis that events occurring between implantation (defined as a positive biochemical pregnancy result) and clinical pregnancy [defined as the detection of a fetal heart beat (FHB)] correlated with the optimal/good/fair/poor prediction using ERICA. This study also investigated whether the artificial intelligence assessment was more predictive for different recipient age groups, and whether correlations improved for embryos known to be euploid following PGT-A screening. If these hypotheses were correct, such information could be

beneficial when counselling couples (or individuals) regarding their embryo quality.

METHODS

A retrospective cohort of patients undergoing ART in two Mexican fertility clinics was included in this study. Only single embryo transfer cases that proceeded to biochemical pregnancy were included in the analysis.

Ethical approval, data collection, definitions and inclusion criteria

Institutional Review Board approval was granted for this project (CONBIOETICA 09-CEI-00120170131, approval date 14 June 2021).

A database of 525 embryo transfers performed between August 2019 and May 2021 at two IVF clinics in Mexico (New Hope Fertility Centers, Guadalajara and Mexico City), for which images had been uploaded into the ERICA web application prior to their transfer (erica.embryoranking.com), was used in this study. A positive biochemical pregnancy was defined as quantitative beta-human chorionic gonadotrophin (β-HCG) ≥20 IU/l 7 days post-transfer. After accounting for 15 cases which were lost to follow-up, the biochemical pregnancy rate per embryo transfer was 39.6% (n = 248/510). FHB was defined as either present or absent, based on week 7–8 transvaginal ultrasonography (TVS) (3–4 weeks after biochemical pregnancy test). Spontaneous abortion was defined as either: an initial positive pregnancy test (β-HCG ≥20 IU/l) followed by a subsequent fall in β-HCG concentration; transvaginal bleeding equal to or heavier than a menstrual period after excluding the possibility of ectopic pregnancy; the absence of a gestational sac at TVS performed 2–3 weeks after first biochemical pregnancy test; or a pregnancy which failed to develop an FHB identifiable with TVS by 7–8 weeks. An ongoing pregnancy was defined as the presence of an identifiable FHB during TVS performed 2–4 weeks following embryo transfer (pregnancy week 8–10).

All single embryo transfer cycles with a positive biochemical pregnancy test, known FHB status (present or absent), and registered in the ERICA web application were included in the analysis. Pregnancies with a confirmed spontaneous abortion following a positive biochemical test were included even if TVS was not performed.

After the exclusion criteria were applied, 172 single embryo transfers were suitable for analysis (FIGURE 1).

Ovarian stimulation and embryo culture

The stimulation, fertilization and culture processes followed standard protocols. In brief, following stimulation and oocyte retrieval (*Zhang et al., 2016*), oocytes were placed in human tubal fluid medium (Life Global, EUA) for 2 h before cumulus stripping. Metaphase II oocytes were inseminated by intracytoplasmic sperm injection, and then cultured in a continuous single culture complete medium with human serum albumin (FUJIFILM IrvineScientific, Inc., EUA), covered with Life-Guard high-viscosity oil (Life Global) for incubation for 5–6 days (CO_2 8.5%, O_2 5%) (Tri-gas; Astec, Japan). The culture medium was renewed after the third day of incubation.

Embryo transfer

Fresh embryo transfers were performed 5 days after oocyte retrieval. Luteal support, started on the night of oocyte retrieval, was 400 mg vaginal progesterone (Geslutin; Asofarma, USA) every 12 h until the pregnancy test, and then until week 8 if pregnant. For frozen embryo transfers, endometrial preparation was started with 4 mg oral oestradiol (Primogyn; Schering AG, Colombia) from day 3 of menstrual flow, and supplemented with 400 mg vaginal progesterone (Geslutin; Asofarma) every 12 h starting on cycle day 14–16; both continued until the pregnancy test. If the pregnancy test was positive, progesterone was continued until week 8, following the same schedule. Blastocysts were thawed 2–4 h before embryo transfer on day 6 from the beginning of luteal support. Vitrification and warming were performed using the Cryotop method and vitrification/thawing kits (Kitazato, Japan), as described elsewhere (*Kuwayama et al., 2005*, *2007*).

Embryo imaging and artificial-intelligence-assisted ranking

Pictures from blastocyst-stage embryos were taken before any interventions were performed (i.e. biopsy, vitrification or transfer). Single static images were uploaded into the ERICA web application (erica.embryoranking.com) for evaluation. Embryos were photographed with total magnification of either 200X or 400X using standard cameras (ZILOS-tk Laser; Hamilton Thorne, USA) installed on inverted microscopes (IX71 and IX73; Olympis, Japan) equipped with Hoffmann modulation contrast.

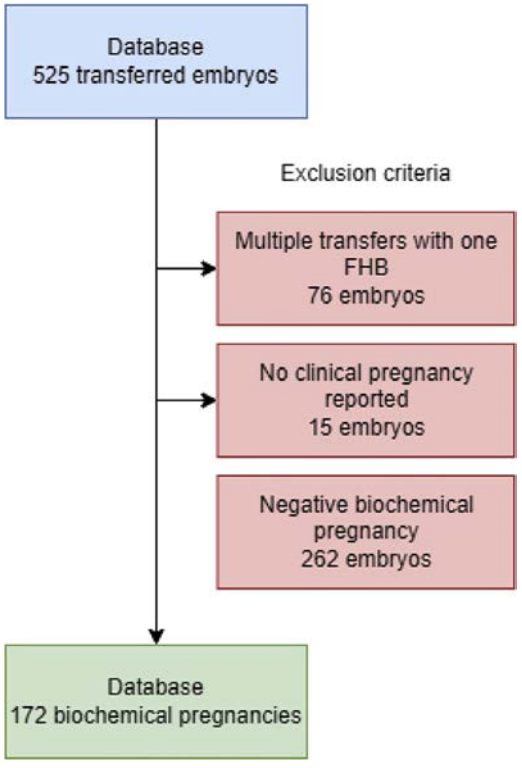

FIGURE 1 Number of embryo transfer cycles performed in the study period (August 2019–May 2021) and detailed inclusion process. FHB, fetal heart beat.

Embryos were assigned an output value ranging from 0 to 1 based on their calculated individual ploidy potential. This score was used to rank all embryos within a given cohort. A technical description of ERICA is given elsewhere (*Chavez-Badiola et al., 2020*). Based on the output of ERICA, embryos were defined arbitrarily as: optimal, score ≥0.70; good, score ≥0.50 and <0.70; fair, score ≥0.30 and <0.50; or poor, score < 0.30

Statistical analysis

Based on the hypothesis that embryos with the lowest scores will have higher spontaneous abortion rates, the ERICA categories were compared for their potential link to outcomes, defined as the presence or absence of an FHB following an initial positive pregnancy test (biochemical pregnancy). The proportion of spontaneous abortions within each category was calculated as the total number of biochemical pregnancies with no FHB, divided by the total number of biochemical pregnancies. Data are presented as percentages with their calculated 95% CI. Due to the low sample size in some comparisons, embryos classified as optimal or good were pooled, as were embryos classified as fair or poor.

Statistical analysis was completed using SPSS v.28 (IBM, USA), and made use of a generalized linear regression model using logit link functions for binary outcomes. The model tested for the presence of any interaction between embryo ranking and female age, as the increased risk of aneuploidy is positively correlated with age (*Hassold and Hunt, 2009*). Age brackets of ≤35, 36–38 and ≥39 years were chosen arbitrarily; however, it should be noted that, due to the use of donor eggs for some patients and incomplete records of the time between egg collection and transfer, it was not possible to make accurate calculations for oocyte age. Thus, recipient age alone was used in this study. Finally, a subset of embryos from this database (n = 50) had a known euploid diagnosis as detected by high-resolution next-generation sequencing PGT-A screening; the effect of this factor was also evaluated.

RESULTS

The demographics and characteristics of the biochemical pregnancies included in this study are described in TABLE 1. A full breakdown of the entire embryo database is presented in Supplementary Table 1 (see online supplementary material).

TABLE 1 DEMOGRAPHIC INFORMATION

Metric	Magnitude
Pregnancies included	172 (100)
Donor eggs	28 (16.3)
Fresh transfers	15 (8.7)
Known euploid transfers	50 (29.1)
Early pregnancy losses[a]	32 (18.6)
Maternal age (years), mean (SD)[b]	36.2 (5.01)

Data are *n* (%) unless otherwise indicated.

[a] All spontaneous abortions before 7–8 weeks, including those with no identifiable fetal heart beat.

[b] For clarity, 'maternal age' refers to recipient age in all cycles (including donor cycles).

Relationship between spontaneous abortion, embryo ranking and recipient age

When a generalized linear model was employed, statistical analysis identified a significant effect for embryo rank. Embryos classified by the artificial intelligence assistant as either optimal or good had an incidence of spontaneous abortion of 16.1% (95% CI 10.7–23.6%, n = 20/124), compared with 25.0% for embryos classified as either fair or poor (95% CI 14.9–38.8%, n = 12/48, OR = 0.46, P = 0.005). The incidence of spontaneous abortion appeared to decrease with recipient age (21.3% for age ≤35 years, 17.9% for age 36–38 years, 16.4% for age ≥39 years; OR = 0.354, P = 0.0181). A significant interaction was detected between embryo ranking and recipient age (P = 0.018). These findings are presented in TABLE 2 and FIGURE 2.

Overall, the results support some correlation between the risk of spontaneous abortion and ERICA rank. The calculated accuracy of this prediction (the overall probability that a biochemical pregnancy was correctly predicted to progress or not progress) was 67.4% (95% CI 59.9–74.4%). The test also achieved good sensitivity (74.3%, 95% CI 66.2–81.3%), but poor specificity (37.5%, 95% CI 21.1–56.3%), suggesting that the artificial intelligence was better at assigning an embryo that was associated with an ongoing pregnancy to the optimal or good rank (sensitivity) than it was at assigning an embryo that was associated with spontaneous abortion to the fair or poor rank (specificity).

Spontaneous abortion in euploid embryos determined by PGT-A

Next, a sub-analysis was performed to compare the same embryo ranks and groups for known euploid embryos (*n* = 50). The incidence of spontaneous abortion was 13.3% (95% CI 5.3–29.7%, *n* = 4/30) in euploid embryos classified as either optimal or good, and 20.0% (95% CI 8.1–41.6%, *n* = 4/20) in embryos classified as either fair or poor; the difference was not significant (*P* = 0.531).

DISCUSSION

Spontaneous abortion is a common complication encountered during fertility treatment, with an incidence rate of 12–22% in IVF cycles (*Bashiri et al., 2018*; *Bu et al., 2020*; *Yang et al., 2019*), although PGT-A has been shown to reduce the risk of spontaneous abortion following the

TABLE 2 INCIDENCE OF SPONTANEOUS ABORTION BY ERICA EMBRYO RANK AND RECIPIENT AGE

Embryo rank	≤35 years		36–38 years		≥39 years	
	Total *n*	SA *n* (%)	Total *n*	SA *n* (%)	Total *n*	SA *n* (%)
Optimal or good	48	7 (14.6)	43	6 (14.0)	33	7 (21.2)
Fair or poor	13	6 (46.2)	13	4 (30.8)	22	2 (9.1)
Overall	61	13 (21.3)	56	10 (17.8)	55	9 (16.4)

SA, spontaneous abortion (early pregnancy loss).

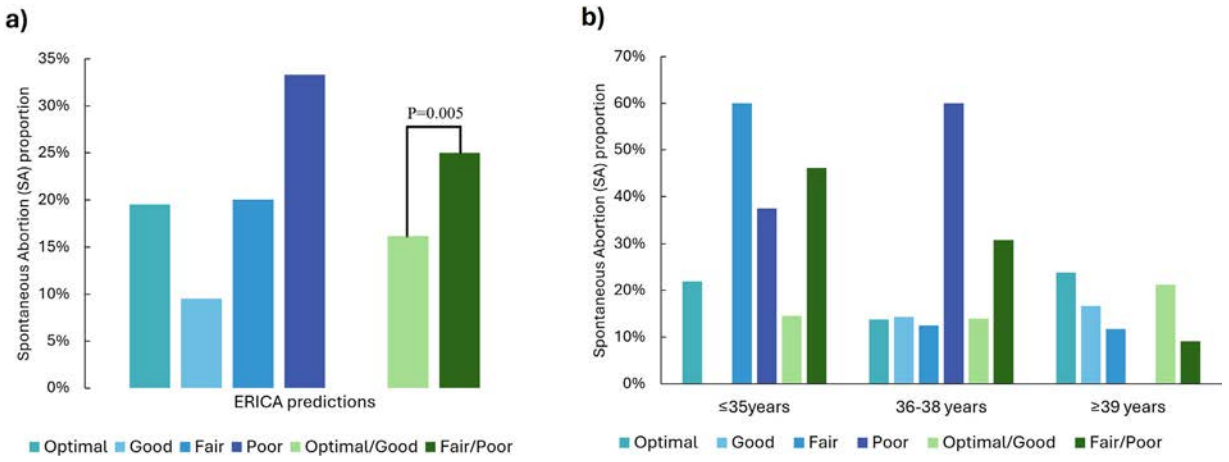

FIGURE 2 Spontaneous abortion proportions by ERICA rank: (a) overall; and (b) stratified by recipient age group (≤35, 36–38 or ≥39 years). ERICA rank: optimal, score ≥0.70; good, score ≥0.50 and <0.70; fair: score ≥0.30 and <0.50; poor: score < 0.30. Optimal/good versus fair/poor compared using generalized linear regression.

transfer of euploid embryos (*Lee et al., 2019*; *Rubio et al., 2017*). Spontaneous abortion imposes a high toll on patients, not only emotionally, financially and physically, but also by increasing the time until the next opportunity for pregnancy, which, for some patients, such as those with a severely compromised ovarian reserve, could further reduce their overall chance of conceiving at all (*Bashiri et al., 2018*; *Cohain et al., 2017*; *Kolte et al., 2015*).

As current ART treatments tend to generate more than one transferrable embryo, use of the embryo selection process as a means to improve time to pregnancy (usually using the subjective judgement of the embryologist) has long merited attention (*Lee et al., 2019*; *Rubio et al., 2017*). As a consequence, approaches to improve this process using more objective and accurate ways of selecting viable embryos have been proposed, and include PGT-A (*Munné et al., 2019*), time-lapse evaluation (*Kovacs et al., 2014*), visual classifications (*Gardner et al., 2000*) and, more recently, artificial intelligence (*Dimitriadis et al., 2022*). Despite the controversial nature of PGT-A in its current form, it is the closest approach to a gold standard that exists for the determination of aneuploidy potential, and hence the likelihood of a pregnancy extending past the first trimester.

Embryo quality is largely considered to be the primary factor responsible for the achievement of a positive pregnancy test, although several other factors, including endometrial receptivity, may contribute (*Ashary et al., 2018*; *Tıras et al., 2014*). Embryos with severe aneuploidy, such as those with monosomies, and chaotic embryos are likely to be lost before a positive pregnancy test is achieved. Beyond this, one could assume that many of the basic factors that might have prevented implantation have been overcome successfully, such as the implantation window and the embryo transfer procedure itself. It is at this point that aneuploidy (principally, trisomy of the autosomes and monosomy X) is relevant. Although the causes of spontaneous abortion are multifactorial, aneuploidy is widely cited as the most common reason (*Hassold and Hunt, 2001*; *Rose et al., 2020*).

The curious incidence of spontaneous abortion appearing to decrease with recipient age bears further scrutiny. Ordinarily, one would expect to see an increase in spontaneous abortion associated with older oocytes (*Hassold and Hunt, 2009*) and, with most recipients (83.7%) receiving their own eggs, a corresponding, although perhaps less strong, association with recipient age. Unfortunately, in this study, it was not possible to determine the age of the oocytes used, partly because the ages of the donor oocytes were not recorded accurately, and partly because records of the time between egg collection and transfer were incomplete. The apparent 'reverse maternal age effect' recorded here could be explained by an increase in the proportion of donor eggs employed in the older patient group(s), and/or increased attention in the treatment of older patients; however, this is not clear at the time of writing, and should be logged more accurately in future studies.

Another factor worthy of consideration is the relatively small sample size of this study (32 spontaneous abortion events). In some ways, it is surprising that such high accuracy was achieved in this study, considering that the algorithm seems to classify most embryos as good or fair. Although sample size calculations were not performed in this study, one argument (which, it is appreciated, is circular) is that significant results were seen, so the sample size must have been sufficient. On the other hand, it is appreciated that, in future studies, the magnitude of the predictive effect of ERICA will be further established. This will be performed on more advanced versions of the programme, and on newly acquired morphokinetic images and videos. These will form part of newly designed studies and future publications.

ERICA was developed as a ranking tool to advise embryologists about the best order in which embryos from a cohort could be transferred. This ranking was developed using PGT-A results as its 'ground truth', with clinical results supporting its performance (*Chavez-Badiola et al., 2020*). Although ERICA was not designed to predict the risk of miscarriage *per se*, aneuploidy is the most common cause of spontaneous abortion. As such, this study aimed to determine whether there was a correlation between ERICA classification

and spontaneous abortion, and found that the ranking assessment was somewhat predictive of spontaneous abortion, as it was able to correctly classify just over two-thirds of the embryos analysed.

Nonetheless, it is acknowledged that before a truly robust statistical evaluation of the hypothesis can be made, the limited sample size in this study should be addressed. Furthermore, while it is not claimed that ERICA is a more accurate assessment of embryo ploidy than the gold standard PGT-A, it was interesting to note a potential application for this tool for the prediction of spontaneous abortion. It should also be considered that PGT-A usually requires cryostorage and subsequent frozen embryo transfer, which are expensive and invasive procedures for the embryos. Not all embryos will survive the biopsy and warming process, and some couples only have a small number of embryos per cohort.

To the best of the authors' knowledge, this is the first study to address the association between pregnancy loss using an artificial intelligence algorithm and static images. Recently, however, *Amitai et al. (2023)* identified six morphodynamic features extracted from time-lapse images that had high prediction capability for spontaneous abortion. These centred around the distribution of nucleolar precursor bodies within the small pronucleus(i) predicting miscarriage at 0.68–0.69. The authors thus claim to have developed a decision-support tool for identifying embryos with a greater chance of miscarriage. With the now-increased interest in PGT-A for reducing the likelihood of miscarriage based on the newly awarded 'green light' by HFEA (https://www.hfea.gov.uk/treatments/treatment-add-ons/pre-implantation-genetic-testing-for-aneuploidy-pgt-a/), the use of tools to predict miscarriage, perhaps associated with aneuploidy, is becoming more topical. Whether artificial intelligence would be used instead of, or as an adjunct to, PGT-A remains to be seen. Indeed, a better understanding of the likelihood of pregnancy and miscarriage for each embryo will assist clinical staff to best manage their patients' expectations during counselling. For future studies focusing on miscarriage, karyotyping of the pregnancy tissue would establish whether ERICA is as good as, or nearly as good as, PGT-A to predict ploidy status, and hence the likely potential for spontaneous abortion of an IVF cycle.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.103934.

DATA AVAILABILITY

Data will be made available on request.

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RBMO

ARTICLE

Artificial shrinkage before fresh blastocyst transfer and IVF outcomes: a pilot randomized controlled study

BIOGRAPHY

Anna Gala has worked as an embryologist for more than 10 years in the Reproductive Biology Department of Montpellier University Hospital, France, on the theme of embryonic development, fertility and environment. Since 2023, she has been associated with the Saint Roch Clinic, a member of the Inovie Fertilité group.

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KEY MESSAGE

This pilot study reported no impact of artificial shrinkage before fresh blastocyst transfer in IVF outcomes. Large-scale randomized control trials are required to confirm these preliminary results.

ABSTRACT

Research question: Does artificial shrinkage before fresh blastocyst transfer improve clinical pregnancy rates in IVF?

Design: In this monocentric prospective, randomized, double-blind, controlled pilot study, 150 couples undergoing fresh single-blastocyst transfer were randomized between 20 May 2018 and 22 February 2022. In the artificial shrinkage group (AS group), a single laser pulse was directed to the cellular junction of the trophectoderm on the opposite side of the inner cell mass in each blastocyst. IVF outcomes were clinical pregnancy, multiple pregnancy and live birth rates. Cell-free DNA (cfDNA) concentration was also measured by quantitative real-time PCR in the blastocyst culture medium.

Results: In total, 142 couples underwent fresh single-blastocyst transfer: control group, no artificial shrinkage, $n = 47$; and AS group, artificial shrinkage, $n = 95$; An intention-to-treat (ITT) analysis was employed. After a reassessment and the exclusion of patients with major protocol deviations, 139 couples underwent fresh single-blastocyst transfer under optimal conditions: control group, $n = 47$; and AS group, $n = 92$; a per-protocol analysis was used here. The clinical and laboratory characteristics were not significantly different between the groups. The clinical pregnancy rate was similar in the control and AS groups (ITT: 48.9% versus 49.5%, $P = 0.97$; per protocol: 48.94% versus 51.1%, $P = 0.89$). The multiple pregnancy rate and the live birth rate were also similar between the groups. No significant differences in gestational age, birthweight or proportion of male/female newborns were observed. The concentration of cfDNA in the blastocyst culture medium was not associated with IVF outcome.

Conclusions: Large-scale randomized controlled trials are required to confirm these preliminary results.

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KEYWORDS
Artificial blastocoel collapse
Artificial shrinkage
Blastocyst transfer
IVF
Live birth
Pregnancy

INTRODUCTION

Artificial shrinkage of the blastocoel cavity is a promising approach to improve IVF outcomes after frozen-thawed blastocyst transfer (*Boyard et al., 2022*). Several studies have reported the significant positive impact of pre-vitrification artificial shrinkage on blastocyst survival after thawing (*Darwish and Magdi, 2016*; *Gala et al., 2014*; *Iwayama et al., 2011*; *Mukaida et al., 2006*; *Van Landuyt et al., 2015*) and on the rate of clinical pregnancy (*Darwish and Magdi, 2016*; *Levi-Setti et al., 2016*; *Mukaida et al., 2006*). It was postulated that the positive effect of artificial shrinkage on frozen-thawed blastocyst transfer outcomes results from the prevention of osmotic changes and ice crystal formation within the collapsed blastocoel during vitrification-thawing (*Boyard et al., 2022*). This technique is now carried out systematically in assisted reproductive technology laboratories worldwide.

Interestingly, one team evaluated the effect of artificial shrinkage on IVF outcomes after fresh blastocyst transfer on day 5 in a prospective non-randomized study (*Hur et al., 2011*). They found that the clinical pregnancy rate was significantly higher when the blastocysts underwent artificial shrinkage before fresh transfer (n = 20/34, 58.8% versus n = 39/100, 39% in the control group; $P < 0.05$). The authors did not investigate the underlying molecular mechanisms associated with this effect in this first paper. Consequently, only hypotheses can currently be put forward to try to explain the observed effect.

One could speculate that this positive effect of artificial shrinkage in fresh transfer could be due to the detoxification of waste products accumulated in the blastocoel cavity during in-vitro culture and expelled from the blastocyst (*Tedeschi et al., 2017*). Indeed, many components are secreted in the blastocoel fluid during blastulation, including cell-free DNA (cfDNA) (*Rule et al., 2018*; *Lal et al., 2022*; *Magli et al., 2019*), microRNA (*Esmaeilivand et al., 2022*) and proteins (*Kavoussi et al., 2022*; *Tedeschi et al., 2017*).

The results of studies evaluating the association between the cfDNA content of the blastocoel fluid and blastocyst quality are contradictory. Rule and colleagues reported higher cfDNA concentrations in the blastocoel fluid of high-quality blastocysts (*Rule et al., 2018*), probably due to the increased number of cells, including apoptotic cells, in fully expanded blastocysts compared with early blastocysts. In agreement with this, a more recent study showed higher apoptotic gene expression in the blastocoel fluid of euploid blastocysts and implanted blastocysts than in aneuploid and non-implanted blastocysts (*Lal et al., 2022*).

Conversely, a third study found lower cfDNA amplification rates in blastocoel fluid from euploid compared with aneuploid blastocysts and also in blastocoel fluid from blastocysts that led to clinical pregnancy after implantation (*Magli et al., 2019*). This last result suggests that the amount of cfDNA might be lower (i.e. below the threshold necessary for successful amplification) in the blastocoel fluid of high-quality blastocysts. Together, these data suggest that cfDNA concentration might be a novel predictive non-invasive biomarker to select the best blastocyst for transfer. However, the cfDNA threshold remains to be clearly defined.

To improve knowledge of the effect of artificial shrinkage before fresh blastocyst transfer, the current authors carried out a pilot randomized prospective study to evaluate the impact of artificial shrinkage on IVF outcomes after the transfer of single fresh day 5 blastocysts. The end-points were clinical pregnancy rate (primary outcome) and multiple pregnancy and live birth rates (secondary outcomes). The cfDNA concentration in the blastocyst culture medium just before fresh blastocyst transfer was also quantified to evaluate its potential as a non-invasive predictive biomarker of blastocyst implantation potential.

MATERIAL AND METHODS

This monocentric, prospective, randomized, double-blind, controlled pilot study was carried out at the Department of Reproductive Medicine of Montpellier University Hospital between 20 May 2018, when the first patient was enrolled, and 22 February 2022. The study was registered on ClinicalTrials.gov (number NCT02988544, trial registration date 9 December 2016).

Ethics

The Montpellier University Hospital Institutional Review Board approved this study (Committee for the Protection of Persons N°2017-T1-20, French National Medicines Safety Agency N°2017-A01256-47, approval date 24 October 2017). All enrolled patients signed an informed consent form for participation in the study and for the use of their clinical and laboratory data. Patients were anonymized and were assigned non-identifying study numbers. All procedures were performed in accordance with the relevant guidelines and regulations.

Clinical study design

The inclusion criteria were as follows: (i) couples with at least one day 5 blastocyst with an expansion grade of ≥3 and trophectoderm quality of grade A or B, according to the Gardner and Schoolcraft classification (*Gardner et al., 2000*); (ii) couples planning to be available for a 12-month follow-up after day 5 blastocyst transfer to collect all serious adverse events and IVF outcomes (clinical pregnancy, first ultrasound, live birth); and (iii) couples who could fully understand the information given and give their informed consent. Exclusion criteria were: (i) undergoing preimplantation genetic testing; (ii) frozen embryo transfer; (iii) participation in another interventional study; and (iv) sperm or oocyte donor.

The sample size was calculated based on the clinical pregnancy rate at the authors' centre (i.e. 45% following single-blastocyst transfer). By assuming a pregnancy rate difference of 30 points between the arms (30% in the control group and 60% in the artificial shrinkage group [AS group]), with an α risk of 5% and a power of 85%, 47 couples were necessary per arm (two-tailed test of equality of two proportions). To take into account possible unobservable data, it was decided to include 50 couples in the control group. A randomization ratio of 1:2 was chosen as a large difference in response rate was expected in favour of the AS group (based on the paper by *Hur et al. [2011]*). Hence, for ethical reasons, it was decide to include 50 couples in the control group and 100 couples in the AS group (n = 150). This imbalance between arms allowed a better estimation of the clinical pregnancy rate in the AS group with a half-width of the 95% confidence interval (95% CI) of ±10 points.

Couples were randomized by taking into account the number of previous IVF attempts, using Clinsight software (Ennov Clinical Version 8.2.320, https://saas7028.

ennov.com/EnnovClinical/) and after confirming that they met the inclusion criteria. The couples and the doctor in charge of the embryo transfer were blinded to the randomization. All artificial shrinkages were performed immediately before fresh blastocyst transfer on day 5 (AS group). Fresh single blastocysts were transferred on day 5 without any additional intervention in the control group.

IVF procedures

After ovarian stimulation (*Brouillet et al., 2021*), when at least three follicles had reached a mean diameter of ≥17 mm, recombinant human chorionic gonadotrophin (HCG) (Ovitrelle) was administered for oocyte triggering and final maturation. Ovarian puncture was performed 35–36 h after α-HCG administration by transvaginal ultrasound-guided needle aspiration of the follicles (Otrieva, Cook Medical, France). This procedure was performed under conscious sedation or occasionally under general anaesthesia.

Culture dish preparation, gamete collection and embryo culture

All the culture dishes were prepared at room temperature 1 day before use and equilibrated overnight in CO_2 incubators (Heracell 240i, Thermo Scientific, France) at 37.0 ± 1°C. The temperature, humidity and gas content inside the incubators were continuously monitored (external monitoring system, Cobalt 2, Oceasoft, France).

Cumulus–oocyte complexes were retrieved from follicular fluid, placed in pre-incubated fertilization medium (G-IVF PLUS, Vitrolife, France) and cultured at 37°C under a gas mixture of 5% O_2, 6% CO_2 and 89% N_2 until insemination in conventional IVF or denudation before intracytoplasmic sperm injection (ICSI). Each semen sample was collected by masturbation in a sterile container after 2–3 days of abstinence. Spermatozoa were prepared by discontinuous density gradient centrifugation. The choice of technique (conventional IVF or ICSI) was based on the sperm parameters on the collection day, according to the World Health Organization criteria (2021). For ICSI, oocytes were denuded using mechanical (Stripper, CooperSurgical, France) and enzymatic (hyaluronidase, HYASE, Vitrolife, France) methods.

Gamete fertilization and embryo culture were individually performed in 20 µl of pre-equilibrated G-1 PLUS medium droplets covered with mineral oil (OVOIL, Vitrolife, France) at 5% O_2, 6% CO_2 and 89% N_2 up to day 3. Then, from day 3 to day 6, embryos were cultured in 20 µl of pre-equilibrated G-2 PLUS medium droplets covered with mineral oil (OVOIL, Vitrolife) at 2% O_2, 6% CO_2 and 92% N_2.

Embryo classification and selection

Following removal from the incubator, the morphology of each embryo was evaluated by two embryologists at the same time. Embryo transfer and cryopreservation were discussed and approved by a medical team. Normal fertilization was confirmed 19–21 h after conventional IVF or ICSI by the presence of two pronuclei and the extrusion of the second polar body.

Embryo growth was monitored daily using morphological and kinetic parameters. Embryo evaluation at day 3 was based on the blastomere number and symmetry, fragmentation percentage, presence of multinucleated blastomeres and degree of compaction. At day 5 and day 6, blastocyst morphology was evaluated according to the Gardner and Schoolcraft grading system (*Gardner et al., 2000*). Usable blastocysts were defined as full (grade 3), expanded (grade 4), partially hatched (grade 5) or fully hatched (grade 6) blastocysts with at least grade B trophectoderm quality. The trophectoderm and inner cell mass of the top-quality blastocysts were grade A. Usable blastocysts were freshly transferred at day 5 or cryopreserved at day 5/6. Early blastocysts (grade 1 or 2) at day 5 were kept in culture until day 6 and cryopreserved if considered to be usable blastocysts.

Artificial shrinkage

A single laser pulse (200 ms) was triggered at the cell junction of the trophectoderm to create a hole between the cells and to induce blastocoel cavity collapse, using a ZILOS-tk laser (Hamilton Thorn Bioscience, USA). The target of the laser pulse was located at the opposite side of the inner cell mass. For hatching embryos (expansion grade ≥5), the laser target was away from both the hernia and the inner cell mass. The reduction in blastocyst size accompanied by the efflux of the blastocoel fluid after the laser pulse and the trophectoderm detachment from the zona pellucida were monitored. To avoid trophectoderm injury no additional laser pulse was applied in cases of partial collapse.

Blastocyst transfer

Single-blastocyst transfer was performed under ultrasound guidance using an embryo transfer catheter (Inventcath Eco Laboratoire CCD, France). Each woman received intravaginal progesterone for luteal phase support from the day of oocyte retrieval until the β-HCG blood test.

cfDNA quantification

Immediately after fresh blastocyst transfer on day 5, an aliquot (6 µl) of the culture medium in which the embryo had been cultured from day 3 to day 5 was pipetted and transferred into a sterile tube (both study arms). To limit the risk of contamination, collection of the medium was performed under a laminar flow hood and by an investigator wearing personal protective equipment. The interval between the artificial shrinkage and medium collection ranged between 30 min and 1 h. The samples were immediately frozen at −20°C. If the analysis could not be carried out in the following days, the samples were transferred to storage at −80°C (*Brouillet et al., 2020*). A negative control (culture medium that had never contained an embryo but had been placed in the same type of culture dish used for the embryos) was systematically analysed (*Capalbo et al., 2018*; *Li et al., 2018*).

To quantify the cfDNA, the mixture (culture medium + buffer) was digested with 16 µg of proteinase K solution (Qiagen, France) at 50°C for 20 min, followed by heat deactivation for 5 min and insolubilization at 95°C (*Umetani et al., 2006*). After centrifugation at 10,000*g* and 4°C, for 5 min, the cfDNA-containing supernatant was immediately stored at −80°C until cfDNA quantification by ALU-quantitativePCR (QuantStudio 7, ThermoFisher Scientific, France). This method quantifies shorter and longer ALU interspersed repeats. Each PCR reaction mixture contained 1 µl of supernatant, 5 µl of 2X LightCycler 480 SYBR Green I master mix (Roche Applied Science, France) and 0.25 µl of each of ALU115 forward and reverse primers (ALU115 forward, 5′-CCTGAGGTCA-GGAGTTCGAG-3′; ALU115 reverse, 5′-CCCGAGTAGCTGGGATTACA-3′). Heating at 95°C for 10 min was followed by 50 cycles in a LightCycler 480 (Roche Applied Science). All assays were repeated four times and a non-template control was included in each run. The cfDNA concentration was determined with the

standard curves obtained for ALU115 using genomic DNA.

Clinical end-points of the study

Biochemical pregnancy was assessed based on serum β-HCG concentrations of over 50 IU/l at day 9 after blastocyst transfer. To confirm the clinical pregnancy, ultrasonography was performed 5 weeks later to visualize the gestational sac and fetal heartbeat. The twin pregnancy rate was defined as the presence of two cardiac activities on this ultrasound scan. The loss of a fetus before 20 weeks of gestation was considered a spontaneous abortion. A live birth was defined as a delivery that resulted in the birth of at least one live-born neonate. Preterm birth was defined as a delivery at less than 37 gestational weeks. A birthweight of less than 2500 g was defined as a low birthweight. The pregnancy duration, delivery mode and weight and sex of the child were recorded as neonatal outcomes.

Statistical analyses

The statistical analysis was performed by the Clinical Research and Epidemiology Unit of Montpellier University Hospital using SAS (SAS Institute, USA) version 9, or R version 2.9.2 (R Development Core Team [2009], R Foundation for Statistical Computing, Austria).

The normality of distribution of the quantitative variables was assessed using the Shapiro–Wilks normality test, and kurtosis and skewness coefficients. Data were presented as means ± standard deviations (SD) for quantitative variables. Qualitative variables were presented using numbers and percentages. The main analysis was based on the intention-to-treat (ITT) principle; per-protocol analysis was performed as a sensitivity analysis. In the ITT analysis, all the randomized participants who had undergone a fresh blastocyst transfer were studied regardless of protocol deviations. In the per-protocol analysis, only data from those who strictly adhered to the study protocol were analysed. The per-protocol analysis was performed to provide an estimate of the true efficacy of the intervention.

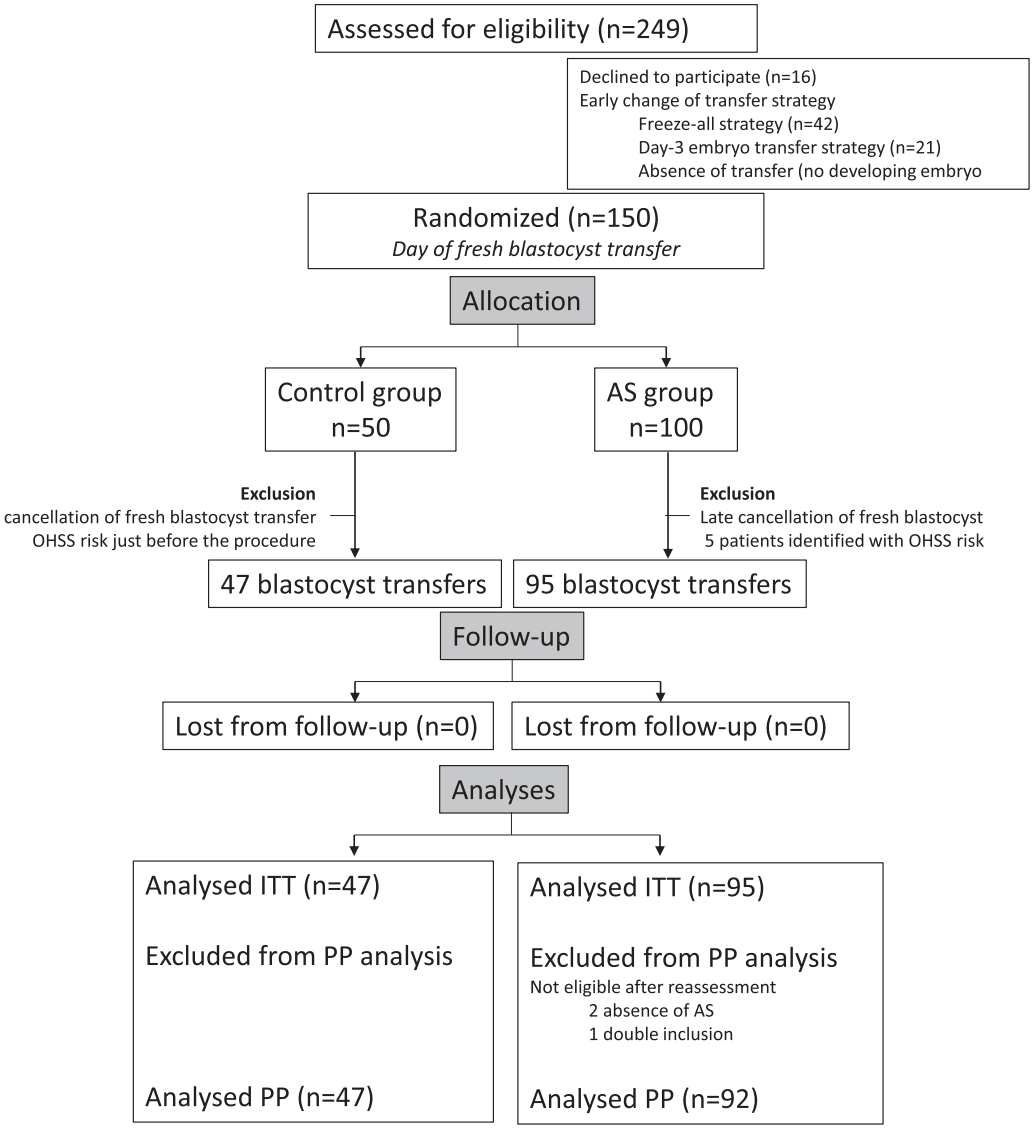

FIGURE 1 The CONSORT flow chart of the study group.
AS, artificial shrinkage; D3, day 3; ITT, intention to treat; OHSS, ovarian hyperstimulation syndrome; PP, per protocol.

The study outcomes were compared as means or percentages. Qualitative variables were compared using the chi-squared test or Fisher's exact test. Quantitative variables were compared between groups using the Wilcoxon–Mann–Whitney test. Differences were considered statistically significant when $P \leq 0.05$.

RESULTS

Patient characteristics

The flow chart of the study group and patient characteristics are shown in FIGURE 1 and TABLE 1, respectively. All the parameters were similar in both groups. In total, eight randomized couples had a late cancellation of their fresh single-blastocyst transfer due to the detection of fluid in the abdominal cavity by ultrasound, suggesting a potential risk of ovarian hyperstimulation syndrome (FIGURE 1; control group, $n = 3$; AS group, $n = 5$). Therefore, 142 couples underwent fresh single-blastocyst transfer: 47 in the control group and 95 in the AS group (FIGURE 1, ITT analysis). In per-protocol analysis, three fresh transfers were excluded after reassessment in the AS group (FIGURE 1; no artificial shrinkage, $n = 2$; double inclusion, $n = 1$).

IVF outcomes

The blastocyst transfers and IVF outcomes are summarized in TABLE 2. The quality of the transferred blastocysts was similar in both groups. The clinical pregnancy rate was similar in the control and AS groups (ITT analysis, 48.9% (23/47) versus 49.5% (47/95); per-protocol analysis, 48.9% (23/47) versus 51.1% (47/92)). Two twin pregnancies were reported in the control group and none in AS group. The live birth rate was similar in the control and AS groups (ITT analysis, 44.7% (21/47) versus 47.4% (45/95); PP analysis, 44.7% (21/47) versus 48.9% (45/92)). The gestational age, birthweight and proportion of male and female newborns were also similar.

The concentration of cfDNA was similar in the two groups (TABLE 2). There was no difference in terms of the outcome (clinical pregnancy or not, live birth or not) in the two groups and in the whole sample (TABLE 3). There was no association between the concentration of cfDNA and the occurrence of clinical pregnancy (OR 1.25, 95% CI 0.82–1.90; $P = 0.3$).

Follow-up

Ten serious adverse events were reported: six concerned the women ($n = 3$ late ovarian hyperstimulation syndrome, $n = 1$ in the control group and $n = 2$ in the AS group; $n = 2$ pre-eclampsia, one in each group; $n = 1$ post-partum haemorrhage on a placenta praevia in the AS group) and four in the fetuses/newborns ($n = 1$ trisomy 13 in the AS group; $n = 1$ biventricular hypertrophic cardiomyopathy in a preterm baby in the AS group; $n = 1$ congenital hypothyroidism in the AS group; $n = 1$ fetal death *in utero* in the control group) (TABLE 2; 6.4% in the control group and 7.6% in the AS group; $P = 1$).

TABLE 1 CLINICAL AND LABORATORY PARAMETERS OF THE ENROLLED COUPLES.

Parameters	Control group ($n = 50$)	AS group ($n = 100$)	*P*-value
Woman's age (years)	32.0 ± 4.8	31.8 ± 4.6	0.81
Woman's BMI (kg/m^2)	23.1 ± 4.9	23.5 ± 3.8	0.15
AMH (ng/ml)	3.5 ± 1.9	3.9 ± 2.8	0.98
Man's age (years)	35.1 ± 6.2	34.4 ± 5.7	0.38
Total gonadotrophin dose (IU)	1947 ± 871	2062 ± 851	0.24
Retrieved oocytes (*n*)	12.6 ± 4.9	12.1 ± 4.6	0.55
Conventional IVF/ICSI (*n*/*n*)	22/28	33/67	0.19
Fertilization rate (%)	75.6 ± 18.6	78.3 ± 17.3	0.42
Embryos on day 3 (*n*)	9.0 ± 3.9	8.9 ± 3.9	0.84
Blastocysts (*n*)	5.2 ± 3.0	5.0 ± 2.8	0.95

All data are presented as mean ± standard deviation or *n*.

The Mann–Whitney test and chi-squared test were used.

AMH, anti-Müllerian hormone; AS, artificial shrinkage; BMI, body mass index; ICSI, intracytoplasmic sperm injection.

DISCUSSION

In this pilot randomized study, there was no significant impact of artificial shrinkage on IVF outcomes after fresh blastocyst transfer. However, the results are preliminary because the sample size was low and calculated on a large benefit, leading to an underpowered trial for detecting small (but potentially clinically significant) differences between the two arms. Therefore, large-scale randomized controlled trials are now required to confirm or inform these preliminary results.

Different artificial shrinkage techniques have been described (micro-needle or laser pulse) (*Boyard et al., 2022*). Due to its simplicity and rapidity, the laser technique is more interesting in an IVF clinical setting. At the authors' centre, the laser technique has been used since 2013 for collapsing blastocysts before cryopreservation. A previous monocentric study has demonstrated that this procedure increases survival and clinical pregnancy rates when performed before vitrification at the authors' centre (*Gala et al., 2014*). This positive impact of artificial shrinkage has been also found in large series and a recent meta-analysis (*Boyard et al., 2022*).

The rationale of using artificial shrinkage before fresh blastocyst transfer is based on the observation of frequent contraction–expansion cycles of blastocysts during in-vitro culture (*Gazzo et al., 2020*). Contraction may be due to fluid leakage through weak tight junctions, and expansion may be explained by an extracellular liquid inflow through aquaporin water channels due to an ion concentration increase via the sodium/potassium pump (*Marcos et al., 2015*). These physiological contraction–expansion cycles might participate in the continuous blastocoel fluid renewal, contributing to the detoxification of waste products accumulated in the blastocoel cavity during in-vitro culture. This hypothesis is consistent with the recently proposed role of spontaneous blastocyst retraction in excluding aneuploid cells or in maintaining inner cell mass integrity (*Cimadomo et al., 2022*; *Coticchio et al., 2021*).

TABLE 2 IVF AND NEONATAL OUTCOMES IN THE CONTROL AND AS GROUPS

Parameters	Control	AS	*P*-value
Couples (*n*)			–
ITT	47	95	
PP	47	92	
Top-quality/fair transferred blastocysts			
ITT	28/19	56/39	0.94
PP	28/19	54/38	0.92
Clinical pregnancy rate			
ITT	23/47 (48.9%)	47/95 (49.5%)	0.97
PP	23/47 (48.9%)	47/92 (51.1%)	0.89
Multiple pregnancy rate			
ITT	2/23 (8.7%)	0/47 (0%)	0.12
PP	2/23 (8.7%)	0/47 (0%)	0.12
Live birth rate			
ITT	21/47 (44.7%)	45/95 (47.4%)	0.85
PP	21/47 (44.7%)	45/92 (48.9%)	0.87
Gestational age of newborns (weeks)			
ITT	38.3 ± 3.3	38.6 ± 3.2	0.77
PP	38.3 ± 3.3	38.6 ± 3.2	0.77
Birthweight of newborn (g)			
ITT	3,130 ± 644	3,218 ± 777	0.41
PP	3,130 ± 644	3,218 ± 777	0.41
Male/female ratio of newborns			
ITT	12/9	24/21	0.77
PP	12/9	24/21	0.77
Serious adverse events			
ITT	3/47 (6.4%)	7/92 (7.6%)	1
PP	3/47 (6.4%)	7/92 (7.6%)	1
cfDNA (pg/μl)			
ITT	0.995 ± 1.322	0.740 ± 0.827	0.41
PP	0.995 ± 1.322	0.762 ± 0.926	0.45

Data are presented as *N*, *n*/*N* (%) or mean ± standard deviation.

The Mann–Whitney test, Fisher's test and chi-squared test were used.

Serious adverse events pertain to both to the women and fetuses/newborns. No multiple pregnancies resulted in a birth.

AS, artificial shrinkage; cfDNA, cell-free DNA; ITT, intention to treat; PP, per protocol.

Moreover, blastocyst collapse by artificial shrinkage before fresh blastocyst transfer could also protect the embryo from osmotic and mechanical shocks during in-vitro micromanipulation. The embryo's re-expansion in the uterine environment would therefore allow the blastocoel to become filled with the medium of the maternal microenvironment. On the basis of these preliminary results, the current authors think that these postulated roles of blastocoel collapse do not mainly contribute to the implantation potential of human blastocysts.

Cell apoptosis occurs very early in the human embryo, playing an essential role in early embryonic development by eliminating abnormal cells. This results in the expulsion of mitochondrial and nuclear cfDNA in the blastocoel fluid and in the embryo culture medium (*Brouillet et al., 2020*). Palini and colleagues first described the presence of cfDNA in the blastocoel fluid (median level of 9.9 pg in approximately 90% of blastocoel fluid samples tested) (*Palini et al., 2013*). A year later, Gianaroli and colleagues detected cfDNA in over 76% of blastocoel fluid samples and showed that the results of the chromosomal analysis of cfDNA from blastocoel fluid could reflect the embryo ploidy (*Gianaroli et al., 2014*). Since then, cfDNA has been frequently detected in the blastocoel fluid of blastocysts, but the quantity and quality are limited (*Brouillet et al., 2020*; *Cai et al., 2022*; *Palini et al., 2013*). In the current study, no significant difference in cfDNA concentration was found between the AS and control groups and between live birth or lack of a live birth.

It has been shown that two blastocyst types with very different implantation potentials show similar cfDNA levels: (i) mosaic blastocysts that have successfully excluded aneuploid cells and become euploid with a

TABLE 3 CFDNA CONCENTRATION IN SPENT CULTURE MEDIUM IN FUNCTION OF THE IVF OUTCOME (PP ANALYSIS)

Parameters	No clinical pregnancy	Clinical pregnancy	*P*-value	No live birth	Live birth	*P*-value
Spent culture medium	69	70	–	73	66	–
cfDNA in the control + AS groups (pg/μl; *n* = 139)	0.77 ± 0.84	0.92 ± 1.24	0.55	0.74 ± 0.83	0.97 ± 1.29	0.26
cfDNA in the control group(pg/μl; *n* = 47)	0.83 ± 0.83	1.13 ± 1.64	0.95	0.79 ± 0.78	1.21 ± 1.73	0.84
cfDNA in the AS group(pg/μl; *n* = 92)	0.73 ± 0.86	0.81 ± 0.98	0.6	0.71 ± 0.86	0.84 ± 1.00	0.28

Data are presented as mean ± standard deviation.

The Mann–Whitney test was used.

AS, artificial shrinkage; cfDNA, cell-free DNA; PP, per protocol.

high chance of pregnancy; and (ii) aneuploid/fragmented blastocysts with a high apoptosis rate and low implantation potential (*Cimadomo et al., 2022*; *Coticchio et al., 2021*). The quantification of total cfDNA concentration does not allow a distinction to be made between these blastocyst types. Therefore, future large prospective studies should assess the relevance of combining total cfDNA concentration measurement and non-invasive preimplantation genetic testing for aneuploidies.

CONCLUSION

In this pilot randomized study, no benefit of artificial shrinkage in fresh blastocyst transfer was observed. However, large-scale randomized controlled trials are required to confirm or inform these preliminary results. If no positive impact of artificial shrinkage on fresh transfer is confirmed, fresh blastocysts on day 5 could be directly transferred without this additional procedure to save time and reduce the risk of cell damage in the event of improper laser use.

DATA AVAILABILITY

Data will be made available on request.

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AUTHOR CONTIBUTIONS

S.B., A.G., F.B., M.A., A.F.-H., A.A., L.G., V. L., T.A. and S.H. participated in the study design. S.B., A.G., F.B., M.A., A.F., A.A., L. G., V.L., T.A. and S.H. participated in the study execution. S.B., A.G., L.G. and S.H. participated in the analysis. S.B., A.G., T.A. and S.H. participated in the manuscript drafting. All authors participated in critical discussion.

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RBMO

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ARTICLE

Vertical transmission of microbiomes into embryo culture media and its association with assisted reproductive outcomes

BIOGRAPHY

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KEY MESSAGE

Microbes from semen and follicular fluid can contaminate embryo culture media during in-vitro culture. Although some of these microbes showed significant correlation with semen quality and the aetiology of female infertility, there was no significant impact of the vertical transmission of microbes to culture media on treatment outcomes.

ABSTRACT

Research question: Can microbes vertically transmit from semen and follicular fluid to embryo culture media during assisted reproductive technology (ART) treatment?

Design: Spent embryo culture media (SECM), seminal fluid and follicular fluid samples were collected from 61 couples with infertility undergoing ART treatment at the Prince of Wales Hospital, Hong Kong SAR, China. Metagenomic analysis was conducted using 16s rRNA sequencing to identify the source of microbes in SECM, correlation between the semen microbiome and male infertility, and correlation between the follicular fluid microbiome and female infertility.

Results: Microbial vertical transmission into SECM was reported in 82.5% of cases, and semen was the main source of contamination in conventional IVF cases. The increased abundances of *Staphylococcus* spp. and *Streptococcus anginosus* in semen had negative impacts on total motility and sperm count, respectively ($P < 0.001$). Significant increases in abundance of the genera *Prophyromonas*, *Neisseria* and *Facklamia* were observed in follicular fluid in women with anovulation, uterine factor infertility and unexplained infertility, respectively ($P < 0.01$). No significant correlation was found between the bacteria identified in all sample types and ART outcomes, including fertilization rate, embryo development, number of available embryos, and clinical pregnancy rate.

Conclusion: Embryo culture media can be contaminated during ART treatment, not only by seminal microbes but also by follicular fluid and other sources of microbes. Strong correlations were found between specific microbial taxa in semen and sperm quality, and between the follicular fluid microbiome and the aetiology of female infertility. However, no significant association was found between the microbiomes of SECM, semen and follicular fluid and ART outcomes.

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KEY WORDS

Spent embryo culture medium contamination
Semen microbiome
Follicular fluid microbiome
Microbiome in assisted reproduction

INTRODUCTION

Assisted reproductive technology (ART) treatment is typically performed in clean environments to reduce the risk of microbial contamination of gametes, embryos or culture media (*Rajput et al., 2021*). Nevertheless, it can occur even when strict standards are maintained (*Borges et al., 2020*). Contamination of embryo culture has been identified in 0.38–8% of conventional IVF cases (*Ben-Chetrit et al., 1996*; *Cottell et al., 1996*; *Kastrop et al., 2007*; *Štšepetova et al., 2020*). However, the true prevalence of microbial colonization is probably underestimated due to the limited evidence discussing this issue, and the fact that most studies reported contamination only when turbidity or bacterial growth was observed.

The source of contamination in culture media usually remains unknown. However, it has been suggested that microbes most likely originate from seminal fluid, and are vertically transmitted to culture media during fertilization (*Cottell et al., 2000*). In recent studies, bacteria have also been found in follicular fluid, indicating that follicular fluid is a possible source of contamination (*Pelzer et al., 2011*, *2013*; *Usman et al., 2021*). The association between contamination of embryo culture media and ART outcomes is not well established, due to limited publications and contradictory results (*Pelzer et al., 2011*, *2013*; *Usman et al., 2021*). To the authors' knowledge, no studies have collectively explored seminal fluid, follicular fluid and spent embryo culture media (SECM) in association with ART outcomes. It was postulated that SECM is not strictly sterile, and microbes can be vertically transmitted from either or both seminal fluid and follicular fluid into embryo culture media during ART treatment. Thus, this study aimed to determine the prevalence of vertical transmission of microbes into embryo culture media; characterize the microbial community of SECM, semen and follicular fluid; and examine the link with ART outcomes.

MATERIALS AND METHODS

Study design

This was a prospective observational study of patients with infertility undergoing ART treatment at the Prince of Wales Hospital, Hong Kong SAR, China. Couples were eligible to participate in this study if the female partner was between the ages of 18–42 years and the male partner was aged >18 years at the time of consent, unless they met any of the exclusion criteria listed in Supplementary Table 1. In total, 108 patients were enrolled between July 2019 and December 2021, of which 61 were analysed in this study. Informed consent was obtained from all subjects, and the protocol for this study was approved by the Institutional Review Board, Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee (CREC Ref. No. 2018.533, approval date 21 December 2018). Bacterial compositions of SECM were examined against the seminal fluid and follicular fluid microbiomes to identify the source of the vertical transmission of microbes. Next, correlations between the seminal fluid microbiome and male infertility, the follicular fluid microbiome and female infertility, and the microbiome profiles of the three sample types and ART outcomes were examined.

ART treatment and specimen collection

Oocyte retrieval was performed under local anaesthesia following a standard ultrasound-guided transvaginal oocyte collection procedure. Briefly, the vaginal wall was cleaned with warmed normal saline prior to the operation. Follicular fluid was aspirated from each ovary using a transvaginal needle, starting from the largest follicle, into a sterile test tube. The fluid was screened immediately in a sterile petri dish for the presence of oocytes. When present, oocytes were collected in a sterile dish containing oocyte-handling media (G-MOPs Plus; Vitrolife, Sweden) (*Kim et al., 2022*; *Pelzer et al., 2013*, *Usman et al., 2021*). The residual follicular fluid from the first aspirated tube of each ovary was placed in a sterile tube and centrifuged at 600 *g* for 5 min to eliminate granulosa cells and other physiological constituents. The pellet was then discarded, and the supernatant was stored at -80°C for microbiome analysis. A mixture of follicular fluid from both ovaries was used for the microbiome analysis.

Semen was obtained after 2–5 days of abstinence from ejaculation in a sterile, wide-capped container. Men were instructed to rinse their glans penis before semen collection. Ejaculates were allowed to liquefy at room temperature, and were then examined according to the 2010 guidelines of the World Health Organization (*WHO, 2010*). Specimens were prepared for insemination using a standard density gradient centrifugation. Briefly, semen samples were layered on top of the bilayered gradient tubes and centrifuged at 1500 rpm for 15 min. After centrifugation, the supernatant was removed and stored at -80°C for microbiome analysis. The pellet was washed and resuspended in 0.5–1.0 ml culture medium (GIVF-plus; Vitrolife), and incubated at 37°C under 5% CO_2 until the time of insemination. Oocytes were inseminated either by injecting a spermatozoon into each mature oocyte [intracytoplasmic sperm injection (ICSI)] or by incubating the oocytes with 20,000–50,000 pre-prepared motile spermatozoa (conventional IVF) in culture media overnight at 37°C under 5% CO_2 in MINC Benchtop Mini incubators (Cook Medical, USA).

Fertilized embryos were transferred into a new culture dish containing fresh drops (25 μl per embryo) of embryo culture media for further culture (GTL; Vitrolife). Embryo grading was performed on cleaved and blastocyst embryos under the microscope, as described by *Veeck (1999)* and *Gardner et al. (2000)*, respectively. Embryos of good quality (Grade 1 or 2 on day 3 or >3BB on day 5) were selected for embryo transfer between day 3 and day 5 of culture or cryopreserved for future use. On day 5, SECM samples from all spent embryo culture drops were stored collectively at -80°C for microbiome analysis. Unused embryo culture medium drops were also collected and utilized as a negative control.

In total, 178 clinical samples and several experimental controls were collected and used in the analysis, including 61 follicular fluid samples, 60 seminal fluid samples and 57 SECM samples (Supplementary Figure 1).

DNA extraction

DNA from all samples was extracted with an identical protocol using a QIAamp DNA Mini Kit (Qiagen, Germany) in accordance with the manufacturer's instructions, with minor modifications. Samples were thawed on ice and centrifuged at 16,700 *g* at 4 °C for 15 min. The supernatant was discarded, and the pellet was resuspended in 180 μl of buffer ATL and 40 μl of proteinase K, and

incubated overnight at 56 °C with shaking at 760 rpm. Next, 200 μl of buffer AL was added to the suspension and incubated at 70 °C for 30 min. After incubation, DNA was purified and eluted using nuclease-free water.

Sequencing and library preparation

The 16S rRNA gene region V3–V4 amplification was performed using forward primer 16S-341F and reverse primer 16S-805R in accordance with the Microbiome Research Centre protocols for sample preparation, with minor modifications. Purified amplicons were sequenced for library preparation using Illumina sequencing adapters with the Nextera XT Index Kit at the Ramaciotti Centre at the University of New South Wales, Australia. The library was normalized with a SequalPrep Normalization Plate Kit (ThermoFisher, USA). The library pool was sequenced on the Illumina MiSeq platform using a v3 2 × 300 bp paired-end sequencing run.

Bioinformatics analysis

Metadata analyses were performed using the open-source QIIME2 (RRID: SCR_021258) workflow to generate amplicon sequence variants (ASV) using demultiplexed FASTQ data obtained from Illumina's BaseSpace cloud storage (*Bolyen et al., 2019*). The 16S V3–V4 sequences went through the DADA2 pipeline, implemented in QIIME2, for quality control and merging. Host contamination was removed by mapping against the host genome, and ASV were produced for downstream analysis. As the samples are low biomass in microbes, a rigorous process for contamination control was applied from sample collection to amplification, negative control samples were collected from type-specific samples, extraction kit blank, and polymerase chain reaction (PCR) process. Firstly, the sample-type-specific negative control and those types of samples were put together in the Decontam R package to identify contaminated ASV, and these were removed. After removing sample-specific contaminations, all samples were put together for decontamination against KIT and PCR control. The clean ASV table was used for downstream analysis. QIIME2 was used to calculate alpha- and beta-diversity metrics. SourceTracker Package in R was used to determine the source of bacteria in SECM samples. The sink was defined as SECM, and the sources were defined as follicular fluid and seminal fluid. 'Unknown' refers to bacteria present in SECM but not in semen or follicular fluid.

Statistical analysis

A non-parametric test (Kruskal–Wallis) was utilized for pairwise comparisons of medians to examine alpha diversity between different sample types. Permutational multi-variate analysis of variance (PERMANOVA) was utilized for the analysis of beta diversity, followed by PERMANOVA-pairwise comparisons between the groups. According to the data descriptive statistics, Chi-squared test and Mann–Whitney test were applied to compare differences in specific taxa abundance. Statistical tests were limited to taxa with at least 1% abundance. For the demographic data, data have been presented as mean ± SD for female age, and median with interquartile range for the rest of the data. The independent sample *t*-test was used to test for significant differences in mean female age between the clinical pregnancy and non-clinical pregnancy groups, and the Mann-Whitney *U*-test was used for the other demographic data. Correlation analysis between the microbial composition in each sample and ART outcomes, and between semen classification groups was performed using the MaAsLin2 R package. Age, body mass index (BMI), smoking and drinking were input because MaAsLin2 treats these confounding factors as covariates. The analyses were run for taxa with a minimum abundance of zero and a minimum prevalence of 10%. Results for statistical tests were considered significant when $P < 0.05$ and the false discovery rate (FDR) was <0.40.

RESULTS

Demographics and reproductive health history

For the female participants, mean age was 34.8 years and median BMI was 22.2 kg/m^2. For the male participants, median age was 36.0 years and median BMI was 23.8 kg/m^2. Male participants showed a higher frequency of tobacco smoking and alcohol consumption compared with female participants. Most of the male participants (85%) presented with defective semen parameters, and 63.9% of the female participants exhibited one or more of the following infertility factors: pelvic adhesions; tubal occlusion; endometriosis; uterine factor; or ovulatory disorders. However, the cause of infertility was unknown in approximately 5% of the study population, and was therefore considered idiopathic (Supplementary Table 2).

Microbiome analysis

The 16S rRNA amplicon analysis generated a median read of 43,800 per sample. In total, 2189 unique ASV were identified after decontamination. Of those, 480 (21.9%) ASV were detected exclusively in SECM, 574 (26.2%) ASV were detected exclusively in semen, and 690 (31.5%) ASV were detected exclusively in follicular fluid, whereas 97 (4.4%) taxa were shared among all specimens (FIGURE 1A). Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria were predominant phyla in all sample types, accounting for >96% of the assigned phyla. Firmicutes was the dominant phylum in all sample types, with a relative abundance of 38.5% in SECM, 56.7% in seminal fluid and 68.2% in follicular fluid (FIGURE 1B). The genus *Lactobacillus* exhibited the highest relative abundance in SECM and follicular fluid samples (11.6% and 48.7%, respectively), while *Streptococcus* was the predominant genera in semen with a relative abundance of 18.7% (FIGURE 1B). SECM samples demonstrated decreased richness compared with seminal fluid ($P = 0.009$) and follicular fluid ($P < 0.0001$) specimens, as demonstrated by the observed number of ASV (FIGURE 1C). Follicular fluid showed significantly lower beta diversity compared with seminal fluid ($P < 0.001$) and SECM ($P < 0.001$) (FIG. 1D).

Vertical transmission of microbiome

Microbial contamination was detected in 82.5% (47/57) of the SECM samples. The source tracking analysis determined that semen was the main source of contamination in SECM, accounting for 55.6% of all sources (FIGURE 2A). Follicular fluid also contributed to the SECM microbiome, but significantly less than semen (0.3% versus 55.6%; $P < 0.001$) (FIGURE 2A). Further analysis of the data identified that specific bacterial taxa originated in significantly different proportions from different sources (Supplementary Figure 2). After grouping samples based on the insemination method (conventional IVF versus ICSI), the data showed no significant difference in the incidence of microbial contamination in SECM between the two groups (Supplementary Figure 3A). However, the contribution of seminal microbes to the bacteria detected in SECM was higher in conventional IVF cases (71.2%) compared with ICSI cases (31.6%) (FIGURE 2B,C).

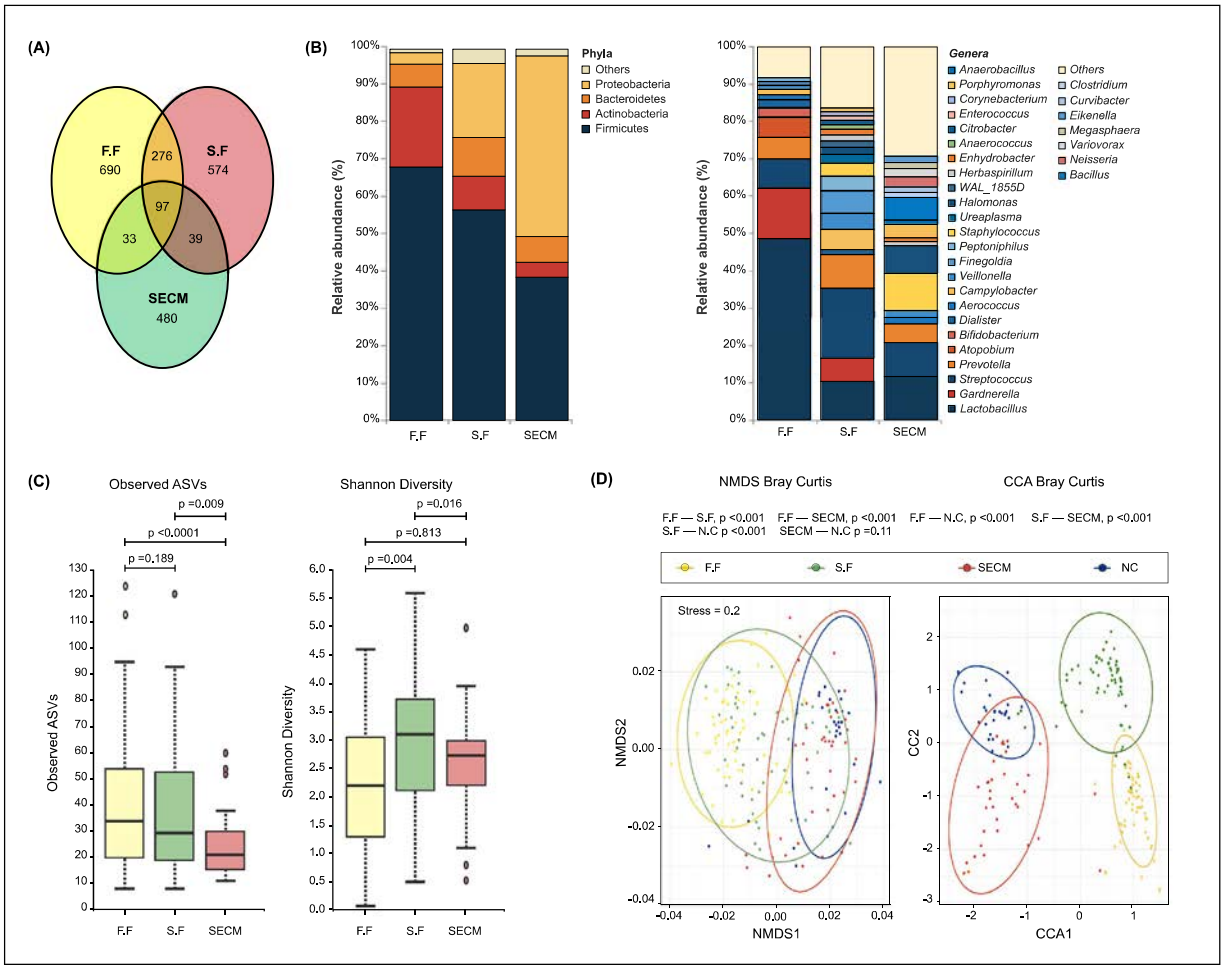

FIGURE 1 Follicular fluid, semen and spent embryo culture media microbiomes in couples undergoing assisted reproductive technology treatment. (A) Venn diagram demonstrating the distribution of taxa between sample sources. (B) Bar plot representing the relative abundance of taxa (phyla and genera) stratified by sample source. (C) Alpha diversity [observed amplicon sequence variants (ASV) and Shannon index]. Box plots indicate median and interquartile range, with outliers marked as individual points. (D) Beta diversity [Bray Curtis by non-metric multi-dimensional scaling (NMDS) and canonical correspondence analysis (CCA)]. The ellipses represent the clusters of different sample types. Differences in alpha and beta diversity between groups were tested using Kruskal–Wallis pairwise and pairwise permutational multi-variate analysis of variance, respectively. F.F, follicular fluid; S.F, seminal fluid; SECM, spent embryo culture media; NC, negative control.

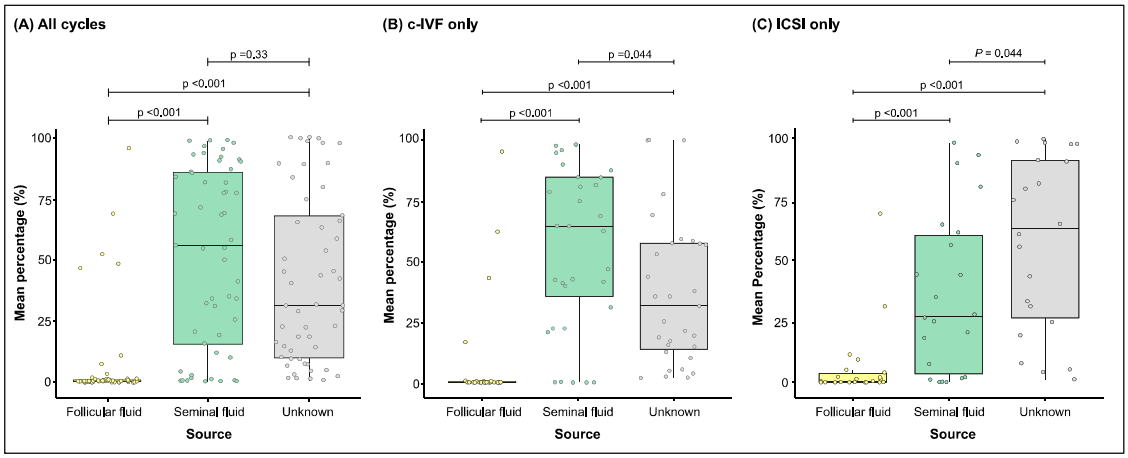

FIGURE 2 Mean percentage contribution of bacteriome from different sample types to the microbiome in spent embryo culture medium. (A) Conventional IVF (c-IVF) and intracytoplasmic sperm infection (ICSI) patients, (B) c-IVF patients alone, and (C) ICSI patients alone. Box plots indicate median and interquartile range, with outliers marked as individual points. *P*-values were calculated using Kruskal–Wallis pairwise comparison test, and values <0.05 were considered to indicate significance.

Microbiome association with male infertility

The results indicated no significant difference in microbial taxa in the semen of normozoospermic men compared with men with defective semen parameters. Further investigation identified a significant inverse association between *Staphylococcus* spp. and sperm total motility, and between *Streptococcus anginosus* and sperm concentration (both $P < 0.001$) (FIGURE 3A,B). Interestingly, the *Paracoccus* genus was found to be directly correlated with male age ($P < 0.001$; FIGURE 3C).

Microbiome association with female infertility

Correlation analysis was conducted to determine whether the follicular fluid microbiome is associated with the aetiology of infertility (FIGURE 4). The relative abundance of *Prophyromonas* spp., *Neisseria* spp. and *Facklamia* spp. was directly associated with the presence of anovulation, uterine factor and unexplained factor infertility, respectively (all $P < 0.001$; FIGURE 4A,B,C). Additionally, a positive association was found between the genus *Facklamia* and BMI in women ($P = 0.01$; FIGURE 4D).

Microbiome association with ART outcomes

Patients were divided into clinical pregnancy and non-clinical pregnancy groups based on the pregnancy scan results, and their demographics, ART procedure and clinical outcomes are compared in Supplementary Table 3. First, potentially confounding variables, such as age, were examined, and no significant association with clinical pregnancy rate was found (Supplementary Table 3). ART outcomes were compared between contaminated and microbe-free SECM patients, and no significant differences were found in the clinical pregnancy rate, number of normally fertilized embryos, number of available embryos, and number of good-quality embryos (Supplementary Figure 3). Alpha-diversity metrics of SECM, seminal fluid and follicular fluid revealed no significant difference between the clinical pregnancy and non-clinical pregnancy groups (Supplementary Figure 4). No correlation was found between the taxa present in those samples and ART outcomes, including clinical pregnancy rate, fertilization rate, embryo development, and number of available embryos (Supplementary Tables 4, 5 and 6).

DISCUSSION

The incidence of bacterial contamination in embryo culture media is believed to be low, and its impact on ART outcomes is not fully understood. Previous reports indicated an incidence of overt contamination of culture media in <1% of IVF cycles using conventional methods (*Ben-Chetrit et al., 1996*; *Kastrop et al., 2007*). Utilizing molecular methods, the prevalence was found to be remarkably higher, as detected by 454-pyrosequencing (8%) and real-time PCR (70%) (*Štšepetova et al., 2020*). The present study explored the microbial composition of SECM samples utilizing 16s rRNA sequencing. Although at low diversity, bacteria were detected in 82.5% of the tested SECM samples, indicating that embryo culture media is not always sterile. The low microbial biomass detected in SECM can be explained by the presence of gentamicin in the culture medium. Gentamicin is a broad-spectrum antimicrobial agent against Gram-positive and Gram-negative aerobic bacteria. Such bactericidal agents are likely to be able to inhibit the growth of potentially large numbers of microbes, and reduce the chance of overt contamination. The prevalence of contamination of SECM may vary between studies depending on the prophylactic regime received by patients prior to oocyte retrieval, vaginal cleansing and semen preparation, and the differences in study designs and methodologies. Two studies were performed using culture-based procedures, and were only conducted when there was a sign of overt contamination, such as turbidity or visible threads in the medium (*Ben-Chetrit et al., 1996*; *Kastrop et al., 2007*). *Štšepetova et al. (2020)* had a similar study design. However, their study employed 454-pyrosequencing technology, producing a lower depth and breadth of coverage than the MiSeq platform used in the present study (*Frey et al., 2014*).

Semen is believed to be the main source of contamination in culture media, especially in conventional IVF cases; however, microbes can also be vertically transmitted

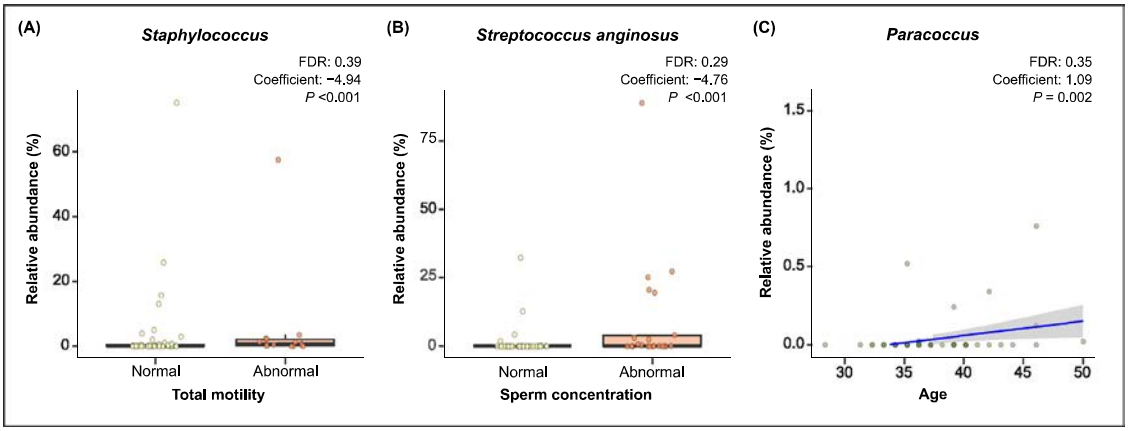

FIGURE 3 Relative abundance of the seminal fluid microbiome in association with semen parameters and demographic data. Comparison between the relative abundance of (A) *Staphylococcus* spp. and sperm total motility, (B) *Streptococcus anginosus* and sperm concentration, and (C) *Paracoccus* spp. and age. Box-whisker plots indicate median, interquartile range and range, with outliers marked as individual points. The blue line in the scatter plot represents the regression line, and the gray area represents the confidence interval. The correlation analysis was performed using a linear-mixed model with the Maaslin2 R package. *P*-values <0.05 and a false discovery rate (FDR) <0.40 were considered to indicate significance.

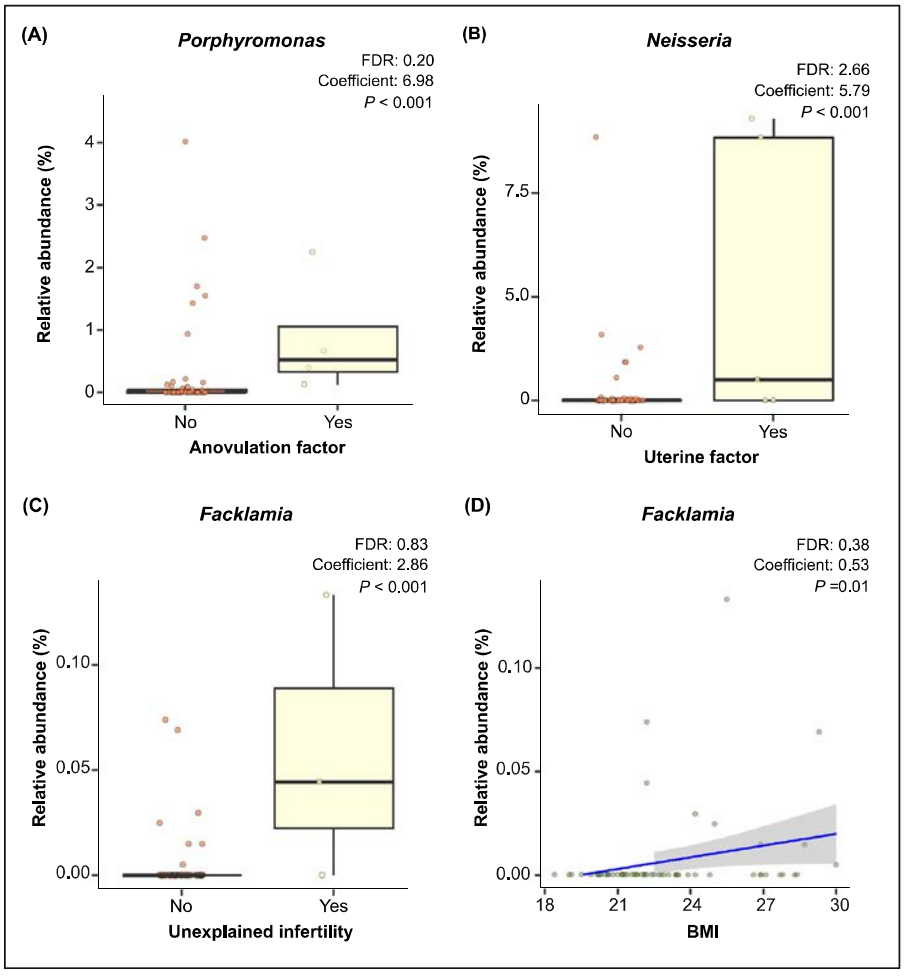

FIGURE 4 Relative abundance of the follicular fluid microbiome in association with female infertility and demographic data. Correlation between the relative abundance of genera: (A) *Prophyromonas* and anovulation infertility, (B) *Neisseria* and uterine factor infertility, (C) *Facklamia* and unexplained infertility, and (D) *Facklamia* and body mass index (BMI). Box-whisker plots indicates median, interquartile range and range, with outliers marked as individual points. The blue line in the scatter plot represents the regression line, and the gray area represents the confidence interval. The correlation analysis was performed using a linear-mixed model with the Maaslin2 R package. *P*-values <0.05 and a false discovery rate (FDR) <0.40 were considered to indicate significance.

from follicular fluid, ambient air and/or non-sterilized materials (*Ben-Chetrit et al., 1996*; *Borges et al., 2020*; *Cottell et al., 1996*; *Du et al., 2021*; *Kastrop et al., 2007*). The present study found that, overall, semen made the greatest contribution to the SECM microbiome, when contribution could be identified. As two methods of insemination were studied (conventional IVF and ICSI), it was expected that semen might affect the SECM samples differently. However, the results showed no significant difference in the incidence of contamination between the two groups. One explanation for this is that contamination can come from many sources other than semen. In addition, differences in the source of contamination between the two groups were investigated. As expected, the contribution of semen to the SECM microbiome was more evident in the conventional IVF group compared with the ICSI group. This could be explained by the fact that, in conventional IVF, oocytes are in contact with the sperm or the residual semen in the prepared sperm for a longer period of time compared with ICSI. Interestingly, in 15.8% (9/57) of cases, SECM samples were contaminated mainly by microbiota present in follicular fluid. This observation highlights that microbial transmission from follicular fluid occurs in ART treatments. One limitation of this study is that 31.0% of bacteria detected in SECM were classed as being of unknown source, as no tests were undertaken for other potential sources of microbial contaminants in SECM.

There is limited evidence regarding the association between bacterial contamination in SECM and ART outcomes. Contamination in SECM by bacteria such as *Candida* spp., *Escherichia coli* and *Enterococcus faecalis* has been linked to poor embryo quality, embryo development, and higher rates of cancellation due to unavailable embryos for transfer (*Du et al., 2021*; *Kastrop et al., 2007*). Two independent groups reported contradictory findings, where contamination was not associated with fertilization rate, cleavage of embryos, embryo quality or pregnancy rate (*Cottell et al., 1996*; *Shu et al., 2016*). The present study did not find a significant association between microbial contamination and ART outcomes, including fertilization rate, embryo development, number of embryos available for transfer, or clinical pregnancy rate. Therefore, it was concluded that SECM contamination did not have an

adverse effect on ART outcomes. However, this may only apply to cases with low bacterial biomass levels, where no visible signs of contamination are observed. Finally, it has been proposed that ICSI can be used to eliminate the risk of culture media contamination in ART cycles. However, the present study challenged previous assumptions, and showed that both conventional IVF and ICSI cycles introduced contamination into embryo culture.

This study used next-generation sequencing (NGS) to explore semen and follicular fluid microbiomes in association with ART outcomes. Two recent studies (*Okwelogu et al., 2021*; *Štšepetova et al., 2020*) investigated the association between the semen microbiome and ART outcomes. *Okwelogu et al. (2021)* reported a significant positive relationship between *Faecalibacterium* and *Lactobacillus jensenii* in semen and clinical pregnancy. *Štšepetova et al. (2020)* found no significant association between the semen microbiome and IVF outcomes, but reported a negative correlation between *Alphaproteobacteria* in washed semen and embryo quality. The present study found no significant relationship between the semen microbiota and ART outcomes, including fertilization rate, embryo development, number of embryos available for transfer, and clinical pregnancy rate.

Next, the microbial composition of semen samples and its relationship with male infertility was examined. Semen was found to be contaminated with microbiota, even at low concentrations. Similar findings were reported in recent metagenomic studies (*Baud et al., 2019*; *Mändar et al., 2015*; *Okwelogu et al., 2021*), collectively supporting the hypothesis that semen is not a microbe-free fluid. The present study found that *Streptococcus*, *Lactobacillus*, *Prevotella*, *Finegoldia*, *Gardnerella*, *Veillonella*, *Campylobacter*, *Staphylococcus*, *Halomonas*, *Peptoniphilus*, *Enhydrobacter*, *Citrobacter*, *Ureaplasma*, *Porphyromonas* and *Dialister* were the most abundant genera in semen. Similar results were reported in several other studies (*Baud et al., 2019*; *Chen et al., 2018*; *Hou et al., 2013*; *Mändar et al., 2015*; *Monteiro et al., 2018*; *Okwelogu et al., 2021*; *Weng et al., 2014*). The present study found a strong negative association between semen abnormality and the relative abundance of the genus *Staphylococcus* and *S. anginosus*. *Staphylococcus* was linked to poor sperm motility. A similar observation has been reported previously (*Filipiak et al., 2015*; *Onemu and Ibeh, 2001*). However, a recent study reported a contradictory result, where normal semen was enriched with *Staphylococcus* spp. (*Baud et al., 2019*). Previous studies have linked the genus *Streptococcus* with reduced sperm motility (*Ivanov et al., 2009*; *Magri et al., 2009*; *Mashaly et al., 2016*; *Yao et al., 2021*). In line with these observations, the present study identified a negative association with *Streptococcus* spp., namely between *S. anginosus* and sperm motility. This species was identified at a significantly higher abundance in asthenozoospermic men compared with controls. In addition, a positive correlation was identified between the prevalence of *Paracocus genus* in semen and age. Finally, this study found that BMI, smoking and alcohol consumption were not significantly associated with the seminal fluid microbiome. To the authors' knowledge, this is the first study to investigate correlations between the seminal fluid microbiome and age, BMI, smoking and alcohol consumption.

Little is known about the association between the follicular fluid microbiome and infertility in women. A few culture-based studies have identified bacteria in follicular fluid, with incidence ranging between 17% and 34.4% of IVF couples (*Ismaeel, 2018*; *Pelzer et al., 2011*, *2013*; *Usman et al., 2021*). The present study explored the follicular fluid microbiome utilizing 16S rRNA sequencing. The findings revealed that follicular fluid is not sterile, but contains several microbes. Microbial DNA was detected in all follicular fluid samples collected at the time of oocyte retrieval. There are two possible explanations for the high incidence rate found in the present study compared with others. First, this could be due to the difference in the microbial detection method (NGS versus culture). The other studies considered follicular fluid samples with shared bacteria with the corresponding vaginal swabs to be contaminated, and excluded them from the analysis (*Ismaeel, 2018*; *Pelzer et al., 2011*; *2013*; *Usman et al., 2021*). Vaginal swabs were not included in the analysis in the present study. The authors believe that there is no evidence to support the assumption of contamination if bacteria are shared between the two sites, or if follicular fluid must have a distinct microbiota. There is still a chance that bacteria colonize both organs independently.

No significant relationship was found between microbial diversity in follicular fluid and ART outcomes in the present study. The most abundant genera in follicular fluid were *Lactobacillus*, *Gardnerella*, *Streptococcus*, *Prevotella*, *Atopobium*, *Bifidobacterium*, *Dialister*, *Aerococcus*, *Campylobacter*, *Veillonella*, *Finegoldia* and *Peptoniphilus*. Some of these were also reported in other parts of the female reproductive tract, such as the endometrium and the vagina (*Babu et al., 2017*; *Benner et al., 2018*). Colonization of follicular fluid by *Lactobacillus* spp. namely, *L. crispatus* and *L. gasseri*, was found to have a positive effect on embryo quality, embryo transfer and pregnancy rate (*Pelzer et al., 2013*). However, a recent metagenomic study found that *L. iners* played a positive role in oocyte maturation, but had a negative impact on pregnancy rate (*Wu et al., 2022*). In the present study, *Lactobacillus* spp. were the most abundant species in follicular fluid, but no significant link was found between the prevalence of *Lactobacillus* spp. and IVF outcomes. These findings were consistent with those of a recent study which found no significant relationship between the presence of bacteria in follicular fluid and fertilization and pregnancy rates following ART treatment (*Usman et al., 2021*). The link between the follicular fluid microbiome and the aetiology of infertility is not yet fully understood. A previous study showed a significant increase in the incidence of microbial contamination of follicular fluid in women with endometriosis (*Pelzer et al., 2013*). The present study found a direct relationship between *Prophyromonas* spp. and anovulation, between *Neisseria* spp. and uterine factor infertility, and between *Facklamia* spp. and unexplained factor infertility. This observation highlighted the complex relationship between the follicular fluid microbiome and infertility in women. Further studies are needed to understand the role of the microbiome in the pathophysiology of female infertility.

CONCLUSION

To the authors' knowledge, this is the first study to collectively investigate follicular fluid, seminal fluid and SECM of ART patients using NGS technology. A high incidence of vertical transmission of microbes to SECM samples was found;

semen and follicular fluid can contaminate culture media during ART, despite not being observable under routine ART procedures. This study identified microbial taxa in semen that were associated with poor semen quality, and in the follicular fluid microbiome linked to aetiologies of infertility in women. However, significant correlation was not found between the microbiomes of all sample sources and ART outcomes. Future studies are needed to understand the complex relationship between microbiomes and infertility.

AUTHOR CONTRIBUTIONS

OAMA, LC and WW were involved in patient recruitment, sample collection, DNA extraction, amplicon sequencing and library preparation. XJ and HY led bioinformatics and statistical analysis. MBW led the guidelines from funding. JPWC, HY, EKLF and DYLC designed the study. OAMA drafted the manuscript. DYLC coordinated the study and helped to draft the manuscript. All the listed authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2024.103977.

DATA AVAILABILITY

Data will be made available on request.

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RBMO

ELSEVIER

ARTICLE

Early versus late follicular phase ovarian stimulation: a randomized controlled trial

BIOGRAPHY

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KEY MESSAGE

Late follicular phase stimulation is as efficient as early follicular phase stimulation in terms of number of oocytes. It is patient-friendly, with reduced cost and reduced number of injections. Further studies are needed to confirm whether these results can be extrapolated to freeze-only cycles in an assisted reproductive technology setting.

ABSTRACT

Research question: Is late follicular phase stimulation as efficient as early follicular phase stimulation in a gonadotrophin-releasing hormone (GnRH) antagonist protocol in oocyte donors in terms of the number of oocytes.

Design: In this open label, phase 3, non-inferiority, randomized controlled trial using a two-arm design with a 1:1 allocation ratio, 84 oocyte donors were allocated to the early follicular start group (control group, $n = 41$) or the late follicular start group (study group, $n = 43$). In the control group, women followed a fixed GnRH antagonist protocol with recombinant FSH (r-FSH) 225 IU. In the study group, r-FSH 225 IU was initiated in the late follicular phase. The primary outcome was the number of oocytes. The secondary outcomes were the number of mature oocytes, consumption of gonadotrophins and GnRH antagonist, and cost of medication.

Results: The number of oocytes did not differ between the control group and the study group (intent-to-treat analysis 15.5 ± 11.0 versus 14.0 ± 10.7, $P = 0.52$; per-protocol analysis 18.2 ± 9.7 versus 18.8 ± 7.8, $P = 0.62$). In addition, the number of mature oocytes did not differ between the groups (14.1 ± 8.1 versus 12.7 ± 8.5, $P = 0.48$). The duration of stimulation was shorter in the control group (10.0 ± 1.4 versus 10.9 ± 1.5 days, $P = 0.01$). The total amount of r-FSH used was lower in the control group (2240.7 ± 313.9 IU versus 2453.9 ± 330.1 IU, $P = 0.008$). A GnRH antagonist was used for approximately 6 days in the control group, while a GnRH antagonist was only prescribed for one woman in the study group (6.0 ± 1.4 days versus 0.13 ± 0.7 days, $P < 0.001$). There was a significant difference in the cost of medication per cycle between the groups (1147.9 ± 182.8€ in control group versus 979.9 ± 129.0€ in study group, $P < 0.001$).

Conclusions: Late follicular phase stimulation is as efficient as early follicular phase stimulation in terms of the number of oocytes.

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KEY WORDS

ART
Oocyte donation
Ovarian stimulation

INTRODUCTION

Human oocyte donation was first introduced in 1983, and has evolved over the past three decades into a relatively common procedure that addresses a variety of reproductive disorders (*Sauer, 2018*). However, it has to be highlighted that oocyte donation is an ethically complex subject that involves young, healthy women undergoing medical procedures for which they do not receive any health benefits. Thus, simplifying treatment protocols could help reduce physical demands and minimize disruption for the donors (*Kalfoglou, 2001*).

Usually, ovarian stimulation is started in the early follicular phase. This is based on the endocrinological concept of folliculogenesis, supporting the recruitment of a single wave of antral follicles with single follicle selection during the early to middle stage of the follicular phase, with the other follicles undergoing atresia (*Pache et al., 1990*). However, more recent studies indicate that multiple waves of follicular recruitment occur within a single interovulatory period (*Baerwald et al., 2003*). Moreover, the antral follicles seen in the late follicular phase may not necessarily be atretic, but may be in early stages of follicular development (*Baerwald et al., 2012*). Regression of the corpus luteum is not mandatory in order to start ovarian stimulation, and luteal phase progesterone and/or the presence of a corpus luteum do not have an adverse effect on synchronized follicular growth (*Cakmak et al., 2013*).

With these concepts in mind, late follicular phase stimulation has been used effectively in women diagnosed with cancer awaiting gonadotoxic treatment, with the advantage of decreasing the total duration of IVF treatment. The numbers of total and mature oocytes obtained, oocyte maturation rate, mature oocyte yield and fertilization rate were similar in late follicular phase ovarian stimulation and conventional early follicular phase ovarian stimulation (*Cakmak et al., 2013*). Recombinant FSH (r-FSH) and/or highly purified human menopausal gonadotrophin (hp-HMG) were used for stimulation, while a gonadotrophin-releasing hormone (GnRH) antagonist was used to prevent a premature surge of LH. A GnRH antagonist could be added in a flexible manner (when serum LH >10 IU/l), resulting in significantly similar outcomes with respect to oocyte yield (*Kolibianakis et al., 2011*). Random start stimulation has now become standard practice for urgent fertility preservation prior to gonadotoxic therapy (*Baerwald and Pierson, 2020*). Similar clinical pregnancy rates and similar or increased live birth rates have been reported, but further research on live birth rates and long-term outcomes in children born after these protocols is needed (*Wang et al., 2016*; *Zhang et al., 2018*).

In view of LH suppression by endogenous progesterone in the luteal phase (*Baerwald et al., 2003*), it may be postulated that administration of a GnRH antagonist could be optional or even omitted when the ovaries are stimulated in the presence of a corpus luteum. This approach may reduce the overall cost of stimulation in oocyte donors. Two retrospective cohort studies (*Zhu et al., 2019*, *2020*), comparing a short GnRH agonist protocol with late follicular phase stimulation with hp-hMG alone, revealed that late follicular phase stimulation could be performed in the absence of an exogenous pituitary modulator.

The rationale for conducting this randomized trial was to enhance cycle flexibility using a protocol with ovarian stimulation started in the late follicular phase. The aim was to assess whether this schedule is as efficient as early follicular phase stimulation in terms of the number of oocytes, but with reduced treatment cost and burden experienced by oocyte donors.

MATERIALS AND METHODS

Institutional review board approval to perform the study was obtained from the Ethical Committee of Universitair Ziekenhuis Brussel, Belgium on 16 May 2018. All women gave written informed consent to participate in this study. The EUDRACT number of the trial was 2017-005005-12, and the study was registered in clinicaltrials.gov (NCT03767218).

Trial design

This was an open label, phase 3, non-inferiority, randomized controlled trial using a two-arm design with a 1:1 allocation ratio.

Participants

The study included 84 oocyte donors, aged 18–36 years, with menstrual cycles of regular length (24–35 days) and body mass index of 19–35 kg/m^2, who had been enrolled in the oocyte donation programme of the Centre for Reproductive Medicine, Universitair Ziekenhuis Brussel (FIGURE 1).

Women underwent ovarian stimulation between November 2018 and May 2022. Each woman was enrolled in the study once. Women with a low ovarian reserve [anti-Müllerian hormone (AMH) <1.1 ng/ml and/or antral follicle count (AFC) <7] and presumed hyper-responders [number of follicles per ovary ≥19 and/or AMH >5 ng/ml (*Fraissinet et al., 2017*)] were excluded, as were women with endometriosis grade 3 or more, oligoamenorrhoea and untreated endocrine abnormalities.

Interventions

Women were allocated to either the early follicular start group (control group) or the late follicular start group (study group) after their eligibility to participate in the study was established (FIGURE 2).

Early follicular start (control group)

Women were evaluated on day 2 of the follicular phase with assessment of serum oestradiol, progesterone, LH, FSH, human chorionic gonadotrophin (HCG) and transvaginal ultrasound. Ovarian stimulation was started with daily subcutaneous injections of r-FSH (Bemfola 225 IU daily; Gedeon Richter Plc, Hungary) from day 2 of the follicular phase onwards. A GnRH antagonist (Ganirelix 0.25 mg/day; Merck Sharp & Dohme, UK) was initiated on stimulation day 6 to prevent premature LH surges. Women were re-evaluated on stimulation day 8 (i.e. cycle day 9) with transvaginal ultrasound and oestradiol, progesterone, FSH and LH sampling, and further treatment was continued as per site protocol until the criteria for oocyte trigger were achieved.

Late follicular start (study group)

Women were first evaluated on day 2 of the follicular phase with measurement of serum oestradiol, progesterone, LH, FSH, HCG and transvaginal ultrasound. Blood samples and ultrasound findings were assessed by the responsible physician. Using the basal oestradiol concentration, the date for the next evaluation was decided by the responsible physician. If a dominant follicle and late follicular hormonal values were observed at the second evaluation (before rise of LH due to impending ovulation), the administration of

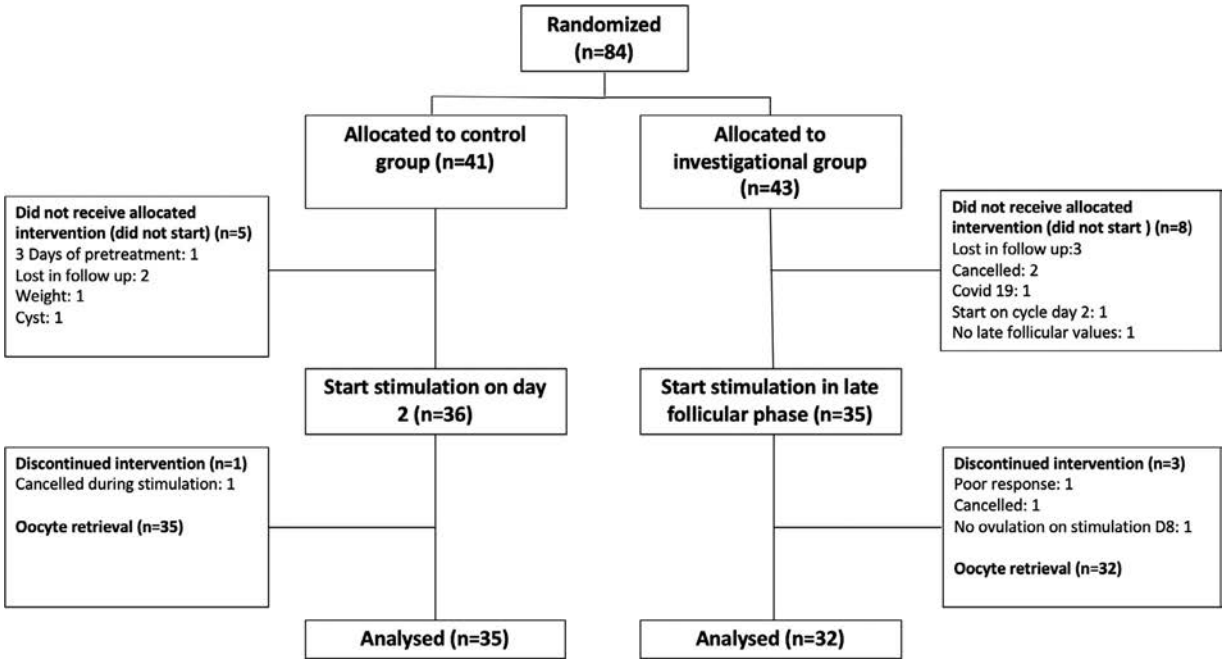

FIGURE 1 Flow diagram.

daily subcutaneous injections of r-FSH (Bemfola 225 IU daily) was started. In the case of a low oestradiol concentration or if no dominant follicle was observed, further evaluation was scheduled until a dominant follicle with late follicular hormonal values was observed, at which point administration of daily subcutaneous injections of r-FSH (Bemfola 225 IU daily) was started. After initiation of r-FSH, the next evaluation with ultrasound and blood sampling was scheduled on stimulation day 8 of r-FSH administration. Daily subcutaneous injection of Ganirelix 0.25 mg was started when the serum LH concentration was >10 IU/l after 8 days of stimulation, and was continued until the day of oocyte trigger.

Both groups

Oocyte maturation trigger was administered with a single subcutaneous injection of GnRH agonist (Gonapeptyl 0.2 mg; Ferring BV, the Netherlands) when transvaginal ultrasound showed at least three follicles >17 mm in diameter. Approximately 34–36 h later, transvaginal oocyte retrieval was performed, retrieved cumulus–oocyte complexes were counted and denuded, and the number of mature oocytes was evaluated.

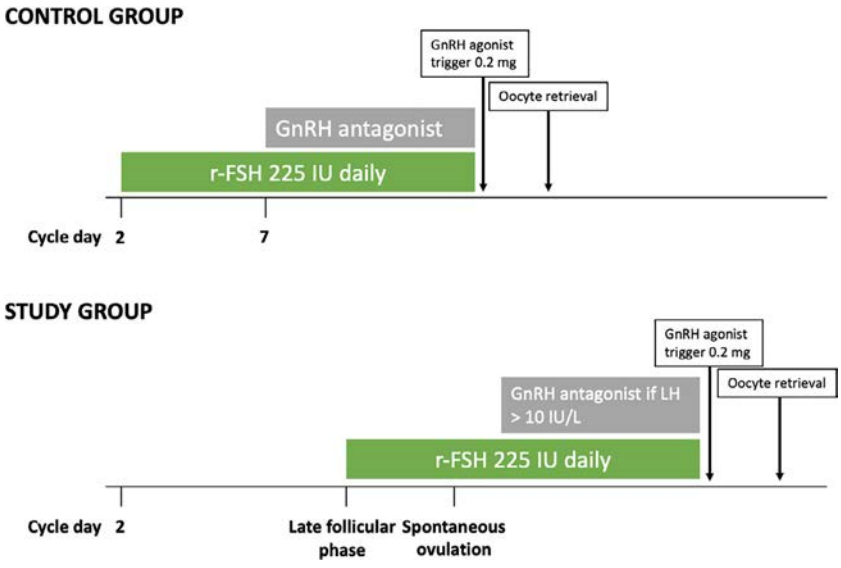

FIGURE 2 Schematic overview of both treatment groups. GnRH, gonadotrophin-releasing hormone; r-FSH, recombinant FSH.

Outcome measures

The primary outcome was the total number of oocytes retrieved after ovarian stimulation. Secondary outcomes included the total number of mature oocytes, consumption of gonadotrophins, duration of ovarian stimulation, total days of use of GnRH antagonist, total cost of medication, and assessment of the endocrine profile in both treatment groups.

Sample size calculation

A sample size of 62 women was required to demonstrate, with 80% power, that the number of oocytes retrieved in the investigational group would be no more than three fewer compared with the number of oocytes retrieved in the reference group. Assuming a one-sided 0.025 significance level and SD of 4.2 retrieved oocytes (*Wikland et al., 2001*), *t*-test yielded a required sample size of 31 women per treatment group.

Randomization and allocation of women to study groups

After assessment of eligibility and signing the informed consent form, women were

TABLE 1 BASELINE CHARACTERISTICS OF THE TWO TREATMENT GROUPS

Characteristic	Control group	Study group
Women, *n*	35	32
Age, years	27.4 (3.7)	26.9 (3.7)
Body mass index, kg/m^2	22.9 (2.4)	22.8 (3.2)
Anti-Müllerian hormone, ng/ml	2.6 (1.1)	2.7 (1.1)
Basal LH, IU/l	6.5 (2.3)	6.6 (2.7)
Basal FSH, IU/l	7.0 (2.0)	7.2 (2.4)
Basal oestradiol, ng/l	36.1 (16.4)	33.1 (16.7)
Basal progesterone, μg/l	0.2 (0.2)	0.4 (0.5)

Data are presented as mean (SD) unless otherwise indicated. There are no *P*-values because this was a randomized controlled trial so the groups were expected to be balanced.

randomized to either the control group or the study group. The randomization sequence was created using STATA Version 13 (StataCorp, USA). Computer-generated randomization codes were used to generate the randomization list. A permutated block size of six was used. Sequentially numbered, identical, opaque, sealed envelopes were used to conceal allocation from clinicians, research personnel and participants, with 1:1 allocation.

Statistical analysis

Analyses were performed in an intent-to-treat fashion and per-protocol. In the intent-to-treat analysis, all women were included in the final analysis provided that, upon meeting the inclusion criteria, they were allocated at random to, and started treatment in, one of the two treatment groups. The per-protocol analysis included only those women who completed the treatment they were allocated at the outset.

Descriptive summary measures expressed as mean ± SD or median (interquartile range) were used for continuous variables, and *n* (%) for categorical variables, in order to provide a summary estimate of donor demographics and baseline characteristics. The analysis and reporting of the results of the clinical outcomes followed the CONSORT guidelines (www.consort-statement.org).

Parametric (independent *t*-test) or non-parametric (Mann–Whitney test) tests were used for continuous variables, depending on the normality of the distribution. Normality was examined using the Shapiro–Wilk test. Results were also summarized with 95% CI, both in absolute terms and as odds ratios for binomial outcomes and 95% difference of means for continuous outcomes, as suggested by the CONSORT statement. All tests were two-sided with *P*-values <0.05 considered to indicate significance.

RESULTS

In total, the data of 84 women were included in the analysis (FIGURE 1). Baseline characteristics of the women are presented in TABLE 1.

Primary outcome measures

Using an intent-to-treat analysis, the total number of oocytes did not differ significantly between the control group (15.5 ± 11.0) and the study group (14.0 ± 10.7) [absolute difference 1.53 (95% CI -3.2 to 5.3)]. In the per-protocol analysis, excluding 17 women (six in the control group and 11 in the study group), no significant difference in the total number of oocytes was found between the control group (18.2 ± 9.7) and the study group (18.8 ± 7.8) [absolute difference -0.6 (95% CI -4.9 to 3.6)] (TABLE 2).

Secondary outcome measures

The number of mature oocytes did not differ significantly between the control group (14.1 ± 8.1) and the study group (12.7 ± 8.5) [absolute difference 1.42 (95% CI -2.6 to 5.5)]. No cases of ovarian hyperstimulation syndrome were observed in either of the treatment arms (TABLE 2).

The stimulation characteristics are presented in TABLE 3. The mean day for r-FSH initiation in the study group was day 13.8 ± 3.4. The duration of stimulation was shorter in the control group (10.0 ± 1.4 days) compared with the study group (10.9 ± 1.5 days) [absolute difference -0.9 (95% CI -1.6 to -0.17); *P* = 0.01]. The total amount of r-FSH used was lower in the control group (2240.7 ± 313.9 IU) compared with the study group (2453.9 ± 330.1 IU) [absolute difference -213.1 (95% CI -370.3 to -56.0); *P* = 0.008]. A GnRH antagonist was used for approximately 6 days in the control group (6.0 ± 1.4 days), whereas a GnRH antagonist was only prescribed for one woman in the study group, and was prescribed for 4 days due to LH concentration >10 IU/l.

TABLE 2 OUTCOME MEASURES OF THE TWO TREATMENT GROUPS

Outcome	Control group	Study group	Difference (95% CI) MH odds ratios (95% CI)	*P*-value
Oocytes retrieved, *n*, intent-to treat[a]	15.5 (11.0)	14.0 (10.7)	1.53 (-3.2 to 5.3)	0.52[c]
Oocytes retrieved, *n*, per-protocol[b]	18.2 (9.7)	18.8 (7.8)	-0.6 (-4.9 to 3.6)	0.62[d]
Mature oocytes, *n*, per-protocol[b]	14.1 (8.1)	12.7 (8.5)	1.42 (-2.6 to 5.5)	0.48[c]
OHSS rate, %	0 (0)	0 (0)		>0.99

Data are presented as mean (SD) unless otherwise indicated.

[a] *n* = 41 for control group and *n* = 43 for study group.

[b] *n* = 35 for control group and *n* = 32 for study group.

[c] Independent *t*-test.

[d] Mann–Whitney test.

OHSS, ovarian hyperstimulation syndrome; MH, Mantel–Haenszel.

TABLE 3 STIMULATION CHARACTERISTICS FOR THE TWO TREATMENT GROUPS

Characteristic	Control group (*n* = 35)	Study group (*n* = 32)	Difference (95% CI)	*P*-value
Duration of ovarian stimulation, days	10 (1.4)	10.9 (1.5)	-0.9 (-1.6 to -0.17)	0.01[c]
Total consumption of rFSH, IU	2240.7 (313.9)	2453.9 (330.1)	-213.1 (-370.3 to -56.0)	0.008[c]
Duration of antagonist use, days	6.0 (1.4)[a]	0.13 (0.7)[b]	5.9 (5.3 to 6.4)	<0.001[d]
Cost of stimulation, €	1147.9 (182.8)	979.9 (129.0)	168 (90.1 to 245.9)	<0.001[c]

Data are presented as mean (SD).
[a] Number of women using antagonist = 35.
[b] Number of women using antagonist = 1.
[c] Independent *t*-test.
[d] Mann–Whitney test.

Consequently, very little GnRH antagonist was used in the study group (0.13 ± 0.7 days) [absolute difference 5.9 (95% CI 5.3–6.4); $P < 0.001$]. There was a significant difference in the total cost of medication per cycle between the two groups, with a cost of 1147.9 ± 182.8€ for the control group and 979.9 ± 129.0€ for the study group [absolute difference 168 (95% CI 90.1–245.9); $P < 0.001$]. This means a cost reduction of 15% for the study group compared with the control group.

Details about the endocrine profile during stimulation of both groups can be found in Supplementary Table 1 (see online supplementary material).

DISCUSSION

According to the results, no significant difference in the number of oocytes was seen between the two groups. This implies that late follicular phase stimulation is not inferior to conventional early follicular phase stimulation in a GnRH antagonist protocol for oocyte donors in terms of the number of oocytes.

In oocyte donors, cycle flexibility is of great importance; avoidance of the unnecessary delay of stimulation until the start of menstruation could improve compliance. Furthermore, random start protocols can allow for better synchronization with the recipient's cycle. A prospective pilot study in nine oocyte donors compared stimulation start on day 2 of the menstrual cycle with start on day 15 of the menstrual cycle in a GnRH antagonist protocol. Good pregnancy rates were achieved with the use of donor oocytes obtained when ovarian stimulation was initiated on day 15 of the cycle (*Martinez et al., 2014*). More recently, the same group published a prospective study in oocyte donors showing a comparable number of oocytes and comparable blastocyst euploidy rates when luteal phase stimulation was compared with follicular phase stimulation in a GnRH antagonist protocol (*Martinez et al., 2022*).

In the present study, the duration of stimulation with r-FSH was approximately 1 day longer when stimulation was started in the late follicular phase, which implies higher total consumption of r-FSH. These findings are in line with previous studies on random start and luteal phase stimulation (*Cakmak et al., 2013*; *Pereira et al., 2017*; *von Wolff et al., 2009*, *2016*). However, very little GnRH antagonist was used when stimulation was started in the late follicular phase because of adequate LH suppression by endogenous progesterone production in the luteal phase. Only one woman required a GnRH antagonist in the present study, which is in line with previous studies (*Zhu et al., 2019*, *2020*; *Kuang et al., 2014*). This resulted in a cost reduction of 15% compared with early follicular phase stimulation in a GnRH antagonist protocol. As very little use of GnRH antagonist was indicated, fewer injections were needed during stimulation, providing a more donor-friendly and less burdensome treatment.

Further studies are needed to confirm if these results can be extrapolated to women undergoing elective freezing (such as patients undergoing preimplantation genetic testing, and women undergoing elective egg freezing). In the setting of oncofertility, the random start of ovarian stimulation (in the luteal and late follicular phase) is already widely adopted, as time is often limited in emergency fertility preservation before initiation of gonadotoxic therapy. Most studies indicate no difference in the number of oocytes retrieved between early and late follicular phase stimulation (*Cakmak et al., 2013*; *Nayak and Wakim, 2011*; *von Wolff et al., 2016*).

Previous studies suggested that an increased progesterone concentration in the last days of ovarian stimulation may have a detrimental effect on oocyte competence and embryo quality. *Racca et al. (2018)* showed that a high serum progesterone concentration at the end of the follicular phase is associated with a decrease in the embryo utilization rate and cumulative live birth rate (CLBR) in patients with infertility. Other studies reported more embryo wastage and lower formation rates of top-quality embryos in the case of elevated progesterone on the day of oocyte trigger (*Huang et al., 2016*; *Vanni et al., 2017*). However, a high progesterone concentration at the end of the follicular phase did not hinder CLBR of the subsequent frozen cycles when a freeze-all approach was used (*Racca et al., 2021*). Prospective data of repeated stimulation cycles in oocyte donors showed that fertilization rates, blastocyst formation rates and embryo euploidy rates were comparable in luteal stimulation cycles and follicular stimulation cycles (*Martinez et al., 2022*). Therefore, it can be assumed that oocyte competence will not be impaired.

This study has several strengths. First, it was a randomized prospective study, which is considered the cornerstone of evidence-based medicine. Second, the authors aimed to minimize confounding bias by using the same stimulation protocol in all women. A fixed starting dose of r-FSH 225 IU/day was used, and no dose adjustments were allowed. In the early follicular stimulation group, a fixed start of the GnRH antagonist on stimulation day 6 was implemented. Last, the only variation

in protocol was the day of start of stimulation in the study group. As the length of the menstrual cycle varies, the day of start of stimulation could not be standardized. Stimulation was initiated only when late follicular hormonal values in the presence of a dominant follicle were observed.

However, this study also had limitations. First, embryological data were lacking. Due to adaptations in the oocyte donation programme (frozen versus fresh donation of oocytes) at the study centre over the years, this study contains a mix of fresh and frozen donation cycles, leading to a very heterogenous group which precludes correct interpretation of the data. Another limitation is that the sample size calculation was based on the data of *Wikland et al. (2001)*, with an estimated SD of 4.2. However, the observed SD values were much larger in this study (9.7 and 7.8 in each group), so a new sample size calculation would have led to a larger sample size.

In conclusion, the findings demonstrate that starting ovarian stimulation with r-FSH in the late follicular phase is as efficient as starting in the early follicular phase in terms of the number of oocytes. Starting r-FSH in the late follicular phase improves cycle flexibility, and the cost and treatment burden experienced by donors undergoing IVF can be reduced. As oocyte donors are young, healthy women, undergoing a medical procedure for which they do not receive any health benefit themselves, there is a clear need for flexible stimulation protocols that reduce physical demands and minimize disruption for donors.

DATA AVAILABILITY

Data will be made available on request.

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AUTHOR CONTRIBUTIONS

CB was responsible for the concept design. SDR wrote the manuscript. SDR and KI performed data extraction. KI, SM, NDM, MDV, HT and CB contributed to the interpretation of the results and editing of the manuscript. All authors approved the final article.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.103889.

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RBMO

ELSEVIER

ARTICLE

The ovarian stimulation regimen does not affect aneuploidy or blastocyst rate

BIOGRAPHY

Mauro Cozzolino completed a Fellowship in Reproductive Medicine at IVI Madrid, Spain in 2017. He obtained a Fellowship at the Department of Obstetrics, Gynecology and Reproductive Sciences of Yale School of Medicine from 2018 to 2021. He got the PhD at University Rey Juan Carlos in 2023. Dr Cozzolino is currently employed at IVIRMA Roma.

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KEY MESSAGE

The total dose of exogenous gonadotrophin is not associated with embryonic aneuploidy, reproductive outcomes or cumulative live birth rates, and the administration of human menopausal gonadotrophin alone during ovarian stimulation does not increase the aneuploidy rate.

ABSTRACT

Research question: Could the total dose (<3000 IU or ≥3000 IU) and type of exogenous gonadotrophin (i.e. recombinant FSH and/or human menopausal gonadotrophin [HMG]) influence aneuploidy and blastulation rates and produce different reproductive outcomes?

Design: This retrospective, observational, multicentre cohort study included a total of 8466 patients undergoing IVF using autologous oocytes and preimplantation genetic testing for aneuploidies. Participants were divided according to the dosage of total gonadotrophins and stratified by maternal age.

Results: The aneuploidy rates, pregnancy outcomes and cumulative live birth rates (CLBR) were similar among women who received total gonadotrophin dosages of <3000 or ≥3000 IU. No statistical differences were reported in the blastulation rate with lower or higher gonadotrophin dosages. Women receiving a higher amount of HMG during ovarian stimulation had a lower aneuploidy rate ($P = 0.02$); when stratified according to age, younger women with a higher HMG dosage had lower aneuploidy rates ($P < 0.001$), while no statistical differences were observed in older women with higher or lower HMG dosages. No significant differences were observed in IVF outcomes or CLBR.

Conclusions: High doses of gonadotrophins were not associated with rate of aneuploidy. However, an increased fraction of HMG in younger women was associated with a lower aneuploidy rate. The study demonstrated that the total gonadotrophin dosage did not influence aneuploidy, reproductive outcomes or CLBR. The increased gonadotrophin and HMG dosages used for ovarian stimulation did not precede aneuploidy, and the use of HMG should be evaluated on a case-by-case basis, according to the individual's characteristics and infertility type.

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KEYWORDS
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Preimplantation genetic testing for aneuploidies
Recombinant FSH

INTRODUCTION

In the last few years, clinical outcomes following assisted reproductive technology (ART) have significantly improved along with advances in embryo selection techniques (*Gardener and Sakkas, 2022*; *Sigalos et al., 2016*). Preimplantation genetic testing for aneuploidies (PGT-A) selects chromosomally normal embryos, improving the success of IVF (per embryo transfer) by excluding abnormal embryos, and reducing the time to pregnancy, especially for older patients (*Dahdouh et al., 2015*). However, the mechanisms underlying aneuploidy are still poorly understood. Apart from advanced maternal age (*Munne et al., 1995*), chromosome abnormalities have been postulated to be attributed to ART treatments (*Munne et al., 2017*). Indeed, previous studies carried out with young fertile oocyte donors showed an alarmingly higher prevalence of embryonic aneuploidy following IVF (*Katz-Jaffe et al., 2005*; *Munne et al., 2006*; *Reis Soares et al., 2003*).

In human ovarian stimulation protocols, the ovarian response plateaus with total daily doses of 450 IU of exogenous gonadotrophin stimulation. Thus, doses over 450 IU are not recommended for prolonged exposure (>12 days) (*Sekhon et al., 2017*), due to the risk of hyperstimulation of the ovaries (*van Tilborg et al., 2017*), possible adverse effects on oocyte quality (*Shapiro et al., 2001*) and increased aneuploidy rates (*Barash et al., 2017*). As ovarian stimulation influences oocyte maturation and the meiosis phase, abnormalities in the underlying mechanisms can potentially mediate chromosomal aneuploidy (*Verpoest et al., 2008*). Furthermore, elevated doses of exogenous gonadotrophins may interfere with the natural selection of the dominant follicle, increasing the selection of poor-quality oocytes that would otherwise have undergone atresia.

This observation supports the hypothesis that mild stimulation protocols (employing lower doses of gonadotrophins) might reduce oocyte and embryonic aneuploidy rates (*Baart et al., 2007*). Indeed, Rubio and colleagues reported decreased rates of aneuploidy in oocyte donors who underwent ovarian stimulation using lower FSH doses (*Rubio et al., 2010*). However, Verpoest and co-workers demonstrated an unexpectedly high rate of chromosomal abnormalities (36.4%) in cleaved embryos derived from 11 unstimulated cycles of young patients (*Verpoest et al., 2008*). Similarly, a prospective cohort study with oocyte donors revealed aneuploidy rates of 38.2% and 34.8%, respectively, following stimulated and natural cycles; this suggests that embryonic aneuploidies are present in humans regardless of ovarian stimulation, and that the use of exogenous gonadotrophins was not solely responsible this type of embryo abnormality (*Labarta et al., 2012*).

In addition, the dose of gonadotrophins was not associated with the number of embryos generated and the aneuploidy rates (*Ata et al., 2012*). The latter finding was corroborated by a recent retrospective study which found that live birth rates (LBR) were significantly improved in high ovarian responders compared with low ovarian responders (who produce 1–5 oocytes) in cycles with PGT-A (*Wu et al., 2018*). Notwithstanding the evidence indicating that overstimulating the ovary does not necessarily improve oocyte quality, milder approaches also have their disadvantages, including a lower number of recovered oocytes and a consequent higher cancellation rate (despite treatment costs being significantly reduced) (*Montoya-Botero et al., 2021*). In this regard, improved euploidy rates can be obtained using a fraction of human menopausal gonadotrophin (HMG) (*McCulloh et al., 2019*). Although previous studies have focused only on LBR, considering the cumulative live birth rate (CLBR) might be a better strategy for analysing the effect of different gonadotrophin dosages.

This retrospective study aimed to determine whether an association exists between the type and total dose of exogenous gonadotrophins and aneuploidy and blastulation rates, based on the maternal age, as well as how different ovarian stimulation protocols reflect on reproductive outcomes.

MATERIALS AND METHODS

Ethical approval and inclusion and exclusion criteria

This retrospective study was approved by the Ethics Committee of the Instituto Valenciano de Infertilidad (no. 1903-FIVI-039-MC, approval date 24 July 2019). Anonymized data were collected from women 18–44 years of age undergoing IVF using autologous oocytes and PGT-A between January 2017 and January 2022, at the authors' IVIRMA clinics in Europe. Only patients with programmed PGT-A were included in the study, and no age limitations were considered in the study. The study included patients who received ovarian stimulation with different doses of gonadotrophin (≥3000 or <3000 IU), and those who underwent trophectoderm biopsy on day 5–6. The analysis did not include only the first cycles. Patients were excluded from the study if they underwent PGT-A on the basis of monogenic disease or translocation or had a partner with severe male infertility (i.e., semen concentration <5 million/ml). The indications for PGT-A were advanced maternal age, recurrent severe male infertility pregnancy loss and recurrent implantation failure. Considering the total amount of gonadotrophins, a subgroup analysis was carried out on patients receiving different dosages of HMG (<1000 or ≥1000 IU).

Ovarian stimulation and oocyte retrieval

Following the standard clinical procedure, the patient's ovarian reserves were evaluated using serum anti-Müllerian hormone concentrations, antral follicle counts and menstrual cycle day 3 FSH concentrations within 3 months of the index IVF cycle, prior to ovarian stimulation. Ovarian stimulation was carried out using the clinic's internal antagonist, short or long protocols, as previously described (*Cozzolino et al., 2021*).

Briefly, treatment with recombinant FSH (Gonal-F) and/or HMG (Menopur) began 2–3 days after menstruation or 5 days after the last contraceptive pill. The first transvaginal ultrasound examination was performed on stimulation day 5, and every 2 days thereafter. A daily dose of 0.25 mg of gonadotrophin-releasing hormone (GnRH) antagonist (Cetrotide) was introduced when the first follicle reached a mean diameter of 13–14 mm. A single dose of recombinant human chorionic gonadotrophin (HCG; 250 μg) alone or in combination with 0.2 mg GnRH agonist (Decapeptyl) was administered to trigger final oocyte maturation when at least three follicles reached a mean diameter of 17–18 mm. Oocytes were retrieved 34–36 h after final oocyte maturation, under ultrasound guidance, and subsequently subjected to intracytoplasmic sperm injection.

Embryo biopsy and chromosomal analysis

According to standard clinical protocol, embryos were cultured in Geri continuous media (Genea Biomedx, Australia) to the blastocyst stage. Trophectoderm biopsies were performed on day 5 or 6, and the embryos were cryopreserved using vitrification (*Cozzolino et al., 2021*). Embryos vitrified using a vitrification kit (Kitazato, Japan) were stored on a Cryotop device, according to the kit instructions. Chromosomal analysis was performed by next-generation sequencing (NGS) at a reputed genetic laboratory, according to their protocols (*Tiegs et al, 2021*).

After PGT-A, frozen embryo transfers (FET) were prepared in natural or stimulated cycles. The blastocyst was warmed using the warming kit (Kitazato, Japan) and cultured in blastocyst medium (Origio, Denmark) before transfer. Blastocysts were thawed on the day of embryo transfer and cultured for 2–4 h before transfer. After inserting a sterile speculum into the vagina in the lithotomy position, the cervical mucus was aspirated using a syringe. The gynaecologist inserted the embryo transfer catheter (Kitazato, Japan) 1 cm below the uterine fundus. The catheter was immediately checked for the presence of a retained embryo inside the catheter. Immediately after embryo transfer, the transfer was scored as easy, moderate or difficult. The endometrium was synchronized with oestrogen patches (150 mg/2 days; Estradot) or oral tablets (6 mg/day; Progynova). On the 10th or 12th day of endometrial preparation, transvaginal ultrasonography was performed to measure the endometrial thickness, and blood samples were collected to test serum oestradiol and progesterone concentrations. When the endometrium was at least 7 mm thick, the luteal phase was stimulated with 800 mg daily of micronized vaginal progesterone (Progeffik). Women with progesterone levels <9.75 ng/mL on the day of SET/FET received a rescue dose of subcutaneous progesterone.

Clinical outcomes

The aneuploidy rate was defined as the number of aneuploid embryos divided by the total number of embryos analysed. The fertilization rate is the percentage of micro-injected oocytes that transformed into two pronuclei oocytes. The blastulation rate was defined as the number of embryos reaching the blastocyst stage out of the total number of fertilized embryos (with two pronuclei). Biochemical pregnancy was diagnosed by a β-HCG concentration of over 10 mIU/ml, measured 2 weeks following the FET. Clinical pregnancy was confirmed by transvaginal ultrasound detection of an intrauterine gestational sac, with a fetal heartbeat present at 6 weeks following FET. Biochemical miscarriage was defined as a decrease in β-HCG after a positive value had been registered. Clinical miscarriage was defined as the spontaneous termination of pregnancy before 22 weeks.

Ongoing pregnancy was characterized by a viable fetus present in the uterus after 22 weeks of gestation. All pregnancy rates were calculated from the number of embryo transfers. LBR was defined per embryo transfer (the percentage of deliveries with at least one baby born at ≥28 weeks) at the first embryo transfer and at all consecutive transfers from each patient, and CLBR (percentage of deliveries with at least one baby born after the subsequent ET, for each transfer) per patient, representing true individual patient data.

Statistical analysis

Categorical and continuous data are respectively presented as proportions or means with confidence intervals for all registered variables. Categorical variables were compared using the chi-squared and Fisher's exact tests. Continuous variables were tested for normality and compared using t-tests.

Multivariate regression was used to assess the association between the gonadotrophin dosage (<3000 or ≥3000 IU) used in the IVF PGT-A cycles and embryonic aneuploidy, while adjusting for the known covariates of maternal age (<37 or ≥37 years old), body mass index (BMI), number of oocytes recovered following ovarian stimulation, days of stimulation, number of embryos biopsied, gonadotrophin dosage and type of gonadotrophin. Odd ratios (OR) with 95% confidence intervals (95% CI) were calculated. Repeated measures were accounted for using generalized estimating equations. In all cases, statistical significance was defined as a value $P < 0.05$, and all statistical analyses were conducted using R software (version 3.5.0; The R Foundation, Austria).

RESULTS

Baseline and cycle characteristics

A total of 8466 patients were included in the study (4945 received <3000 IU of total gonadotrophins, and 3521 received ≥3000 IU of total gonadotrophins, during a given cycle). Together, these patients underwent 8957 IVF cycles before PGT-A, with 5206 and 3751 cycles, respectively, eligible for analysis. A total of 37,093 trophectoderm biopsies were included in the study; 19,519 were euploid blastocysts while 17,574 were aneuploid blastocysts. The principal indication for IVF in both groups was advanced maternal age, with 74.72% of women receiving ≥3000 IU and 64.23% receiving <3000 IU of total gonadotrophins. All indications for treatment are reported in Supplementary Table 1, and each couple could present multiple indications for IVF.

The clinical characteristics of the patients and the cycle parameters (stratified by gonadotrophin dosages) are presented in TABLE 1. The ovarian stimulation was conducted using three protocols. In the group receiving <3000 IU of gonadotrophin most of the patients received an antagonist GnRH protocol (87.12%), followed by progestogens (8.54%), a long GnRH protocol (2.17%) and a short GnRH protocol (2.17%) ($P < 0.001$). In the group with ≥3000 IU of gonadotrophin, most patients received an antagonist GnRH protocol (83.04%), followed by progestogens (11.36%), a long GnRH protocol (4.79%) or a short GnRH protocol (0.81%) ($P < 0.001$). Of the 37,093 embryos genetically screened using PGT-A, 17,574 aneuploid embryos were identified, so the overall embryonic aneuploidy rate was 47.37%.

A total of 12,265 euploid blastocysts was recorded for the group receiving <3000 IU and 7254 for those receiving ≥3000 IU. There was a total of 10,976 aneuploid blastocysts in the group given <3000 IU and 6598 in the ≥3000 IU group.

In relation to the number of embryo transfers, there were 7023 in the group receiving <3000 IU and 4745 in the ≥3000 IU group. The study included

TABLE 1 PATIENT CLINICAL CHARACTERISTICS AND CYCLE PARAMETERS STRATIFIED BY GONADOTROPHIN DOSAGE

Variable	Total gonadotrophin dosage <3000 IU (*n* = 4945)	Total gonadotrophin dosage ≥3000 IU (*n* = 3521)	*P*-value
Age (years)	37.05 (37.01–37.09)	38.06 (38.01–38.11)	<0.001
BMI (kg/m^2)	22.9 (22.74–22.98)	23.7 (23.49–23.90)	<0.001
Number of years of infertility	2.41 (2.34–2.49)	2.34 (2.29–2.31)	0.14
Number of days of stimulation	10.35 (10.24–10.46)	12.06 (11.87–12.25)	<0.001
Amount of rFSH (IU)	1488.12 (1480.63–1495.6)	2452.7 (2442.39–2461.93)	<0.001
Amount of HMG (IU)	753.5 (749.13–757.87)	1187.1 (1079.12–1195.18)	<0.001
Peak oestradiol (pg/ml)	2804 (20780–2827)	2304.3 (2624–2678)	<0.001
Number of oocytes	17.31 (17.18–17.44)	14.42 (14.27–14.53)	<0.001
Number of MII oocytes	14.77 (14.67–14.88)	12.79 (12.68–12.90)	<0.001
Number of biopsied embryos	6.55 (6.50–6.60)	5.46 (5.41 – 5.51)	<0.001
Number of euploid embryos	3.31 (3.28–3.34)	2.64 (2.61–2.68)	<0.001
Euploidy rate (%)	52.77 (52.13–53.42)	52.37 (51.53–53.20)	0.09
Blastocyst rate (%)	61.19 (60.84–61.53)	61.33 (60.89–61.76)	0.63
Fertilization rate (%)	74.9 (74.40–75.46)	73.6 (72.88–74.40)	0.006
Aneuploidy rate (%)	47.23 (46.58–47.87)	47.63 (46.80–48.47)	0.45
Number of previous cycles	0.9 (0.85–0.91)	0.9 (0.82–0.90)	0.37
Number of trophectoderm biopsies	23,241	13,852	<0.001

Data are reported as mean (95 CI).

Groups were compared using t-tests. Data were reported for all cycles.

BMI, body mass index; HMG: human menopausal gonadotrophin; MII, metaphase II; rFSH, recombinant FSH.

11,327 single-embryo transfers, 440 double-embryo transfers and one triple-embryo transfer. Endometrial preparation was conducted using hormone replacement therapy (HRT), natural cycles or stimulation with gonadotrophin. The group given <3000 IU of gonadotrophin was divided into 5201 (74.06%) patients receiving HRT, 1772 (25.23%) with natural cycles and 50 (0.71%) receiving gonadotrophins, while for the group given ≥3000 IU the figures were 3731 (78.63%) for HRT, 980 (20.65%) for natural cycles and 34 (0.72%) with gonadotrophins.

Effect of total gonadotrophin dosage on aneuploidy rates

The embryonic aneuploidy rates were comparable between women who received the lower and higher gonadotrophin doses (47.23% [95% CI 46.58–47.87] versus 47.63% [95% CI 46.80–48.47]; $P = 0.45$). The euploidy rate was also similar across the groups, being 52.77% (95% CI 52.13–53.42%) in the women in the lower gonadotrophin group and 52.37% (95% CI 51.53–53.20%) in the higher gonadotrophin group ($P = 0.09$). The univariate analysis showed no difference in the aneuploidy rate ($P = 0.84$). However, the multivariate analysis after controlling for potential covariates showed that a higher number of oocytes retrieved influenced the aneuploidy rate (OR 0.26 [95% CI 0.19–0.34]; $P < 0.001$), while the BMI, number of days of stimulation and gonadotrophin dosage did not affect it ($P = 0.20$, $P = 0.18$ and $P = 0.98$).

In the group of younger patients (<37 years), the aneuploidy rate was not statistically different in patients who received lower gonadotrophin dosages (38.2% [95% CI 37.16–39.32]) compared with higher gonadotrophin doses (36.7% [95% CI 35.04–38.51]) ($P = 0.16$). The univariate analysis showed no difference in the embryo aneuploidy rate according to the gonadotrophin dosage (OR 2.06 [95% CI −0.18 to 4.3]; $P = 0.07$).

However, after controlling for potential covariates, the odds ratio to evaluate the relation between dosages and aneuploidy rate adjusted for covariables in younger women showed that the number of oocytes retrieved influenced the aneuploidy rate (adjusted OR [aOR] 0.13 [95% CI 0.09–0.26]; $P = 0.03$), while the BMI, number of days of stimulation, number of embryos biopsied, gonadotrophin dosage and type of gonadotrophin did not affect the aneuploidy rate.

Meanwhile, aneuploidy rates in the group of older patients (≥37 years) were not statistically different with the lower or higher gonadotrophin dosage (51.8% [95% CI 50.73–51.95] versus 50.7% [95% CI 49.72–51.61]; $P = 0.07$). However, after controlling for potential covariates, the multivariate analysis showed that the number of oocytes retrieved influenced the aneuploidy rate (aOR 0.48 [95% CI 0.39–0.57]; $P < 0.001$), while the BMI, number of days of stimulation, number of embryos biopsied, gonadotrophin dosage and type of gonadotrophin did not.

Effect of HMG dosage on aneuploidy rates

In the 6900 patients who received HMG (4544 with a low dosage [<1000 IU] and 2356 patients with a high dosage [≥1000 IU]) of HMG, resulting in the analysis of 29,249 embryos genetically screened using PGT-A, there were 14,098 aneuploid blastocysts and 15,151 euploid blastocysts. A significant difference was found in the aneuploidy rate between the groups of patients receiving low or high HMG dosages (48.61% [95% CI

TABLE 2 REPRODUCTIVE OUTCOMES STRATIFIED BY GONADOTROPHIN DOSAGE AND MATERNAL AGE CALCULATED FOR EMBRYO TRANSFERS

Variable	<3000 IU	≥3000 IU	Crude odds ratio (95% CI), *P*-value	Adjusted odds ratio (95% CI), *P*-value
Biochemical pregnancy				
Total	4462/7023 (63.5)	2954/4745 (62.3)	1.4 (−0.40 to 3.34), *P* = 0.12	0.15 (−1.86 to 2.16), *P* = 0.88
<37 years old	1325/2038 (65.0)	549/901 (60.9)	2.9 (−1.03 to 6.83), *P* = 0.14	5.4 (1.26 to 9.64), *P* = 0.09
≥37 years old	3137/4985 (62.9)	2405/3844 (62.6)	0.79 (−1.32 to 2.92), *P* = 0.46	−0.26 (−2.53 to 2.00), *P* = 0.81
Biochemical miscarriage				
Total	539/7018 (7.1)	351/4742 (7.4)	0.44 (−0.57 to 1.46), *P* = 0.38	0.77 (−0.32 to 1.88), *P* = 0.16
<37 years old	166/2037 (8.1)	64/901 (7.1)	1.01 (−1.16 to 3.19), *P* = 0.36	2.36 (−0.04 to 4.78), *P* = 0.05
≥37 years old	373/4981 (7.5)	287/3841 (7.5)	0.19 (−0.95 to 1.34), *P* = 0.74	0.45 (−0.79 to 1.70), *P* = 0.47
Clinical pregnancy				
Total	3889/6989 (55.6)	2569/4711 (54.5)	1.01 (−1.04 to 3.07), *P* = 0.33	−0.59 (−2.79 to 1.60), *P* = 0.59
<37 years old	1152/2031 (56.7)	478/894 (53.5)	2.16 (−2.12 to 6.45), *P* = 0.32	1.46 (−3.24 to 6.17), *P* = 0.32
≥37 years old	2737/4958 (55.2)	2091/3817 (54.8)	0.56 (−1.74 to 2.87), *P* = 0.63	−0.72 (−3.16 to 1.71), *P* = 0.56
Clinical miscarriage				
Total	578/6726 (8.2)	384/4555 (8.4)	0.04 (−1.04 to 1.3), *P* = 0.93	−0.25 (−1.44 to 0.92), *P* = 0.67
<37 years old	181/1949 (9.3)	76/865 (8.8)	0.04 (−2.36 to 2.46), *P* = 0.96	0.21 (−2.52 to 2.95), *P* = 0.87
≥37 years old	397/4777 (8.3)	308/3690 (8.3)	−0.11 (−1.34 to 1.11), *P* = 0.85	−0.28 (−1.62 to 1.04), *P* = 0.67
Ongoing pregnancy				
Total	3036/6714 (45.2)	2021/4547 (44.4)	0.54 (−0.93 to 2.02), *P* = 0.47	−0.22 (−1.66 to 1.20), *P* = 0.75
<37 years old	885/1945 (45.5)	372/864 (43.1)	1.3 (−1.68 to 4.32), *P* = 0.38	0.95 (−1.99 to 3.91), *P* = 0.52
≥37 years old	2151/4769 (45.1)	1649/3683 (44.8)	0.41 (−1.38 to 2.20), *P* = 0.65	−0.32 (−1.95 to 1.29), *P* = 0.69
Live birth				
Total	2568/6246 (41.1)	1676/4202 (39.9)	1.0 (−0.82 to 2.83), *P* = 0.28	−0.08 (−2.03 to 1.85), *P* = 0.92
<37 years old	742/1802 (41.2)	303/795 (38.1)	2.31 (−1.74 to 6.36), *P* = 0.26	1.2 (−3.21 to 5.64), *P* = 0.59
≥37 years old	1826/4444 (41.1)	1373/3407 (40.3)	0.86 (−1.25 to 2.98), *P* = 0.42	−0.22 (−2.45 to 2.00), *P* = 0.84

Data are given as *n*/*N* (%).

Adjustment was made for maternal age, body mass index, number of oocytes recovered following ovarian stimulation, number of days of stimulation, number of embryos biopsied, gonadotrophin dosage and type of gonadotrophin, using generalized estimating equations.

Biochemical pregnancy was diagnosed from a β-HCG concentration of over 10 mIU/ml. Clinical pregnancy was defined by the presence of an intrauterine gestational sac with a fetal heartbeat present. Biochemical miscarriage was defined as a decrease in β-HCG after a positive value. Clinical miscarriage was defined as spontaneous termination of pregnancy before 22 weeks. Ongoing pregnancy was characterized by a viable fetus present in the uterus after 22 weeks of gestation.

HCG, human chorionic gonadotrophin.

47.92–49.30] versus 47.14% [95% CI 46.12–48.17]; $P = 0.02$). After controlling for potential covariates, multivariate analysis evaluating the relation between dosages and aneuploidy rate showed no statistical differences.

Considering the dosing of HMG according to the age of the patients, younger women (<37 years) receiving a higher dosage of HMG presented a lower aneuploidy rate than women with a lower dosage (34.8% [95% CI 32.79–36.90] and 39.2% [95% CI 37.87–40.54]; $P < 0.001$). Further analysis controlling for possible confounders showed that the number of embryos biopsied influenced the relation between the dosage and aneuploidy rate (aOR 6.60 [95% CI 5.71–7.49]; $P < 0.001$) and the dosage of HMG (aOR −2.82 [95% CI −5.35 to 0.29]; $P = 0.02$).

Meanwhile, aneuploidy rates in the group of older patients (≥37 years) were on a par regardless of HMG dosing (51.88% [95% CI 51.08–52.69] with low doses versus 50.83% [95% CI 49.66–52.01] with high dosages; $P = 0.388$). The multivariate analysis showed no statistical difference in embryo aneuploidy rate according to the dosage of HMG.

Effect of total gonadotrophin dosage on blastulation rate

The overall blastulation rate was 61.2% (95% CI 60.97–61.51). Women who received <3000 IU total gonadotrophins showed a blastocyst development rate of 61.19% (95% CI 60.84–61.53) while women who received ≥3000 IU total gonadotrophins had a blastocyst rate of 61.33% (95% CI 60.89–61.76; $P = 0.63$). The univariate analysis showed that the blastulation rate according to gonadotrophin dosage had an odds ratio of −0.12 (95% CI −0.85 to 0.61; $P = 0.73$). In the multivariate analysis, the regression coefficient to evaluate the relation between dosage and blastulation rate adjusted for several covariables showed that the number of aspirated oocytes and the number of embryos analysed were statistically significant (aOR −0.83 [95% CI −0.89 to 0.77];

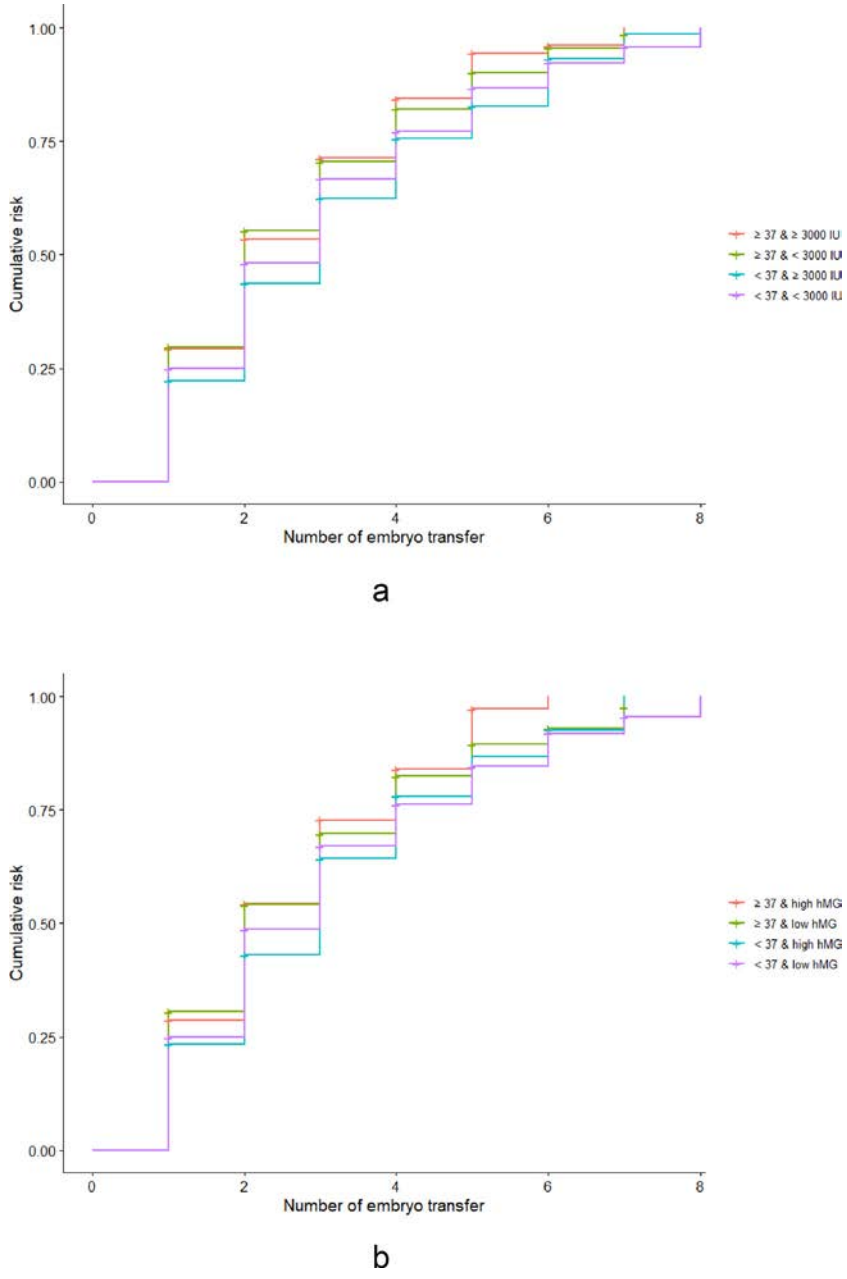

FIGURE 1 Cumulative live birth rate after consecutive frozen embryo transfers with euploid blastocysts according to maternal age and total gonadotrophins (a), and maternal age and dosage of human menopausal gonadotrophin (low <1000 or high ≥1000 IU) (b).

$P < 0.001$) and aOR 2.87 [95% CI 2.67–3.07]; $P < 0.001$).

No difference in the blastocyst rate was seen in the younger patients with gonadotrophin dosages of <3000 IU rather than ≥3000 IU (61.01% [95% CI 60.39–61.63] versus 60.13% [95% CI 59.15–61.71]; $P = 0.13$). The regression coefficient for the blastulation rate according to gonadotrophin dosage gave an odds ratio of 1.18 (95% CI −0.39 to 2.76; $P = 0.14$). However, after controlling for potential covariates, the adjusted odds ratio to evaluate the relationship between dosage and blastulation rate showed statistical significance for the number of aspirated oocytes and the number of embryos biopsied (aOR −0.83 [95% CI −0.91to 0.75], $P < 0.001$ and OR 2.87 [95% CI 2.63–3.11], $P < 0.001$). The same trend was observed with blastocysts from older participants, where the ones who received lower total gonadotrophin dosages had a comparable blastulation rate to those with higher dosages (61.27% [95% CI 60.85–61.69] versus 61.61% [95% CI 61.14–62.13]; $P = 0.26$. After controlling for potential covariates, the adjusted odds ratio to evaluate the relationship between dosage and blastulation rate showed statistical significance for aspirated oocytes and the number of embryos biopsied (aOR −0.89 [95% CI −0.96 to 0.81], $P < 0.001$ and aOR 3.06 [95% CI 2.83–3.29], $P < 0.001$).

The effect of total gonadotrophin and HMG dosage on reproductive outcomes

Reproductive outcomes stratified by gonadotrophin dosage and maternal age are presented in TABLE 2. The LBR was similar in the eligible FET of young women (<37 years) receiving a low or high total gonadotrophin dosage (41.1% [95% CI 39.9–42.3 versus 39.9% [95% CI 38.4–41.4]; $P = 0.28$). The dosage of gonadotrophin in young women did not affect the LBR (OR 2.31 [95% CI −1.74 to 6.36]; $P = 0.26$). The multivariate analysis showed that the number of embryos biopsied influenced the relationship between dosage and LBR (aOR 1.94 [95% CI 0.285–3.02]; $P < 0.00$).

In the older women (≥37 years) with a lower or higher total gonadotrophin dosage (41.1% [95% CI 39.64–42.55] versus 40.3% [95% CI 38.65–41.87]; $P = 0.49$) there was no difference in the LBR. The dosage of gonadotrophin did not affect the LBR (OR 0.86 [95% CI −1.25 to 2.98]; $P = 0.42$). The multivariate analysis showed that the number of embryos biopsied influenced the relationship between the dosage and the LBR (aOR 1.07 [95% CI 0.46–1.68]; $P < 0.001$).

Notably, the CLBR (FIGURE 1a) observed in older women who received higher gonadotrophin dosages in the fourth embryo transfer (84.3%, 95% CI 80.87–87.14) were comparable to those of older women with lower gonadotrophin dosages in the fourth transfer (82.1% [95% CI 79.06–84.75]; $P = 0.61$). The CLBR in younger women (FIGURE 1a) who received higher gonadotrophin dosages in the fourth embryo transfer (75.6% [95% CI 69.12–80.76]) were comparable to those of women with lower gonadotrophin dosages in the fourth (77.1% [95% CI 73.35–80.47]; $P = 0.84$).

Reproductive outcomes stratified by HMG dosage and maternal age are presented in TABLE 3. The LBR in young (<37 years) women receiving a lower HMG dosage was 40.9%

TABLE 3 REPRODUCTIVE OUTCOMES STRATIFIED BY HMG DOSAGE AND MATERNAL AGE CALCULATED FOR EMBRYO TRANSFERS

Variable	Low dose (<1000 IU)	High dose (≥1000 IU)	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Biochemical pregnancy				
Total	3913/6288 (62.2)	2000/3154 (63.4)	1.1 (−1.05 to 3.2), *P* = 0.27	1.5 (−0.77 to 3.69), *P* = 0.20
<37 years old	881/1394 (63.2)	396/635 (62.4)	−0.6 (−5.24 to 3.99), *P* = 0.79	−2.0 (−6.95 to 2.96), *P* = 0.43
≥37 years old	3032/4894 (62.0)	1604/2519 (63.7)	1.5 (−0.82 to 3.84), *P* = 0.420	2.1 (−0.32 to 3.84), *P* = 0.10
Biochemical miscarriage				
Total	463/6284 (7.4)	244/3153 (7.7)	0.35 (−0.78 to 1.50), *P* = 0.53	0.5 (−0.72 to 1.77), *P* = 0.41
<37 years old	107/1394 (7.7)	44/635 (6.9)	−0.66 (−3.15 to 1.83), *P* = 0.60	−1.76 (−4.40 to 0.87), *P* = 0.19
≥37 years old	356/4890 (7.3)	200/2518 (7.9)	0.62 (−0.66 to 1.90), *P* = 0.34	0.97 (−0.43 to 2.39), *P* = 0.17
Clinical pregnancy				
Total	3411/6284 (54.3)	1742/3153 (55.2)	0.86 (−1.28 to 3.02), *P* = 0.43	1.04 (−1.26 to 3.35), *P* = 0.37
<37 years old	769/1394 (55.2)	351/635 (55.3)	0.16 (−4.56 to 4.89), *P* = 0.94	−0.2 (−5.24 to 4.85), *P* = 0.94
≥37 years old	2642/4890 (54.0)	1391/2518 (55.2)	1.01 (−1.40 to 3.44), *P* = 0.41	1.21 (−1.39 to 3.81), *P* = 0.32
Clinical miscarriage				
Total	500/6090 (8.2)	274/3041 (9.0)	0.76 (−0.47 to 1.99), *P* = 0.22	0.9 (−0.43 to 2.20), *P* = 0.2
<37 years old	116/1347 (8.6)	63/610 (10.3)	1.72 (−1.14 to 4.60), *P* = 0.23	1.91 (−1.21 to 5.04), *P* = 0.23
≥37 years old	384/4743 (8.1)	211/2431 (8.7)	0.52 (−0.84 to 1.89), *P* = 0.45	0.46 (−0.99 to 1.92), *P* = 0.53
Ongoing pregnancy				
Total	2708/6090 (44.5)	1350/3041 (44.4)	−0.15 (−2.36 to 2.06), *P* = 0.89	0.16 (−2.36 to 2.06), *P* = 0.89
<37 years old	604/1347 (44.8)	262/610 (43.0)	−1.83 (−6.76 to 3.08), *P* = 0.46	−2.17 (−7.49 to 3.13), *P* = 0.42
≥37 years old	2104/4743 (44.4)	1088/2431 (44.8)	0.27 (−2.20 to 2.76), *P* = 0.82	0.79 (−1.86 to 3.45), *P* = 0.55
Live birth				
Total	2284/5666 (40.3)	1124/2815 (39.9)	−0.44 (−2.71 to 1.81), *P* = 0.69	−0.03 (−2.44 to 2.37), *P* = 0.97
<37 years old	515/1258 (40.9)	218/566 (38.5)	−2.41 (−7.40 to 2.57), *P* = 0.34	−2.4 (−7.80 to 3.04), *P* = 0.39
≥37 years old	1769/4408 (40.1)	906/2249 (40.3)	0.06 (−2.48 to 2.60), *P* = 0.96	0.64 (−2.06 to 3.36), *P* = 0.64

Data are given as *n/N* (%).

Adjustment was made for maternal age, body mass index, number of oocytes recovered following ovarian stimulation, number of days of stimulation, number of embryos biopsied, gonadotrophin dosage and type of gonadotrophin, using generalized estimating equations.

Biochemical pregnancy was diagnosed from a β-HCG concentration of over 10 mIU/ml. Clinical pregnancy was defined by the presence of an intrauterine gestational sac with a fetal heartbeat present. Biochemical miscarriage was defined as a decrease of β-HCG after a positive value. Clinical miscarriage was defined as spontaneous termination of pregnancy before 22 weeks. Ongoing pregnancy was characterized by a viable fetus present in the uterus after 22 weeks of gestation.

HCG, human chorionic gonadotrophin; HMG, human menopausal gonadotrophin.

(95% CI 39.03–41.60) and in the higher HMG dosage was 38.5% (95% CI 38.12–41.77) (*P* = 0.75). The dosage of gonadotrophin did not affect the LBR (OR −2.41 [95% CI −7.40 to 2.57]; *P* = 0.34). The multivariate analysis showed that the number of embryos biopsied (aOR 2.19 [95% CI 0.81–3.58]; *P* = 0.002) influenced the relationship between the dosage and the LBR. In the older women (≥37 years) the LBR with a lower HMG dosage was 40.1% (95% CI 38.68–41.60) versus 40.3% (95% CI 38.25–42.35) with the higher dosage (*P* = 0.92). The dosage of gonadotrophin did not affect the LBR (OR 0.06 [95% CI −2.48 to 2.60]; *P* = 0.96). The multivariate analysis showed that the number of embryos biopsied influenced the relation between the dosages and the LBR (aOR 1.42 [95% CI 0.65–2.20]; *P* < 0.001).

Notably, the CLBR observed in older women (FIGURE 1b) who received higher gonadotrophin dosages in the fourth embryo transfer (83.9% [95% CI 79.77–87.23]) were comparable to those of older women with lower gonadotrophin dosages in the fourth transfer (82.4%, 95% CI 79.06–85.17]) (*P* = 0.92). The CLBR in younger women (FIGURE 1b) who received higher gonadotrophin dosages in the fourth embryo transfer (77.9% [95% CI 70.06–83.82]) were comparable to those of women receiving a lower gonadotrophin dosage in the fourth transfer (76.2% [95% CI 71.60–80.08]) (*P* = 0.56).

DISCUSSION

These findings reinforce the fact that ovarian stimulation with a high dose of exogenous gonadotrophins is not a risk factor for embryonic aneuploidy. Furthermore, the total gonadotrophin dosage did not influence the embryo ploidy rate, CLBR or other IVF outcomes of euploid embryos, regardless of the maternal age at oocyte retrieval. Likewise, when considering cycles with HMG it seems that younger patients receiving a higher dosage of HMG had a lower aneuploidy rate than women receiving lower dosages; however, the HMG dosage did not affect the blastulation rate, CLBR or other IVF outcomes. The number of

euploid embryos progressively decreases with maternal age, and post-meiotic chromosomal abnormalities may be induced by ovarian stimulation (*Munne et al., 2017*). Thus, more oocytes, and subsequent good-quality blastocysts, are necessary to obtain at least one euploid embryo in women with an advanced maternal age (*Franasiak et al., 2014*). These premises are, at least in part, based on the compromised chromosomal status observed in the oocytes of older patients who received high doses of gonadotrophin during ovarian stimulation (*Bosch et al., 2016*). In this regard, the use of PGT-A has improved IVF outcomes by reducing the risk of miscarriage, particularly for women of advanced maternal age.

The effect of conventional versus mild stimulation is controversial. Studies investigating the effects of exogenous gonadotrophins to enhance oocyte yield present conflicting results for oocyte quality, embryo ploidy and implantation potential (*Baart et al., 2007*; *Gianaroli et al., 2010*; *Hong et al., 2019*; Katz-Jaffe et al.2005; *Labarta et al., 2012*). High doses of gonadotrophins are cautiously administered for ovarian stimulation due to the increased risk of inducing ovarian hyperstimulation syndrome, but the current study reliably validated that they do not increase the rate of blastocyst aneuploidy. Indeed, the study found lower aneuploidy rates in the group of younger women (<37 years) who used a higher amount of HMG; however, it is not possible to confirm the possible effect of HMG on meiotic division.

The effects of ovarian stimulation and oocyte yield on CLBR may indirectly reflect embryo development and implantation potential. The CLBR rises according to the number of oocytes retrieved, without reaching a plateau (*Polyzos et al., 2018*). Indeed, retrieving more than 20 mature oocytes was correlated with a comparable implantation of euploid embryos and ongoing pregnancy rate to women who produced fewer mature oocytes (i.e. 6–10 or 10–20 mature oocytes) (*Unal et al., 2009*). Together, these findings suggest that more aggressive ovarian stimulation protocols eliciting elevated oocyte yields are not embryotoxic and corroborate the comparable aneuploidy rates and CLBR that were observed between groups with different oocyte yields.

In corroboration, a previous study found that the number of euploid embryos, but not aneuploid ones, was positively correlated to the oocyte yield (*Labarta et al., 2017*; *McCulloh et al., 2019*). Sekhon and colleagues retrospectively evaluated the effects of varying doses of gonadotrophins on embryonic aneuploidy and showed an increased aneuploidy rate with cumulative exogenous gonadotrophin exposure. However, the participants who required high doses of gonadotrophins were those with a declining ovarian reserve and consequent intrinsic risk of embryonic aneuploidy (*Sekhon et al., 2017*). Another analysis of 12,298 trophectoderm biopsies confirmed that euploidy rates and LBR after the transfer of euploid embryos were not significantly influenced by gonadotrophin dosage, but in this case the authors did not report the CLBR (*Irani et al., 2020*). Finally, oocyte donor euploidy rates were significantly associated with the HMG fraction of total gonadotrophin during ovarian stimulation, but not with other cycle parameters (*McCulloh et al., 2019*). Recently, a retrospective study including cycles of first PGT-A conducted in women aged 35 years old or more with their own metaphase II oocytes inseminated in the absence of severe male factor infertility were clustered based on whether recombinant FSH (112 patients) or HMG (127 patients), was given; the authors showed no association between the gonadotrophin adopted for ovarian stimulation and the euploid rate per cohort of metaphase II oocytes (*Vaiarelli et al. 2023*). In contrast, the current study found an association between the fraction of HMG in younger women and the embryonic aneuploidy rate. HMG induces specific gene expression profiles in human cumulus cells during ovarian stimulation that are different from those with recombinant FSH (*Cruz et al., 2017*).

This study presents some notable limitations, including the retrospective nature and the heterogeneity of the included patients, with different infertility and indications for PGT-A. Furthermore, the patients received low or high doses of gonadotrophins, based on various underlying causes of infertility. Finally, and most importantly, the findings of this study should not be misinterpreted as promoting aggressive ovarian stimulation in IVF patients (especially those with a low response), since hyperstimulation of the ovaries is associated with a higher risk of complications (e.g. ovarian hyperstimulation syndrome). However, the study included only trophectoderm biopsy using NGS technology excluding array comparative genomic hybridization (aCGH), and the authors acknowledge that aCGH is less sensitive than more recent NGS technology.

In conclusion, this study provides strong support that total gonadotrophin dosage does not influence aneuploidy rates or CLBR for patients younger or older than 37 years old. Nevertheless, increased the HMG dosage seems to protect against the risk of aneuploidy during ovarian stimulation in younger women, and the use of HMG should be evaluated on a case-by-case basis, according to the patient's characteristics and infertility type.

DATA AVAILABILITY

Data will be made available on request.

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SUPPLEMENTARY MATERIALS

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REVIEW

Revitalizing female fertility: platelet-rich plasma – hype or hope?

BIOGRAPHY

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KEY MESSAGE

There is a need for standardization of platelet-rich plasma (PRP) protocols in reproductive medicine, with identification of the patient populations that would gain greatest benefit from treatment. This review provides a timely update on the potential of PRP as a promising approach in reproductive medicine.

ABSTRACT

Platelet-rich plasma (PRP) has gained popularity as an experimental tool in regenerative medicine, with potential applications in reproductive medicine. This review will assess the existing literature on the role of PRP in female fertility enhancement, focusing on ovarian rejuvenation and increased endometrial thickness. PRP is being explored as a treatment for recurrent implantation failure, primary ovarian insufficiency and poor ovarian response. While the influence of PRP on endometrial thickness and implantation success is postulated, its effectiveness remains the subject of debate due to protocol variability and unclear patient selection criteria.

This narrative review includes 36 articles published before December 2022, and highlights the lack of comprehensive molecular studies examining the impact of PRP on reproductive capacity. This review underscores the importance of standardizing PRP preparation protocols in reproductive medicine. However, challenges persist, and there is a need for well-planned randomized controlled trials and a deeper understanding of the patient population that would gain the greatest benefit from PRP treatment. Clarifying these aspects is crucial to improve outcomes for low-prognosis patients undergoing assisted reproductive technology.

INTRODUCTION

Among the multifarious cellular constituents synthesized from megakaryocytes in bone marrow, platelets serve a critical role in clotting and wound repair, possessing an array of proteins, growth factors, cytokines and other bioactive molecules that facilitate these functions (*Lin et al., 2021*). As an approach to promote successful implantation, platelet-rich plasma (PRP) preparation, involving centrifugation of the patient's peripheral blood to elevate the platelet concentration, is believed to stimulate endometrial proliferation, instigate neoangiogenesis, and exert anti-inflammatory effects (*Bos-Mikich et al., 2018*). In other clinical fields, it has been shown that PRP-induced angiogenesis

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KEY WORDS

Platelet-rich plasma
Female infertility
Ovarian rejuvenation
Endometrial thickness
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Recurrent implantation failure

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results in an improvement in oxygen and nutrient delivery to the cells at the site of injection (*Bir et al., 2011*), and that PRP can also induce an anti-inflammatory response by activating signalling pathways involved in extracellular matrix synthesis and remodelling (*Hudgens et al., 2016*). Both of these characteristics are consistent with the induction of a wound healing response. PRP classification is determined based on leukocyte and fibrin content, with each category having distinct biological impacts (Dohan *Ehrenfest et al., 2009*). Despite its minimal invasiveness, relatively low cost and easy administration, the efficacy of PRP in medically assisted reproduction remains the subject of debate due to discrepancies in protocols and a lack of standardization in patient selection. Moreover, varying availability and effectiveness of bioactive molecules may arise due to differences in cell types, protein concentrations, release kinetics and platelet activation (*Foster et al., 2009*). Therefore, it is critical to consider the method of PRP activation, dosage and timing (*Cavallo et al., 2016*).

Recently, several novel technologies and adjuvant therapies have been introduced to augment fertility treatment success rates. However, the benefits offered by many of these adjuncts are marginal, and robust evidence supporting their effectiveness remains wanting. Therefore, a rigorous evaluation of the safety and efficacy of any emerging technology offered to patients is paramount. This review will endeavour to conduct a thorough examination of the existing literature on PRP in medically assisted reproduction, comparing various protocols and patient groups. The aim is to provide a comprehensive understanding of the current state of knowledge, while objectively assessing the outcomes and implications of PRP use in this field.

MATERIALS AND METHODS

Electronic database platforms including PubMed, Web of Science and Scopus were searched to identify studies reporting the use of PRP in ovarian rejuvenation and endometrial thickness using the key words: 'platelet-rich plasma', 'female infertility', 'ovarian rejuvenation', 'endometrial thickness', 'endometrial receptivity', 'premature ovarian insufficiency', 'recurrent implantation failure' and 'assisted reproduction techniques'. The time frame for the publications was up to December 2022, resulting in the inclusion of a total of 36 original articles. The selection primarily encompassed original research papers. It is important to highlight that, for the purposes of this study, abstracts, case reports and case series were excluded from the analysis, specifically in the context of endometrial thickness, ovarian rejuvenation and recurrent implantation failure (RIF), with the exception being made for Asherman syndrome and chronic endometritis. Case report studies were included and described for Asherman syndrome and chronic endometritis as no prospective studies exist in the published literature. Only full-text articles were considered to ensure comprehensive and accurate data extraction. The language criterion was restricted to English, and search parameters were adjusted according to the guidelines of each database. The process involved the independent extraction of studies by two reviewers to ensure impartial evaluation and reporting (FIGURE 1).

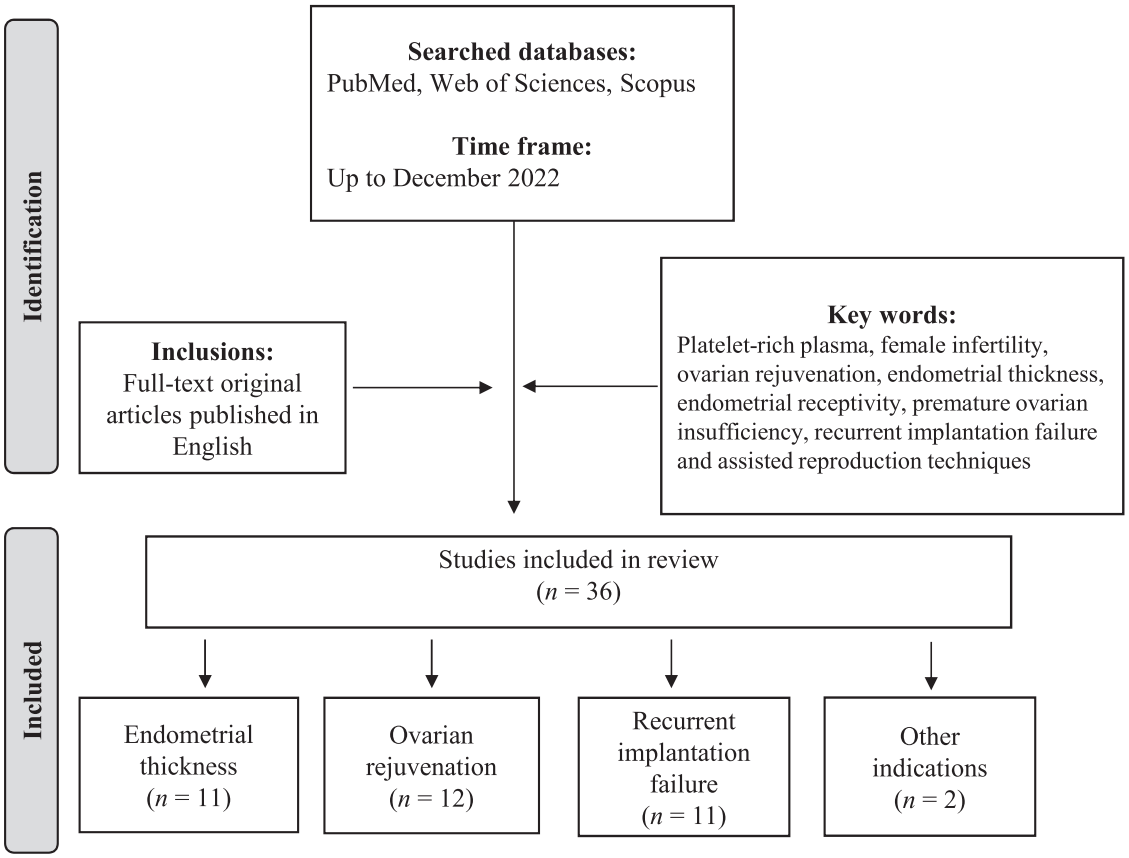

FIGURE 1 Flow diagram of the included studies.

MECHANISTIC UNDERPINNING OF PRP

Platelets, anucleate disc-shaped cellular components found in blood, serve as a critical element within the clotting system, orchestrating the prevention of excessive haemorrhage following injuries. Originating from membrane fragments of megakaryocytes, platelets are equipped with an array of molecules on their surface, including G-protein coupled receptors, integrins and glycoproteins. These molecular structures facilitate the response of platelets to injury, initiating specific signalling pathways that lead to activation (*Li et al., 2010*). Various extracellular ligands, such as thrombin, collagen, adenosine diphosphate, and even divalent cations such as Ca^{2+}, are capable of instigating platelet activation (*Li et al., 2010*; *Lopez-Vilchez et al., 2009*; *Shen et al., 2017*). Following activation, platelets experience a surge in cytoplasmic Ca^{2+} concentrations coupled with enhanced cytoskeletal activity, leading to a morphological transformation characterized by a spiky and irregular shape that fosters aggregation (*Aslan et al., 2012*), and subsequent release of molecules from cytoplasmic granules (*Jurk Kehrel, 2005*). Within the cytoplasm, platelets house two distinct types of granules: (i) dense granules, comprising histamine, serotonin, adenosine diphosphate and divalent cations; and (ii) α-granules, rich in an assortment of molecules including cytokines and growth factors such as interleukin-8, chemokine-ligand 5, platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), insulin-like growth factor, and others (*Anitua et al., 2004*; *El-Sharkawy et al., 2007*). Beyond their well-established function in clotting, these platelet-derived growth factors and cytokines exhibit a vital role in various biological processes, including cell proliferation, differentiation, angiogenesis and regulation of apoptosis (*Au et al., 2014*; *Bakacak et al., 2016*; *Bir et al., 2011*; *Golebiewska Poole, 2015*). Such attributes underscore their potential application in assisted reproduction, where they are believed to contribute to tissue repair, wound healing and blood vessel regeneration (*Atkinson et al., 2021*; *Pellicer et al., 2023*).

The role of VEGF-induced angiogenesis is well documented in both normal ovarian function and during endometrial thickening, as highlighted in studies by *Smiths (1998)*, *Kaczmarek et al. (2005)* and *Araújo et al. (2013)*. Conversely, the down-regulation of VEGF has been associated with decreased angiogenesis, leading to reduced development of antral follicles in primates (*Wulff et al., 2002*), and an abnormally thin endometrium in patients (*Alfer et al., 2017*; *Miwa et al., 2009*). VEGF is known to stimulate the formation of new capillaries and enhance the delivery of oxygen and nutrients to cells. It is noteworthy that intra-ovarian injection of PRP has been shown to modulate angiogenesis in an animal model and restore ovulation (*Ahmadian et al., 2020*). However, while these findings suggest that PRP therapy could potentially stimulate neoangiogenesis and improve reproductive outcomes in infertile patients, it is crucial to approach these results with caution. The study was preliminary and conducted in an animal model, underscoring the need for further research to validate these findings in human clinical settings.

PRP therapy is a medical approach that capitalizes on the inherent healing capabilities of platelets and growth factors derived from an individual's blood. This process entails the collection of a modest blood sample from the patient, followed by centrifugation to segregate the PRP based on density. The resultant concentrated PRP is then assembled, typically activated, and subsequently administered into the specified region of the body in need of therapeutic intervention (*Lacci Dardik, 2010*). PRP therapy is posited to facilitate organ regeneration through several mechanisms, including (i) exerting anti-inflammatory effects by modulating the secretion of inflammatory cytokines and the activities of inflammatory cells (*El-Sharkawy et al., 2007*); (ii) orchestrating extracellular matrix remodelling via the synthesis of collagen and fibronectin (*Sills Wood, 2019*); (iii) promoting angiogenesis (*Kakudo et al., 2014*); and (iv) directing cellular recruitment (including mesenchymal stem cells) to the injection site, thereby promoting differentiation and reparative processes (*Holmes et al., 2018*). Notwithstanding its therapeutic promise, the current landscape of experimental research dissecting the impact of PRP at cellular and molecular strata remains scant. This paucity of comprehensive studies hinders the elucidation of the critical steps that govern the mechanisms of action of PRP in patients, warranting further investigative efforts.

PRP IN THE TREATMENT OF FEMALE INFERTILITY: A CRITICAL EXAMINATION OF THE POTENTIAL IMPACT OF PRP

Specific indications where PRP has been implemented include thin endometrial lining, poor ovarian response (POR), primary ovarian insufficiency (POI), RIF, Asherman syndrome and chronic endometritis (*Bos-Mikich et al., 2018*; *Lin et al., 2021*; *Maleki-Hajiagha et al., 2020*; *Puente Gonzalo et al., 2021*). Its application in these areas represents a recognition of its potential to tackle intricate reproductive challenges. However, its efficacy and optimal use in these contexts remain to be defined more clearly, necessitating further research through well-designed studies.

PRP as a treatment modality for thin endometrial lining in women

An increase in endometrial thickness has been identified by multiple studies as a critical factor in both implantation and pregnancy, with indications that it may contribute to a higher pregnancy rate (*Chang et al., 2015*; *Eftekhar et al., 2018*). Typically, an endometrial thickness ≥7 mm is considered ideal for embryo transfer, while a thin endometrium, usually <7 mm, is associated with decreased likelihood of pregnancy (*Bos-Mikich et al., 2018*; *Eftekhar et al., 2018*; *Maleki-Hajiagha et al., 2020*). Within the existing body of research, 11 studies specifically addressed intrauterine PRP infusion treatment of infertile women diagnosed with a thin endometrium, two of which were randomized controlled trials (RCT) (**TABLE 1**).

Non-randomized studies on PRP for thin endometrium

Most studies addressing the effect of PRP on thin endometrium found favourable results upon application of the treatment. *Chang et al. (2015)* reported positive outcomes following PRP infusion. This study involved five women aged 31–39 years who underwent IVF and had endometrial thickness <7 mm after standard hormone replacement therapy (HRT). These women had previously experienced cancellation of an embryo transfer cycle. Following intrauterine infusion of PRP, all patients exhibited an increase in endometrial thickness and achieved clinical pregnancy. *Tandulwadkar et al. (2017)* investigated the efficacy of autologous PRP in enhancing endometrial thickness and vascularity among infertile women with suboptimal

TABLE 1 STUDIES THAT USED PLATELET-RICH PLASMA TO INCREASE ENDOMETRIAL THICKNESS

Study	Study design	Age (years)	Characteristics	Sample size		Outcome measures	Key findings	Time frame from intervention to cycle	Endometrial preparation used	Site of injection	Route of injection
				Case	Control						
Chang et al., 2015	Prospective	31–39	Women with a thin endometrium (<7 mm) after standard HRT	5	0	Endometrial thickness Pregnancy Live birth	PRP successfully facilitated endometrial growth and enhanced pregnancy outcomes in all patients with a thin endometrium in the study, and four of five patients had live births	Same cycle, day 10 of HRT cycle	HRT	Cavity	Transvaginal
Tandulwadkar et al., 2017	Prospective	22–40	Women who had suboptimal endometrial growth and endometrial thickness <7 mm despite standard dose of oestradiol valerate (up to 16 mg/day), or suboptimal endometrial vascularity [fewer than five vascular signals reaching the central zone (Zones 3 and 4 as per Applebaum grading) of the endometrium], and repeated cycle cancellations	64	0	Endometrial thickness Vascularity Positive serum β-HCG Implantation rate Clinical pregnancy rate Outcome of pregnancy	Utilization of autologous PRP shows promise in the treatment of women with suboptimal endometrial thickness and vascularity for ET	Same cycle, day 15–16 of HRT cycle	HRT	Cavity	Transvaginal
Wang et al., 2019	Prospective	27–43	Women with RIF and thin endometrium (<7 mm) or suboptimal endometrial vascularity, and fewer than five vascular signals reaching the central zone (Zones 3 and 4 as per Applebaum grading)	20	0	Endometrial thickness Pregnancy rate	Intrauterine PRP infusion presents a novel approach for female patients with a thin endometrium and a poor response	Not reported	HRT	Cavity	Transvaginal
Eftekhar et al., 2018	RCT	18–42	Women with a thin endometrium (<7 mm)	40	43	Endometrial thickness Clinical pregnancy Implantation rate Ongoing pregnancy	PRP has the potential to be effective in enhancing endometrial growth and, consequently, may have a positive influence on pregnancy outcomes in women with a thin endometrium	Same cycle, day 11–12 followed by day 13–14 of HRT cycle	HRT	Cavity	Transvaginal
Molina et al., 2018	Prospective	33–45	Women with a history of refractory endometrium (characterized by atrophy with endometrial interface measurements <6 mm by ultrasound, and/or Asherman syndrome) and at least one failed IVF attempt	19	0	Positive serum β-HCG Live birth Ongoing pregnancy Biochemical pregnancy Anembryonic pregnancy Endometrial thickness	PRP and its biostimulatory effects on the endometrial microvasculature appear to be advantageous for individuals with refractory endometrium, providing enhanced endometrial receptivity, leading to a subsequent increase in implantation rates	Same cycle, day 10 followed by day 13 of HRT cycle	HRT	Cavity	Transvaginal
Nazari et al., 2019	RCT		Women with a history of cancelled ET due to thin endometrium (<7 mm)	30	30	Endometrial thickness Chemical pregnancy Clinical pregnancy	PRP demonstrated effectiveness in promoting endometrial thickening in patients with refractory thin endometrium	Same cycle, day 11–12 followed by day 13–14 of HRT cycle	HRT	Cavity	Transvaginal
Chang et al., 2019	Prospective	<40	Women with a thin endometrium (<7 mm)	34	30	Endometrial thickness Clinical pregnancy Implantation rate	PRP had a positive impact on fostering endometrial proliferation, thereby enhancing embryo implantation rates and clinical pregnancy rates in women with a thin endometrium during FET cycles	Same cycle, day 10 of HRT cycle	HRT	Cavity	Transvaginal

(*continued on next page*)

TABLE 1 (*Continued*)

Study	Study design	Age (years)	Characteristics	Sample size		Outcome measures	Key findings	Time frame from intervention to cycle	Endometrial preparation used	Site of injection	Route of injection
				Case	Control						
Kim et al., 2019	Prospective	31–44	Women with a history of two or more failed IVF cycles and refractory thin endometrium (<7 mm)	20	0	Endometrial thickness Clinical pregnancy Live birth Implantation rate	Utilization of autologous PRP resulted in improvements in implantation, pregnancy and live birth rates for patients with refractory thin endometrium	Same cycle, day 10 of HRT cycle followed by every 3 days until endometrium ≥7 mm	HRT	Cavity	Transvaginal
Agarwal et al., 2020	Prospective	27–39	Women with primary or secondary infertility with a thin endometrium (<7 mm)	32	0	Endometrial thickness Positive serum β-HCG Clinical pregnancy	Enhancements in endometrial thickness and increased pregnancy rates were observed in cases where ET had been cancelled previously due to thin endometrium	Day 16 of OCP, 22–27 days before ET	OCP	Cavity	Transvaginal
Frantz et al., 2020	Retrospective	23–41	Women with endometrial thickness <5 mm during preparation for FET	21	0	Clinical pregnancy Ongoing pregnancy Miscarriage rate Live birth rate	PRP enhanced intrauterine receptivity for embryo implantation, irrespective of whether the endometrium achieved the necessary growth for ET	Same cycle, day 14–17 of HRT cycle, every 2 days, total of three times	HRT	Cavity	Transvaginal
Dogra et al., 2022	Prospective	<38	Women with endometrial thickness <7 mm on baseline evaluation before IVF despite standard HRT and hysteroscopically normal endometrial cavity, and history of cycle cancellation during FET cycles due to persistent thin endometrium	20		Endometrial thickness Implantation rate Clinical pregnancy rate Live birth rate	PRP enhanced endometrial thickness significantly during fresh ET and FET cycles in thin endometrium associated with tuberculosis, polycystic ovary syndrome and diminished ovarian reserve, thus improving clinical pregnancy and live birth rates in these patients with a poor prognosis	48 h before ET	HRT	Cavity	Transvaginal

HRT, hormone replacement therapy; β-HCG, beta-human chorionic gonadotrophin; PRP, platelet-rich plasma; RCT, randomized controlled trial; RIF, recurrent implantation failure; ET, embryo transfer; FET, frozen embryo transfer; OCP, oral contraceptive pill.

endometrium undergoing frozen embryo transfer (FET) cycles. According to their results, the mean pre-PRP endometrial thickness was 5 mm, which increased significantly to 7.22 mm post-PRP treatment. Moreover, the positive beta-human chorionic gonadotrophin (β-HCG) rate was 60.93% and the clinical pregnancy rate was 45.31%, suggesting that autologous PRP may serve as a potential therapeutic intervention for women presenting with suboptimal endometrial thickness in preparation for embryo transfer. *Molina et al. (2018)* performed a prospective study on 19 patients aged 33–45 years with refractory endometrium. These patients received PRP infusion into the uterine cavity on day 10 of HRT and 72 h later. In all cases, patients showed an increase in endometrial thickness, reaching >9 mm after the second administration of PRP. The entire group qualified for embryo transfer, resulting in 73.7% positive pregnancy tests expressed as implantation rate. This study suggests that PRP may enhance endometrial receptivity and increase implantation rates, making it a cost-effective and easily obtainable resource for inclusion in endometrial preparation protocols, including natural cycles. *Wang et al., (2019)* conducted a study to investigate the influence of PRP on the proliferation and migratory activity of endometrial mesenchymal stem cells (EnMSC), as well as evaluating its efficacy in treating patients with a thin endometrium. These EnMSC (extracted from human menstrual blood) were incubated with PRP and applied to a cohort of 20 patients, each engaged in IVF and with a history of intractable thin endometrium and RIF. The treatment resulted in increased thickness of the endometrium along with an increase in pregnancy rates. *Chang et al. (2019)* conducted a study focusing on the FET programme in women with a thin endometrium. Their results also indicated that PRP injection was associated with increased endometrial thickness, decreased cycle cancellation rates, and enhancements in both embryo implantation and clinical pregnancy rates compared with a control group. A cross-sectional study by *Agarwal et al. (2020)* of 32 patients aged 27–39 years with primary or secondary infertility with a thin endometrium (<7 mm) found that 75% of patients achieved an endometrial thickness >7 mm following PRP injection combined with progesterone therapy, also indicating a potential benefit of PRP in endometrial thickening. Finally, *Dogra et al. (2022)* found that PRP injections improved

endometrial thickness significantly in women with a thin endometrium, particularly those affected by tuberculosis and polycystic ovary syndrome, as well as those with diminished ovarian reserve, in both fresh embryo transfer and FET cycles. Notably, this increase in endometrial thickness was associated with higher clinical pregnancy and live birth rates within this patient group, which traditionally has a lower prognostic outlook. While these studies collectively highlight the potential of PRP to improve endometrial health during fertility treatment, it is crucial to note that these studies were constrained by limitations in the number of patients involved, undermining their statistical power and the strength of their evidence.

More recently, results were published contradicting the efficiency of PRP for the treatment of thin endometrium. A prospective study by *Kim et al. (2019)* reported no significant differences between the PRP-treated group and the control group in terms of endometrial thickness in women with refractory endometrium. Furthermore, *Frantz et al. (2020)* conducted a clinical retrospective analysis of 24 IVF cycles, involving 21 patients with various infertility factors who received intrauterine PRP infusions before embryo transfer. They did not find any significant improvement in endometrial thickness attributable to PRP treatment, despite reporting enhanced uterine receptivity for embryo implantation, regardless of whether or not the endometrium reached the conventionally desired thickness for embryo transfer. Furthermore, and in a similar fashion, *Ata et al. (2023)* conducted a retrospective analysis to explore whether endometrial thickness influences the live birth rate independently following embryo transfer. They were unable to determine a specific endometrial thickness threshold that either precluded live birth or reduced the live birth rate significantly. Their findings suggest that the common clinical practice of cancelling embryo transfers when the endometrial thickness is <7 mm may lack justification, and imply that an increase in endometrial thickness does not necessarily correlate with an improvement in the live birth rate.

Randomized controlled trials on PRP for thin endometrium

In an RCT conducted by *Eftekhar et al. (2018)*, 83 women aged 18–42 years with poor endometrial response were allocated to one of two groups: the intrauterine PRP therapy group and the control group. The application of PRP treatment led to a significant increase in endometrial thickness compared with the control group, suggesting its potential efficacy in improving endometrial response. Similarly, *Nazari et al. (2019)* conducted a double-blind, randomized sham-controlled trial involving 60 women who had previously cancelled FET cycles due to inadequate endometrial thickness. The participants received either an intrauterine PRP infusion or a sham catheter procedure. Both groups experienced an increase in endometrial thickness 48 h after the intervention. After a second intervention, the authors observed an increase in endometrial thickness in the PRP group, indicating the potential effectiveness of PRP in promoting endometrial thickness in patients with a refractory thin endometrium after subsequent treatment. Nevertheless, it is crucial to note that these RCT were conducted with small sample sizes, which may limit the generalizability of their findings.

In summary, most studies on the use of PRP to treat thin endometrium in patients undergoing IVF suggest that this intervention may improve implantation rates. However, these studies had relatively small sample sizes, and the evidence they present is of rather low strength. Furthermore, the most recent studies contradict the efficiency of PRP in patients undergoing IVF. The only two RCT designed to test the efficacy of PRP treatment also had low sample sizes, rendering them less generalizable.

PRP for the rejuvenation of ovarian function in women: a critical examination of the potential impact of PRP

In addition to promoting endometrial proliferation, PRP has also been employed to enhance the response to ovarian stimulation in patients with a poor prognosis (*Cakiroglu et al., 2020*, *2022*). Low-prognosis patients, often referred to as those with the 'Patient-Oriented Strategies Encompassing IndividualizeD Oocyte Number' (POSEIDON) classification, are characterized by factors such as advanced female age, diminished ovarian reserve, underlying female infertility, and responsiveness to stimulation regimens (*Poseidon et al., 2016*). In 1% of women of reproductive age, the ovarian reserve diminishes to such an extent that it results in menopausal serum gonadotrophin hormone concentrations and menstrual irregularity or amenorrhoea, a condition named 'premature ovarian insufficiency' (POI) (*Ferraretti et al., 2011*). At present, there are no effective treatment protocols for women diagnosed with POI, and live birth rates remain dishearteningly low, even with the application of various experimental strategies (*Ben-Nagi Panay, 2014*). Recent developments have revealed that through the utilization of PRP, isolated human primordial and primary follicles can develop to the preantral stage. For instance, *Hosseini et al. (2017)* performed a study using donated ovaries procured from three females aged <35 years after brain death. Follicles and ovarian cells from these ovaries were cultured in a medium enriched with fetal bovine serum (FBS), PRP, PRP + FBS, or human serum albumin for 10 days. The rates of growth and survival for follicles cultured in media supplemented with PRP were notably higher compared with the rates observed in other culture conditions. This experimental study demonstrated the potential of PRP in supporting the development of human primordial and primary follicles to the preantral stage. However, it is crucial to note that these findings, while promising, are based on follicles from donors with a normal ovarian reserve. Patients with a diminished ovarian reserve may present more complex and varied dynamics in ovarian follicle development, which could influence the efficacy and outcomes of PRP treatment in these cases. The findings by *Hosseini et al. (2017)* led to the belief that PRP may enhance ovarian reserve parameters specifically for patients with diminished ovarian reserve (*Cakiroglu et al., 2022*). To date, 12 studies have been conducted to investigate the effect of PRP on ovarian rejuvenation within the context of IVF, although none of these studies were RCT (TABLE 2).

Non-randomized studies on PRP for rejuvenation of ovarian function

Sills et al. (2018) investigated four patients diagnosed with diminished ovarian reserve. Their mean ± SD age was 42 ± 4 years, and they were selected based on specific criteria, including cancelled IVF cycles, or abnormal serum concentrations of anti-Müllerian hormone (AMH) and FSH. The treatment involved the administration of autologous PRP injections bilaterally into the ovaries. The study outcomes consistently displayed an increase in serum AMH concentration and a decrease in FSH concentration, or both, across all cases examined. A mean of 5.3 mature oocytes were retrieved from each patient. Subsequent IVF procedures were carried

TABLE 2 STUDIES THAT USED PLATELET-RICH PLASMA FOR REJUVENATION OF OVARIAN FUNCTION

Study	Study design	Age (years)	Patient characteristics	Sample size		Outcome measures	Key findings	Time frame from intervention to cycle
				Case	Control			
Sills et al., 2018	Prospective	>35	Women with at least one ovary, history of infertility for >1 year, at least one prior failed (or cancelled) IVF cycle, or amenorrhoea for at least 3 months	4		FSH AMH Oestradiol	All individuals who underwent intra-ovarian PRP experienced enhanced ovarian function	Twice-monthly assessments of FSH and oestradiol, and IVF cycle/s following improved hormone measurements on two consecutive tests
Stojkovska et al., 2019	Prospective	35–42	Poor ovarian reserve as per Bologna criteria, all patients had IVF with embryo transfer	20	20	FSH Oestradiol AMH Fertilization rate Implantation rate Clinical pregnancy rate Live birth rate	Application of intra-ovarian autologous PRP injection in patients with POR before undergoing IVF did not significantly improve clinical pregnancy and live birth rates	PRP on cycle before IVF
Cakiroglu et al., 2020	Prospective	24–40	Women diagnosed with POI (not defined), with history of infertility for >1 year, and with at least one ovary	311		FSH Oestradiol AMH AFC	For women diagnosed with POI, intra-ovarian injection of autologous PRP could be contemplated as a potential experimental alternative for treatment	N/R
Petryk and Petryk, 2020	Prospective	31–45		38		Serum LH FSH AMH Oestradiol Live birth rate Pregnancy rate Oocyte retrieval number	Injecting PRP into the ovaries is a safe, effective and natural treatment that holds promise for assisting women with POI in conceiving and having their own child	N/R
Tandulwadkar et al., 2020	Prospective	20–45	Poor responders as per Poseidon criteria groups 3 and 4	20		AMH AFC Oocyte number Embryo number	Intra-ovarian instillation of ABMDSC combined with PRP is safe and enhances the activation of dormant primordial follicles, leading to improved oocyte yield	IVF cycle 6 weeks after intervention
Hsu et al., 2021	Prospective	40–50	Women aged 40–50 years, with amenorrhoea for >1 year and FSH>35 IU/l	12		Oestradiol Oocyte retrieval Mature oocytes Fertilization Embryo number and grading Outcome of IVF procedure	Subcortical ovarian administration of PRP along with gonadotrophin was able to restore ovarian function temporarily	N/R
Aflatononian et al. 2021	Prospective	35.47 ± 4.34 and 33.66 ± 4.84 for POR and POI, respectively	Women with POI diagnosed according to ESHRE guidelines: onset prior to 40 years of age, oligo/amenorrhoea for ≥4 months, and elevated FSH level >25 IU/l on two occasions >4 weeks apart. Women with POR	26		FSH LH Oestradiol AMH Chemical pregnancy Clinical pregnancy	Intra-ovarian injection of autologous PRP could be contemplated as an alternative treatment for individuals with POR	Up to 1 year after intervention

(*continued on next page*)

TABLE 2 (*Continued*)

Study	Study design	Age (years)	Patient characteristics	Sample size		Outcome measures	Key findings	Time frame from intervention to cycle
				Case	Control			
			were selected based on Bologna criteria: age >40 years or any other risk factor for POR, history of POR detected by three or fewer oocytes in previous conventional stimulation protocols, and low ovarian reserve tests including AMH <1.1 ng/ml or AFC <5 follicles			Abortion Ongoing pregnancy		
Tülek and Kahraman, 2022	Retrospective	<45		71		Live birth rate Oocytes retrieved Number of MII oocytes Fertilization rate (two pronuclear embryos/MII oocytes) Number of cleavage-stage embryos Implantation rate (gestational sacs observed/transferred embryos)	As it stands, the current form of intra-ovarian PRP injections does not seem to increase live birth or clinical pregnancy rates, particularly among women classified as poor responders	N/R
Cakiroglu et al., 2022	Prospective	40.3 ± 4.0	History of poor ovarian response based on Poseidon criteria	510		AFC FSH AMH	PRP treatment may be considered in women with POR	N/R
Tremellen and Ince, 2022	Retrospective	<45	Poor ovarian reserve as per Bologna criteria and minimum of two failed IVF cycles	20		Number of mature oocytes Peak serum oestradiol Total dose of FSH Total number of embryos Total number of high-quality blastocysts	Ovarian PRP treatment seems to carry low risk and holds promise in supporting pregnancy, both through natural means and in the context of IVF	IVF cycle 2–3 months after intervention
Barad et al., 2022	Prospective	28–54	Patients with LFOR, defined as AMH <1.1 ng/ml, FSH >12 mIU/ml, or at least one prior IVF cycle with retrieval of three oocytes within 1 year	80		AFC Maximal lead follicle Day 2 FSH Day 2 oestradiol AMH Peak oestradiol Oocytes retrieved	No clinically significant effects of PRP treatment on ovarian function were observed over 1 year of follow-up	IVF cycle 1 month after intervention
Merhi et al., 2022	Prospective		Patient with at least one previous failed IVF cycle and women who produced fully developed embryos (blastocysts) before and after intra-ovarian PRP administration	12		FSH AFC Number of oocytes retrieved Number of good-quality embryos formed at blastocyst stage Percentage of euploid embryos	PRP may demonstrate a nearby paracrine impact, potentially enhancing meiotic abnormalities in human oocytes, consequently enhancing euploidy rates	IVF cycle within 3 months after intervention

AMH, anti-Müllerian hormone; PRP, platelet-rich plasma; POR, poor ovarian response; POI, premature ovarian insufficiency; AFC, antral follicle count; N/R, not reported; ABMDSC, autologous bone-marrow–derived stem cells; ESHRE, European Society of Human Reproduction and Embryology; MII, metaphase II; LFOR, low functional ovarian reserve.

out approximately 78 days post-PRP injections, and the results appeared to be independent of factors such as patient age, duration of infertility, baseline platelet concentration, or antral follicle count (AFC). These observations compellingly indicate enhanced ovarian function in all the recipients of intra-ovarian PRP, with potential improvements discernible as early as 2 months following the treatment. *Tandulwadkar and Karthwick (2020)* conducted a small-scale study using POSEIDON classification to treat 20 patients with POR. The participants received a combination of bone-marrow-derived stem cells and PRP injected into their ovaries. The findings indicated that this procedure is safe and potentially improves the quantity and quality of embryos obtained after ovarian stimulation. *Petryk and Petryk (2020)* conducted a study to investigate the effectiveness of intra-ovarian PRP injection in restoring ovarian function in women with low ovarian reserve and a history of at least two IVF cycles with no oocytes collected. The study involved 38 women aged 31–45 years who received PRP injections into their ovaries. Following PRP treatment, significant improvements in hormone concentrations were observed, resulting in the birth of six healthy babies and 10 pregnancies, four of which occurred naturally. *Cakiroglu et al. (2020)* investigated the potential of intra-ovarian PRP injection to improve reproductive outcomes in a larger study involving 311 women aged 24–40 years diagnosed with POI based on the criteria of the *European Society for Human Reproduction and Embryology (ESHRE)* (European Society for Human Reproduction and Embryology Guideline Group on POI, 2016). Among the women treated with PRP, 8% experienced successful live birth or sustained implantation (either spontaneously or after IVF), and another 8% were able to cryopreserve embryos. *Hsu et al. (2021)* conducted a study including 12 women in the early stages of menopause with a mean ± SD age of 44.42 ± 2.84 years, and showed that direct administration of PRP in conjunction with gonadotrophin to the subcortical ovarian region has the potential to rejuvenate ovarian function temporarily. *Merhi et al. (2022)* evaluated the influence of intra-ovarian PRP administration on embryo genetics in infertile women who had previously experienced failed IVF cycles. Twelve participants underwent two cycles of ovarian stimulation and preimplantation genetic testing for aneuploidy, with PRP being administered between the cycles. They found a significant increase in the proportion of euploid embryos, from 8.11% in the first cycle to 39.28% in the second cycle. Moreover, three clinical pregnancies were achieved. *Aflatononian et al. (2021)* studied 17 women with POR and nine women with POI who received multifocal intramedullary PRP injections into each ovary, with a second injection 3 months later. The mean ± SD age of the women was 35.47 ± 4.34 years in the POR group and 33.66 ± 4.84 years in the POI group. Among the POR group, 47% achieved spontaneous pregnancies after PRP injection, with varying outcomes, while 22.2% of women in the POI group experienced menstrual restoration, but none became pregnant. Interestingly, evaluation of hormone concentrations (AMH, FSH, LH and oestradial) showed no significant differences over time between treatments. *Tremellen and Ince (2022)* investigated the potential of ovarian PRP in 20 women aged 35–42 years with severely diminished ovarian reserve and a history of unsuccessful IVF attempts. Although the number of oocytes retrieved did not increase significantly after PRP treatment, there was a noticeable increase in the number of embryos compared with previous IVF cycles without PRP. Additionally, four patients achieved successful pregnancies with genetically normal embryos following IVF, and two patients conceived naturally within 4 months of PRP treatment. Notably, five of these pregnancies occurred in women aged ≥40 years. Finally, *Cakiroglu et al. (2022)* conducted a larger study involving 510 women of reproductive age, all diagnosed with POR as per the POSEIDON classification (mean age ± SD 40.3 ± 4.0 years). Interestingly, their findings showed increased AFC, elevated serum AMH concentration, decreased serum FSH concentration, an increase in the number of mature oocytes, and enhanced embryo quality in women who underwent PRP treatment.

Studies have also been published with negative or contradictory results regarding the efficacy of PRP on the rejuvenation of ovarian function. *Stojkovska et al. (2019)* evaluated the effectiveness of PRP in patients with POR undergoing IVF or intracytoplasmic sperm injection (ICSI) with low-dose ovarian stimulation. The study enrolled 40 patients, 20 of whom received PRP treatment and 20 served as the control group. The mean ± SD age of the PRP group was 37.47 ± 3.87 years, compared with 37.64 ± 3.20 years for the control group. Their results showed no significant differences in clinical pregnancy and live birth rates between the PRP group and the control group. However, there was a noticeable trend towards higher implantation and live birth rates among patients who received prior PRP treatment. *Tülek and Kahraman (2022)* studied two specific groups of women: women with POR, with a mean ± SD age of 38.1 ± 4.4 years; and women with POI, with a mean ± SD age of 37.9 ± 1.9 years. The results indicated that intra-ovarian injections of PRP resulted in a greater number of retrieved oocytes among women with POR. Nevertheless, despite this increase, no substantial enhancement was noted in the clinical pregnancy or live birth rates in this group. Conversely, for women diagnosed with POI, despite a total of eight grade 1 and 2 embryos obtained and transferred subsequent to PRP injections, no clinical pregnancies were realized. Finally, *Barad et al. (2022)* investigated whether intra-ovarian injection of PRP could improve ovarian function in patients with extremely low functional ovarian reserve (LFOR). The study followed 80 patients with LFOR aged 28–54 years for 1 year after undergoing the PRP procedure. PRP was injected into the ovaries. The study found no clinically significant effects of PRP treatment on ovarian function over the 1-year follow-up period. However, two patients with a very poor prognosis, aged 40 years, who had previously failed IVF cycles at other centres, achieved pregnancy after PRP treatment. However, this study failed to establish a causal relationship conclusively between the intra-ovarian injection of PRP and the observed pregnancies.

In summary, the studies on the efficacy of PRP in ovarian rejuvenation present extreme differences in study design, patient populations, interventions and primary endpoints, making direct comparison challenging. Thus, each study should be interpreted within the context of its specific methodologies and outcomes. As no RCT have been published on the topic and most non-randomized studies present limited evidence, it is difficult to ascertain definitive conclusions about the effectiveness of PRP treatment.

PRP for the treatment of women with repeated implantation failure: a critical examination of the potential impact of PRP

One of the common unsolved problems in medically assisted reproduction that has

led to the plateau of success rates is RIF. According to the ESHRE Preimplantation Genetic Diagnosis Consortium, RIF is the absence of implantation following more than three embryo transfers with high-quality embryos or the transfer of at least 10 embryos in multiple transfers, with exact numbers defined by each centre (*Thornhill et al., 2005*). RIF is a complex problem that could arise from several different aetiologies, with variables ranging from advanced maternal age, smoking, body mass index, stress, immunological factors, infection and uterine pathologies (*Bashiri et al., 2018*). Substandard endometrial receptivity and inadequate poor embryo–endometrium communication account for approximately two-thirds of implantation failures (*Craciunas et al., 2019*). In the last few years, autologous PRP has been used in clinical cases of patients with RIF (*Bos-Mikich et al., 2018*; *Maleki-Hajiagha et al., 2020*). The research on this topic includes six observational cohort studies and five RCT (TABLE 3).

Non-randomized studies on PRP for patients with repeated implantation failure

Coksuer et al. (2019) conducted a retrospective study to evaluate the effects of intrauterine PRP treatment on FET cycles. Patients were aged between 21 and 39 years with a history of at least three consecutive failed IVF attempts due to unexplained infertility and a history of RIF. The authors compared the pregnancy outcomes of two groups: 34 patients who received PRP treatment during FET due to a suboptimal endometrial lining; and 36 patients who underwent FET without PRP treatment because they had an optimal endometrial lining. The results showed that endometrial thickness increased significantly 48 h after PRP treatment compared with before (10 mm versus 6.25 mm). Furthermore, the clinical pregnancy and live birth rates were significantly higher in the PRP group compared with the control group. *Mehrafza et al. (2019)* conducted a retrospective study on the effectiveness of intrauterine infusion of autologous PRP and systemic administration of granulocyte colony-stimulating factor (G-CSF) in patients with RIF. This study involved 123 patients divided into two groups: the PRP group (67 patients) with a mean ± SD age of 31.85 ± 5.22 years; and the G-CSF group (56 patients) with a mean ± SD age of 33.46 ± 5.17 years. The results revealed a significantly higher clinical pregnancy rate in the PRP group compared with the G-CSF group (40.3% versus 21.4%). A further retrospective study was conducted by *Xu et al. (2022)* to evaluate whether intrauterine perfusion of PRP before FET improves pregnancy outcomes in patients with RIF. The study included 288 infertile women who underwent IVF or ICSI treatment. The patients were divided into two groups based on whether or not they received intrauterine PRP perfusion before embryo transfer in FET cycles. The results revealed that a significantly higher number of women in the PRP group achieved live birth (29.71%) and clinical pregnancy (36.23%) compared with the control group (18% for live birth and 24.67% for clinical pregnancy). Overall, these retrospective studies support the efficacy of PRP in patients with RIF.

Nevertheless, some other studies during the same period showed contradictory results. For instance, *Dieamant et al. (2019)* conducted a study to evaluate the effectiveness of a novel treatment protocol called PRIMER (Protocol for Endometrial Receptivity Improvement) in enhancing ongoing pregnancy rates among patients with RIF. The research involved women undergoing IVF/ICSI who were divided into two groups: the PRIMER/RIF group, consisting of 33 patients with a mean ± SD age of 37.8 ± 3.8 years, and the control group, comprising 33 patients with a mean ± SD age of 37.8 ± 3.9 years. Patients in the PRIMER/RIF group received an intrauterine injection of PRP and a subcutaneous injection of G-CSF. In contrast, the control group did not receive these infusions. The outcomes of the research demonstrated that there were no significant disparities in the implantation, clinical pregnancy or miscarriage rates between the two groups. Furthermore, a study was conducted by *Aghajanzadeh et al. (2020)* on patients with RIF treated with PRP. In this study, 30 women with RIF undergoing FET cycles received intrauterine infusions of autologous purified PRP preparations 48 h before embryo transfer. The study found an implantation rate of 6.7%, with no significant differences in implantation, clinical pregnancy, ongoing pregnancy and miscarriage rates compared with cycles without PRP infusion. Finally, *Tehraninejad et al. (2021)* conducted a prospective study to examine the impact of intrauterine infusion of PRP on pregnancy outcomes in patients experiencing RIF with normal endometrial thickness. A total of 85 participants with RIF and normal endometrial thickness (≥7 mm) were enrolled and divided into two groups based on the patient's preference: the PRP intervention group; and the control group. Among the 85 participants, 42 individuals with a mean ± SD age of 32.9 ± 3.0 years received PRP, while the remaining 43 individuals with a mean ± SD age of 33.5 ± 2.5 years were assigned to the control group. The authors concluded that PRP does not demonstrate efficacy as an adjunctive treatment for IVF in patients with RIF and normal endometrial thickness undergoing embryo transfer.

Randomized controlled trials on PRP for patients with repeated implantation failure

Five clinical trials have been published to date, all of which support the use of PRP as a potential treatment for patients with RIF. A single-arm preliminary study of an RCT was conducted by *Nazari et al. (2016)*. The study included 20 women with a mean ± SD age of 33.4 ± 5.7 years who had a history of RIF and were undergoing FET. Forty-eight hours prior to the transfer of blastocysts, the participants received an intrauterine infusion of autologous PRP. The study found that 18 of the 20 participants achieved clinical pregnancies. One participant experienced an early miscarriage and another had a molar pregnancy. The same research group conducted another RCT in 2020 on 97 patients who had undergone three or more unsuccessful embryo transfers with high-quality embryos and were eligible for FET. The PRP group exhibited a higher chemical pregnancy rate compared with the control group (53.06% versus 27.08%, respectively), as well as a higher clinical pregnancy rate (44.89% versus 16.66%, respectively) (*Nazari et al., 2020*). An RCT by *Zamaniyan et al. (2021)* assessed the effectiveness of PRP in improving the clinical pregnancy rate among patients with RIF. Ninety-eight women who had experienced unsuccessful pregnancies after three or more high-quality embryo transfers underwent FET, with or without an intrauterine infusion of PRP 48 h before. The study found that the intervention group had a higher proportion of cases with secondary infertility and thicker endometrium 96 h before embryo transfer. Notably, the intervention group exhibited significantly higher clinical pregnancy rates (48.3% versus 23.26%), ongoing pregnancy rates (46.7% versus 11.7%) and implantation rates (58.3% versus 25%) compared with the control group. Furthermore, *Rageh et al. (2020)* conducted a prospective RCT involving

TABLE 3 STUDIES THAT USED PLATELET-RICH PLASMA FOR RECURRENT IMPLANTATION FAILURE

Study	Study design	Age (years)	Characteristics	Sample size		Outcome measures	Key findings	Time frame from intervention to cycle	Endometrial preparation used	Site of injection	Route of injection
				Case	Control						
Nazari et al., 2016	Single arm preliminary study of an RCT	<40	Women who failed to conceive after three or more ET cycles with high-quality embryos	20		Clinical pregnancy Ongoing pregnancy Molar pregnancy	PRP demonstrated effectiveness in achieving clinical pregnancy for patients with RIF	48 h before ET	HRT	Cavity	Transvaginal
Coksuer et al., 2019	Retrospective	21–39	Women with a history of at least three consecutive failed IVF attempts	34	36	Endometrial thickness Clinical pregnancy Live birth	PRP was found to be a safe, cost-effective supplementary treatment to enhance the endometrium, particularly beneficial for patients with a history of RIF	48 h before ET	HRT	Cavity	Transvaginal
Mehrafza et al., 2019	Retrospective	31.85 ± 5.22 and 33.46 ± 5.17 for women in PRP and G-CSF groups, respectively	Women with history of more than two failed ET cycles	67	56	Chemical pregnancy Clinical pregnancy Implantation rate	PRP was shown to have a positive impact on pregnancy outcomes in patients with RIF compared with systemic administration of G-CSF	48 h before ET	HRT	Cavity	Transvaginal
Dieamant et al., 2019	Prospective	37.8 ± 3.8 and 37.8 ± 3.9 for women in PRIMER and control groups, respectively		33	33	Clinical pregnancy Implantation rate Miscarriage rate Ongoing pregnancy Live birth	Therapeutic protocol (PRIMER) could be used as a feasible treatment based on biological rationale for patients with RIF	48 h before ET	HRT	Cavity	Transvaginal
Rageh et al., 2020	RCT	<40	Women who failed to conceive after three or more ET cycles with high-quality embryos	75	75	Positive serum β-HCG	PRP improved the pregnancy rate significantly and may be a new hope in patients with RIF	48 h before ET	Blastocyst ET – unknown if fresh or frozen	Cavity	Transvaginal
Zamaniyan et al. 2021	RCT	20–40	Women who failed to conceive after three or more ET cycles with high-quality embryos	55	43	Clinical pregnancy Ongoing pregnancy Implantation rate	Intrauterine infusion of PRP 48 h before FET may improve IVF outcomes in patients with RIF	48 h before ET	HRT	Cavity	Transvaginal
Nazari et al., 2020	RCT	<40	Women who failed to conceive after three or more ET cycles with high-quality embryos, and candidates for FET	49	48	Chemical pregnancy Clinical pregnancy	Intrauterine PRP may improve the clinical pregnancy rate in patients with RIF	48 h before ET	HRT	Cavity	Transvaginal
Aghajanzadeh et al. (2020)	Prospective	18–40	Women with a history of four or more episodes of implantation failure submitted to FET with excellent or good embryos	30	0	Positive chemical pregnancy Clinical pregnancy Implantation rate Ongoing pregnancy	PRP may yield beneficial effects as a safe therapeutic option offered alongside other treatments designed to improve the reproductive outcomes of patients with RIF	48–72 h before ET	HRT	Cavity	Transvaginal
Tehraninejad et al., 2021	Prospective	<35		42	43	Biochemical pregnancy Clinical pregnancy Ongoing pregnancy	PRP is not an effective adjuvant treatment for IVF for patients with RIF and normal endometrial thickness undergoing ET	48 h before ET	HRT	Cavity	Transvaginal
Bakhsh et al., 2021	Single arm preliminary study of an RCT	<40	Women with at least two previous unexplained RIF	100		Positive serum β-HCG Implantation rate Clinical pregnancy	PRP may improve pregnancy outcomes in patients with RIF, but more studies are needed	48 h before ET	ICSI or FET (endometrial preparation type not defined)	Cavity	Transvaginal

(*continued on next page*)

TABLE 3 (Continued)

Study	Study design	Age (years)	Characteristics	Sample size	Outcome measures	Key findings	Time frame from intervention to cycle	Endometrial preparation used	Site of injection	Route of injection
Case Control										
Xu et al., 2022	Retrospective	23–40	Women with three or more consecutive failed embryo implantations with good-quality embryos (at least six cleavage-stage embryos or three blasto-cysts)	138 150	Positive serum β-HCG Implantation rate Clinical pregnancy rate Spontaneous miscarriage Live birth rate	Intrauterine perfusion of PRP before embryo transfer in FET cycles can significantly increase live birth and clinical pregnancy rates in patients with RIF	48–72 h before ET	HRT	Cavity	Transvaginal

RCT, randomized controlled trial; ET, embryo transfer; PRP, platelet-rich plasma; RIF, recurrent implantation failure; HRT, hormone replacement therapy; G-CSF, granulocyte colony-stimulating factor; β-HCG, beta-human chorionic gonadotrophin; FET, frozen embryo transfer; ICSI, intracytoplasmic sperm injection.

150 infertile women with a history of RIF. The women in the study were divided into two groups, with the study group receiving a PRP infusion 48 h before blastocyst transfer. According to their results, the positive β-HCG rate in the group that received the PRP infusion was significantly higher (43%) compared with the control group (15%). Finally, in a randomized, double-blind, single-arm, controlled clinical trial conducted by *Bakhsh et al. (2022)*, the primary objective was to assess the impact of intrauterine infusion of PRP on pregnancy outcomes in women undergoing ICSI. The study enrolled 100 women with a history of at least two unexplained cases of RIF who met the eligibility criteria for FET. The participants were divided at random into two groups: the intervention group, with a mean age of 35 years; and the control group, with a mean age of 32.7 years. The intervention group received an intrauterine infusion of PRP, and the control group underwent intrauterine catheterization without PRP treatment. The results showed a notable disparity in clinical pregnancy rates, with the intervention group achieving a rate of 20%, whereas the control group exhibited a rate of 13.33%.

Most of these studies reported positive outcomes with PRP treatment in terms of improved pregnancy rates in patients with RIF, suggesting a potential role for PRP in enhancing fertility outcomes. However, the studies vary in their design (single-arm, double-blind and prospective RCT), scale and specific patient demographics, which can influence the interpretation and comparability of results. While most studies showed a positive trend, the degree of efficacy varied. Despite the generally positive outcomes, the variability in results and methodologies underscores the need for further, more standardized research to conclusively determine the efficacy of PRP and optimal application in treating RIF.

PRP for the treatment of Asherman syndrome and chronic endometritis: a critical examination of the potential impact of PRP

In addition to the well-established indications for PRP usage, emerging research has highlighted its potential application in addressing Asherman syndrome and chronic endometritis. Asherman syndrome, characterized by the formation of intrauterine adhesions resulting from endometrial trauma, radiation exposure or infection, presents with a diverse range of clinical symptoms, including menstrual irregularities, infertility, and pregnancy complications such as recurrent pregnancy loss (RPL) or abnormal placentation (*Aghajanova et al., 2018*; *Puente Gonzalo et al., 2021*). *Aghajanova et al. (2018)* conducted a notable study that reported two case reports showcasing the clinical use of PRP as a potential therapeutic intervention for Asherman syndrome. In both cases, ongoing pregnancies were achieved with PRP therapy, shedding light on the promising prospects of PRP in addressing this challenging medical condition. Additionally, the study found that PRP may exert a more extensive influence than merely augmenting endometrial growth, as substantiated by the enhancements in endometrial thickness discernible on ultrasound examinations. The findings also suggest that PRP could enhance the functional attributes of the endometrium, culminating in successful pregnancies, even when the endometrial lining continues to be thin after treatment. This conjecture infers that PRP could exert a positive effect on both the receptivity and functionality of the endometrial lining, ultimately facilitating favourable pregnancy outcomes, despite challenges in endometrial thickness. Another case report by *Puente Gonzalo et al. (2021)* reported successful treatment of severe Asherman syndrome, resulting in a live birth. The study's findings indicate that autologous intrauterine PRP has potential for promoting endometrial regeneration, especially in cases of moderate-to-severe endometrial atrophy, and could serve to establish an optimal endometrial environment necessary for successful embryo implantation (*Puente Gonzalo et al., 2021*).

Chronic endometritis is characterized by prolonged, mild inflammation in the endometrial tissue, with the presence of plasma cells in the stromal region. The prevalence of the condition is often underestimated due to diagnostic challenges. PRP contains various essential components, including cytokines, hormones and growth factors, conferring mitogenic, chemotactic, neovascular and anti-inflammatory effects. Despite these properties, the use of PRP for treating chronic endometritis has been limited, possibly reflecting an underestimation of its potential to manage this complex condition (*Puente Gonzalo et al., 2021*; *Sfakianoudis et al., 2019*; *Singh Sethi, 2022*; *Sklyarova et al., 2020*). *Sfakianoudis*

et al. (2019) reported a case of a woman with chronic endometritis and POI who faced multiple implantation failures after receiving donated embryos. After unsuccessful attempts at curing chronic endometritis with antibiotics and a failed embryo transfer, the woman achieved a twin pregnancy and successful birth following PRP treatment, which treated the chronic endometritis effectively. In another case report involving five infertile women with chronic endometritis, the application of intrauterine PRP infusions every 2 days for three cycles of infusion led to endometrial histological recovery and positive serum β-HCG results after embryo transfer in all cases (*Li et al., 2021*).

PRP therapy for other indications

Two studies utilized PRP in patient groups distinct from those in the studies described above. A pilot retrospective study was conducted by *Madhavan et al. (2018)* to investigate the potential effects of intrauterine PRP on implantation rates in patients undergoing FET. The study analysed data from 98 patients who had experienced at least one failed FET and subsequently underwent another FET cycle. The study found that intrauterine PRP did not increase implantation or clinical pregnancy rates significantly in patients who had previously experienced a failed FET cycle (*Madhavan et al., 2018*). On the other hand, *Nazari et al. (2022)* undertook an RCT to investigate the effectiveness of PRP in improving the live birth rate of women with RPL undergoing IVF. Sixty-three patients with a history of at least two previous pregnancy losses and no specific cause for RPL were included in the study, and were divided into two groups. The PRP group received an intrauterine infusion of autologous PRP 48 h before embryo transfer, while the control group received standard treatment. The clinical pregnancy rate appeared to be higher in the PRP group (35%) compared with the control group (20%); however, the difference was not significant ($P = 0.288$). The live birth rate was 15% in the PRP group, with no live births recorded in the control group. While both studies investigated the efficacy of PRP, they targeted different patient groups (failed FET versus RPL) and had different primary endpoints. The retrospective nature of the study by *Madhavan et al. (2018)* contrasts with the prospective RCT design of the study by *Nazari et al. (2022)*, which could impact the strength of the evidence provided by each study. The conclusions of the two studies differ, as *Madhavan et al. (2018)* found no significant benefit of PRP in their context, while *Nazari et al. (2022)* observed positive outcomes in their specific patient group. These variations highlight the complexity of the role of PRP in reproductive treatments, and the need for further research tailored to specific patient groups and clinical scenarios.

PROTOCOLS FOR THE PREPARATION OF PRP FOR APPLICATION IN OVARIAN AND ENDOMETRIAL PROCEDURES

A comprehensive examination of the protocols employed across various studies is essential in order to evaluate the efficacy of PRP within the domain of reproductive medicine. This review aims to discern the level of coherency and ascertain the quality characteristics inherent in these methodologies. Before breaking down the protocols, the intended use of PRP post-preparation was scrutinized for the 36 included studies. Ten studies used PRP for ovarian rejuvenation, while 11 studies used PRP to increase endometrial thickness. There were also 11 studies which used PRP for RIF, with definitions of RIF ranging from one to three failed embryo transfers, and minimal information on endometrial thickness and quality of embryos transferred. There were also two studies that used PRP for other indications, such as RPL, defined as patients who had two or more pregnancy losses, and patients who had experienced at least one failed FET. From the preceding information, it is evident that the applications of PRP are markedly diverse. Considering the complexity of an autologous product such as PRP and the need for quality control in clinical applications, the ability to reproduce consistent results must be shown. The similarities and differences in PRP preparation per intended use are described below (TABLE 4 and FIGURE 2).

Blood collection volume and initial platelet concentration

For ovarian injection, the initial blood volume aspirated from infertile women ranged from 8 to 50 ml. Regarding uterine cavity injections, the initial blood volume from infertile women ranged from 8 to 20 ml. However, four studies did not report the initial blood volume collected in the context of endometrial preparation. Furthermore, none of the included studies mentioned the initial PRP concentration obtained from the participants (TABLE 4). At the physiological level, human platelet counts range from 150×10^9 to 400×10^9/l (*Daly, 2011*). On the one hand, platelet counts can exhibit variations among individuals, attributable to a multitude of factors including sex, ethnicity, age, genetic determinants (such as single nucleotide polymorphisms), inflammatory conditions and oncological diseases (*Biino et al., 2011*; *Daly 2011*; *Segal Moliterno 2006*). On the other hand, *Fadadu et al. (2019)* demonstrated that the concentration of PRP obtained subsequent to blood processing exhibits a moderate positive correlation with the initial volume of blood aspirated.

Anticoagulants

In the context of PRP preparation for ovarian rejuvenation, two studies omitted to mention the specific type of anticoagulant employed, while five studies utilized 3.2% sodium citrate and one study used anticoagulant citrate dextrose solution A (ACD-A) (TABLE 4). Concerning the protocols for PRP preparation pertaining to endometrial preparation, seven studies did not identify the anticoagulant type, and 16 studies opted for ACD-A solution, albeit without consistent specification of the percentage used. Additionally, one study used 3.8% sodium citrate during the PRP preparation process. Two studies embarked on comparative analyses of different anticoagulants, such as ACD-A, sodium citrate, EDTA and heparin, with respect to their impact on PRP quality, specifically concerning concentration and functional activity. Notably, sodium citrate and ACD-A solutions were delineated as more efficacious in maintaining platelet morphology and physiology compared with other anticoagulant options (*Aizawa et al., 2020*; *Singh, 2018*). Consequently, when preparing PRP, the anticoagulant must be chosen carefully to minimize platelet aggregation/platelet damage and to avoid suppression of the regenerative activity of cells in the target tissue (*Aizawa et al., 2020*; do *Amaral et al., 2016*; *Singh, 2018*). Nevertheless, the determination of which anticoagulant might induce minimal or reversible adverse effects at the surrounding implantation site during PRP injections for ovarian or endometrial rejuvenation remains an unresolved question.

Centrifugal force

For the purpose of ovarian rejuvenation, four studies implemented a two-step centrifugation process in the preparation of PRP. The centrifugal force employed

TABLE 4 PLATELET-RICH PLASMA PREPARATION PROTOCOLS

Study	Patient group	Blood volume collected (ml)	Commercial/ non-commercial	Substrate activation	Anti-coagulant	Centrifugation step 1		Centrifugation step 2		Initial platelet concentration	Final PRP concentration	PRP factor increase from whole blood	PRP volume injected (ml)	PRP timing of injection in patient's cycle	PRP timing of injection post preparation	PRP frequency of injection
						Speed (g)	Time (min)	Speed (g)	Time (min)							
Sills et al., 2018	Ovary	8–10	NC	Calcium gluconate	N/A	250	15	300[a]	10[a]	N/A	N/A	N/A	1.5	N/A	N/A	N/A
Stojkovska et al., 2019	Ovary	10	Regen	Calcium gluconate	Sodium citrate	KIT	KIT	KIT	KIT	N/A	N/A	N/A	3–5	N/A	Immediately	1
Cakiroglu et al., 2020	Ovary	20	T-lab	KIT	Sodium citrate	830	8	1 step	1 step	N/A	N/A	N/A	2–4	Day 10	2 h	1
Petryk and Petryk, 2020	Ovary	8.5	NC	N/A	Sodium citrate	830	3	1400	15	N/A	1,000,000/μl	N/A	0.7[c]	N/A	N/A	1
Hsu et al., 2021	Ovary	40	NC	Calcium gluconate	N/A	800	8	1500	5	N/A	N/A	N/A	5	N/A	N/A	1
Aflatoonian et al., 2021	Ovary	20	Rooyagen	Calcium gluconate	ACD-A	1600	10	3500	5	N/A	N/A	3–4-fold	1.5	Day 10	Incubation 1 h at 4°C	1
Tülek and Kahraman, 2022	Ovary	20	T-lab	KIT	Sodium citrate	1500	8	1 step	1 step	N/A	N/A	N/A	2[c]	N/A	2 h	1
Cakiroglu et al., 2022	Ovary	20	T-lab	KIT	Sodium citrate	830	8	1 step	1 step	N/A	N/A	N/A	4–8	Day 1–10	N/A	1
Tremellen and Ince, 2022	Ovary	50	Pro-PRP	KIT	KIT	1700	8	1 step	1 step	N/A	N/A	N/A	3[c]	Day 6–9	N/A	1
Tandulwadkar et al., 2020	Ovary	20[b]	NC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Chang et al., 2015	ET	15	NC	N/A	ACD-A	200	10	500	10	N/A	N/A	N/A	0.5–1	Day 10	Immediately	1–2
Tandulwadkar et al., 2017	ET	10	NC	N/A	N/A	200	15	600	6	N/A	N/A	N/A	0.5–0.8	Day 15–16	N/A	1–2
Wang et al., 2019	ET	N/A	NC	Thrombin	ACD-A	200	15	300	10	N/A	1,000,000/μl	N/A	0.5–0.8	Day 15–16	N/A	1
Eftekhar et al., 2018	ET	8.5	NC	N/A	ACD-A	1600	10	350	5	N/A	N/A	4–5-fold	0.5–1	Day 13	N/A	1
Molina et al., 2018	ET	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1	Day 10	N/A	1
Nazari et al., 2019	ET	17.5	NC	N/A	ACD-A	150	12	1000	7	N/A	N/A	N/A	0.5	Day 11–12	N/A	1–2
Chang et al., 2019	ET	15	NC	Thrombin	ACD-A	300	10	700	15	PDGF-AB/ PDGF-BB/ TGF-β	PDGF-AB/ PDGF-BB/ TGF-β	4.21-fold	0.5–1	Day 10	N/A	1
Kim et al., 2019	ET	18	PROSYS PRP	KIT	N/A	1000	3	1 step	1 step	N/A	717.000–1,565.,000/μl[e]	N/A	0.7–1	Day 10	N/A	2–3
Agarwal et al., 2020	ET	8	GEO PRP kit	KIT	KIT	1200	6	1 step	1 step	N/A	N/A	N/A	4	Day 23–26 OCP	N/A	1
Frantz et al., 2020	ET	N/A	NC	N/A	Sodium citrate	280	12	280	10	N/A	N/A	N/A	0.5	Day 14–17	Immediately[d]	3
Dogra et al., 2022	ET	15	NC	N/A	ACD-A	175	12	1300	7	N/A	1,000,000/μl	N/A	0.5–1	Day 8	1 h	1–2
Coksuer et al., 2019	RIF	8	NC	N/A	N/A	1500	5	1 step	1 step	N/A	992.45 ± 212.85/μl[e]	N/A	1	48 h before ET	N/A	1
Mehrafza et al., 2019	RIF	8.5	Arya Mabna Tashkhis	KIT	ACD-A	KIT	KIT	KIT	KIT	N/A	N/A	4–5-fold	1	48 h before ET	N/A	1
Dieamant et al., 2019	RIF	N/A	NC	N/A	ACD-A	200	12	1000	7	N/A	N/A	N/A	0.7	48 h before ET	N/A	1
Rageh et al., 2020	RIF	17.5	NC		ACD-A	161	12	1220	7	N/A	N/A	4–5-fold	0.5–1	48 h before ET	N/A	1
Zamaniyan et al. 2021	RIF	17.5	Arya Mabna Tashkhis	KIT	ACD-A	150	12	1000	7	N/A	N/A	4–7-fold	0.5	48 h before ET	N/A	1
Nazari et al., 2020	RIF	8.5	Arya Mabna Tashkhis	KIT	ACD-A	175	12	1300	5	N/A	N/A	N/A	0.5	48 h before ET	N/A	1
Aghajanzadeh et al. (2020)	RIF	15–20	NC	N/A	N/A	175	12	1300	7	N/A	N/A	N/A	0.5–0.7	Day 9–10	N/A	1
Tehraninejad et al., 2020	RIF	10	NC	N/A	ACD-A	175	12	1300	5	N/A	N/A	4–5-fold	1	48 h before ET	N/A	1
Bakhsh et al., 2021	RIF	8.5	Arya Mabna Tashkhis	KIT	ACD-A	175	10	1300	6	N/A	N/A	4–5-fold	0.5	48 h before ET	N/A	1
Xu et al., 2022	RIF	20	NC	N/A	N/A	500	10	500	10	N/A	1.513.000 ± 322.18/μl[e]	N/A	1	48 h before ET	<24 h	1
Nazari et al., 2016	RIF	17.5	NC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.5	Day 11–12	N/A	1–2

(continued on next page)

TABLE 4 (*Continued*)

Study	Patient group	Blood volume collected (ml)	Commercial/ non-commercial	Substrate activation	Anti-coagulant	Centrifugation step 1		Centrifugation step 2		Initial platelet concentration	Final PRP concentration	PRP factor increase from whole blood	PRP volume injected (ml)	PRP timing of injection in patient's cycle	PRP timing of injection post preparation	PRP frequency of injection
						Speed (g)	Time (min)	Speed (g)	Time (min)							
Madhavan et al., 2018	Endometrium 15	NC		N/A	ACD-A	200	15	500	8	N/A	N/A	N/A	0.3–0.4	Day 8–9	N/A	1
Nazari et al., 2022	Endometrium 8.5	Arya Mabna Tashkhis KIT		ACD-A		175	12	1300	5	N/A	N/A	4–5-fold	0.5	48 h before ET	N/A	1

[a] Additional third centrifugation step 2000 *g* for 10 min.

[b] Plus bone marrow.

[c] Per ovary.

[d] One used immediately after preparation, and the other two kept at room temperature and used 2 and 4 days later.

[e] Data expressed as mean ± SD.

NC, non-commercial; PRP, platelet-rich plasma; N/A, not available; KIT, commercially available kit; ACD-A, anticoagulant citrate dextrose solution-A; ET, embryo transfer; PDGF-AB, platelet-derived growth factor-AB; PDGF-BB, platelet-derived growth factor-BB; TGF-*β*, transforming growth factor-beta; OCP, oral contraceptive pill; RIF, repeated implantation failure.

during the initial centrifugation varied from 830 to 1600 *g*, while the subsequent centrifugation was conducted within a range of 1400–3500 *g* (TABLE 4). Four studies employed a one-step centrifugation process, utilizing a centrifugation force that ranged between 830 and 1700 *g* for ovarian rejuvenation (TABLE 4). With respect to endometrial preparation, 19 studies applied a two-step centrifugation procedure, with forces ranging from 150 to 1600 *g* in the initial centrifugation, and from 280 to 3500 *g* in the subsequent step. Conversely, three studies opted for a single-step centrifugation strategy, with forces ranging between 1000 and 1500 *g*. Additionally, two studies did not provide specific details concerning the centrifugation steps utilized (TABLE 4). In this particular context, an article from the Indian Association of Dermatologists and Venereologists that evaluated 45 scholarly articles, including both observational studies and review articles, suggested that the first spin should be performed between 100 and 300 *g* for 5–10 min, and the second spin should be performed between 400 and 700 *g* for 10–17 min (*Dashore et al., 2021*). This recommendation was formulated on the findings of prior research, where investigators identified manifestations of spontaneous and premature platelet activation – a condition interpreted as a loss of platelet integrity – at relative centrifugal forces of ≥800 *g*. As a consequence, the utilization of lower relative centrifugal forces and extended spin durations is advocated over the reverse approach (*Dashore, 2021*; *Perez, 2014*).

Centrifugation time

In the context of PRP preparation specifically for ovarian rejuvenation, three studies employed a two-step centrifugation process. The duration of the initial centrifugation varied between 3 and 15 min, while the subsequent centrifugation ranged from 5 to 15 min. Furthermore, four investigations employed a single-step centrifugation process lasting for 8 min (TABLE 4). Concurrently, in the application of PRP injection into the uterine cavity, 19 studies utilized a two-step centrifugation process. The duration of the initial centrifugation step varied between 3 and 15 min, while the subsequent centrifugation step ranged from 5 to 15 min. Contrarily, three studies employed a single centrifugation step, with the duration ranging between 3 and 6 min (TABLE 4). As delineated in a prior optimization investigation, centrifugation conducted for an extended period and at a reduced spin rate appears to enhance platelet recovery and diminish the number of white blood cells (WBC) present in the superficial layer (*Perez et al., 2014*). On the one hand, the inclusion of WBC in the PRP prepared for injection might augment the concentrations of growth factors and cytokines (*Zimmermann et al., 2001*). Conversely, under particular circumstances, these WBC could exert deleterious effects due to their pro-inflammatory and catabolic properties (*Kim et al., 2021*).

Commercial kits

In the context of ovarian injections, four studies did not use commercial kits for PRP preparation, whereas six studies used various commercially available kits. One study used the RegenKit A-PRP (Regen Laboratory, Switzerland), approved by the US Food and Drug Administration, with Class IIb CE certification. Three studies used the T-LAB PRP kit (T-Biotechnology Laboratory, Turkey) with Class IIb CE certification. In addition, one study used the Pro-PRP kit (Alocuro, Australia), approved by the Therapeutic Goods Administration, and one study employed the Rooyagen kit (Tharan, Iran) (TABLE 4). Employing commercial kits may enhance the precision and repeatability of platelet concentration and leukocyte inclusion, potentially leading to standardization through the utilization of specialized tubes for blood collection. However, an investigation comparing PRP commercial kits in common use revealed variations in several blood components within PRP extractions, including platelet, red blood cell, leukocyte, pH and glucose concentrations (*Fitzpatrick et al., 2017*). These variations necessitate careful consideration when evaluating the outcomes of clinical trials, and selecting the most suitable preparation methodology. An ancillary concern involves the concentration of WBC, which exhibit a positive correlation with VEGF and PDGF. While these correlations can be beneficial, they may also lead to detrimental consequences. Such consequences can include inflammation caused by the release of oxygen-free radicals, catabolic cytokines, matrix metalloproteinases and interleukin, all of which can potentially affect the desired therapeutic outcomes (*DeLong et al., 2011*; *Zimmermann et al., 2001*). Furthermore, different provider protocols have resulted in different final PRP concentrations, and

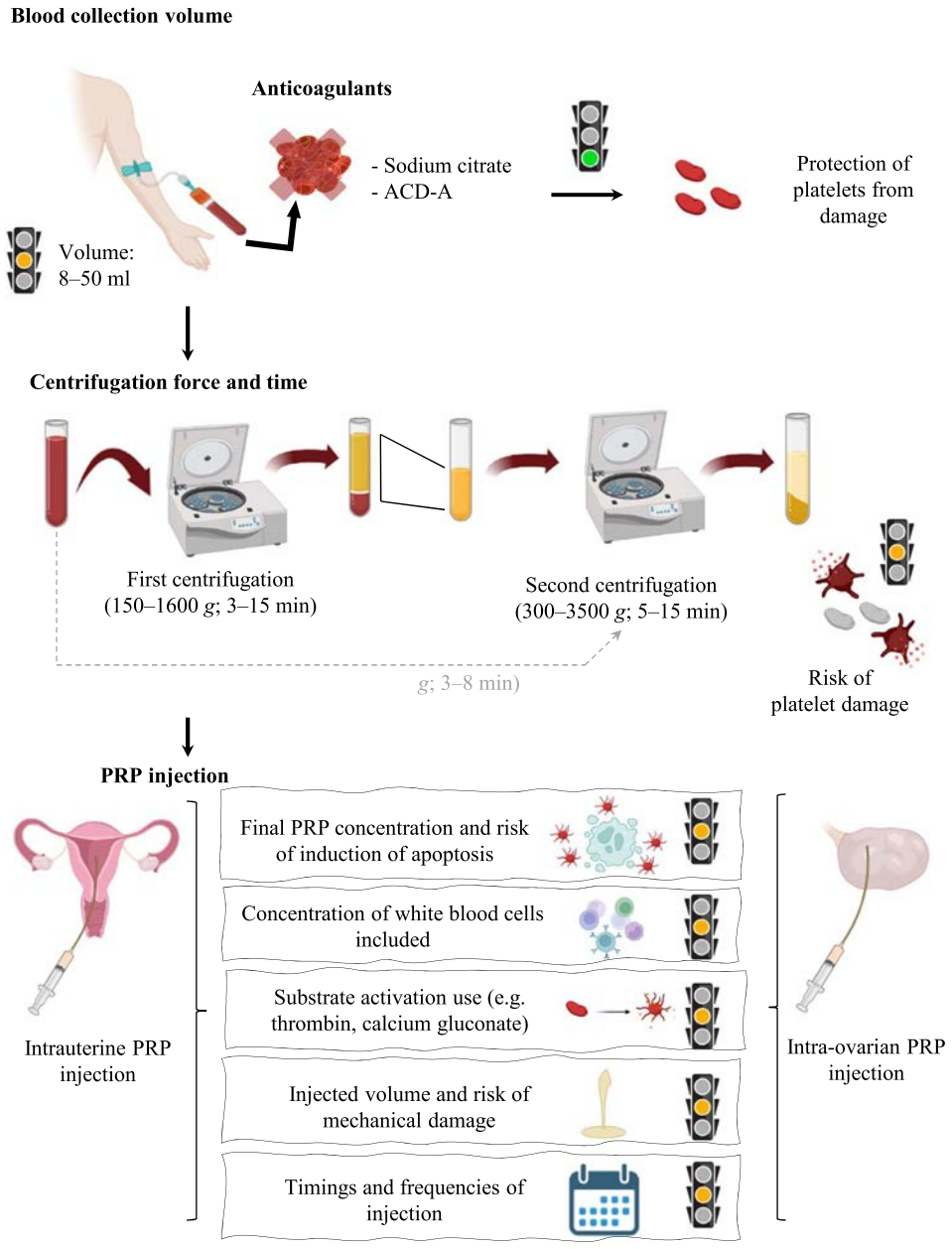

FIGURE 2 Protocols for the preparation of platelet-rich plasma (PRP) for use in treating female infertility. The diagram provides an overview of the uniformity and quality aspects of PRP preparation methods as detailed in studies examining the effects of PRP on female infertility. It emphasizes the congruencies and variations in PRP preparation techniques tailored for specific applications, employing a 'traffic light' colour-coding system: green, this step is adhered to uniformly across all protocols; yellow, there is variation in this step between protocols, with the implications of these variations remaining uncertain; and red, there is consensus among studies that this step may have adverse effects. ACD-A, anticoagulant citrate dextrose solution A.

separate studies using the same commercial kits have shown significantly different results (*Fadadu et al., 2019*).

Initial and final concentrations of platelets before and after PRP preparation

Concerning the initial platelet concentration, one study employing PRP to enhance endometrial thickness specifically reported the initial concentrations of PDGF-AB, PDGF-BB and TGF-β as quantified in whole blood (*Chang et al., 2019*). Without the establishment of an initial measurement, the ascertainment of an increase in the platelet concentration factor following PRP preparation is unattainable. As a result, an assessment of the effectiveness of individual protocols is precluded.

The final concentration following PRP in ovarian injection was reported in one study as 1,000,000 platelets/μl (*Petryk Petryk 2020*). Five studies reported the final platelet concentration utilized for endometrial preparation, with values ranging from approximately 992.45 ± 212.85 to 1,565,000 ± 322.18/μl (TABLE 4). However, the available literature does not clarify sufficiently whether these reported concentrations were measured individually for each patient, determined in accordance with initial control measurements, or predicated on the manufacturer's guidelines when

commercial kits were employed. Measurements of platelet concentrations for PDGF-AB, PDGF-BB and TGF-β only took place in one study (*Chang et al., 2019*). It is apparent that without precise assessments of platelet concentrations, the effectiveness of this technology remains indefinable across various studies. Moreover, the determination of the optimal platelet concentration for PRP presents an unresolved issue. An excessive concentration is not necessarily advantageous, as an overly high platelet concentration may prove deleterious, leading to cellular apoptosis. As demonstrated by a study assessing proliferation in human tenocytes, a concentration of 500,000 and 1,000,000/μl resulted in a positive effect, while a concentration of 5,000,000/μl induced cell death (*Giusti et al., 2014*).

PRP factor increase from whole blood

It is acknowledged that the lack of studies evaluating both initial and final platelet concentrations precludes assessment of the increase in PRP factor derived from whole blood. Six studies reported a 4–5-fold increase in concentration following preparation, without presenting specific values, while a single study indicated a 4–7-fold increase in PRP factor. In one study, the factor increase was calculated precisely as 4.21-fold higher in PRP compared with whole blood ($889.42 \pm 64.41 \times 10^3$/ml versus $211.37 \pm 46.20 \times 10^3$/ml; $P < 0.01$) (*Chang et al., 2019*). It can be concluded that, without determining the factor increase, a precise evaluation of the efficacy of each preparation protocol remains unachievable.

PRP volume of injection

Considerable variation was observed in the injection volume across the studies, regardless of the patient group and intended use. In the context of ovarian rejuvenation, reported volumes ranged from 0.7 to 8 ml per ovary, whereas for endometrial preparation, volumes ranged from 0.3 to 4.0 ml. This variation underscores the lack of standardization concerning the protocol and the optimal concentration to be employed for both ovarian and endometrial treatments.

Timing of PRP injection in patient's cycle

With regard to the timing of injection in relation to the patient's menstrual cycle for the purpose of ovarian rejuvenation, one study documented that the procedure was conducted without regard to the menstrual cycle phase in women experiencing amenorrhoea. For women presenting with oligomenorrhoea, PRP was administered within 10 days following the cessation of menstrual bleeding. In the studies listed in TABLE 4 pertaining to ovarian rejuvenation, one study indicated that the injection was administered during the mid-follicular phase of the cycle (days 6–9), while another study suggested its administration within 10 days of the completion of menstrual bleeding. Additionally, two studies documented the procedure being performed on day 10 of the menstrual cycle. Additionally, six studies omitted to specify the timing of injection for ovarian rejuvenation. When considering endometrial preparation, the timing of PRP injection within the patient's cycle demonstrated even greater variability across the reported studies. Nine studies documented the injection as occurring 48 h prior to embryo transfer, and two studies reported injection on day 8–9 following menstrual bleeding. Additional reports included one study reporting injection on days 9–10 of the cycle, four studies reporting injection on day 10 and one study reporting injection on day 13. Two studies, focusing on women who were unable to achieve an endometrial thickness of 7 mm despite receiving an optimal dose of oestradiol valerate for 15–16 days, coupled with poor vascularity, reported injection on day 15 or 16. One study reported injections on two or three separate occasions, commencing on day 10 of the menstrual cycle, and at 3-day intervals until the endometrium reached 7 mm, with a maximum of three injections. Embryo transfer occurred 3 days subsequent to the final injection in the protocols outlined, with one study failing to report this particular aspect. The divergence in the timing of injections relative to the patient's cycle prohibits a comparative analysis of methodological efficacy. Furthermore, with regard to endometrial preparation, the majority of studies did not specify the developmental stage of the embryo at the time of embryo transfer. If the injection is administered 48 h prior to embryo transfer, the implantation timing will vary depending on whether the embryo or embryos being transferred are at day 2, day 3 or day 5 of in-vitro culture and development.

Timing of PRP injection following preparation

Most studies did not include detailed information regarding the time of PRP injection after preparation. The limited available data include three studies where the injection was carried out immediately after preparation, one with a lapse of 1 h, and two studies involving a 2-h wait post preparation (TABLE 4). Furthermore, one study documented a unique three-step injection process for each patient, with the first injection at the time of preparation, and the others maintained at room temperature and administered 24 and 48 h later. In contrast, one study specified that the injection was conducted within 24 h of preparation. The research findings emphasize the critical importance of PRP storage conditions. One investigation elucidated that storing PRP at either 4°C or room temperature triggers modifications in platelet morphology and activation (*Watts et al., 1986*). A separate study illustrated that the storage of PRP precipitated increased release of PDGF from platelet-bound α-granules into the plasma. This release was found to be independent of the storage temperature, with PDGF concentrations reaching a peak during chilled or frozen storage, specifically at −20°C. This temperature facilitated an accelerated release of PDGF in PRP over a storage duration of up to 1 month (*Düregger et al., 2016*).

Frequency of PRP injection

In the studies concerning the use of PRP for ovarian rejuvenation, a single injection was administered per ovary, as reported in eight studies. However, two studies did not report the frequency of PRP injection. In contrast, in the context of endometrial preparation, the methodologies varied. Eighteen studies documented a single injection, five reported one to two injections, one reported two to three injections, and another reported three injections. An area that presents itself as an intriguing subject for exploration involves the execution of clinical trials designed to compare the effects of single-dose and multi-dose intra-ovarian/endometrial injections of PRP in infertile women, focusing on distinct aetiologies of female infertility. Such a comparative study could yield essential insights into optimal treatment modalities tailored to the individual underlying aetiological factors contributing to infertility.

CONCLUSIONS

Protocols for PRP require standardization

Much of the current research is devoted to applying PRP preparations to enhance

the endometrial environment for successful embryo transfer. Only a few studies have reported comprehensive protocols for using PRP injections into the ovaries to augment follicular function. Thirteen of 35 studies employed commercial PRP preparation kits with standardized compositions and easy reproducibility. On the other hand, in-house PRP preparation protocols were less standardized, utilizing diverse anticoagulants or substrate activating agents, centrifugation steps and administration strategies, which may impact reproducibility and treatment efficiency. The most notable discrepancy lies in the centrifugation stage, a vital element of the PRP preparation protocol. Most in-house and some commercial protocols entail two rounds of blood centrifugation. The first centrifugation separates red blood cells and the buffy coat from PRP. In contrast, the second centrifugation concentrates platelets found in the bottom phase, with the upper phase containing the platelet-poor plasma. Nonetheless, certain commercial preparations have opted for a one-stage centrifugation process at 830 *g* for 8 min. It is worth noting that the range of centrifugal force values during the initial phase was extensive, varying from 150 to 1600 *g*, and extending to a maximum duration of 15 min. When detailed, the second phase of centrifugation exhibited a range from 300 to 3500 *g*, with a maximum time limit of 15 min. It is crucial to note that certain centrifugation conditions pose considerable risks, such as platelet damage and premature activation, which can compromise their therapeutic effectiveness (*Seidel et al., 2019*). Centrifugation strategies must be optimized to reduce outcome variability and establish a credible route for retrieving PRP.

A notable dimension in examining the reported protocols is the recurrent omission of any assessment of platelet concentration at any phase of the procedure. Only a few studies reported the exact amount of PRP administered, which remained consistent among them. The reported values in these articles range between 992.45 ± 212.85 and 1.5 million platelets/μl. Additionally, for the studies reporting injection into the endometrium, 11 reported injection volumes of up to 1 ml. For injection into the ovaries, volumes ranged from 0.7 to 8 ml across the discussed protocols. The time interval between PRP preparation and administration did not exceed 24 h in the reported studies with detailed protocols. A concerning issue for the ovarian PRP protocols arises regarding the lack of information about the specific day of the menstrual cycle when PRP was administered. Of the 10 studies, **TABLE 4** indicates that eight studies administered a single injection, while this information was not available for two studies. However, the underlying rationale for choosing the particular day for PRP administration remains undefined, constituting a void in the scientific foundation.

In relation to the preparation of PRP for endometrial use, although there is a more consistent description of the injection timing, significant variation persists concerning the day of the menstrual cycle that women receive intrauterine PRP. Some research protocols involve administration during the mid-follicular phase, whereas others have chosen a 2-day window before embryo transfer. The discrepancy underscores the absence of a clear scientific foundation, leaving determination and optimization of the ideal time for PRP administration unresolved.

The discrepancies and unrecorded practices in the protocols itemized in **TABLE 4** are conspicuous. Given the escalating interest in PRP as a promising therapeutic instrument for augmenting ovarian function and endometrial receptivity, the absence of standardized procedures for using PRP is unexpected, rendering its incorporation into clinical practice inadvisable at this stage. The existing landscape reveals that both researchers and clinicians operate with a multiplicity of protocols, thereby obstructing the ability to derive decisive conclusions from studies and impeding comparisons across disparate research efforts. The urgency for protocol standardization is palpable, as it would enable RCT that aim to bolster reproductive function with PRP. By reaching a consensus on the appropriate procedures, the scientific community can pave the way towards more consistent research findings, leading ultimately to the creation of empirically supported therapeutic approaches. Such consensus would facilitate harnessing the full potential of PRP in enhancing both ovarian rejuvenation and endometrial receptivity, with an assurance of safety and efficacy.

Short- and long-term biosafety of PRP requires assessment

While the existing literature predominantly delves into the therapeutic efficacy of PRP, it is imperative to acknowledge and scrutinize potential adverse outcomes arising from the intricate technical challenges associated with the injection of PRP into the ovaries and the endometrium, alongside the underlying mechanisms governing its action. Despite not being endorsed by the recently published Good Clinical Practice recommendations for IVF add-ons, to date, no harm has been reported in association with PRP utilization (*ESHRE Add-ons Working Group, 2023*). It remains indispensable to implement meticulously designed studies to assess efficacy and establish a safety profile for PRP administration, a prerequisite before contemplating its integration into routine clinical practice. Nonetheless, extant studies administering PRP to the reproductive tract report an absence of side effects or notable complications following the procedure (*Fraidakis et al., 2023*; *Gangaraju et al., 2023*; *Pantos et al., 2019*; *Puente Gonzalo et al., 2021*), and recent evidence spotlighting PRP as an antimicrobial agent serves to allay concerns regarding potential infections stemming from PRP injections (*Ghallab et al., 2023*; *Segabinazzi et al., 2021*). Furthermore, multiple studies affirm that PRP growth factors exhibit non-mutagenic characteristics and lack the capability to induce tumour generation (*Marx, 2001*; *Schmitz et al., 2001*). However, a conspicuous dearth of safety evidence persists concerning the exposure of ovaries, endometrium and hosted embryos to PRP injection. Indeed, a comprehensive and robust assessment of the long-term safety of novel treatments such as PRP in assisted reproductive technology (ART) is imperative, particularly considering ongoing concerns that ART itself may elevate the risk of birth defects (*Atkinson et al., 2021*).

DATA AVAILABILITY

No data was used for the research described in the article.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used CHATGPT-4 PLUS in order to improve readability and language of the work. After using this tool, the authors reviewed and edited the content as needed, and take full responsibility for the content of the publication.

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ARTICLE

Ongoing pregnancy rates in single euploid frozen embryo transfers remain unaffected by female age: a retrospective study

BIOGRAPHY

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KEY MESSAGE

Female age in itself does not have a substantial impact on ongoing pregnancy rates in single euploid frozen embryo transfer cycles, but the outcome is impacted significantly by embryo quality, body mass index, previous parity, and a natural cycle endometrial preparation protocol.

ABSTRACT

Research question: Is female age a significant factor in the likelihood of an ongoing pregnancy in single euploid frozen embryo transfers (FET)?

Design: Retrospective study of 1923 single euploid FET cycles in 1464 women, either in a natural cycle or a hormone replacement therapy cycle. The primary outcome was the ongoing pregnancy rate (OPR).

Results: There were 990 (51.48%) ongoing pregnancies among 1923 included transfers. The OPR were 51.4%, 49.1%, 53.3% and 52.3% for women aged ≤35, >35−≤37, >37−≤40 and >40 years at oocyte retrieval (OCR), without a significant trend for decreasing OPR ($P = 0.679$). No significant differences in female age at embryo transfer ($P = 0.609$) and female age at OCR ($P = 0.816$) were found between the groups (ongoing pregnancy versus no pregnancy or miscarriage). Women who received good-quality embryos ($P < 0.001$), had a lower body mass index (BMI) ($P < 0.001$), had achieved at least one pregnancy previously ($P < 0.001$), and underwent natural cycle endometrial preparation ($P < 0.001$) were more likely to achieve an ongoing pregnancy. Multivariable regression analysis (adjusted for BMI, embryo quality and endometrial preparation) did not show a significant effect of female age at OCR on achieving an ongoing pregnancy. Compared with women aged ≤35 years, none of the age groups had significantly higher or lower OPR. A multinomial regression analysis showed that BMI, embryo quality and endometrial preparation were associated with miscarriage/no pregnancy versus ongoing pregnancy ($P = 0.001$, 0.001 and 0.001, respectively). Female age had no significant association with either outcome.

Conclusions: Female age in itself does not have a substantial impact on the OPR in single euploid FET cycles, but the OPR is impacted significantly by embryo quality, BMI, previous parity, and a natural cycle endometrial preparation protocol.

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KEY WORDS
Female age
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Natural cycle
BMI

INTRODUCTION

The decrease in female fecundity with increasing age is undisputed (*American College of Obstetricians and Gynecologists Committee on Gynecologic Practice and Practice Committee, 2014*; *'Female age-related fertility decline', 2014*). The most evident reason for the decrease in fertility is the increase in embryo aneuploidy with aging (*Capalbo et al., 2017*; *Franasiak et al., 2014*; *Park et al., 2021*). Infertile couples with an advanced female age frequently turn to assisted reproductive technology (ART) to overcome infertility. However, in large datasets, the age of the female remains an important predictor for the likelihood of a live birth with IVF or intracytoplasmic sperm injection (ICSI) treatment (*Nelson and Lawlor, 2011*).

Implantation and maintenance of a pregnancy depends on maternal (*Guzeloglu-Kayisli et al., 2007*; *Melado Vidales et al., 2023*) and embryonic factors such as embryo quality, the pace of embryo development (*Irani et al., 2019*; *Melado Vidales et al., 2023*; *Shapiro et al., 2001*; *Wang et al., 2021*) and, most importantly, euploidy (*Cimadomo et al., 2018*; *Tiegs et al., 2021*). For a successful ART outcome, the availability of a euploid embryo is pivotal, and implementation of preimplantation genetic testing for aneuploidy (PGT-A), allowing the selection of euploid embryo(s), was thought to mitigate the age-related decrease in fertility in ART treatment (*Sacchi et al., 2019*).

The routine use of PGT-A as a tool for embryo selection is an ongoing controversy (*Gleicher et al., 2021*; *Morales, 2024*; *Neal et al., 2018*; *Orvieto, 2016*; *Sabbagh et al., 2023*). Whereas no improvements in pregnancy rates and live birth rates through implementation of PGT-A as a routine selection tool were seen in unselected patient populations, performance of PGT-A with the transfer of a euploid embryo seems to be beneficial in patients with advanced maternal age (>35 years) (*Munné et al., 2019*; *Simopoulou et al., 2021*). However, whether euploid embryo transfer can compensate for the assumed age-related decline in female fertility is controversial, and recently published studies (*Reig et al., 2020*; *Vitagliano et al., 2023*) point towards a persistent negative impact of female age on the ongoing pregnancy rate (OPR) and live birth rate. As other factors, aside from embryo ploidy, might impact implantation and the OPR, this study intends to add data to the ongoing controversy, assessing factors beyond embryo ploidy which might influence the age-related decline in fertility.

MATERIALS AND METHODS

This retrospective study, conducted at a tertiary referral fertility unit, included women who underwent single euploid frozen embryo transfer (FET) in a natural cycle or a hormone replacement therapy (HRT) cycle between April 2017 and August 2023 with a known outcome of the cycle. Cycles in which the embryos had been biopsied and cryopreserved on day 7, preimplantation genetic testing for monogenic disorder cycles and downregulated cycles were excluded from the analysis. The primary outcome was the OPR (*Braakhekke et al., 2014*); this group included patients with an ongoing pregnancy (>12 weeks of gestation) and patients who had experienced a live birth at the time of data extraction and analysis. Patients who did not conceive, or had a biochemical pregnancy or early pregnancy loss were defined as no pregnancy or miscarriage.

Ovarian stimulation

Patients underwent ovarian stimulation using standard protocols with gonadotrophin dosage adjustments for age, body mass index (BMI), anti-Müllerian hormone (AMH), antral follicle count (AFC), ovarian response and hormonal measurements during stimulation (*Lawrenz et al., 2022*, *2021*; *ESHRE Guideline Group on Ovarian Stimulation et al., 2020*). Final oocyte maturation was triggered with either human chorionic gonadotrophin (HCG), gonadotrophin-releasing hormone agonist or both, and oocyte retrieval was conducted 34–36 h later when at least three follicles had a diameter ≥17 mm, or at smaller follicle sizes in women with a severely reduced ovarian reserve (*Lawrenz et al., 2024b*).

Oocyte insemination and embryo culture

Oocytes underwent insemination through conventional IVF or ICSI, in accordance with standard clinical procedures, at 40 h post trigger. Following fertilization, the embryos were cultured in Life Global single-step medium (Global Total LP, CooperSurgical, USA). Embryos were cultured individually in a 30-μl droplet of culture medium covered with mineral oil, and incubated for up to 7 days at 37°C, 6% CO_2 and 5% O_2. On day 3, the medium was refreshed for all cleaved embryos, and embryo morphology was assessed in line with the Istanbul consensus guidelines (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology et al., 2011*). Post warming, embryos were cultured in a 30-μl droplet of SAGE Quinn's Advantage Sequential medium (Quinn's Advantage Protein Plus Blastocyst media, CooperSurgical) covered with 8 ml of oil, and incubated until embryo transfer in a benchtop incubator (K-SYSTEM, CooperSurgical) at 37°C, 6% CO_2 and 5% O_2.

Classification of embryo quality

Embryos with Gardner criteria (*Gardner and Schoolcraft, n.d.*) of AA, AB or BA for inner cell mass (ICM) and trophectoderm were classified as 'good' quality, when trophectoderm biopsies and cryopreservation were performed on day 5. Embryos of 'fair' quality were classified as those with Gardner criteria of AA, AB or BA for day 6 embryo biopsy, and BB, BC, CA or CB for day 5 and/or day 6 biopsy. Embryos with Gardner criteria of AC, BC, CB or CC were classified as 'poor' quality, regardless of the day of biopsy.

Blastocyst biopsy

Blastocysts classified as ≥BL3CC using the Gardner modified scoring system (*Gardner and Schoolcraft, n.d.*) underwent trophectoderm biopsy for PGT-A, analysed by next-generation sequencing (NGS) on day 5 or day 6. The biopsy involved the use of a pipette with an internal diameter of 30 μm (Origio, CooperSurgical) to aspirate between three and 10 trophectoderm cells, as described earlier (*Capalbo et al., 2014*). The cells were loosened delicately using a 2.2-ms intensity laser pulse, followed by complete detachment using a mechanical flicking technique. Subsequently, the trophectoderm biopsies were washed, placed in 0.2-ml Eppendorf polymerase chain reaction tubes containing 2.5 μl of phosphate-buffered saline, and stored at -20°C until further processing.

PGT-A of trophectoderm samples

A whole-genome amplification protocol was applied on all trophectoderm samples, employing PicoPlex technology (Rubicon Genomics, USA). The process involved the preparation of individual libraries, wherein distinct barcodes were integrated to label

the amplified DNA from each sample. For sequencing, a 316 or 318 chip was employed in conjunction with Personal Genome Machine sequencing technology (Thermo Fisher Scientific, USA) after amplification and enrichment of the DNA. Analysis and interpretation of sequencing data were conducted using ion Reporter software (Thermo Fisher Scientific).

HRT cycle endometrial preparation approach

Patients were assessed on day 2/3 of their menses by vaginal ultrasound and measurement of serum oestradiol and progesterone concentations. Patients commenced oral oestradiol valerate 4 mg (2 × 2 mg) for 2 days, and the dosage was increased to 6 mg on day 3 of treatment. Monitoring included ultrasonographic measurement of the lining, and serial measurements of serum LH, oestradiol and progesterone to exclude spontaneous ovulation. Luteal phase support (LPS) was started when a triple lining appearance without a minimal thickness of the endometrium was achieved (*Ata et al., 2023*). During the study period, different regimens of LPS were used, including cycles in which LPS comprised micronized vaginal progesterone (MVP) alone, and other cycles in which oral progesterone (Dydrogesterone) was added to MVP as clinical standard (*Lawrenz et al., 2024a*).

Natural cycle endometrial preparation approach

Patients underwent intermittent ultrasound scans to monitor follicular growth, and serial measurements of serum LH, oestradiol and progesterone concentrations were performed throughout the cycle to determine ovulation. An LH surge was identified when an increase of 180% above the previous level occurred, and ovulation was confirmed with a decrease in oestradiol concentration, and an increase in progesterone concentration to ≥1.0 ng/ml (*Irani et al., 2017*). Embryo transfer was scheduled approximately 120 h after ovulation was confirmed, and MVP was started with a dosage of 200 mg/day on the same day, and increased to 300 mg/day from the day after embryo transfer until the day of the pregnancy test (*Lawrenz et al., 2024a*).

When patients achieved pregnancy, LPS was continued in both endometrial preparation approaches until 7 weeks of pregnancy in the natural cycle or until 12 weeks of pregnancy in the HRT cycle.

Pregnancy was confirmed by measurement of serum HCG 10 days after embryo transfer, and a concentration >15 IU was regarded as a positive result.

Embryo transfer procedure

Embryo transfers were performed in a lithotomy position under abdominal ultrasound guidance with a full bladder. Key performance indicators demonstrate that there is no significant difference in the pregnancy rates after embryo transfer between the physicians at the study centre.

Ethical approval

The study was approved by the Ethics Committee of ART Fertility Clinics, Abu Dhabi, UAE on 7 November 2023 (REFA108).

Statistical analysis

Continuous variables are presented as median and interquartile range, and categorical variables are presented as count and percentage of the total, unless otherwise specified. Group comparisons were made using either Wilcoxon rank-sum test or chi-squared test. To account for multiple transfers in the same couple, mixed effects were employed in the regression analyses, and intercepts were allowed to vary for the transfer. Regression analyses were undertaken for ongoing pregnancy versus miscarriage or no pregnancy (logistic), and ongoing pregnancy versus miscarriage and ongoing pregnancy versus no pregnancy (multinomial). Results of the regression analyses are reported as odds ratios with 95% CI. All analyses were performed with R for Statistical Computing Software (v4.1.2), and *P*-values <0.05 were considered to indicate significance.

RESULTS

In total, there were 990 (51.48%) ongoing pregnancies among 1923 included transfers from 1464 couples. The OPR were 51.8%, 47.4%, 52.3% and 52.8% for women aged ≤35, >35–≤37, >37–≤40 and >40 years at embryo transfer, and 51.4%, 49.1%, 53.3% and 52.3% for women aged ≤35, >35–≤37, >37–≤40 and >40 years at oocyte retrieval (OCR), respectively. The Cochrane Armitage test did not show a significant trend for decreasing pregnancy rates with increasing female age at OCR ($P = 0.679$) or at embryo transfer ($P = 0.866$). Group comparison of women with ongoing pregnancy versus those with no pregnancy or miscarriage did not reveal a significant difference in terms of female age at embryo transfer ($P = 0.609$), female age at OCR ($P = 0.816$), male age at OCR ($P = 0.146$), AMH concentration ($P = 0.578$) or storage duration ($P = 0.997$). Women who received good-quality embryos ($P < 0.001$), had a lower BMI ($P < 0.001$), had achieved at least one pregnancy previously ($P < 0.001$), and underwent natural cycle endometrial preparation ($P < 0.001$) were more likely to achieve an ongoing pregnancy (TABLE 1).

Multivariable regression analysis, after adjusting for the effect of BMI, embryo quality and endometrial preparation protocol, did not show a significant effect of female age at OCR on achieving an ongoing pregnancy (TABLE 2). Compared with women aged ≤35 years, none of the age groups had significantly higher or lower pregnancy rates (TABLE 2). A sensitivity analysis was performed to see whether age would make a difference in different embryo-quality groups or endometrial preparation protocols by allowing interactions between these variables in the regression analysis. The marginal effect plots (FIGURE 1) did not suggest a decrease in the OPR with increasing female age.

A multinomial regression analysis was performed to see whether age would have a specific effect on miscarriage as opposed to not achieving pregnancy at all. The multinomial regression analysis showed no pregnancy versus ongoing pregnancy was associated with overweight and obese BMI ($P = 0.021$ and 0.019, respectively), good-quality embryos ($P < 0.001$) and natural cycle endometrial preparation ($P = 0.029$); miscarriage versus ongoing pregnancy was associated with overweight BMI ($P = 0.039$) and natural cycle endometrial preparation ($P < 0.001$). Female age was not significantly associated with either outcome (Supplementary Table S1 and FIGURE 2).

DISCUSSION

The data presented herein clearly demonstrate that female age at the time of OCR or at the time of embryo transfer does not impact the OPR after single euploid FET. The OPR was reduced significantly by elevated BMI, lower embryo quality and HRT cycle endometrial preparation. The OPR was increased significantly when the patient had achieved

TABLE 1 COMPARISON OF BASELINE AND TRANSFER CHARACTERISTICS IN CYCLES RESULTING IN NO PREGNANCY OR MISCARRIAGE VERSUS CYCLES WITH ONGOING PREGNANCY

Variable	No pregnancy or miscarriage (*n* = 933)	Ongoing pregnancy (*n* = 990)	*P*-value[a]
Female age at embryo transfer, years	34.0 (29.0–38.0)	34.0 (30.0–38.0)	0.553
Female age at OCR, years	33.0 (29.0–37.0)	33.0 (29.0–37.0)	0.589
Female age at embryo transfer, years			
≤35	565 (48.2)	608 (51.8)	0.609
36–37	121 (52.6)	109 (47.4)	
38–40	145 (47.7)	159 (52.3)	
>40	102 (47.2)	114 (52.8)	
Female age at OCR, years			
≤35	600 (48.6)	634 (51.4)	0.816
36–37	115 (50.9)	111 (49.1)	
38–40	134 (46.7)	153 (53.3)	
>40	84 (47.7)	92 (52.3)	
Male age at OCR, years	36.0 (31.0–40.0)	36.0 (32.0–41.0)	0.146
Storage duration, years			0.997
≤1	809 (48.5)	858 (51.5)	
>1–≤2	42 (47.7)	46 (52.3)	
>2–≤3	48 (50.0)	48 (50.0)	
>3–≤4	22 (46.8)	25 (53.2)	
>4	12 (48.0)	13 (52.0)	
Parity			
Nulliparous	316 (65.6)	166 (34.4)	<0.001
Primiparous	230 (41.4)	325 (58.6)	
Multiparous	387 (43.7)	499 (56.3)	
AMH, ng/ml	2.4 (1.2–4.3)	2.4 (1.2–3.8)	0.578
Body mass index at OCR, kg/m^2	26.7 (23.6–30.1)	26.0 (22.7–29.6)	0.001
Quality of transferred embryos			
Good	689 (44.5)	859 (55.5)	<0.001
Fair	86 (60.1)	57 (39.9)	
Poor	158 (68.1)	74 (31.9)	
FET protocol			
HRT	565 (53.4)	493 (46.6)	<0.001
Natural cycle	368 (42.5)	497 (57.5)	

Values reported as median (interquartile range) or *n* (%).

[a] Wilcoxon rank sum test or chi-squared test.

AMH, anti-Mullerian hormone; OCR, oocyte retrieval; FET, frozen embryo transfer; HRT, hormone replacement therapy.

at least one pregnancy previously or underwent FET in a natural cycle.

The lack of effect of female age on the OPR supports the results of *Irani et al. (2019)*, but contrasts with the results of *Reig et al. (2020)* (8175 single euploid blastocyst transfers) and the systematic review and meta-analysis by *Vitagliano et al. (2023)*, including seven studies with 11,335 single euploid blastocyst transfers. Registry-based studies, such as the study by Vitagliano et al., permit the inclusion of high numbers of patients, but detailed, patient-specific information may not be captured due to heterogeneity of the included studies, and the lack of individual participant data for possible confounders.

Nonetheless, it is critical to evaluate further patient features as they may be critical to the implantation process and the maintenance of pregnancy. This gap was covered, in part, by *Reig et al. (2020)* through inclusion of BMI, male age, ovarian reserve parameters (AFC, AMH and basal FSH), day of embryo transfer and embryo quality as well as female age. Their findings are partially in line with the present results, confirming the significant impact of embryo quality. *Reig et al. (2020)* categorized embryo quality as 'excellent', 'good' or 'poor', whereas, in the present study, the detailed analysis of expansion grade, trophectoderm/ICM quality, and the day of sufficient blastocyst quality for performing the trophectoderm biopsy provides more insight into the different aspects of embryo quality. In the adjusted

TABLE 2 RESULTS OF MULTIVARIABLE LOGISTIC REGRESSION ANALYSIS

Predictor	OR	95% CI	*P*-value[a]
Female age at OCR, years			
≤35	Reference	–	–
36–37	0.93	0.68–1.27	0.640
38–40	1.09	0.82–1.44	0.559
>40	1.12	0.79–1.57	0.534
Body mass index, kg/m^2			
Normal weight (≥18–<25)	Reference		
Underweight (<18)	0.71	0.29–1.74	0.457
Overweight (≥25–<30)	0.74	0.59–0.92	0.008
Obese (≥30)	0.71	0.55–0.92	0.009
Embryo quality			
Fair			
Good	1.94	1.34–2.80	<0.001
Poor	0.70	0.45–1.11	0.133
Endometrial preparation protocol			
HRT	Reference	–	–
Natural cycle	1.50	1.23–1.83	<0.001

[a] Mixed-effects logistic regression analysis.

OCR, oocyte retrieval; FET, frozen embryo transfer; HRT, hormone replacement therapy.

analysis, biopsy day and trophectoderm/ICM quality remained significant factors for the likelihood of ongoing pregnancy. Compromised mitochondrial DNA integrity, altered mitochondrial morphology or an altered follicular micro-environment might constitute the mechanism leading to lower embryo quality in women of advanced maternal age (*Cimadomo et al., 2018*). Associations between ovarian aging and a reduced number of mitochondrial copies, oxidative damage and apoptosis was seen in mouse models (*Marchante et al., 2023*). More research is required to fully understand the factors associated with the loss of embryo quality.

The absence of effect of male age on the OPR is a further common finding between the present results and the study by *Reig et al. (2020)*. This contrasts with the study by *Farabet et al. (2024)*, which reported a lower live birth rate following single euploid FET when the male partner was >45 years old. Contrary to *Reig et al. (2020)*, the present study did not find that AMH, an ovarian reserve parameter, affected the OPR, and there were also disparities regarding the influence of BMI between the studies. Whereas no influence was seen by *Reig et al. (2020)*, the present study found a highly significant negative impact of BMI on the OPR, which is in line with several recent publications, including large datasets (*Bakkensen et al., 2023*; *Melado Vidales et al., 2023*; *Zheng et al., 2024*).

A history of at least one previous pregnancy significantly increased the likelihood of ongoing pregnancy after single euploid FET compared with women without a history of previous pregnancy. This was reported previously (*Nelson and Lawlor, 2011*) based on data from the UK Human Fertilization and Embryology Authority database. The precise reasons for this remain unknown, but could point towards a 'functioning' reproductive system of the prospective mother.

Endometrial proliferation and secretory transformation depend on the presence of oestrogen and progesterone, and their actions are mediated through the corresponding receptors in endometrial tissue. This function might be compromised by age-related down-regulation of both receptors, and diminished capacity of the endometrium to absorb the steroids, leading to changes in endometrial receptivity (*Pathare et al., 2023*). Studies in oocyte donation cycles, where oocyte quality can be controlled, attempted to evaluate the effect of age on endometrial receptivity, but the results did not consistently indicate a decrease in the rate of conception with increasing age of the recipient (*Gupta et al., 2012*; *Kelada et al., 2008*; *Pathare et al., 2023*; *Paulson, 2014*). In FET cycles, endometrial receptivity can be reached by an HRT cycle or a natural cycle. According to the literature (*Ghobara et al., 2017*; *Groenewoud et al., 2013*), neither approach is superior to the other in terms of achieving pregnancy, but recent evidence indicates a lower risk for early miscarriage (*Roelens and Blockeel, 2022*) and for the development of pregnancy-induced hypertension and pre-eclampsia in natural cycles with the presence of a corpus luteum (*von Versen-Höynck et al., 2019*). Given that women of advanced maternal age are at increased risk for pre-eclampsia (*Lopian et al., 2023*), the preferred endometrial preparation – if feasible – should be the natural cycle approach. The present study found no difference in implantation rate between the HRT and natural cycle approaches, but found a higher miscarriage rate associated with the HRT cycle approach. This led to a highly significant increase in the likelihood of ongoing pregnancy for the natural cycle approach. On the other hand, *Reig et al. (2020)* described a decreased implantation rate as the cause of the lower clinical and live birth rates and not a higher pregnancy loss rate; however, they did not mention the endometrial preparation protocol in their study. More research is required to fully understand the endometrial aging process, its potential effects on endometrial receptivity, and whether a natural cycle approach might mitigate age-related changes in endometrial receptivity.

Since the development of PGT-A, platforms have evolved and their accuracy has increased. While all platforms can accurately assess aneuploidy of full chromosomes while displaying low error rates, not all platforms can reliably identify unbalanced translocations, segmental aneuploidies, polyploidy or mosaicism. Compared with other platforms, NGS offers the advantage of being able to detect and screen for partial aneuploid and mosaic embryos, which are less viable, and enhance the OPR by not transferring these embryos (*Friedenthal et al., 2018*). While NGS was the platform of choice for the present analysis, the meta-analysis of *Vitagliano et al. (2023)* comprised three studies which used NGS (*Munné et al., 2019*; *Reig et al., 2020*; *Tong et al., 2021*), three studies which used microarray comparative genomic hybridization (CGH) (*Harton et al., 2013*; *Irani et al., 2019*;

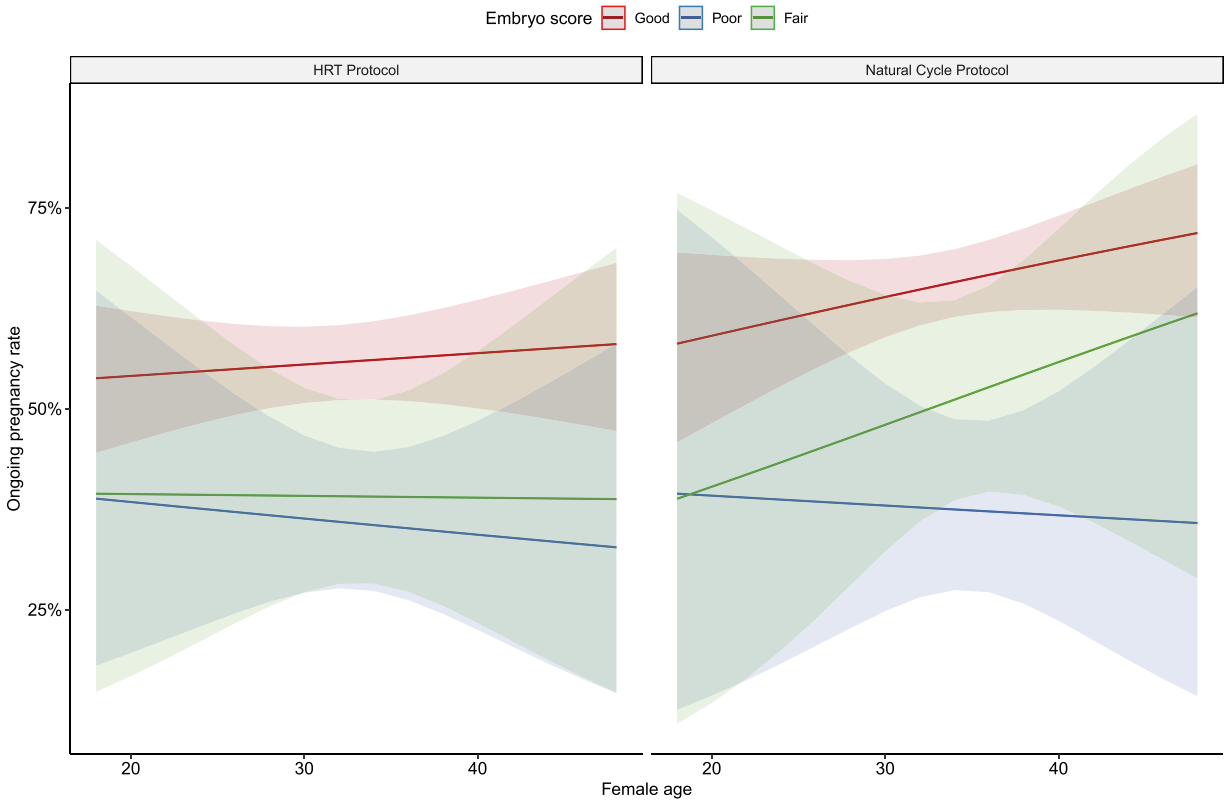

FIGURE 1 Association of female age at oocyte retrieval with ongoing pregnancy rate after allowing interaction between female age, embryo quality and endometrial preparation protocol. The graph depicts the association between ongoing pregnancy rate and female age for each endometrial preparation and according to embryo quality. Solid lines show the predicted ongoing pregnancy rate, and shaded areas depict the 95% prediction intervals.

Whitney et al., 2014) — of note, the data by *Whitney et al. (2014)* were only published as an abstract — and one multicentre study with no information regarding the platform (*Yan et al., 2021*). The discrepancy between the findings of *Vitagliano et al. (2023)* and the present study could theoretically be attributed to differing accuracy of the platforms in terms of the detection of subtle chromosomal abnormalities by microarray CGH, which would lead to a lower OPR/live birth rate (*Zhang et al., 2020*).

The current data contradict the findings of *Reig et al. (2020)* and *Vitagliano et al. (2023)* by clearly demonstrating no decrease in the OPR with increasing female age in single euploid FET cycles. While there is no doubt that ovarian aging may contribute to a decline in embryo quality, which is a major contributor to the reduced OPR, the age of the women did not, in itself, appear to have a substantial

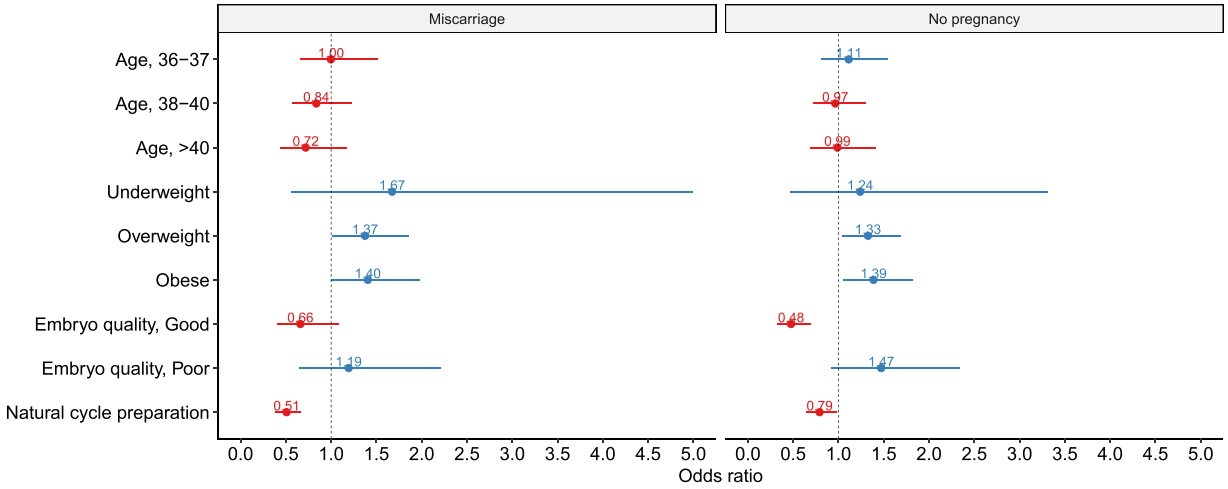

FIGURE 2 Multinomial regression analyses with ongoing pregnancy as the reference group. Odds ratios in blue (>1) indicate that the variable increases the odds of response (miscarriage or no pregnancy) compared with the reference group (ongoing pregnancy), and odds ratios in red (<1) indicate the opposite. Horizontal lines depict the 95% prediction intervals.

impact. The retrospective design of this study may be viewed as a limitation, as well as the subjectivity of classification of embryo quality, but the use of a single NGS platform for PGT-A and thorough analysis of patient-specific characteristics are strengths, as some of the factors that significantly affect the OPR have not been considered in previous research studying the influence of female age on the outcomes of single euploid FET cycles. Given the prospect that the trend towards delaying motherhood/parenthood may continue in future, further research into the aging process of human reproduction is required to better understand the key components of this process.

DATA AVAILABILITY

Data will be made available on request.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.104074.

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ARTICLE

Association between endometrial thickness and birthweight of singletons from vitrified-warmed cycles: a retrospective cohort study

BIOGRAPHY

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KEY MESSAGE

An inverted U-shaped non-linear association was observed between continuous endometrial thickness (EMT) and neonatal birthweight in frozen-thawed embryo transfer cycles. EMT is more closely associated with the birthweight in hormone replacement therapy cycles. An appropriate EMT could benefit neonatal health but more research is needed to clarify the relationship between EMT and perinatal outcomes.

ABSTRACT

Research question: What is the association between endometrial thickness (EMT) and the birthweight of singleton infants born from frozen-thawed embryo transfer cycles?

Design: This retrospective cohort study was conducted from January 2016 to December 2019. Participants were categorized into a natural cycle (NC, $n = 8132$) group and hormone replacement therapy (HRT, $n = 4975$) group. Only singleton deliveries were included. The primary outcomes were measures of birthweight and relevant indexes. Multivariable logistic regression and multivariable-adjusted linear regression models that incorporated restricted cubic splines were used.

Results: In the HRT group, the risk of delivering a small for gestational age (SGA) infant was increased in women with an EMT <8.0 mm (adjusted odds ratio [aOR] 1.85, 95% confidence interval [CI] 1.17–2.91) compared with women with an EMT of 8.0 to <12.0 mm, and increased with an EMT ≥12.0 mm (aOR 1.85, 95% CI 1.03–3.33). An inverted U-shaped relationship was found between EMT and birthweight in women with HRT. No significant differences were shown in

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KEY WORDS
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Hormone replacement
Natural cycle
Small for gestational age

birthweight z-score, or being SGA or large for gestational age, in singletons among the three EMT groups in the natural cycles.

Conclusions: A thinner endometrium seen in women undergoing HRT cycles was associated with a lower birthweight z-score, as well as a higher risk of SGA. However, no significant association was observed between EMT and birthweight z-score or SGA in the NC group. It is noteworthy that a thicker endometrium was not associated with a higher birthweight in frozen-thawed embryo transfer (FET) cycles. Women with a thin endometrium who achieve pregnancy require specialized attention, particularly if they are undergoing FET with HRT cycles.

INTRODUCTION

Over the past decade, the use of frozen-thawed embryo transfers (FET) has consistently increased, comprising over 30–40% of all transfers in numerous regions worldwide (*De Geyter et al., 2020*; *Roque et al., 2019*). Previous studies have demonstrated that FET with a single frozen blastocyst results in a higher rate of singleton live births compared with fresh single-blastocyst transfer in ovulatory women with a favourable prognosis (*Wei et al., 2019*). However, FET has been associated with an elevated risk of abnormal birthweight (*Wei et al., 2019*), which may lead to subsequent infections (*Collins et al., 2018*; *Fleiss et al., 2022*; *Levy 2007*), premature mortality (*Brumbaugh et al., 2019*; *Manuck, 2017*) and future adverse chronic diseases in adulthood (*Nishizaki and Shimizu, 2022*). Nevertheless, the mechanisms underlying the observed neonatal outcome risks in FET cycles remain unclear and require further elucidation.

Endometrial preparation is widely acknowledged as a crucial factor in the success of FET (*Rosalik et al., 2022*). Complete development of the endometrium is considered to be one of the most important aspects affecting early embryo implantation and placental formation (*Norwitz et al., 2001*; *Park et al., 2022*). Endometrial thickness (EMT) has been previously investigated as a potential predictor of endometrial receptivity (*Cheng et al., 2021*). Moreover, studies have shown that a thin endometrium is associated with lower rates of implantation, pregnancy and cumulative pregnancy in fresh transfer cycles following IVF and intracytoplasmic sperm injection (ICSI) (*Liao et al., 2021*). Du and colleagues discovered that an EMT ≤7.5 mm on the day of human chorionic gonadotrophin (HCG) triggering was an independent risk factor for low birthweight (LBW) in singleton pregnancies resulting from fresh single-blastocyst transfer (*Du et al., 2021*). The current authors' previous research, conducted through a retrospective cohort study of 9273 singleton births, revealed that a thinner endometrium was linked to a lower birthweight and birthweight z-score, as well as a higher risk of small for gestational age (SGA) in fresh IVF/ICSI embryo transfer cycles (*Liu et al., 2021*).

However, few studies have addressed the impact of EMT on neonatal outcomes in FET, particularly the effect of increased EMT, which remains inconclusive and controversial (*Liao et al., 2021*; *Rombauts et al., 2014*; *Weissman et al., 1999*; *Zhang et al., 2005*; *Zhang et al., 2019*). Additionally, most previously published studies have employed EMT as a categorical variable to determine an optimal range, thereby impeding the exploration of a clear dose–response relationship between EMT and birthweight.

Currently, the most commonly employed endometrial preparation regimens for FET include the natural cycle and hormone replacement therapy (HRT) regimens. According to a Cochrane review published in 2017, which encompassed 18 randomized controlled trials, several studies have indicated that both natural cycle and HRT regimens can achieve favourable EMT with similar clinical outcomes, including live birth rates and miscarriage rates (*Ghobara et al., 2017*). HRT cycles are widely used in clinical settings due to their scheduling flexibility, while also requiring less monitoring and enabling a more reliable determination of the optimal implantation window.

However, a recent systematic review and meta-analysis demonstrated that endometrial preparation protocols involving HRT were associated with poorer obstetric and perinatal outcomes (*Busnelli et al., 2022*). Moreover, Zhou and colleagues found that singletons resulting from HRT-cycle FET had a higher risk of being large for gestational age (LGA), suggesting that the natural cycle regimen may be more beneficial in women who are ovulating (*Zhou et al., 2022*). As both EMT and endometrial preparation regimens can impact newborn birthweight, it is necessary to separately analyse the effect of EMT on neonatal outcomes after FET based on different endometrial preparation regimens.

In this retrospective cohort study, the aim was to assess the association between EMT prepared through either natural cycle or HRT cycles and infant birthweight. EMT was analysed as both a categorical and a continuous variable. The authors anticipate that their findings will contribute to the enhancement of perinatal outcomes by providing more evidence-based information for effective clinical management.

MATERIALS AND METHODS

Study design and participants

This retrospective cohort study was undertaken at the Center for Reproductive Medicine affiliated to Shandong University. The inclusion criteria were patients with a maternal age of ≤45 years who underwent FET and achieved singleton delivery after 28 weeks of gestation. The exclusion criteria comprised women undergoing sperm and oocyte donation cycles, women with congenital uterine malformations, women with multiple births and those with missing core data (e.g. unknown EMT, infant birthweight or body mass index [BMI]). In cases where a single patient had multiple deliveries, only the first pregnancy was included.

A total of 19,561 patients presented between January 2016 and December 2019; once the inclusion and exclusion criteria had been applied, 6454 (32.99%) patients were excluded. Among the excluded cases, 2566 (39.76%) were associated with a failure of data extraction related to EMT, 34 (0.53%) had no BMI data, and 412 (6.38%) lacked infant birthweight information. Finally, 13,107 patients were selected for the study. Approximately 99.5% of the patients

underwent vitrified-warmed blastocyst transfer.

Endometrial preparation and EMT assessment

The decision to use a natural cycle or HRT cycle for endometrial preparation was based on factors such as regular ovulation cycles, patient and physician preferences and cost considerations. After applying the inclusion and exclusion criteria, a sample of 13,107 patients remained, comprising 8132 who underwent the natural cycle protocol and 4975 who underwent the HRT protocol.

In the natural cycle protocol, ultrasound monitoring was initiated between days 8 and 12 of the menstrual cycle, with urine LH and serum hormone concentrations used to predict the timing of ovulation. Ovulation occurred spontaneously or was induced by HCG, followed by the transfer of cryopreserved blastocysts 5 days after ovulation. Assessment of EMT in natural cycles was performed on the day when the (theoretical) ovulation day and the transfer day could be decided. The maximal distance from one interface between the endometrium and myometrium to the other in the mid-sagittal plane of the uterus including the cervical canal was measured using ultrasonography (GE Medical Systems, Co., Ltd). The minimum thickness accepted for embryo transfer in natural cycles was 4 mm in the present study.

In the HRT cycle protocol, exogenous oestradiol and progesterone were administered to induce endometrial proliferation and transformation while preventing follicular development. The day of progesterone administration was designated as day 0, and blastocyst transfer was scheduled for the 5th day of progesterone administration. In HRT cycles, EMT was assessed before starting progesterone support. The minimum thickness accepted for embryo transfer in HRT cycles was 0.5 mm in present study.

Patient follow-up

As mentioned in a previous study (*Zhang et al., 2022*), the first follow-up was performed around the 14th day after embryo transfer, and biochemical pregnancy was assessed by measuring the serum concentration of the HCG beta subunit. The second follow-up was performed at 5 and 6 weeks after embryo transfer, and clinical pregnancy was detected if gestational sacs were confirmed by transvaginal ultrasonography. Ongoing pregnancy was confirmed at the third follow-up, which was performed at the 12th week of gestation (around 9–10 weeks after ET (embryo transfer)). Subsequently, participants received telephone surveys and standardized questionnaires from trained nurses. The information collected included perinatal complications, number of gestational weeks, birth date, delivery mode, gender and birthweight of the newborn and neonatal diseases, treatment and prognosis. All follow-up information was recorded in the electronic medical records.

Outcome measures

The primary outcomes of this study were birthweight measures, including absolute birthweight, *z*-score, LBW (birthweight <2500 g), high birthweight (HBW; birthweight ≥4000 g), SGA (birthweight <10th percentile of the reference birthweights), LGA (birthweight >90th percentile of the reference birthweights) and incidence of preterm birth (gestational week <37 weeks). Birthweight percentiles and *z*-scores were calculated based on the Chinese singleton birthweight reference (*Dai et al., 2014*). The *z*-score was used to standardize the birthweight after adjusting for gestational age and neonatal sex, and the formula for calculating the *z*-score was obtained from a previous article (*Liu et al., 2021*).

Covariates

Covariates included maternal and paternal age, BMI, educational level, type and cause of infertility, polycystic ovary syndrome, type of assisted reproductive technology (ART), number of embryos transferred, number of previous fresh embryo transfer attempts, parity, history of Caesarean section, pregnancy complications (including hypertensive disorders of pregnancy, gestational diabetes mellitus, placenta praevia and placental abruption) and sex of the neonate.

Statistical analysis

All data were analysed using SAS 9.4 (SAS institute Inc., Cary, NC, USA). Continuous variables were presented as mean ± standard deviation (SD), while categorical variables were expressed as numbers and percentages. Differences in variables across groups were examined using Independent samples t-test (for comparisons between two groups), one-way analysis of variance (for comparisons between three groups) or chi-squared tests. Post-hoc pairwise comparison was performed using Bonferroni's correction. Statistical significance was defined as a two-tailed $P < 0.05$. The relationship between the exposure (EMT) and the outcome (neonatal birthweight) was investigated using the following approaches.

Categorized EMT values

Continuous EMT values were divided into three groups based on the 10th and 90th percentiles of the EMT (thin, <8 mm; moderate, 8 ≤ EMT <12 mm; thick, ≥12 mm). Multivariable multinomial logistic regression models adjusted for covariates were used to examine the associations between the categories of EMT and LBW, HBW, SGA and LGA. The reference group consisted of patients with a moderate EMT (8–12 mm). Effect estimates are presented as odds ratios (OR) with 95% confidence intervals (CI).

Continuous EMT values

Restricted cubic splines (RCS) were employed to model non-linear associations in linear regression models (*Harrell, Lee, and Mark, 1996*; *Marrie, Dawson, and Garland, 2009*). RCS models with four knots placed at the 5th, 35th, 65th and 95th percentiles of EMT were used to flexibly analyse the non-linear relationship between continuous EMT and neonatal birthweight. An EMT of 8 mm was set as the reference value. In the preliminary analysis, receiver operating characteristic curves were plotted for each birthweight-related binary outcome (LBW, SGA and LGA) and the optimal cut-off threshold for 'thin' endometrium to discriminate LBW, SGA and LGA was found to be 8.08, 8.12 and 7.86 mm, respectively. The findings of several previous studies (*Mahutte et al., 2022*, *Shalom-Paz et al., 2021*, *Zhang et al., 2019*) were also referred to. Finally, 8 mm was set as the reference value.

Sensitivity analysis

The birthweight *z*-score was used instead of birthweight in linear regression models with RCS to conduct sensitivity analyses to assess the robustness of the findings.

Ethical approval

The study was approved by the Institutional Review Board of the Centre for Reproductive Medicine affiliated to Shandong University on 16 May 2023 (reference number 2023-40). Written informed consent was obtained from all the participants.

TABLE 1 BASELINE CHARACTERISTICS OF THE STUDY POPULATION, CLASSIFIED ACCORDING TO ENDOMETRIAL THICKNESS (EMT)

Characteristics	Overall (*n* = 13,107)	EMT			*P*-value
		<8 mm (*n* = 917)	8 to <12 mm (*n* = 10,226)	≥12 mm (*n* = 1964)	
Maternal age (years)	30.40 ± 4.24	31.15 ± 4.38	30.37 ± 4.23	30.23 ± 4.19	<0.001
Maternal BMI (kg/m^2)	23.34 ± 3.50	23.06 ± 3.35	23.34 ± 3.51	23.45 ± 3.54	0.021
Paternal age (years)	31.46 ± 4.85	32.13 ± 5.19	31.44 ± 4.85	31.21 ± 4.68	<0.001
Paternal BMI (kg/m^2)	25.89 ± 4.04	25.95 ± 3.97	25.87 ± 4.01	26.00 ± 4.22	0.360
Maternal educational level, *n* (%)					0.986
Primary school or below	4996 (38.1)	351 (38.3)	3892 (38.1)	753 (38. 3)	
High school	5387 (41.1)	379 (41.4)	4211 (41.2)	797 (40.6)	
College or above	2724 (20.8)	187 (20.4)	2123 (20.8)	414 (21.1)	
Paternal educational level, *n* (%)					0.976
Primary school or below	4336 (33.1)	308 (33.6)	3371 (33.0)	657 (33.5)	
High school	5902 (45.0)	405 (44.2)	4616 (45.1)	881 (45.0)	
College or above	2869 (21.9)	204 (22.3)	2239 (21.9)	426 (21.7)	
Type of infertility, *n* (%)					<0.001
Primary	6561 (50.1)	292 (31.9)	5152 (50.4)	1117 (56.8)	
Secondary	6546 (49.9)	625 (68.2)	5074 (49.6)	847 (43.1)	
Polycystic ovary syndrome, *n* (%)					<0.001
No	10,410 (79.4)	702 (76.6)	8032 (78.5)	1676 (85.3)	
Yes	2697 (20.6)	215 (23.4)	2194 (21.5)	288 (14.7)	
Parity, *n* (%)					<0.001
1	10,311 (78.7)	660 (72.0)	8088 (79.1)	1563 (79.6)	
2 or more	2796 (21.3)	257 (28.0)	2138 (20.9)	401 (20.4)	
History of Caesarean section, *n* (%)					<0.001
No	12,011 (91.6)	818 (89.2)	9361 (91.5)	1832 (93.3)	
Yes	1096 (8.4)	99 (10.8)	865 (8.5)	132 (6.7)	
Type of ART treatment, *n* (%)					0.002
IVF	7607 (58.0)	550 (60.0)	5952 (58.2)	1105 (56.2)	
ICSI	5252 (40.1)	350 (38.2)	4062 (39.7)	840 (42.8)	
IVF+ICSI	248 (1.9)	17 (1.9)	212 (2.1)	19 (1.0)	
Number of embryos transferred, *n* (%)					0.110
1	12,538 (95.7)	882 (96.2)	9762 (95.5)	1894 (96.4)	
2	569 (4.3)	35 (3.8)	464 (4.5)	70 (3.6)	
Number of previous fresh embryo transfer attempts, *n* (%)					<0.001
0	8737 (66.7)	660 (72.0)	6828 (66.8)	1249 (63.6)	
1	4370 (33.3)	257 (28.1)	3398 (33.2)	715 (36.4)	
Pregnancy complications, *n* (%)	2090 (15.9)	185 (20.2)	1584 (15.5)	321 (16.3)	0.001
Hypertensive disorders of pregnancy	969 (7.4)	96 (10.5)	734 (7.2)	139 (7.1)	0.001
Gestational diabetes mellitus	1136 (8.7)	90 (9.8)	864 (8.4)	182 (9.3)	0.220
Placenta praevia	132 (1.0)	12 (1.3)	101 (1.0)	19 (1.0)	0.636
Placental abruption	14 (0.1)	2 (0.2)	8 (0.1)	4 (0.2)	0.168
Gender of neonate, *n* (%)					0.165
Male	6924 (52.8)	496 (54.1)	5428 (53.1)	1000 (50.9)	
Female	6183 (47.2)	421 (45.9)	4798 (46.9)	964 (49.1)	

Values are presented as mean ± standard deviation or *n* (%).

Differences in variables across EMT categories were examined using analysis of variance (for continuous variables) or chi-squared test (for categorical variables), and the *P*-values for these tests are presented.

ART, assisted reproductive technology; BMI, body mass index; EMT, endometrial thickness; ICSI, intracytoplasmic sperm injection.

RESULTS

Baseline characteristics

A total of 13,107 patients who met the inclusion criteria were included in this analysis. The baseline characteristics of the study population are presented in TABLE 1. The study included 917 (7.0%) women with a thin endometrium (EMT <8 mm), 10,226 (78.0%) with a moderate endometrium (8 ≤ EMT <12 mm) and 1964 (15.0%) with a thick endometrium (EMT ≥ 12 mm). The overall mean (SD) maternal and paternal ages were 30.40 (4.24) and 31.46 (4.85) years, respectively. The mean (SD) maternal and paternal BMI were 23.34 (3.50) and 25.89 (4.04) kg/m^2, respectively. Primary infertility was observed in 50.1% of the patients, while 49.9% had secondary infertility. The majority of patients were primipara. Additionally, 8.4% of the women had a history of Caesarean section, and 15.9% had pregnancy complications. Further details can be found in TABLE 1.

In the sample, 8132 and 4975 live-born singletons were conceived through natural cycle and HRT cycle FET, respectively. The detailed baseline characteristics for each group are presented in TABLE 2, revealing significant differences in many baseline characteristics between the two groups, indicating imbalances. Since endometrial preparation is considered a crucial factor in the success of FET, a subgroup analysis stratified by natural cycle or HRT cycles was conducted, and thus the relationship between EMT and outcomes should be investigated in both groups.

NC group

Among the natural cycle (NC) group, 369, 6136 and 1627 women had thin (EMT <8 mm), moderate (8 ≤ EMT <12 mm) and thick (EMT ≥ 12 mm) endometrium, respectively, as shown in TABLE 3. Women with a thin endometrium had a higher proportion of secondary infertility (69.6% versus 52.4%, $P < 0.001$), a greater proportion of previous deliveries (32.8% versus 23.4%, $P < 0.001$), a higher proportion of history of Caesarean sections (14.9% versus 9.4%, $P < 0.001$) and a higher incidence of pregnancy complications (17.9% versus 13.3%, $P = 0.02$) compared with women with a moderate endometrium. On the other hand, women with a thick endometrium had a lower proportion of secondary infertility (44.1% versus 52.4%, $P < 0.001$) and a lower proportion of history of Caesarean section (7.1% versus 9.4%, $P < 0.001$) compared with those with a moderate endometrium.

TABLE 2 COMPARISONS OF BASELINE CHARACTERISTICS BETWEEN WOMEN WITH A NATURAL CYCLE AND THOSE RECEIVING HORMONE REPLACEMENT THERAPY

Characteristics	NC group (*n* = 8132)	HRT (*n* = 4975)	*P*-value
Maternal age (years)	30.74 ± 4.32	29.85 ± 4.04	<0.001
Maternal BMI (kg/m^2)	22.92 ± 3.33	24.01 ± 3.67	<0.001
Paternal age (years)	31.76 ± 4.96	30.96 ± 4.63	<0.001
Paternal BMI (kg/m^2)	25.88 ± 4.87	26.86 ± 4.54	0.291
Maternal educational level, *n* (%)			<0.001
Primary school or below	2986 (36.7)	2010 (40.4)	
High school	3340 (41.1)	2047 (41.1)	
College or above	1806 (22.2)	918 (18.5)	
Paternal educational level, *n* (%)			<0.001
Primary school or below	2551 (31.4)	1785 (35.9)	
High school	3660 (45)	2242 (45.1)	
College or above	1921 (23.6)	948 (19.1)	
Type of infertility, *n* (%)			<0.001
Primary	3944 (48.5)	2617 (52.6)	
Secondary	4188 (51.5)	2358 (47.4)	
Polycystic ovary syndrome, *n* (%)			<0.001
No	7434 (91.4)	2976 (59.8)	
Yes	698 (8.6)	1999 (40.2)	
Parity, *n* (%)			<0.001
1	6223 (76.5)	4088 (82.2)	
2 or more	1909 (23.5)	887 (17.8)	
History of Caesarean section, *n* (%)	747 (9.2)	349 (7.0)	<0.001
Type of ART treatment, *n* (%)			<0.001
IVF	4621 (56.8)	2986 (60.0)	
ICSI	3372 (41.5)	1880 (37.8)	
IVF+ICSI	139 (1.7)	109 (2.2)	
Number of embryos transferred, *n* (%)			0.008
1	7809 (96)	4729 (95.1)	
2	323 (4)	246 (4.9)	
Number of previous fresh embryo transfer attempts, *n* (%)			<0.001
0	5018 (61.7)	3719 (74.8)	
1	3114 (38.3)	1256 (25.2)	
Pregnancy complications, *n* (%)	1127 (13.9)	963 (19.4)	<0.001
Gender of neonate, *n* (%)			0.037
Male	4238 (52.1)	2686 (54.0)	
Female	3894 (47.9)	2289 (46)	

Values are presented as mean ± standard deviation or *n* (%).

Differences in variables between the NC and HRT groups were examined using a Independent samples t-test (for continuous variables) or chi-squared test (for categorical variables), and the *P*-values for these tests are presented.

ART, assisted reproductive technology; BMI, body mass index; HRT, hormone replacement therapy; ICSI, intracytoplasmic sperm injection; NC natural cycle.

HRT group

Among the HRT cycle group, 548, 4090 and 337 women had a thin, moderate and

TABLE 3 BASELINE CHARACTERISTICS OF WOMEN WITH NATURAL CYCLES, CLASSIFIED ACCORDING TO EMT

		EMT			
Characteristics	**Overall (*n* = 8132)**	**<8 mm (*n* = 369)**	**8 to <12 mm (*n* = 6,136)**	**≥12 mm (*n* = 1,627)**	***P*-value**
Maternal age (years)	30.74 ± 4.32	32.00 ± 4.47	30.76 ± 4.33	30.39 ± 4.22	<0.001[a,b,c]
Maternal BMI (kg/m^2)	22.92 ± 3.33	23.11 ± 3.20	22.84 ± 3.32	23.19 ± 3.37	<0.001[c]
Paternal age (years)	31.76 ± 4.96	33.00 ± 5.37	31.80 ± 4.98	31.36 ± 4.73	<0.001[a,b,c]
Paternal BMI (kg/m^2)	25.88 ± 4.87	25.88 ± 4.15	25.85 ± 5.07	25.96 ± 4.19	0.731
Maternal educational level, *n* (%)					0.986
Primary school or below	2986 (36.7)	133 (36.0)	2254 (36.7)	599 (36.8)	
High school	3340 (41.1)	154 (41.7)	2526 (41.2)	660 (40.6)	
College or above	1806 (22.2)	82 (22.2)	1356 (22.1)	368 (22.6)	
Paternal educational level, *n* (%)					0.747
Primary school or below	2551 (31.4)	105 (28.5)	1926 (31.4)	520 (32.0)	
High school	3660 (45.0)	176 (47.7)	2755 (44.9)	729 (44.8)	
College or above	1921 (23.6)	88 (23.8)	1455 (23.7)	378 (23.2)	
Type of infertility, *n* (%)					<0.001[a,b,c]
Primary	3944 (48.5)	112 (30.4)	2922 (47.6)	910 (55.9)	
Secondary	4188 (51.5)	257 (69.6)	3214 (52.4)	717 (44.1)	
Polycystic ovary syndrome, *n* (%)					0.131
No	7434 (91.4)	347 (94.0)	5610 (91.4)	1,477 (90.8)	
Yes	698 (8.6)	22 (6.0)	526 (8.6)	150 (9.2)	
Parity, *n* (%)					<0.001[a,b]
1	6223 (76.5)	248 (67.2)	4702 (76.6)	1273 (78.2)	
2 or more	1909 (23.5)	121 (32.8)	1434 (23.4)	354 (21.8)	
History of Caesarean section, *n* (%)	747 (9.2)	55 (14.9)	577 (9.4)	115 (7.1)	<0.001[a,b,c]
Type of ART treatment, *n* (%)					0.035[c]
IVF	4621 (56.8)	220 (59.6)	3504 (57.1)	897 (55.1)	
ICSI	3372 (41.5)	144 (39.0)	2515 (41.0)	713 (43.8)	
IVF+ICSI	139 (1.7)	5 (1.4)	117 (1.9)	17 (1.0)	
Number of embryos transferred, *n* (%)					0.328
1	7809 (96.0)	356 (96.5)	5881 (95.8)	1572 (96.6)	
2	323 (4.0)	13 (3.5)	255 (4.2)	55 (3.4)	
Number of previous fresh embryo transfer attempts, *n* (%)	0.425				
0	5018 (61.7)	234 (63.4)	3762 (61.3)	1022 (62.8)	
1	3114 (38.3)	135 (36.6)	2374 (38.7)	605 (37.2)	
Pregnancy complications, *n* (%)[a]	1,127 (13.9)	66 (17.9)	819 (13.3)	242 (14.9)	0.02

Values are presented as mean ± standard deviation or *n* (%).

Differences in variables across EMT categories were examined using analysis of variance (for continuous variables) or chi-squared test (for categorical variables), and the *P*-values for these tests are presented. Post hoc pairwise comparison was performed using Bonferroni's correction.

[a] refers to the comparison between "EMT < 8 mm and 8 ≤ EMT < 12 mm" is significant.

[b] refers to the comparison between "EMT < 8 mm and EMT ≥ 12 mm" is significant.

[c] refers to the comparison between "8 ≤ EMT < 12 mm and EMT ≥ 12 mm" is significant.

Due to the large number of results, the P-value of the post-hoc pairwise comparison results is not shown in the table.

ART, assisted reproductive technology; BMI, body mass index; EMT, endometrial thickness; ICSI, intracytoplasmic sperm injection.

thick endometrium, respectively, as shown in TABLE 4. Women with a thin endometrium had a lower BMI (23.03 versus 24.09, $P < 0.001$), a higher proportion of secondary infertility (67.1% versus 45.5%, $P < 0.001$) and a greater proportion of previous deliveries (24.8% versus 17.2%, $P < 0.001$) compared with those with a moderate endometrium. Conversely, women with a thick endometrium had a higher BMI (24.74 versus 24.09, $P < 0.001$), a lower proportion of

TABLE 4 BASELINE CHARACTERISTICS OF WOMEN WITH HORMONE REPLACEMENT THERAPY CYCLE CLASSIFIED ACCORDING TO (EMT)

Characteristics	Overall (*n* = 4975)	EMT			*P*-value
		<8 mm (*n* = 548)	8 to <12 mm (*n* = 4090)	≥12 mm (*n* = 337)	
Maternal age (years)	29.85 ± 4.04	30.58 ± 4.24	29.78 ± 4.01	29.46 ± 3.98	<0.001[a,b]
Maternal BMI (kg/m^2)	24.01 ± 3.67	23.03 ± 3.46	24.09 ± 3.65	24.74 ± 4.02	<0.001[a,b,c]
Paternal age (years)	30.96 ± 4.63	31.54 ± 4.98	30.92 ± 4.60	30.46 ± 4.39	0.001[a,b]
Paternal BMI (kg/m^2)	26.86 ± 4.54	29.69 ± 4.66	26.54 ± 3.84	26.13 ± 4.48	0.299
Maternal educational level, *n* (%)					0.13
Primary school or below	2010 (40.4)	218 (39.8)	1638 (40.1)	154 (45.7)	
High school	2047 (41.1)	225 (41.1)	1685 (41.2)	137 (40.7)	
College or above	918 (18.5)	105 (19.2)	767 (18.8)	46 (13.7)	
Paternal educational level, *n* (%)					0.048[b,c]
Primary school or below	1785 (35.9)	203 (37.0)	1445 (35.3)	137 (40.7)	
High school	2242 (45.1)	229 (41.8)	1861 (45.5)	152 (45.1)	
College or above	948 (19.1)	116 (21.2)	784 (19.2)	48 (14.2)	
Type of infertility, *n* (%)					<0.001[a,b,c]
Primary	2617 (52.6)	180 (32.9)	2230 (54.5)	207 (61.4)	
Secondary	2358 (47.4)	368 (67.1)	1860 (45.5)	130 (38.6)	
Polycystic ovary syndrome, *n* (%)					0.043
No	2976 (59.8)	355 (64.8)	2422 (59.2)	199 (59.1)	
Yes	1999 (40.2)	193 (35.2)	1668 (40.8)	138 (40.9)	
Parity, *n* (%)					<0.001[a,b]
1	4088 (82.2)	412 (75.2)	3386 (82.8)	290 (86.1)	
2 or more	887 (17.8)	136 (24.8)	704 (17.2)	47 (13.9)	
History of Caesarean section, *n* (%)	349 (7.0)	44 (8.0)	288 (7.0)	17 (5.0)	0.237
Type of ART treatment, *n* (%)					0.349
IVF	2986 (60.0)	330 (60.2)	2448 (59.9)	208 (61.7)	
ICSI	1880 (37.8)	206 (37.6)	1547 (37.8)	127 (37.7)	
IVF+ICSI	109 (2.2)	12 (2.2)	95 (2.3)	2 (0.6)	
Number of embryos transferred, *n* (%)					0.49
1	4729 (95.1)	526 (96.0)	3881 (94.9)	322 (95.6)	
2	246 (4.9)	22 (4.0)	209 (5.1)	15 (4.4)	
Number of previous fresh embryo transfer attempts, *n* (%)					0.002[b,c]
0	3719 (74.8)	426 (77.7)	3066 (75.0)	227 (67.4)	
1	1256 (25.2)	122 (22.3)	1024 (25.0)	110 (32.6)	
Pregnancy complications, *n* (%)[c]	963 (19.4)	119 (21.7)	765 (18.7)	79 (23.4)	0.036

Values are presented as mean ± standard deviation or *n* (%).

Differences in variables across EMT categories were examined using analysis of variance (for continuous variables) or chi-squared test (for categorical variables), and the *P*-values for these tests are presented. Post hoc pairwise comparison was performed using Bonferroni's correction.

[a] refers to the comparison between "EMT < 8 mm and 8 ≤ EMT < 12 mm" is significant.

[b] refers to the comparison between "EMT < 8 mm and EMT ≥ 12 mm" is significant.

[c] refers to the comparison between "8 ≤ EMT < 12 mm and EMT ≥ 12 mm" is significant.

Due to the large number of results, the P-value of the post-hoc pairwise comparison results is not shown in the table.

ART, assisted reproductive technology; BMI, body mass index; EMT, endometrial thickness; ICSI, intracytoplasmic sperm injection.

secondary infertility (38.6% versus 45.5%, $P < 0.001$) and a higher proportion of previous fresh embryo transfer attempts (32.6% versus 25%. $P = 0.002$) compared with women with a moderate endometrium.

Neonatal outcomes

The neonatal outcomes categorized by the three EMT groups are presented in TABLE 5.

NC group

In women with natural cycles, no statistically significant differences were observed in the sex of the neonates, birthweight *z*-score, SGA or LGA across the three EMT groups. However, the birthweight in the EMT <8 mm group was significantly lower than in the EMT ≥12 mm group (3383 ± 584 g versus 3456 ± 474 g, $P = 0.032$), and the rates of LBW and preterm birth in the EMT <8 mm group were significantly higher than those in the 8 ≤ EMT <12 mm group and the EMT ≥12 mm group (7.0% versus 3.2% and 2.3%, $P < 0.001$; 8.9% versus 5.7% and 4.9%, $P = 0.009$, respectively). Additional information is provided in TABLE 5.

Furthermore, multivariable multinomial logistic regression analyses, using the 8 ≤ EMT <12 mm group as a reference, revealed that the risk of having an LBW infant was significantly increased in the EMT <8 mm group (adjusted odds ratio [aOR] 2.03, 95% CI 1.31–3.15) and significantly decreased in the EMT ≥12 mm group (aOR 0.70, 95% CI 0.49–1.00). However, no significant differences were found in the risk of having an HBW, SGA or LGA infant in the EMT <8 mm and EMT ≥12 mm groups after adjusting for confounders (including maternal and paternal age, BMI, educational level, type

TABLE 5 NEONATAL OUTCOMES IN GROUPS WITH NORMAL CYCLES AND HRT-INDUCED CYCLES CATEGORIZED ACCORDING TO EMT

		EMT			
Characteristics	**Overall**	**<8 mm**	**8 to <12 mm**	**≥12 mm**	***P*-value**
NC group					
n	8132	369	6136	1627	
Gender of neonate, *n* (%)					0.27
Male	4,238 (52.1)	196 (53.1)	3,223 (52.5)	819 (50.3)	
Female	3,894 (47.9)	173 (46.9)	2,913 (47.5)	808 (49.7)	
Birthweight (g)	3,436 ± 498	3,383 ± 584	3,434 ± 498	3,456 ± 474	0.032[b]
Birthweight *z*-score	0.37 ± 0.67	0.37 ± 0.67	0.36 ± 0.67	0.39 ± 0.66	0.596
LBW, *n* (%)	261 (3.2)	26 (7.0)	197 (3.2)	38 (2.3)	<0.001[a,b]
HBW, *n* (%)	1054 (13.0)	42 (11.4)	801 (13.1)	211 (13.0)	0.650
SGA, n (%)	233 (2.9)	11 (3.0)	181 (2.9)	41 (2.5)	0.647
LGA, *n* (%)	2053 (25.2)	90 (24.4)	1545 (25.2)	418 (25.7)	0.848
Preterm birth, *n* (%)	464 (5.7)	33 (8.9)	352 (5.7)	79 (4.9)	0.009[a,b]
HRT group					
n	4975	548	4090	337	
Gender of neonate, *n* (%)					0.93
Male	2686 (54.0)	300 (54.7)	2205 (53.9)	181 (53.7)	
Female	2289 (46.0)	248 (45.3)	1885 (46.1)	156 (46.3)	
Birthweight (g)	3457 ± 557	3352 ± 583	3472 ± 549	3447 ± 588	<0.001[a,b]
Birthweight *z*-score	0.45 ± 0.70	0.32 ± 0.68	0.47 ± 0.70	0.46 ± 0.70	<0.001[a,b]
LBW, *n* (%)	208 (4.2)	36 (6.6)	153 (3.7)	19 (5.6)	0.003[a]
HBW, *n* (%)	792 (15.9)	70 (12.8)	671 (16.4)	51 (15.1)	0.085
SGA, *n* (%)	137 (2.8)	28 (5.1)	95 (2.3)	14 (4.1)	<0.001[a,c]
LGA, *n* (%)	1,419 (28.5)	115 (21.0)	1200 (29.3)	104 (30.9)	<0.001[a,b]
Preterm birth, *n* (%)	413 (8.3)	53 (9.7)	331 (8.1)	29 (8.6)	0.443

Values are presented as mean ± standard deviation or *n* (%).

Differences in variables across EMT categories were examined using analysis of variance (for continuous variables) or chi-squared tests (for categorical variables), and the *P*-values for these tests are presented.

[a] refers to the comparison between "EMT < 8 mm and 8 ≤ EMT < 12 mm" is significant.

[b] refers to the comparison between "EMT < 8 mm and EMT ≥ 12 mm" is significant.

[c] refers to the comparison between "8 ≤ EMT < 12 mm and EMT ≥ 12 mm" is significant.

Due to the large number of results, the P-value of the post-hoc pairwise comparison results is not shown in the table.

EMT, endometrial thickness; HBW, high birthweight; HRT, hormone replacement therapy; LBW, low birthweight; LGA, large for gestational age; NC, natural cycle; SGA, small for gestational age.

TABLE 6 RELATIONSHIPS BETWEEN EMT AND BIRTHWEIGHT PARAMETERS IN THE NATURAL CYCLE AND HRT GROUPS: MULTIVARIABLE LOGISTIC REGRESSION ANALYSES

EMT	LBW		HBW		SGA		LGA	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Natural cycles								
Unadjusted (*n* = 8132)								
<8 mm	2.25 (1.47–3.45)	<0.001	0.90 (0.64–1.25)	0.512	1.00 (0.54–1.86)	0.999	0.96 (0.75–1.23)	0.736
8 to <12 mm	1 (Reference)		1 (Reference)		1 (Reference)		1 (Reference)	
≥12 mm	0.72 (0.51–1.02)	0.067	0.98 (0.84–1.16)	0.829	0.86 (0.61–1.21)	0.375	1.02 (0.90–1.16)	0.740
Adjusted (*n* = 8132)								
<8 mm	2.03 (1.31–3.15)	0.002	0.86 (0.61–1.20)	0.362	1.03 (0.55–1.92)	0.938	0.91 (0.71–1.16)	0.439
8 to <12 mm	1 (Reference)		1 (Reference)		1 (Reference)		1 (Reference)	
≥12 mm	0.70 (0.49–1.00)	0.048	0.96 (0.81–1.14)	0.645	0.85 (0.60–1.20)	0.348	1.00 (0.88–1.14)	0.968
Hormone replacement cycles								
Unadjusted (*n* = 4975)								
<8 mm	1.74 (1.19–2.53)	0.004	0.77 (0.59–1.01)	0.055	2.03 (1.32–3.14)	0.001	0.66 (0.53–0.82)	<0.001
8 to <12 mm	1 (Reference)		1 (Reference)		1 (Reference)		1 (Reference)	
≥12 mm	1.52 (0.93–2.49)	0.096	0.93 (0.68–1.27)	0.646	1.88 (1.06–3.35)	0.032	1.11 (0.87–1.41)	0.416
Adjusted (*n* = 4975)								
<8 mm	1.84 (1.24–2.73)	0.003	0.83 (0.63–1.09)	0.188	1.85 (1.17–2.91)	0.008	0.70 (0.56–0.87)	0.002
8 to <12 mm	1 (Reference)		1 (Reference)		1 (Reference)		1 (Reference)	
≥12 mm	1.33 (0.80–2.21)	0.270	0.90 (0.66–1.24)	0.523	1.85 (1.03–3.33)	0.039	1.06 (0.83–1.36)	0.626

Adjustment for the multivariate logistic regression analysis included maternal and paternal age, body mass index, educational level, type of infertility, cause of infertility, polycystic ovary syndrome, type of ART treatment, number of embryos transferred, number of previous fresh embryo transfer attempts, parity, history of Caesarean section, pregnancy complications (including hypertensive disorders of pregnancy, gestational diabetes mellitus, placenta praevia and placental abruption) and gender of the neonate.

ART, assisted reproductive technology; CI, confidence interval; EMT, endometrial thickness; HBW, high birthweight; HRT, hormone replacement therapy; LBW, low birthweight; LGA, large for gestational age; OR, odds ratio; SGA, small for gestational age.

and cause of infertility, polycystic ovary syndrome, type of ART, number of embryos transferred, number of previous fresh embryo transfer attempts, parity, history of Caesarean section, pregnancy complications and sex of the neonate). Detailed information is available in TABLE 6.

TABLE 7 shows the overall associations between birthweight, birthweight *z*-score and continuous EMT. Adjusted multivariate results indicated significant non-linear associations between EMT and birthweight ($P < 0.001$), and EMT and birthweight *z*-score ($P = 0.021$) among the total population studied. Significant non-linear associations were observed in women with HRT cycles for both birthweight ($P < 0.001$) and birthweight *z*-score ($P = 0.0021$) with continuous EMT. However, in the NC group, the non-linear association is only present between birthweight and continuous EMT ($P = 0.004$), and not between birthweight *z*-score and continuous EMT ($P = 0.077$).

FIGURE 1 illustrates the relationship between continuous EMT and neonatal birthweight in women with natural cycles, indicating a non-linear inverted U-shaped association. Both too thin and too thick an EMT were relatively associated with trends of decreased birthweight. In this analysis, four knots of the RCS model were placed at an EMT of 8, 9.5, 11 and 13 mm. From the lowest EMT to an EMT of approximately 12 mm in the cohort, the birthweight data showed an increased birthweight with increased EMT. However, when the EMT exceeded 12 mm, neonatal birthweight exhibited a gently decreasing decline. The results of the birthweight *z*-score in women with natural cycles were consistent with the main findings.

HRT group

In women with HRT cycles, no statistically significant differences were observed in the sex of the neonate or the rate of preterm birth across the three EMT groups. However, mean birthweight and birthweight *z*-score varied significantly among the three EMT groups. The mean birthweight was significantly lower in the EMT <8 mm group than in the 8 ≤ EMT <12 mm and EMT ≥ 12 mm groups (3352 g versus 3472 g, $P < 0.001$; 3352g versus 3447 g, $P < 0.001$). The LBW rate in the EMT <8 mm group was significantly higher than that in the 8 ≤ EMT <12 mm group (6.6% versus 3.7%, $P = 0.003$). The rates of SGA were higher in the EMT <8 mm group and the EMT ≥ 12 mm group compared with the 8 ≤ EMT <12 mm group (5.1% versus 2.3%, $P < 0.001$; 4.1% versus 2.3%, $P < 0.001$). Furthermore, the rates of LGA were significantly lower in the EMT <8 mm group than in the 8 ≤ EMT <12 mm group and the EMT ≥ 12 mm group (21% versus 29.3%, $P < 0.001$; 21% versus 30.9%, $P < 0.001$). Additional information is available in TABLE 5.

The comparisons between natural cycles and HRT cycles are also presented in TABLE 5. The birthweight *z*-score was significantly higher in the HRT group than the NC group (0.45 ± 0.70 versus

TABLE 7 ASSOCIATION BETWEEN BIRTHWEIGHT, BIRTHWEIGHT *Z*-SCORE AND CONTINUOUS EMT AMONG THE TOTAL POPULATION, AND NC AND HRT GROUPS

Type of analysis	Parameter	Type of association	NC group *P*-value	HRT group *P*-value	Overall *P*-value
Univariate	Birthweight	Overall association	<0.001	0.012	<0.001
		Non-linear association	0.013	<0.001	<0.001
Multivariate	Birthweight	Overall association	0.002	0.032	<0.001
		Non-linear association	0.004	<0.001	<0.001
Univariate	Birthweight *z*-score	Overall association	0.008	<0.003	0.047
		Non-linear association	0.108	0.002	0.023
Multivariate	Birthweight *z*-score	Overall association	0.014	0.063	0.029
		Non-linear association	0.077	0.0021	0.021

Overall association refers to the result when a linear association is assumed. The non-linear association test is from the Wald chi-squared test.

Adjustment for multivariate models included maternal and paternal age, body mass index, educational level, type of infertility, cause of infertility, polycystic ovary syndrome, type of ART treatment, number of embryos transferred, number of previous fresh embryo transfer attempts, parity, history of Caesarean section, pregnancy complications (including hypertensive disorders of pregnancy, gestational diabetes mellitus, placenta praevia and placental abruption) and gender of the neonate.

ART, assisted reproductive technology; EMT, endometrial thickness; HRT, hormone replacement therapy; NC, natural cycle.

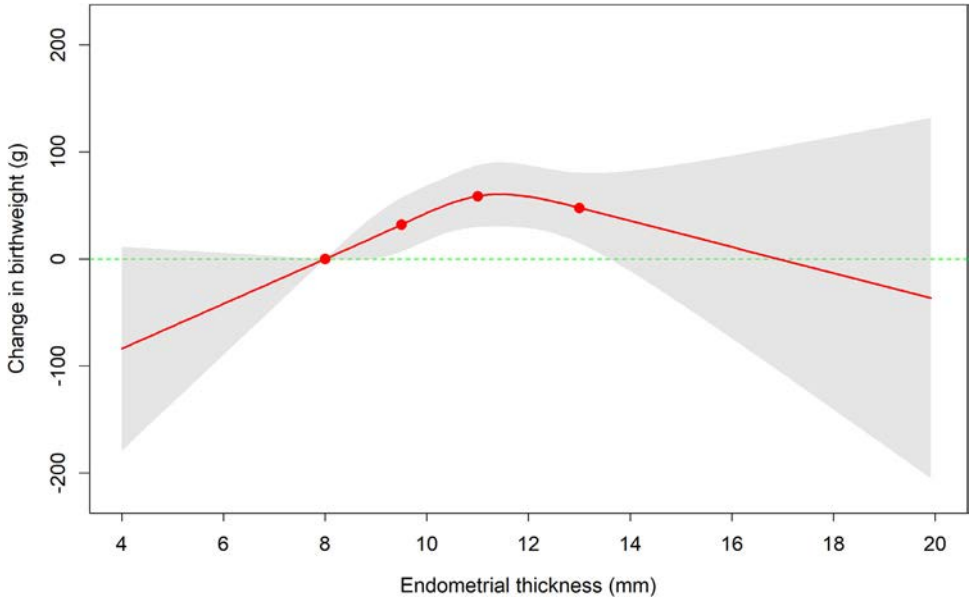

FIGURE 1 Association between birthweight and continuous endometrial thickness in women with natural cycles. The dotted green line indicates the set reference value (8 mm) for endometrial thickness. A restricted cubic spline incorporated in the multivariable-adjusted linear regression model was used to fit the association.

0.37 ± 0.67, $P < 0.001$). Additionally, the HRT group exhibited higher rates of LBW, HBW, LGA and preterm birth compared with the NC group (4.2% versus 3.2%; 15.9% versus 13.0%; 28.5% versus 25.2%; 8.3% versus 5.7%; $P < 0.001$).

Additionally, when using the 8 ≤ EMT < 12 mm group as a reference, the risk of having an LBW infant (aOR 1.84, 95% CI 1.24–2.73) or SGA infant (aOR 1.85, 95% CI 1.17–2.91) was significantly increased in the EMT <8 mm group. Similarly, the risk of having an SGA infant was also significantly increased in the EMT ≥12 mm group (aOR 1.85, 95% CI 1.03–3.33), and the risk of having an LGA infant was significantly decreased in the EMT <8 mm group (aOR 0.70, 95% CI 0.56–0.87). Detailed information is available in TABLE 6.

FIGURE 2 demonstrates an inverted U-shaped association between EMT and neonatal birthweight in women undergoing HRT cycles. In particular, too low an EMT was associated with a trend of decreased birthweight. In this analysis, the four knots of the RCS model were placed at an EMT of 7.5, 8.5, 9.5 and 12 mm, respectively. Birthweight decreased in neonates with a lower EMT compared with those with an EMT of 8 mm, suggesting that individuals with a thin endometrium had poorer neonatal birthweights. From the lowest EMT to an EMT of approximately 8.5 mm, the birthweights presented increased birthweight with increased EMT. The birthweight reached a plateau between 8.5 and 9.5 cm, and then decreased slowly as the EMT exceeded 9.5 mm. The results for the birthweight *z*-score in women with

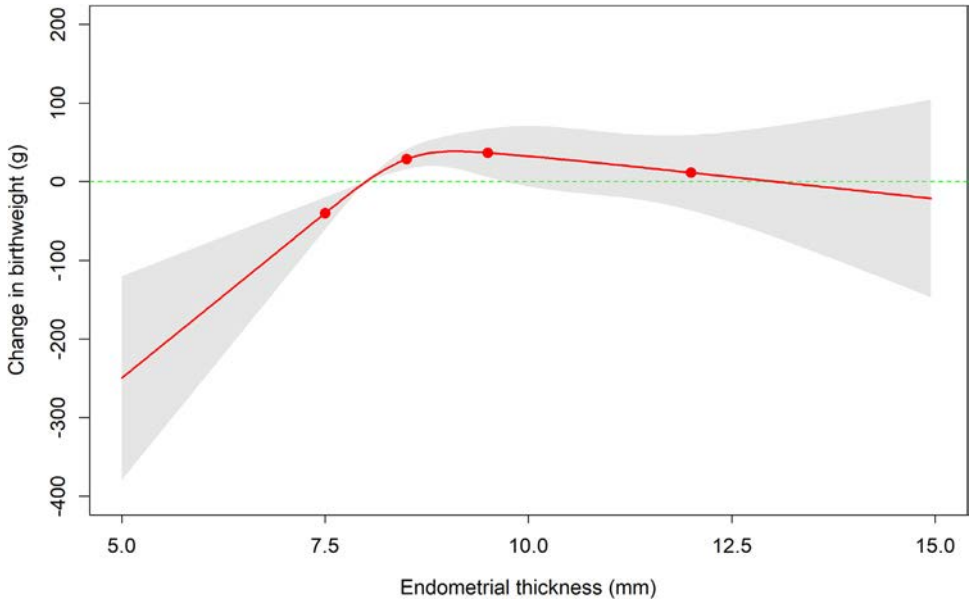

FIGURE 2 Association between birthweight and continuous endometrial thickness in women undergoing hormone replacement therapy cycles. The dotted green line indicates the set reference value (8 mm) for endometrial thickness. A restricted cubic spline incorporated in the multivariable-adjusted linear regression model was used to fit the association.

HRT cycles were consistent with the main findings.

DISCUSSION

In this retrospective cohort study, there were several key findings regarding the association between EMT and neonatal outcomes in women undergoing HRT cycles. First, a thin endometrium (EMT <8 mm) was associated with an increased risk of LBW and SGA compared with women with a moderate endometrium (8 ≤ EMT < 12 mm) in HRT cycles. Second, a thick endometrium (EMT ≥12 mm) was associated with an increased risk of SGA in HRT cycles. Finally, using RCS regression analysis, an inverted U-shaped relationship was identified between continuous EMT and neonatal birthweight or birthweight *z*-score in HRT cycles.

ART has been associated with higher risks of adverse obstetric and perinatal outcomes, which can be attributed to factors such as multiple gestations and underlying risk factors in the patient population. Previous studies have examined the impact of various ART parameters on pregnancy outcomes, aiming to identify risk factors and explore the underlying molecular mechanisms during implantation and placentation. The current team has previously investigated the correlation between EMT and SGA during fresh embryo transfer cycles, revealing a non-linear relationship (*Liu et al., 2021*). Consequently, it was important to examine whether EMT is associated with birthweight and other birth outcomes during FET cycles. EMT is routinely monitored as a key clinical indicator in embryo transfer cycles, with a particular focus on FET cycles over ovarian stimulation cycles (*Tian et al., 2022*). While several studies have demonstrated the association between EMT and pregnancy outcomes in FET cycles, limited research has explored obstetric and perinatal outcomes (*Liu et al., 2018*).

A recent study reported a link between thin endometrium and reduced birthweight, but it did not analyse the association in the context of HRT and natural cycle regimens, nor did it examine the potential linear or non-linear relationship between EMT and birthweight in terms of continuous variables (*Zhang et al., 2019*). A previous study using linear or logistic regression models assumed a linear relationship, which would not detect a non-linear association (*Richardson et al., 2018*). To address this limitation, the current study employed RCS models to assess non-linear relationships. Surprisingly, an inverted U-shaped association between continuous EMT and neonatal birthweight or birthweight *z*-score in FET cycles was discovered, which differed from the rising curve with a middle platform observed in fresh embryo cycles. This inverted U-shaped non-linear relationship between EMT and birthweight was consistent with the U-shaped relationship observed between EMT and hypertensive disorders of pregnancy in the authors' previous study on FET cycles (*Zhang et al., 2022*).

Several studies have reported worse obstetric and perinatal outcomes associated with endometrial preparation protocols involving HRT. However, there have been inconsistent conclusions regarding birthweight, SGA and LGA (*Ishii et al., 2018*; *Li et al., 2021*; *Zong et al., 2020*). The findings of the current study demonstrated that neonates born from HRT cycles exhibited a higher birthweight *z*-score and an increased risk of LBW, HBW or LGA compared with those born from natural cycles. More importantly, the impact of EMT on neonatal birthweight varied between the two preparation regimens. While the values of birthweight *z*-score, SGA and LGA remained relatively stable among the three EMT groups in the natural cycle regimen after adjusting for covariates, EMT exhibits a significant association with these birthweight indices in HRT cycles (**TABLE 7**).

This finding provides important insights into the interaction between different ART parameters and perinatal outcomes. The neonatal outcomes were more susceptible to the fluctuations in EMT in HRT cycles. Optimizing endometrial development and providing increased surveillance for

women with a thin endometrium are crucial for improving infant health. Clinicians should pay particular attention to endometrial adjustments during HRT cycles to enhance maternal and infant outcomes.

The significance of a healthy endometrium as a baseline condition for fertility is widely acknowledged, but few studies have explored the pathogenesis of thin or thick endometrium leading to adverse perinatal outcomes, particularly regarding thick endometrium. It has been proposed that a healthy pregnancy is established during endometrial decidualization before pregnancy implantation (*Ng et al., 2020*). Transcriptomic analysis of human endometrial cells in thin endometrium has indicated compromised cell cycle signalling pathways, high rates of cellular senescence in the stroma and epithelium, collagen overdeposition around blood vessels and decreased numbers of macrophages and natural killer cells, all contributing to insufficient proliferation and subsequent decidualization of the thin endometrium (*Lv et al., 2022*).

Deficient decidualization and an abnormal local microenvironment in the thin endometrium can impact trophoblast invasion and spiral artery remodelling during implantation (*Staff et al., 2022*), affecting oxygen tension and local anatomical structure. In the presence of a thin endometrial lining, the invasive extravillous trophoblast may be closer to the high vascularity and oxygen concentrations of the basal layer, potentially influencing the differentiation of extravillous trophoblast cells (*Casper 2011*; *Catt and Henman, 2000*; *Robins et al., 2007*). Additionally, a thin endometrium with a local discontinuous basal layer and junctional zone caused by mechanical injury may affect the remodelling of spiral arteries, resulting in defective deep or shallow placentation associated with a wide range of pregnancy complications (*Brosens et al., 2002*; *Brosens et al., 2011*). Further investigations are required to provide stronger and more direct evidence to support these speculations (*Huang et al., 2021*). Furthermore, additional research is necessary to elucidate the association and pathogenesis of a thick endometrium in relation to perinatal outcomes.

In the current team's previous studies on the relationship between EMT and birthweight in fresh embryo transfer cycles, a non-linear positive relationship was identified between EMT and birthweight (*Liu et al., 2021*). However, in FET cycles, the association between EMT and birthweight is not consistently positive, particularly in HRT FET cycles, where a thin or thick endometrium has a significant negative impact on birthweight after adjusting for covariates. The absence of a corpus luteum in HRT cycles has been identified as one of the primary causes of adverse obstetric outcomes (*Ginstrom Ernstad et al., 2019*; *Zong et al., 2020*).

In the authors' study, although the low EMT NC group exhibited lower birthweights and an increased risk of LBW risk, the inclusion of gestational age resulted in relatively stable SGA, LGA and birthweight *z*-score across the three EMT groups in natural cycles. Furthermore, no significant non-linear relationship between EMT and birthweight *z*-score was observed in natural cycles. These findings suggest that a low EMT is more likely to be directly associated with preterm birth rather than LBW in natural cycles, while EMT appears to be directly associated with LBW in HRT cycles.

The differential effects of EMT on birthweight indices in different transfer regimen cycles may be attributable to the presence or absence of a corpus luteum, as well as the numbers and function of the corpus luteum. The corpus luteum may act as a 'buffer' in regulating decidualization, trophoblast invasion and spiral artery remodelling in endometria with varying thicknesses. Understanding the aetiology and pathogenesis of this phenomenon could help identify critical elements for establishing a healthy pregnancy before implantation and improve pregnancy and neonatal outcomes for women with an excessively thin or thick endometrium.

The strengths and limitations of this study should be considered. In terms of the strengths, first, the sample size was sufficiently large to provide a high statistical power. Second, EMT was evaluated both as a categorical and as a continuous variable to ensure sensitivity in the measurements. Stratifying continuous variables may limit the assessment of the true effect of a predictor, assuming that values in different categories have different influences, even if they are closely related, and that values may fall within extremes (*Ribeiro et al., 2018*). Third, the analyses were conducted in the context of two endometrial preparations and revealed the different patterns of association between EMT and birthweight. Fourth, EMT measurements were performed by trained doctors from the same team, reducing the potential bias. Finally, the study was adjusted for various potential confounders to enhance the reliability of the results.

Despite these strengths, a couple of limitations should be acknowledged. First, the retrospective design of the study may introduce bias. Moreover, as the original allocation process was not randomized, the sample size of the two groups differed. Second, the study did not analyse other factors associated with neonatal birthweight, such as previous uterine surgery, severe fibroids or adenomyosis, maternal dietary pattern and exercise during pregnancy.

CONCLUSIONS

In conclusion, this retrospective study, with a sufficient sample size, demonstrates that a thinner endometrium resulting from preparation in HRT cycles is associated with a lower birthweight *z*-score, as well as a higher risk of SGA. However, no significant association was observed between EMT and birthweight *z*-score or SGA in the NC group. It is noteworthy that a thicker endometrium was not associated with a higher birthweight in FET cycles. A non-linear relationship, resembling an inverted U-shaped curve, was observed between continuous EMT and neonatal birthweight in FET cycles. Further research is needed to verify the underlying pathogenesis. Women with a thin endometrium who achieve pregnancy require specialized attention, particularly those undergoing FET with HRT cycles.

DATA AVAILABILITY

Data will be made available on request.

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AUTHOR CONTRIBUTIONS

Jiwei Sun and Xiaojie Liu: conceptualization, data curation, investigation, methodology, roles/writing – original draft; Shengnan Guan: conceptualization, methodology; Tong Wu and Xiao Fu: data curation, formal analysis, methodology; Linlin Cui: investigation, project administration; Shanshan Gao: conceptualization, funding acquisition, investigation, methodology, supervision; Zi-Jiang Chen: resources, supervision.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2023.103736.

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ELSEVIER

ARTICLE

Value of 2D ultrasonography in the diagnosis and evaluation of intrauterine adhesions – a prospective study

BIOGRAPHY

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KEY MESSAGE

Although ultrasonography examination cannot replace hysteroscopy in the diagnosis of intrauterine adhesions, it provides useful clinical information regarding severity to help in planning hysteroscopy to optimize management.

ABSTRACT

Research question: What is the value of 2D ultrasonography in the diagnosis and assessment of intrauterine adhesions (IUA)?

Design: This was a prospective study conducted at a hysteroscopy centre.

Results: Of a total of 600 subjects recruited, 41 dropped out and 559 were finally enrolled and analysed. The observed 2D ultrasonography features, in decreasing order of frequency, were 'irregular endometrium' (37.9%), 'broken endometrial echo' (23.4%), 'thin endometrium' (13.7%), 'loss of endometrial echo' (13.1%,), 'hyperechoic focus' (12.5%) and 'fluid in the cavity' (8.8%). The sensitivity of individual ultrasound features ranged from 8.8% to 37.9%, whereas the specificity of individual ultrasound features ranged from 78.9% to 100%. When all the six ultrasound features were considered together, the sensitivity and specificity were 71.7% and 66.2% respectively. The sensitivity, specificity and accuracy of ultrasound diagnosis in the mid-proliferative phase, peri-ovulatory phase and mid-luteal phase did not appear to be significantly different statistically, although the results in the mid-proliferative phase appeared to be consistently higher than those in the mid-luteal phase. In women confirmed to have IUA, the likelihood of the adhesions being severe in nature in the presence of zero, one, two or three or more ultrasound features was 8.7%, 23.0%, 40.2% and 80.5%, respectively ($P < 0.001$).

Conclusions: The findings in this study support the notions that ultrasonography examination in women suspected to have IUA cannot replace hysteroscopy in the diagnosis of the condition. However, it does provide useful clinical information regarding severity and could help in the planning of hysteroscopy to optimize management.

INTRODUCTION

Intrauterine adhesions (IUA) were first reported by Heinrich Fritsch in 1894. Later Joseph G. *Asherman (1966)* described the frequency, aetiology, symptoms and X-ray features of this condition; more recently, the risk factors, classification and diagnosis of this condition based on ultrasonography have been reviewed (*Doroftei et al., 2020*; *Khan, 2023*; *Shen et al., 2022*). The incidence of Asherman syndrome was found to be 1.6% in women who had undergone surgical abortion (uterine evacuation and curettage) before the 20th gestational week (*Sevinç et al., 2021*).

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KEYWORDS

Diagnosis and assessment
Intrauterine adhesions
Ultrasonography

Historically, hysterosalpingography was the main tool for the diagnosis of IUA, but nowadays hysteroscopy is considered the 'gold standard' for diagnosis (*Di Guardo et al., 2020*) as well as treatment (*Doroftei et al., 2020*). More recently, the value of ultrasonography in the diagnosis of IUA has generated considerable interest, especially as it is frequently included in the initial evaluation of women with infertility, oligomenorrhoea or amenorrhoea and recurrent miscarriage, which are common presenting symptoms of IUA. It may also be useful in mapping the uterine cavity when complete obstruction of the cervix precludes hysterosalpingography or hysteroscopy being carried out (*Di Guardo et al., 2020*). Moreover, ultrasonography findings appear to have prognostic value (*Fouks et al., 2022*).

A number of ultrasonographic features have been reported to be associated with IUA, including an irregular endometrium (*Confino et al., 1985*; *Salle et al., 1999*), broken endometrial echo (*Amin et al., 2015*; *Confino et al., 1985*; *Fedele et al., 1996*; *Leone et al., 2010*; *Narayan and Goswamy, 1993*), thin endometrium (*Amin et al., 2015*; *Confino et al., 1985*; *Lo et al., 2008*; *Movilla et al., 2020*; *Salle et al., 1999*), hyperechoic focus (*Kim et al., 2015*), fluid in the cavity (*Amin et al., 2015*; *Yu et al., 2008*) and loss of the endometrial echo (*Yu et al., 2008*).

With the introduction of high-resolution transvaginal ultrasonography (TVS) and continuing improvements in the quality of imaging of the endometrium, the potential use of ultrasonography to replace hysteroscopy as a diagnostic tool has been the subject of a number of investigations (*Amin et al., 2015*). The diagnostic value of ultrasonography appears to vary considerably in the literature, with the sensitivity reported to range from as low as 0% to as high as 91% (*Fedele et al., 1996*; *Soares et al., 2000*) and the specificity ranging from 95.2% to 100% (*Fedele et al., 1996*; *Soares et al., 2000*). The rather wide range of results reported by various investigators has, not surprisingly, generated uncertainty regarding the genuine usefulness of ultrasonography in diagnosing IUA. The main reasons for the conflicting results relate to the small sample size in many of the reported studies, many of them consisting of fewer than 50 cases (*Lo et al., 2008*; *Salle et al., 1999*; *Schlaff and Hurst, 1995*), in addition to the retrospective nature of the studies (*Lo et al., 2008*; *Salle et al., 1999*), which were prone to selection bias.

In this prospective study involving a sufficiently large sample size, the aims were to investigate the value of ultrasonography in the diagnosis and evaluation of Asherman syndrome, by examining: (i) how often various ultrasonographic features can be detected; (ii) the observer variability of the measurement; (iii) the diagnostic accuracy and whether it is affected by the timing of the measurement in the menstrual cycle; and (iv) to what extent the ultrasonographic features correlate with the severity of the IUA.

MATERIALS AND METHODS

Study design

This was a prospective cohort study of a consecutive series of patients referred to the hysteroscopy centre at Fuxing Hospital, Beijing, China, a national referral centre, with a provisional diagnosis of Asherman syndrome for management of the disease. The centre has performed over 1000 cases of hysteroscopic surgery per year for Asherman syndrome over the last 5 years. All recruited participants underwent 2D TVS (TVS-2D), before or within 1 week of diagnostic hysteroscopy to ascertain the presence or absence of IUA and the severity of IUA according to the American Fertility Society (AFS) scoring system (*The American Fertility Society, 1988*).

Participants

Six hundred participants were recruited between June 2020 and December 2022, upon referral for suspected IUA. The inclusion criteria were: (i) women aged 20–40 years, (ii) who were presenting with oligomenorrhoea or amenorrhoea, and (iii) from whom written consent had been obtained. The exclusion criterion was noteworthy co-existing uterine pathology such as fibroids or adenomyosis that interfered with scanning for the endometrial echo.

Ultrasonographic measurement

The date of the last menstrual period was recorded during the ultrasonography measurement. TVS-2D was performed by one of two observers (S.L. and R.H.) using the same ultrasound machine (Voluson E10; GE, USA) and transvaginal probe (RIC5-9-D, frequency 5–9 MHz, depth 5.0–7.0 cm, angle 180°; GE, USA).

The specific ultrasonographic features being looked for in each case are shown in FIGURE 1: (a) thin endometrium, defined as an endometrial thickness of less than 3 mm, as measured on the longitudinal section of the uterus, from the echogenic interface of the junction of the endometrium and myometrium; (b) irregular endometrium, defined as an irregular endometrial thickness or irregular outline of the endometrial echo; (c) fluid in the cavity, defined as the presence of one or more echo-lucent (cystic-like) areas interrupting the endometrium, or inside the uterine cavity; (d) a broken endometrial echo, defined as interruptions of the endometrial echo by one or more hypoechoic bridge-like bands traversing the centre of the uterine cavity; (e) a hyperechoic focus within the endometrial echo; and (f) the loss of a clearly defined endometrial echo. In each case, the presence or absence of each of these six ultrasonographic features was recorded.

Observer variability of ultrasonographic measurement

A total of 60 ultrasonographic video-recordings were randomly selected and assessed by one experienced ultrasonographer (S.L.) on two different occasions, and two different experienced ultrasonographers (S.L. and R.H.) to measure intraobserver and interobserver variability.

Diagnostic hysteroscopy

During the same menstrual cycle as the ultrasonography measurement, office hysteroscopy was performed by one of five experienced endoscopists (Y. T. Zhao, Q. Y. Zhou, F. Q. Zhou, W. Xie and L. L. Yang), who were blinded to the ultrasound results.

A 4.5 mm diameter diagnostic hysteroscope (Olympus, Japan) was introduced into the external cervical os under direct vision. Normal saline solution was used as a distension medium and was instilled under a pressure of 80–100 mmHg. The hysteroscope was then gently introduced through the cervical canal and internal os, and into the uterine cavity. Once it had entered the uterine cavity, a systematic inspection was performed, including the uterine cornua, tubal ostia, uterine fundus and lateral, anterior and posterior uterine walls. In women with confirmed IUA, the AFS classification system was used to assess the severity: mild (score 1–4), moderate (score

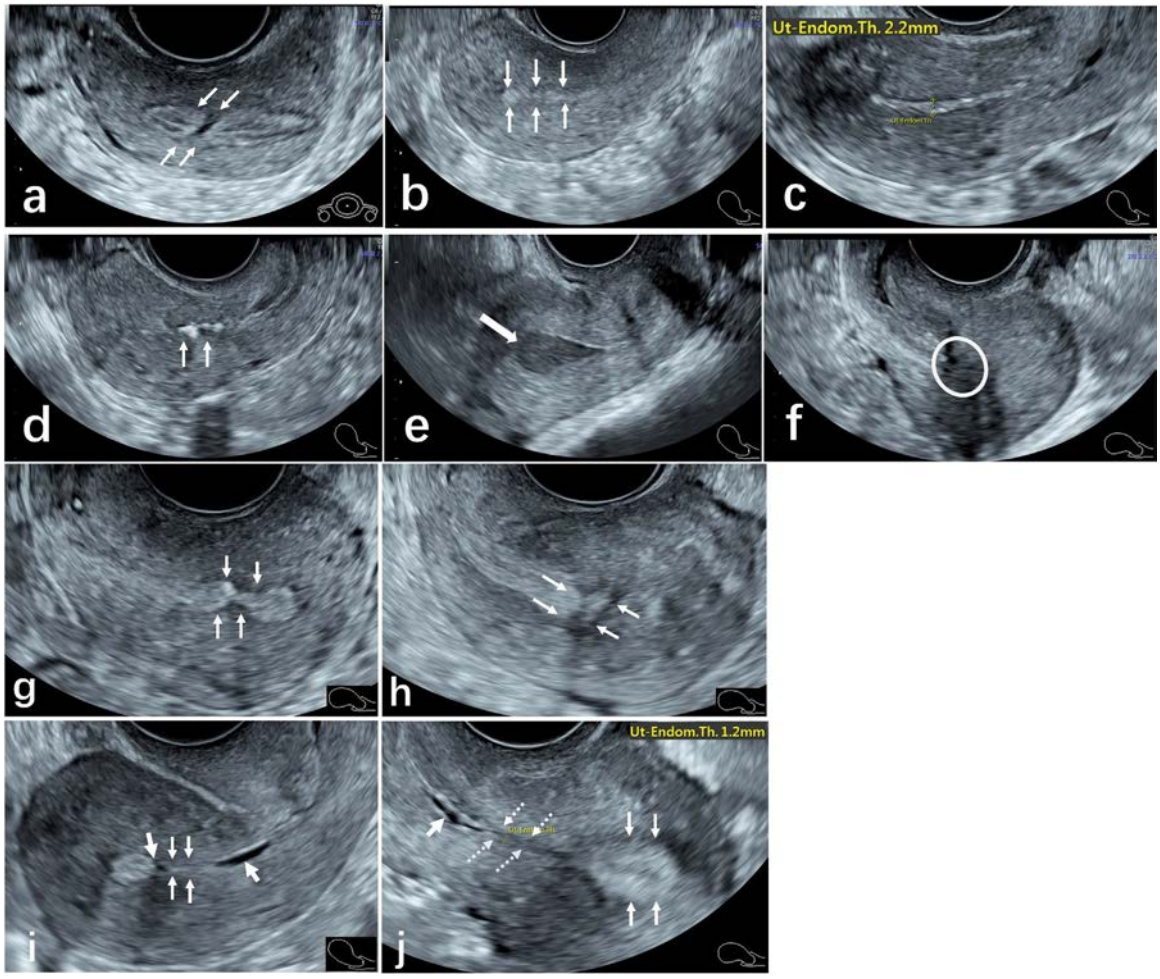

FIGURE 1 Examples of 2D ultrasonography features associated with intrauterine adhesions. (a) Broken endometrial echo in the transverse plane (arrows (b) Endometrium with an irregular margin (arrows). (c) Thin endometrium (<3 mm), measured on the longitudinal section of the uterus, from the echogenic interface of the junction of the endometrium and myometrium. (d) Multiple hyperechoic foci in the middle segment of the cavity (arrows). (e) Fluid distending the upper uterine cavity (arrow). (f) Loss of the endometrial echo (circle). (g) Constriction of the endometrial echo in the upper uterine cavity (arrows). (h) A broken or interrupted endometrial echo in the upper part of the uterine cavity (arrows). (i) Two features indicative of intrauterine adhesions, i.e. a small amount of fluid in the upper and lower halves of the endometrial cavity (thick arrows), and irregular endometrium (thin arrows). (j) Multiple features suggestive of intrauterine adhesions: the upper third of the endometrial echo exhibits a fuzzy margin (thin arrows); the middle third of the echo is thin and poorly defined (dotted arrows); and the lower third contains a small amount of intracavity fluid (thick arrow).

5–8) or severe (score 9–12) (*The American Fertility Society, 1988*).

Ethics

This study was approved by the Ethics Committee Board of Fu Xing Hospital, Capital Medical University (number 2019FXHEC-KY006, 1 November, 2019). Informed consent was obtained from each of the individuals participating in the study.

Statistical analysis

Prior to the study, the sample size was calculated using PASS 15.0 software (ncss.com/software/pass), based on the sensitivity (0.8) and specificity (0.4) reported in the authors' preliminary experiment (n = 100) on 2D ultrasonography in the diagnosis of IUA. A sample size of 387 produced a two-sided 95% confidence interval with a width equal to 0.09988 when the sample proportion was 0.4. Assuming a drop-out rate of 20%, the drop-out-inflated sample size was at least484. In this study, a sample size of 600 was judged sufficient.

Using SPSS 27 statistical software (IBM, USA), all normally distributed measurement data were expressed as the mean and standard deviation. A group t-test and chi-squared test were used to compare the distribution of data between the findings when IUA or no IUA was identified. The sensitivity, specificity, accuracy, positive predictive value and negative predictive value of TVS-2D for identifying the presence of IUA were calculated. The chi-squared test was used to correlate the results of TVS-2D and hysteroscopy. A P-value <0.05 was considered statistically significant.

RESULTS

A total of 600 participants were recruited, of whom 41 dropped out and 559 completed the study protocol. The demographic features of individuals who completed the protocol are presented in TABLE 1. Hysteroscopy confirmed the presence of IUA in 488 subjects but its absence in the remaining 71.

TABLE 1 COMPARISON OF CLINICAL CHARACTERISTICS BETWEEN WOMEN WITH AND WITHOUT IUA

Clinical characteristics	IUA confirmed by HS (*n* = 488)	Absence of IUA confirmed by HS (*n* = 71)	*P*-value
Age (years)	32.6 ± 4.0	33.1 ± 3.3	0.257[a]
BMI (kg/m^2)	21.8 ± 2.9	20.6 ± 2.5	<0.001[a]
Period pattern			
Oligomenorrhoea	433 (88.7)	70 (98.6)	0.018[b]
Amenorrhoea	55 (11.3)	1 (1.4)	
IUA classification by AFS			
Mild	6 (1.2)		
Moderate	350 (71.7)		
Severe	132 (27.0)		

Results are given as mean ± standard deviation, or *n* (%).

[a] 2-sample t-test.

[b] 2 × 2 contingency table analysis.

AFS, American Fertility Society; BMI, body mass index; HS, hysteroscopy; IUA, intrauterine adhesions.

Observer variability

The intraobserver and interobserver variability of the various ultrasonographic features are presented in TABLE 2. The mean kappa values for the paired measurements made by the same observer and by two different observers were 0.78 and 0.62, respectively.

The occurrence of 2D features

Among the women with IUA, overall 28.3% (138/488) did not have any ultrasonographic features, whereas 71.7% (350/488) had one or more ultrasound features suggestive of the diagnosis. The occurrence of the various individual ultrasonographic features in the study population is summarized in TABLE 3. Among participants confirmed as having IUA, the following individual ultrasonographic features, in decreasing order of frequency, were seen: 'irregular endometrium' (37.9%,), 'broken endometrial echo' (23.4%), 'thin endometrium' (13.7%), 'loss of endometrial echo' (13.1%,), 'hyperechoic focus' (12.5%) and 'fluid in the cavity' (8.8%). Among the women confirmed by hysteroscopy as having IUA, 217 (44.5%) had only one ultrasonography features, 92 (18.9%) subjects had two ultrasonography features, and the remaining 41(8.4%) subjects had three or more ultrasonography features.

Sensitivity, specificity and accuracy of 2D ultrasonography findings in the diagnosis of IUA

The sensitivity and specificity of individual 2D-TVS features in the diagnosis of IUA are summarized in TABLE 3. The sensitivity of individual ultrasonography features in the

TABLE 2 KAPPA VALUES FOR INTRAOBSERVER AND INTEROBSERVER AGREEMENT WITH RESPECT TO THE VARIOUS ULTRASONOGRAPHY FEATURES ON 60 RANDOMLY SELECTED SCANS

Ultrasonography features	Intraobserver agreement	Interobserver agreement
Irregular endometrium	0.571	0.4
Broken endometrial echo	0.848	0.688
Thin endometrium	0.957	0.734
Loss of endometrial echo	0.713	0.516
Hyperechoic focus	0.868	0.689
Fluid in the cavity	0.746	0.667
Mean kappa value for all six features	0.78	0.62

TABLE 3 COMPARISON OF THE OCCURRENCE OF VARIOUS ULTRASONOGRAPHY FEATURES AMONG WOMEN WITH AND WITHOUT IUA CONFIRMED ON HYSTEROSCOPY, AND THE SENSITIVITY AND SPECIFICITY OF THESE FEATURES FOR THE DIAGNOSIS OF IUA

Ultrasonography features	No IUA (*n* = 71)	IUA (*n* = 488)	*P*-value[a]	Sensitivity (%)	Specificity (%)
Irregular endometrium	15	185	0.006	37.9	78.9
Broken endometrial echo	0	114	<0.001	23.4	100
Thin endometrium	1	67	0.001	13.7	98.6
Loss of endometrial echo	5	64	0.146	13.1	93
Hyperechoic focus	4	61	0.112	12.5	94.4
Fluid in the cavity	2	43	0.101	8.8	97.2
Any one feature	21	217	0.018	44.5	70.4
Any two features	3	92	0.001	18.9	95.8
Three or more of the six features	0	41	0.006	8.4	100
One or more features	24	350	<0.001	71.1	66.2

[a] Comparison between cases with or without IUA using contingency table analysis or a Fisher's exact test (in cases when one or more cells contains fewer than five cases).

IUA, intrauterine adhesions.

diagnosis of IUA was generally low (8.8–37.9%) while the specificity appeared higher (78.9–100%). However, when the diagnosis was based on the presence of any one of the ultrasonography features, the sensitivity increased to 44.5% but the specificity decreased to 70.4%. When any two of the ultrasonography features were present, the specificity became higher again, at 95.8%, while the sensitivity became lower, at 18.9%. When three or more ultrasonography features were present, the specificity reached 100% but the sensitivity was as low as 8.4%. On the other hand, when any one or more ultrasonography features was considered, the sensitivity and specificity were 71.1% and 66.2%, respectively.

In the total of 559 participants, ultrasonography was performed in the mid-proliferative phase in 150, in the peri-ovulatory phase in 125, and in the mid-luteal phase in 146; in the remaining 138, the ultrasound assessment took place outside these three defined phases. The 2D ultrasonography diagnostic performance parameters for IUA, based on the presence of one or more ultrasonographic features, during the mid-proliferative, periovulatory or mid-luteal phase of the menstrual cycle are compared in TABLE 4. The results for all the indices appear best when ultrasonography is performed in the mid-proliferative phase, but the differences between the groups did not reach statistical significance ($P > 0.05$).

The correlation of 2D ultrasonography findings and severity of IUA

TABLE 5 shows the correlation between the 2D ultrasonography findings and severity of IUA, when IUA was present. Overall, in women confirmed to have IUA, the likelihood of the IUA being severe in nature when there were three or more features, two features, one feature or no features was 80.5%, 40.2%, 23.0% and 8.7%, respectively ($P > 0.001$).

DISCUSSION

This prospective study examined the clinical value of ultrasonography examination in women suspected to have Asherman syndrome in a cohort of 559 participants and correlated the observations with the results of hysteroscopy, which has been considered the gold standard in the diagnosis of this syndrome.

Several ultrasonographic features have been recognized to be associated with IUA, including an irregular endometrium, broken endometrial echo, thin endometrium, hyperechoic focus, fluid in the cavity and loss of the endometrial echo (see TABLE 3); however, to the best of the current authors' knowledge, the frequency of their occurrence in Asherman syndrome has not previously been examined in a relatively large cohort of subjects. The most common 2D ultrasonography feature was found to be 'irregular endometrium' (37.9%), followed by 'broken endometrial echo' (23.4%), 'thin endometrium' (13.7%), 'loss of endometrial echo' (13.1%,), 'hyperechoic focus' (12.5%) and 'fluid in the cavity' (8.8%).

These various ultrasonographic features could all be explained by the underlying pathophysiology of IUA. The irregular endometrium results from patchy damage to the basal layer of the endometrium preventing regeneration of the endometrium and causing it to have an uneven thickness. A broken endometrial echo or loss of the endometrial echo reflects the loss of normal endometrial tissue resulting from damage to the basal layer. Endometrial thinning occurs when the damage is more widespread, rendering most of the endometrium unresponsive to oestrogenic stimulation. A hyperechoic focus in the endometrium suggests localized fibrosis or calcification. The presence of one or more pockets of fluid-filled spaces results from the trapping of menstrual blood within adhesion bands.

Among the six commonly recognized features, it was found that an irregular endometrial echo was the most sensitive but least specific. On the other hand, a broken endometrial echo was the most specific ultrasonographic feature. It was also found that the diagnostic value of each of the six ultrasonographic features in isolation was somewhat limited, but the diagnostic value become significantly higher when multiple features were present. Overall, it appears that individual ultrasonographic features had a rather low sensitivity (ranging from 8.8% to 37.9%) but a higher specificity (ranging from 78.9% to 100%) in the diagnosis of IUA.

The intraobserver and interobserver agreements for the various ultrasonography measurements were modestly high, with a mean kappa value of 0.78 and 0.62, respectively. Among the six features, thin endometrium, a broken endometrial echo and a hyperechoic focus had higher kappa values than the remaining three features.

TABLE 4 COMPARISON OF THE 2D ULTRASONOGRAPHY DIAGNOSTIC PERFORMANCE PARAMETERS FOR IUA, BASED ON THE PRESENCE OF ONE OR MORE ULTRASONOGRAPHY FEATURES, DURING THE MID-PROLIFERATIVE, PERI-OVULATORY OR MID-LUTEAL PHASE OF THE MENSTRUAL CYCLE

Diagnostic performance parameters	Mid-proliferative phase ($n = 150$)	Peri-ovulatory phase ($n = 125$)	Mid-luteal phase ($n = 146$)
Sensitivity	71.7 (91/127)	70.5 (79/112)	68.9 ¯(84/122)
Specificity	73.9 (17/23)	61.5 (8/13)	54.2 (13/24)
Accuracy	72 (108/150)	69.6 (87/125)	66.4 (97/146)
Positive predictive value	93.8 (91/97)	94 (79/84)	88.4 (84/95)
Negative predictive value	32.1 (17/53)	19.5 (8/41)	25.5 (13/51)

Results are given as % (*n*/*N*).

Paired comparison for all indices showed no significant difference ($P > 0.05$) between any of the three groups. Data from a total of 421/559 participants are presented; 138 participants were not included in the analysis as the ultrasonography assessment was performed outside these three defined phases.

IUA, intrauterine adhesions.

TABLE 5 COMPARISON OF THE NUMBER OF 2D ULTRASONOGRAPHY FEATURES SUGGESTIVE OF IUA BETWEEN WOMEN WITH MILD TO MODERATE ADHESIONS, AND THOSE WITH SEVERE ADHESIONS

Number of ultrasonography features	Mild to moderate IUA (*n* = 356)	Severe IUA (*n* = 132)	Likelihood of adhesion being severe (%)	*P*-value
No feature (*n* = 138)	126	12	8.7	<0.001[a]
Any one feature (*n* = 217)	167	50	23.0	
Any two features (*n* = 92)	55	37	40.2	
Three or more features (*n* = 41)	8	33	80.5	

Data are *n*.

[a] 4 × 2 contingency table analysis.

IUA, intrauterine adhesions.

The diagnostic value of ultrasonography examination has been examined in several earlier studies. *Fedele et al. (1996)* reported a sensitivity of 91% and a specificity of 100% among 77 cases, with 11 cases confirmed by hysteroscopy to have IUA. The ultrasonography diagnosis was solely based on the finding of an interruption of the endometrial echo by hypoechoic bridge-like bands (one of six recognized ultrasonographic features associated with IUA examined in the current study). In another study, Salle and colleagues found that TVS examination, based on the finding of a thin endometrium measuring less than 2 mm in the luteal phase, an asymmetrical uterine cavity on a transverse scan, or an echogenic area seen inside the uterine cavity, was able to detect 10 out of 19 cases of IUA (sensitivity 52%) (*Salle et al., 1999*). In a further study conducted by Soares and collaborators involving 65 patients, four of whom were confirmed by hysteroscopy to have IUA, TVS did not detect any of the cases of IUA and three false-positive diagnoses were made (sensitivity and positive predictive value of 0%) (*Soares et al., 2000*). The authors concluded that it was possible to occasionally detect adhesions by TVS on the basis of an echogenic, irregular eccentric line within the endometrium or a narrowing of the endometrial echo but did not think that TVS was a reliable method to investigate IUA.

It is difficult to directly compare the results of these earlier studies mainly because the specific individual ultrasonographic features used to detect IUA were not the same in these reports. Second, many of the case series have consisted of a relatively small number of cases with IUA, and so are more prone to selection bias. The current study included a large sample size and analysed each of the six ultrasonographic features separately as well as in combination. It was found that, overall, the sensitivity of individual ultrasonographic features was low (ranging from 8.8% to 37.9%) but that this could be significantly improved if the six features were considered in combination (71.7%). On the other hand, the specificity of the ultrasonography features is high, whether considered individually (range 78.9–100%) or combined when two or more features are present (95.8–100%).

It can be noted that several investigators have expressed a preference for examining the endometrium in the mid-luteal phase in patients suspected to have IUA (*Fedele et al., 1996*; *McCormack et al., 2016*; *Salle et al., 1999*). In the current study, the authors took the opportunity to examine the impact on the results of the timing of the ultrasonography examination in different parts of the menstrual cycle and found no significant difference between the mid-proliferative, peri-ovulatory and mid-luteal phases; however, ultrasonography examination in the mid-proliferative phase appeared to consistently produce slightly better results (see TABLE 4).

This study found that the ultrasonographic findings are of value not only in diagnosis, but also in predicting the severity of IUA as measured according to the AFS score. When two or more ultrasonographic features are present, the likelihood of encountering severe IUA becomes significantly higher. Such an observation may have practical clinical implications for planning how hysteroscopy should be conducted. For example, in women with no or only one detectable ultrasonography feature, in whom the likelihood of severe disease is relatively low, it is reasonable to proceed with a see-and-treat strategy in the outpatient setting. However, in women with multiple ultrasonographic features, indicating a relatively high likelihood of severe disease, it seems better to proceed with hysteroscopy under intravenous sedation or general anaesthesia. It is worth mentioning that ultrasound-guided balloon distension therapy has been used in the outpatients setting to prevent a recurrence of adhesions (*Ludwin et al., 2019*; *Shi et al., 2023*).

One possible weakness of the current study is the choice of imaging technique used to study the endometrial morphology, namely, conventional 2D ultrasonography. It is likely that the additional use of more refined imaging techniques such as sono-hysterography (*Dreisler and Kjer, 2019*; *Salle et al., 1999*), 3D ultrasonography (*Burjoo et al., 2020*; *Jiang et al., 2021*; *Zhao et al., 2023*) or 3D ultrasonography combined with sono-hysterography (*Sabry et al., 2018*) may improve the diagnostic accuracy. However, the findings of the current study are of greater relevance to day-to-day clinical practice as 2D ultrasonography is routinely used in many parts of the world, whereas sono-hysterography and 3D ultrasonography are not routinely performed other than in specialized units. Nevertheless, a future study is warranted to investigate to what extent sono-hysterography or other more sophisticated imaging techniques, including the use of machine learning (*Zhao et al., 2023*), can improve the accuracy of diagnosis.

In conclusion, it was found that individual ultrasonography features in women suspected to have IUA have low sensitivity but high specificity in terms of diagnostic value. This confirms the notion that ultrasonography cannot replace hysteroscopy in the diagnosis of IUA, but it does provide useful clinical information regarding its severity and could help in

planning hysteroscopy to optimize the management of Asherman syndrome.

DATA AVAILABILITY

Data will be made available on request.

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AUTHOR CONTRIBUTIONS

Tin-Chiu Li conceived and designed the study with Xiaowu Huang and Enlan Xia. Rui Huang drafted the manuscript and formatted the article. Sijing Li, Yuting Zhao and Sotirios Saravelos analysed the data. Xiaodan Lv, YingTao Li and Qi Cheng produced the figures and table. All authors contributed to the article and approved the submitted version.

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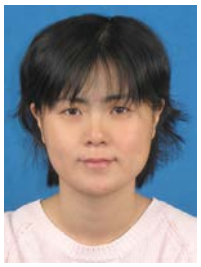
RBMO

ARTICLE

Effect of vitamin D on pregnancy in women with polycystic ovary syndrome: retrospective and prospective studies



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KEY MESSAGE

Serum vitamin D concentration is associated with pregnancy rate in women with polycystic ovary syndrome (PCOS) undergoing ovulation-induction treatment. Vitamin D supplementation can improve the pregnancy rate of ovulation induction, and improve the high LH and testosterone concentrations in women with PCOS who lack vitamin D.

ABSTRACT

Research question: Does vitamin D affect the pregnancy rate of patients with polycystic ovary syndrome (PCOS) receiving ovulation-induction therapy?

Design: The retrospective study included 200 patients with PCOS and 200 healthy women. The prospective study included 160 patients with PCOS receiving vitamin D or placebo supplementation. Pregnancy rates were assessed after a maximum of three cycles of ovulation induction. Serum concentrations of 25-hydroxycalciferol [25-(OH)D3], LH, FSH, progesterone, oestradiol, testosterone and fasting insulin; LH/FSH ratio; and body mass index were evaluated.

Results: In the retrospective study, patients with PCOS had lower 25-(OH)D3 concentrations than healthy women, pregnant patients with PCOS had higher 25-(OH)D3 concentrations than non-pregnant patients with PCOS (both $P = 0.000$), and the pregnancy rate was lower in the vitamin-D-deficient group compared with the non-vitamin-D-deficient group ($P = 0.022$). In the prospective study, compared with placebo supplementation, vitamin D supplementation increased the serum concentration of 25-(OH)D3 ($P = 0.000$), and reduced the LH/FSH ratio, and concentrations of LH and testosterone significantly (all $P \leq 0.049$). After the intervention, it was found that the LH/FSH ratio, and concentrations of LH and testosterone were significantly lower in both groups compared with pre-intervention ($P = 0.000$). After ovulation induction, the pregnancy rate was higher in patients in the vitamin D supplementation group compared with the placebo supplementation group ($P = 0.049$).

Conclusions: Vitamin D deficiency is common in patients with PCOS, and vitamin-D-deficient patients with PCOS have lower pregnancy rates after ovulation induction compared with non-vitamin-D-deficient patients with PCOS. Vitamin D supplementation can improve the pregnancy rate and mitigate basic hormone disorders. Therefore, monitoring vitamin D supplementation and checking vitamin D concentrations before and during interventions are essential for patients with PCOS.

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KEY WORDS
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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common gynaecological endocrine disease characterized by long-term anovulation and hyperandrogenism (*Al Wattar et al., 2021*; *Lizneva et al., 2016*), and is usually accompanied by insulin resistance, and glucose and lipid metabolic disorders. The incidence of PCOS in women of childbearing age is approximately 5–10% (*Clark et al., 2014*; *De Leo et al., 2016*), and studies on its aetiology (*Cooney and Dokras, 2021*; *Escobar-Morreale, 2018*; *Wikiera et al., 2017*) have shown that the reproductive potential in patients with PCOS is decreased mainly due to poor ovulatory status and a decrease in oocyte–embryo–endometrial competence (*Palomba, 2021*). Currently, follicular stimulation and assisted reproductive technology (*Garg and Tal, 2016*; *Legro et al., 2014*) are widely used to treat infertility in patients with PCOS. However, the pregnancy success rate and the length of treatment required are not entirely satisfactory.

The pathogenesis of PCOS is complex, and several factors affect its development (*De Leo et al., 2016*). In healthy women, various hormones, such as FSH and LH, periodically change and interact with each other during the menstrual cycle, with eventual ovulation of the dominant follicles, which is the basis of pregnancy (*Baerwald and Pierson, 2020*). However, patients with PCOS often encounter difficulties in this process, which can lead to persistent ovulation dysfunction, polycystic ovaries and hyperandrogenism. Although some international guidelines consider ovulatory dysfunction in PCOS to be a risk factor for infertility, a large proportion of patients with PCOS struggle to conceive after ovulation induction and require IVF treatment (*Palomba et al., 2017*); this indicates that infertility in patients with PCOS is not linked solely to ovarian status, but also encompasses factors such as oocyte, embryo and endometrial quality (*Palomba, 2021*). Therefore, various interventions have been implemented in clinical settings to enhance female fertility and increase the pregnancy rate. Vitamin D, or cholecalciferol, is an important nutrient involved in female reproductive function. The vitamin D receptor is expressed in the endometrium and placenta, and low serum concentrations of vitamin D are present in a high proportion of patients with PCOS, as well as in patients who experience recurrent miscarriage (Muscogiuri et al., 2017; *Trummer et al., 2018*). Vitamin D deficiency is common in patients with PCOS, with approximately 67–85% of patients affected (*Thomson et al., 2012*). Vitamin D deficiency has been found to be correlated with PCOS symptoms such as ovulatory dysfunction and infertility (*Cappy et al., 2016*; *Ngo et al., 2011*), and vitamin-D-deficient patients with PCOS exhibit a lower pregnancy rate after ovulation induction compared with those with normal concentrations of vitamin D (*Xia et al., 2019*). Vitamin D may exert a positive effect on the ovulation rate by sensitizing the ovaries to clomiphene citrate, and improving oocyte and endometrial quality (Pal et al., 2016). Two recent comprehensive reviews highlighted the potential influence of vitamin D on alterations in oocyte competence and endometrial dysfunction in patients with PCOS (*Palomba et al., 2017*, *2021*). According to *Dennis et al. (2012)*, vitamin D interferes with oocyte competence in patients with PCOS through anti-Mullerian hormone (AMH). Furthermore, vitamin D improves the intrafollicular endocrine milieu (*Irani et al., 2015*) and the endometrial immune environment of patients with PCOS (*Schroder-Heurich et al., 2020*). These studies demonstrate that vitamin D is not only involved in follicular formation, but also in oocyte competence and the endometrium, thus improving endometrial receptivity and embryo implantation (*Parikh et al., 2010*). Nevertheless, some studies have demonstrated that the differential effects on pregnancy rate between vitamin-D-deficient and non-vitamin-D-deficient patients with PCOS were not significant (*Corcoy et al., 2020*; *Harreiter et al., 2022*). The question of whether vitamin D supplementation can improve the pregnancy rate in patients with PCOS as routine clinical treatment is controversial and requires further study.

This study compared serum concentrations of vitamin D in patients with PCOS and healthy women, and clarified the correlation between vitamin D concentration and pregnancy rate in patients with PCOS after ovulation-induction treatment in a retrospective cohort. In addition, this study demonstrated that vitamin D supplementation can improve the pregnancy rate and alleviate basic hormonal disorders in patients with PCOS. This study adds to understanding of the association between vitamin D concentration and pregnancy rate in patients with PCOS, and the effects of vitamin D intervention on the pregnancy rate in patients with PCOS. This provides a novel rationale and conceptual framework for personalized treatment in the diagnosis and treatment of infertile patients with PCOS.

MATERIALS AND METHODS

Population

A retrospective cohort study (200 patients with PCOS and 200 healthy women, from 2016 to 2018) and a prospective cohort study (160 patients with PCOS, from 2018 to 2019) were conducted in Beijing Anzhen Hospital (FIGURE 1). Ethical approval was granted by the Ethics Committee of Beijing Anzhen Hospital (2023114X, approval date 20 January 2023). Before the study, informed consent was obtained from all participants. This study was conducted according to the relevant guidelines and regulations. The inclusion and exclusion criteria for patients with PCOS were consistent with the Rotterdam Diagnostic Criteria (*Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004a*,*b*).

Measurement of serum 25-(OH)D3, sex hormone and fasting insulin, calculation of LH/FSH ratio and body mass index

Serum samples were collected 2–3 days after menstruation or withdrawal bleeding in all registered participants. Serum concentrations of 25-hydroxy-calciferol [25-(OH)D3] were detected using chemiluminescence immunoassay kits (LIAISON 25-OH Vitamin D total assay kit; DiaSorin, Italy) in the retrospective study, and high-performance liquid chromatography-tandem mass spectrometry in the prospective study.

Serum concentrations of FSH, LH, progesterone, oestradiol and testosterone were also ascertained using chemiluminescence immunoassay kits (Roche Diagnostics GmbH, Germany). The fasting insulin (FINS) concentration was determined using an enzyme-linked immunosorbent assay (Roche Diagnostics GmbH). The LH/FSH ratio and body mass index (BMI) were calculated, and the formula used for calculating BMI was: body weight (kg)/height (m^2).

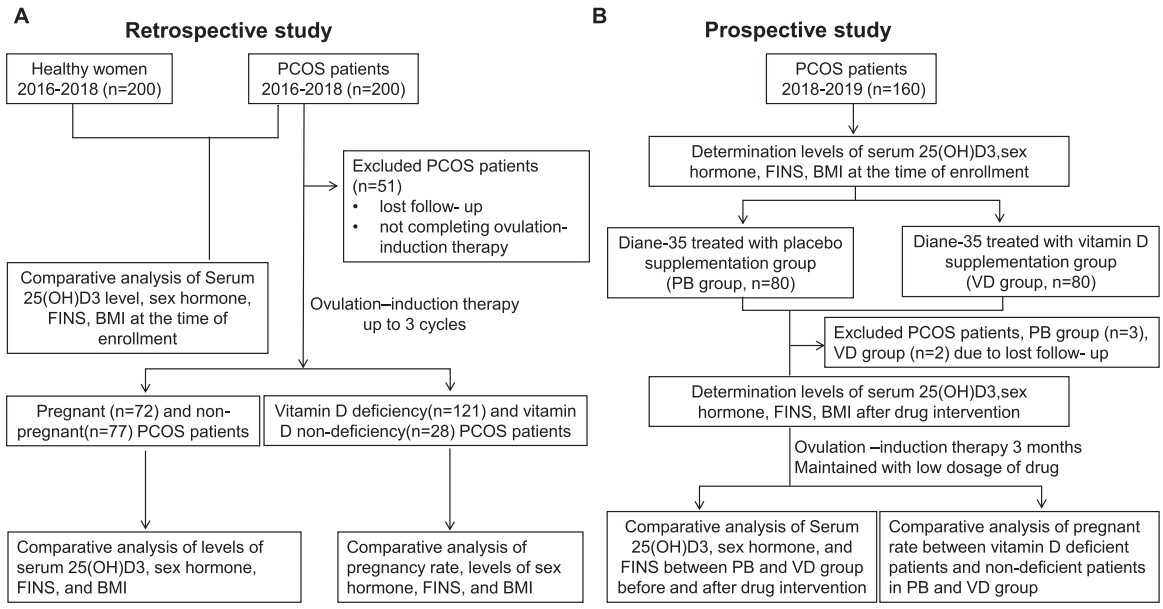

FIGURE 1 Flowcharts of (A) retrospective and (B) prospective studies. PCOS, polycystic ovary syndrome; 25-(OH)D3, 25-hydroxycalciferol; FINS, fasting insulin; BMI, body mass index; VD group, patients receiving Diane-35 treatment with vitamin D supplementation; PB group, patients receiving Diane-35 treatment with placebo supplementation.

Ovulation-induction therapy

Letrozole (Hengrui Pharmaceuticals, China) 2.5–5 mg/day was administrated orally for 5 consecutive days in the retrospective study, while clomiphene citrate tablets (Codal Synto, France) 50–100 mg/day were used in the prospective study according to the same oral administration schedule. Subsequently, human chorionic gonadotrophin (HCG) (Livzon, China) 10,000 units was administered by intramuscular injection for triggering when ultrasonography detected one or two mature follicles (i.e. follicles with diameter ≥18 mm), and the couple was instructed to engage in sexual intercourse 36 h after HCG administration. Two days after HCG injection, participants were checked for ovulation through transvaginal ultrasonography. The serum concentration of HCG was checked 2 weeks after ovulation, and if HCG was positive (>5.3 IU/l), a transvaginal ultrasonography was conducted 4–5 weeks later to confirm clinical pregnancy. Up to three cycles were observed per patient in this study.

Drug intervention in patients with PCOS in the prospective study

All enrolled patients began to take the short-acting contraceptive Diane-35 (Bayer Schering Pharma AG, Germany) orally on day 5 of menses or withdrawal bleeding (one tablet per day; each tablet contained 2 mg cyproterone acetate and 0.035 mg ethinylestradiol). Drug use was halted for 7 days after 21 consecutive days of treatment to constitute one cycle, and then halted after two cycles. Subsequently, patients with PCOS in the vitamin D supplementation (VDS) group were orally administered vitamin D 4000 IU/day (Shuangjing Pharmaceutical Co., China) continuously for 2 months, while patients with PCOS in the placebo supplementation group (PBS) were provided with a placebo (Shuangjing Pharmaceutical Co.) continuously for 2 months. Patients in both groups continued to receive vitamin D or a placebo at the maintenance dose of 1200 IU/day (i.e. three tablets/day) after 2 months of administration of vitamin D 4000 IU/day.

Statistical analysis

All statistical analyses were conducted using SPSS Version 22.0 (IBM Corp., USA), and measurement data are expressed as mean ± SD. All data conformed to a normal distribution when assessed using a homogeneity test of variance, and means of the two samples were compared using Student's *t*-test. F-test was used to determine whether variability between group means was significant within the groups. Counting data were expressed as the number of cases and ratios (*n*, %), and intergroup comparisons were performed using Chi-squared tests. $P < 0.05$ was considered to indicate significance.

RESULTS

Results of the retrospective cohort study

Patients with PCOS had a mean age of 29.66 ± 3.80 years and a mean BMI of 22.91 ± 3.42 kg/m^2, while healthy women had a mean age of 29.08 ± 3.594 years and a mean BMI of 23.57 ± 3.77 kg/m^2. Among the patients with PCOS, the number of patients with vitamin D deficiency [25-(OH) D3 <20 ng/ml] or relative vitamin D insufficiency [25-(OH)D3 ≥20–< 30 ng/ml] was significantly higher ($P = 0.000$; TABLE 1), and the vitamin D concentration was lower compared with healthy women ($P = 0.000$; FIGURE 2A); the mean serum concentration of 25-(OH)D3 in patients with PCOS was 14.57 ng/ml compared with 22.24 ng/ml in healthy women. Next, patients with PCOS were divided into four clinical phenotypes according to their clinical characteristics of hyperandrogenism, ovarian dysfunction and polycystic ovarian morphology (PCOM), as defined by the Rotterdam consensus conference (2003): phenotype A (hyperandrogenism and ovarian dysfunction and PCOM); phenotype B (hyperandrogenism and ovarian dysfunction without PCOM); phenotype C (hyperandrogenism and PCOM with ovulatory cycles); and phenotype D (ovarian dysfunction and PCOM without hyperandrogenism). No significant difference in vitamin D concentration was

TABLE 1 SERUM 25-HYDROXYCALCIFEROL CONCENTRATIONS IN HEALTHY WOMEN AND PATIENTS WITH POLYCYSTIC OVARY SYNDROME

Serum 25-(OH)D3 (ng/ml)	Patients with PCOS *n* (% of total)	Healthy women *n* (% of total)	Chi-squared	*P*-value
<20	165 (82.5)	75 (37.5)	82.5	0.000
20–<30	33 (16.5)	97 (48.5)	45.23	0.000
30–≤100	2 (1)	28 (14)	22.52	0.000
Total	200 (100)	200 (100)		

PCOS, polycystic ovary syndrome; 25-(OH)D3, 25-hydroxycalciferol.

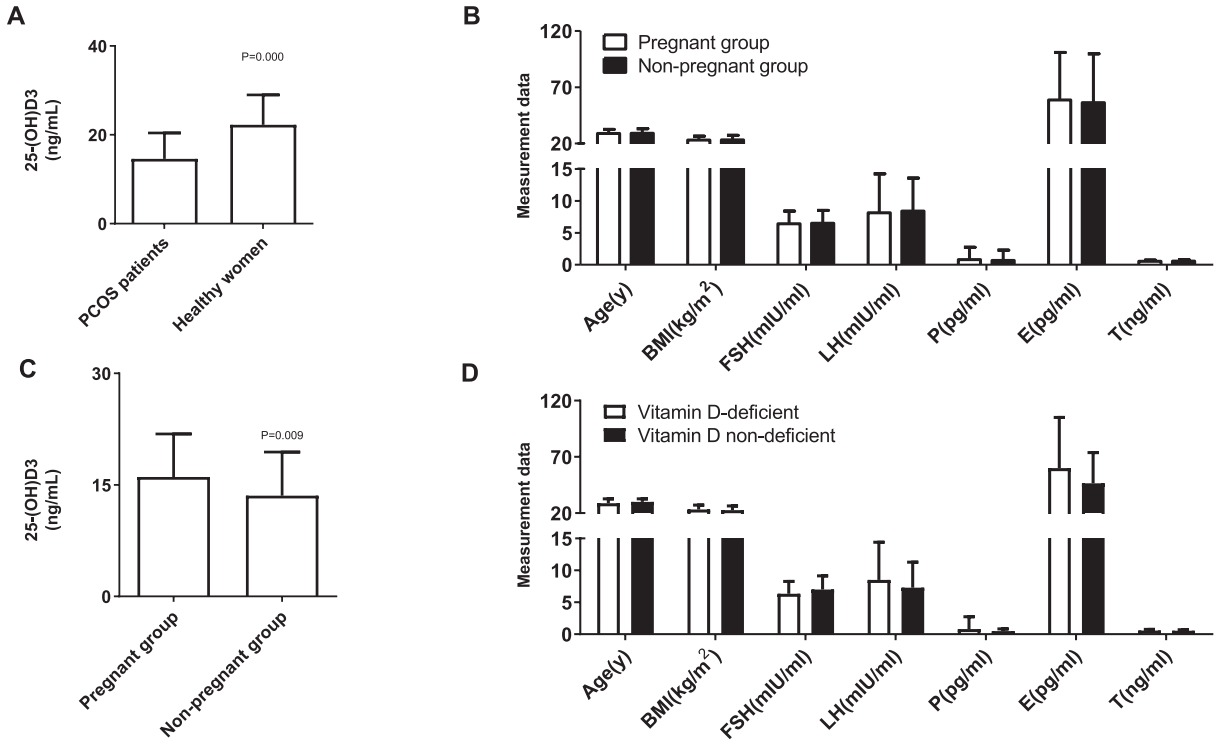

FIGURE 2 Comparison of concentrations of vitamin D, basal sex hormones and fasting insulin between patients with polycystic ovary syndrome (PCOS) and healthy women. (A) Comparison of serum 25-hydroxycalciferol [25-(OH)D3] concentrations between patients with PCOS ($n = 200$) and healthy women ($n = 200$). (B) Comparison of age (years), body mass index (BMI, kg/m^2), concentrations of sex hormones [luteinizing hormone (LH), follicle-stimulating hormone (FSH), progesterone (P), oestradiol (E) and testosterone (T)], and FSH/LH ratio between pregnant and non-pregnant patients with PCOS. (C) Comparison of serum concentrations of 25-(OH)D3 between pregnant ($n = 72$) and non-pregnant ($n = 77$) patients with PCOS. (D) Comparison of age (years), BMI (kg/m^2), concentrations of sex hormones (LH, FSH, E, P and T), and FSH/LH ratio between vitamin-D-deficient ($n = 28$) and non-vitamin-D-deficient ($n = 121$) patients with PCOS. All data are expressed as mean ± SD. Student's *t*-test was used to compare the groups.

found between the four phenotypes (Supplementary Table 1).

Of the 200 patients with PCOS, 51 patients were lost to follow-up by March 2019. The remaining 149 patients who completed the ovulation-induction regimen were divided into two subgroups based on whether or not the cycles resulted in a clinical pregnancy. Among them, 72 patients became pregnant and 77 patients did not (pregnancy rate 48.32%) (TABLE 2). No significant differences in age, BMI or baseline sex hormone concentrations were found between the two groups (FIGURE 2B). Serum concentrations of 25-(OH)D3 were compared between the pregnant and non-pregnant groups, and the concentration was found to be lower in the non-pregnant group (13.54 versus 16.06 ng/ml) ($P = 0.009$; FIGURE 2C).

Next, according to vitamin D concentrations, 149 patients with PCOS who completed the ovulation-induction regimen were divided into two subgroups: a vitamin-D-deficient group, with 25-(OH) D3 <20 ng/ml (121 patients); and a non-vitamin-D-deficient group, with 25-(OH)D3 ≥20 ng/ml (53 patients). There were no significant differences in age, BMI or baseline sex hormone concentrations between the vitamin-D-deficient and non-vitamin-D-deficient patients with PCOS, as shown in FIGURE 2D. The clinical pregnancy rate of vitamin-D-deficient patients with PCOS was 43.8% (53/121), and that of non-vitamin-D-deficient patients with PCOS was 67.86% (19/28). The pregnancy rate for non-vitamin-D-deficient patients with PCOS was therefore significantly higher

TABLE 2 COMPARISON OF PREGNANCY RATES BETWEEN VITAMIN-D-DEFICIENT AND NON-VITAMIN-D-DEFICIENT PATIENTS WITH POLYCYSTIC OVARY SYNDROME

	n	Pregnant (*n*)	Not pregnant (*n*)	Pregnancy rate (%)	Chi-squared	*P*-value
Vitamin D deficient [25-(OH)D3 <20 ng/ml]	121	53	68	43.80		
Non-vitamin D deficient [25-(OH)D3 ≥20 ng/ml]	28	19	9	67.86	5.240	0.022
Total	149	72	77	48.32		

Clinical pregnancy was confirmed by human chorionic gonadotrophin concentration >5.3 IU/l and transvaginal ultrasonography.

25-(OH)D3, 25-hydroxycalciferol.

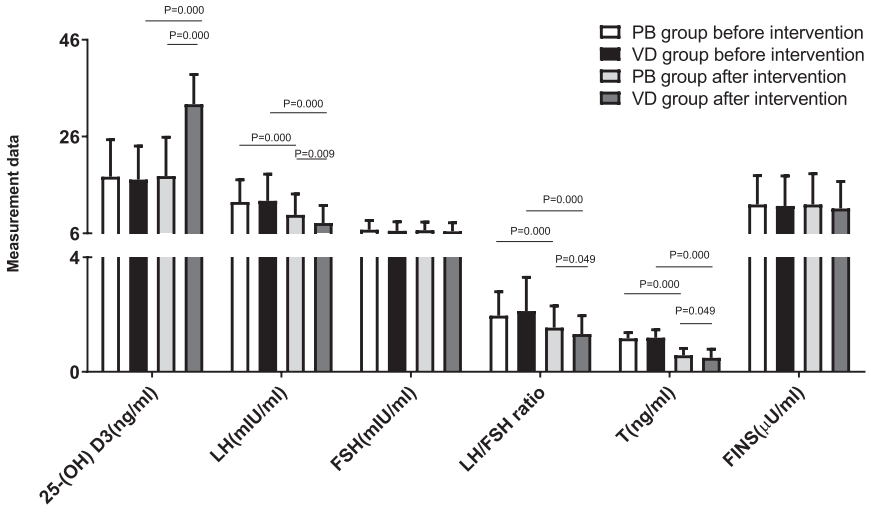

FIGURE 3 **Comparison of concentrations of vitamin D, basal sex hormones and fasting insulin in patients with polycystic ovary syndrome (PCOS) between patients receiving vitamin D supplementation or placebo supplementation before and after drug intervention. Comparison of 25-hydroxycalciferol [25(OH)D3], concentrations of sex hormones [luteinizing hormone (LH), follicle-stimulating hormone (FSH), progesterone (P), oestradiol (E) and testosterone (T)], FSH/LH ratio and fasting insulin (FINS) between patients receiving vitamin D supplementation (VD group) and patients receiving placebo supplementation (PB group) before and after intervention. Data are expressed as mean ± SD. Student's *t*-test was used to compare the groups. All significant comparisons are shown in the figure; further *P*-values are shown in Supplementary Table 3.**

than that for vitamin-D-deficient patients with PCOS (Chi-squared = 5.240, *P* =0.022; TABLE 2).

Results of the prospective cohort study

The mean age of patients in the VDS group (receiving Diane-35 treatment with vitamin D supplementation) was 28.45 ± 3.64 years, and their mean BMI when entering the group was 22.91 ± 3.42 kg/m^2. The mean age of patients in the PBS group (receiving Diane-35 treatment group with placebo supplementation) was 29.34 ± 3.80 years, and their mean BMI when entering the group was 23.57 ± 3.77 kg/m^2. There were no significant differences in age; BMI; concentrations of 25-(OH)D3, basal sex hormones or FINS; or LH/FSH ratio (FIGURE 3) between the VDS and PBS groups at the time of enrolment (FIGURE 3). At the time of enrolment, there were no significant differences in the rates of vitamin D deficiency, vitamin D insufficiency or normal vitamin D concentration between the VDS and PBS groups (TABLE 3).

Over 2 months of drug intervention, two patients in the VDS group and three patients in the PBS group were lost to follow-up. Therefore, a total of 78 patients in the VDS group and 77 patients in the PBS group completed the analysis at each endpoint. After 2 months of drug intervention, concentrations of LH and testosterone and the LH/FSH ratio in the VDS group were lower compared with the PBS group (*P* = 0.009, 0.049 and 0.049, respectively; FIGURE 3). Although the mean concentrations of FSH and FINS in the VDS group were lower compared with the PBS group, the differences were not significant (FIGURE 3). The data also revealed that, after 2 months of drug intervention, the serum concentration of 25-(OH)D3 was significantly higher in the VDS group (32.68 ng/ml) compared with the PBS group (17.83 ng/ml) (*P* = 0.000; FIGURE 3). After 2 months of drug intervention, the rate of vitamin D deficiency (2.6%) was decreased, and the rates of vitamin D insufficiency (34.6%) and normal vitamin D concentration increased (62.8%) in the VDS group; these findings differed significantly from those in the PBS group (TABLE 3).

After 2 months of drug intervention, the serum concentration of 25-(OH)D3 in the VDS group increased significantly compared with pre-intervention (*P* < 0.001; FIGURE 3). The serum concentrations of LH and testosterone and the LH/FSH ratio were significantly reduced (*P* < 0.001; FIGURE 3). The mean FINS concentration showed a tendency to decrease after vitamin D supplementation, but the difference between the VDS group and the PBS group was not significant (FIGURE 3). The concentrations of LH and testosterone and the LH/FSH ratio in the PBS group decreased after 2 months of placebo supplementation (*P* < 0.001; FIGURE 3), while the serum concentrations of 25-(OH)D3, FSH and FINS did not differ (FIGURE 3).

During ovulation-induction treatment, one patient in the VDS group and eight patients in the PBS group were lost to follow-up. In the VDS group, after three cycles of ovulation-induction treatment, 32 patients became pregnant and 45 did not (pregnancy rate 41.6%). In the PBS group, after three cycles of ovulation-induction treatment, 18 women became pregnant and 51 did not (pregnancy rate 26.1%). These results showed that the pregnancy rate for ovulation induction was significantly higher in the VDS group compared with the PBS group (Chi-squared = 3.868, *P* = 0.049; TABLE 4).

TABLE 3 VITAMIN D CONCENTRATIONS IN PATIENTS WITH POLYCYSTIC OVARY SYNDROME TREATED WITH DIANE-35 WITH VITAMIN D SUPPLEMENTATION OR DIANE-35 WITH PLACEBO SUPPLEMENTATION AT THE TIME OF ENROLMENT AND AFTER DRUG INTERVENTION

25-(OH)D3 concentration (ng/ml)	VDS group at time of enrolment (*n*, %)	PBS group at time of enrolment (*n*, %)	Chi-squared	*P*-value	VDS group after intervention (*n* cases, %)	PBS group after intervention (*n* cases, %)	Chi-squared	*P*-value
<20	54 (67.5)	59 (73.8)	0.753	0.385	2 (2.6)	54 (70.1)	73.756	0.000
20–<30	22 (27.5)	15 (18.8)	1.723	0.189	27 (34.6)	14 (18.2)	5.379	0.020
30–≤100	4 (5.0)	6 (7.5)	0.427	0.514	49 (62.8)	9 (11.7)	43.262	0.000
Total (*n*)	80	80			78	77		

VDS group, Diane-35 treatment with vitamin D supplementation; PBS group, Diane-35 treatment with placebo supplementation.

P-values show comparisons between VDS and PBS groups.

25-(OH)D3, 25-hydroxycalciferol.

TABLE 4 COMPARISON OF SUCCESS RATES OF OVULATION INDUCTION PREGNANCIES BETWEEN PATIENTS WITH POLYCYSTIC OVARY SYNDROME TREATED WITH DIANE-35 WITH VITAMIN D SUPPLEMENTATION AND PATIENTS TREATED WITH DIANE-35 WITH PLACEBO SUPPLEMENTATION

Group	*n*	Pregnancy (*n*)	No pregnancy (*n*)	Rate of pregnancy (%)	Chi-squared	Correlation index	*P*-value
Vitamin D deficient, PBS group	55	14	41	25.5			
Vitamin D deficient, VDS group	53	17	36	32.1	0.578	0.161	0.447
Non-vitamin D deficient, PBS group	14	4	10	28.6			
Non-vitamin D deficient, VDS group	24	15	9	62.5	4.071	0.613	0.044
Total VDS group	77	32	45	41.6			
Total PBS group	69	18	51	26.1	13.247	0.564	0.049

Vitamin D deficient, 25-(OH)D3 <20 ng/ml; non-vitamin D deficient, 25-(OH)D3 ≥20 ng/ml; VDS group, Diane-35 treatment with vitamin D supplementation; PBS group, Diane-35 treatment with placebo supplementation.

Vitamin D deficiency stratified by level at enrolment.

Clinical pregnancy was confirmed by human chorionic gonadotrophin concentration >5.3 IU/l and transvaginal ultrasonography.

25-(OH)D3, 25-hydroxycalciferol.

Changes in the pregnancy rates of vitamin-D-deficient and non-vitamin-D-deficient patients were analysed further to clarify the influence of the difference in vitamin D concentration at the time of enrolment on the results. The pregnancy rate of patients who were non-vitamin D deficient at the time of enrolment was significantly higher in the VDS group compared with the PBS group (Chi-squared = 4.07143, $P = 0.04361$). Additionally, among the patients who were vitamin D deficient at the time of enrolment, there was no difference in pregnancy rates between the VDS and PBS groups (Chi-squared = 0.57816, $P = 0.44703$; TABLE 4). Next, patients with PCOS were divided into four clinical phenotypes to compare the effects of vitamin D supplementation on pregnancy in different phenotypes. However, no significant differences in pregnancy outcomes between vitamin D supplementation and placebo supplementation were found between the four phenotypes of PCOS (Supplementary Table 2).

DISCUSSION

Patients with PCOS demonstrate low fertility potential, which may be related to ovulation status, endometrial function and oocyte quality (*Trummer et al., 2018*). Although some medical treatments have been adopted for ovulation induction, the pregnancy rate remains relatively low. This study compared the serum concentrations of 25-(OH)D3 of patients with PCOS and healthy women, and studied the correlation between the serum concentration of 25-(OH)D3 and the pregnancy rate in patients with PCOS. Furthermore, the effect of vitamin D supplementation on pregnancy and related sex hormone disorders in patients with PCOS was analysed, with the aim of demonstrating that vitamin D supplementation may be a therapeutic approach for treating infertility in patients with PCOS.

Vitamin D plays an important role in fertility by binding with its receptor in the female reproductive system (*Lerchbaum and Obermayer-Pietsch, 2012*; *Voulgaris et al., 2017*; *Xia et al., 2019*). Vitamin D not only improves endometrial quality, but is also related to endometrial receptivity and embryonic implantation (*Agic et al., 2007*; *Bagot et al., 2000*). In addition, vitamin D itself is involved in the regulation of calcium ion concentrations in the placenta, and is critical in promoting endometrial decidualization (*Shahrokhi et al., 2016*). Moreover, vitamin D regulates ovarian and placental functions, and participates in follicle formation, and vitamin D deficiency exerts an adverse impact on sex hormone concentrations, thus interfering with formation of the dominant follicle (*Parikh et al., 2010*). Furthermore, vitamin-D-

receptor-deficient mice showed uterine hypoplasia and impaired folliculogenesis (*Voulgaris et al., 2017*; *Yoshizawa et al., 1997*). Vitamin D deficiency or insufficiency is very common in patients with PCOS (*Mogili et al., 2018*; *Thomson et al., 2012*). A retrospective study (*Krul-Poel et al., 2013*) revealed explicitly that the serum concentration of vitamin D in patients with PCOS was significantly lower than that in healthy women. In the present study, the serum concentration of 25-(OH)D3 was lower in patients with PCOS compared with healthy women, with 82.5% of patients with PCOS manifesting vitamin D deficiency and only two of the 200 patients with PCOS showing normal vitamin D concentrations. It is noteworthy that the mean vitamin D concentration was below the normal limit in both patients with PCOS and healthy women of childbearing age, indicating that vitamin D deficiency is prevalent in women of childbearing age in Beijing. In addition, in this prospective cohort, most of the patients with PCOS had vitamin D deficiency and vitamin D insufficiency, with few having normal concentrations of vitamin D. These results are consistent with previous studies in the literature. Given the common occurrence of vitamin D deficiency in healthy women of childbearing age and patients with PCOS, it is reasonable to consider testing vitamin D concentration as part of preparation for pregnancy.

Recent studies (*Fung et al., 2017*; *Trummer et al., 2018*; *Usadi and Legro, 2012*) have revealed that vitamin D concentration is closely related to pregnancy outcomes in patients with PCOS. A clinical study found that subjects with serum 25-(OH)D3 sufficiency exhibited a higher pregnancy rate than those with 25-(OH)D3 insufficiency (*Rudick et al., 2014*). In the present retrospective study, the mean serum concentration of 25-(OH)D3 was much higher in pregnant patients with PCOS compared with non-pregnant patients with PCOS. In addition, subjects were stratified by vitamin D concentration, and the pregnancy rate of non-vitamin-D-deficient patients was found to be significantly higher compared with vitamin-D-deficient patients. The above data indicate that vitamin D concentration is related to pregnancy rate after ovulation-induction therapy in patients with PCOS, which is congruent with extant studies (*Rudick et al., 2014*; *Usadi and Legro, 2012*). According to the retrospective study, the vitamin D concentrations in both the pregnant and non-pregnant groups were significantly lower than the classical limit, and it is suggested that vitamin D supplementation in patients with PCOS can improve the pregnancy rate. After 2 months of drug intervention, the serum concentration of 25-(OH)D3 increased in the VDS group compared with the PBS group, and the pregnancy rate increased within three cycles of ovulation induction. In a clinical study, *Butts et al. (2019)* found that vitamin-D-deficient patients with PCOS had impaired chances of ovulation, pregnancy and live birth with letrozole or clomiphene citrate ovulation stimulation. These data show that vitamin D supplementation can improve the pregnancy rate after ovulation induction in patients with PCOS to a certain degree. In the present study, patients with PCOS were further stratified by vitamin D concentration at the time of enrolment, and the pregnancy rate of non-vitamin-D-deficient patients in the VDS group was found to be higher compared with non-vitamin-D-deficient patients in the PBS group. In addition, the pregnancy rate of vitamin-D-deficient patients in the VDS group was slightly higher compared with vitamin-D-deficient patients in the PBS group, although the result was non-significant. It is suggested that vitamin D supplementation can improve fertility outcomes in non-vitamin-D-deficient patients with PCOS, but may have a limited effect in vitamin-D-deficient patients with PCOS. A recent randomized controlled trial on infertile women undergoing IVF demonstrated that a single oral dose of vitamin D had no effect on pregnancy (*Somigliana et al., 2021*). Some studies (*Asemi et al., 2015*; *Dastorani et al., 2018*; *Karadag et al., 2018*; *Seyyed Abootorabi et al., 2018*) have discussed the effects of vitamin D on fertility, and found that vitamin D supplementation improves insulin resistance and lipid metabolism; reduces testosterone, inflammatory indicator and serum AMH concentations; and regulates the menstrual cycle in patients with PCOS. In contrast, these effects were not significant in vitamin-D-insufficient women without PCOS. In addition, *Butts et al. (2019)* found that vitamin-D-deficient patients with PCOS are less likely to ovulate and have a lower live birth rate, whereas in women with unexplained infertility, vitamin D deficiency does not reduce the live birth rate significantly. Based on the above study and the present authors' observations, it is suggested that treatment with vitamin D may have an effect on fertility in patients with PCOS, but the effect on vitamin-D-deficient patients with PCOS may be limited. Taken together, it is suggested that vitamin D supplementation should be monitored, and vitamin D concentration should be checked before and during intervention in patients with PCOS undergoing ovulation induction.

Regarding the supplementary dose of vitamin D, there is no detailed treatment plan for patients with PCOS who are deficient or insufficient in vitamin D. The dose of vitamin D used in this study is the safe dose recommended by the American Endocrinology Society in June 2011 in the Clinical Practice Guide of the *Journal of Clinical Endocrine and Metabolism* (*Holick et al., 2011*). In the present prospective study, patients with PCOS in the VDS group were treated with oral vitamin D 4000 IU/day for 2 months, and then continued maintenance at 1200 IU/day, while the IVF randomized controlled trial (*Somigliana et al., 2021*) supplemented a single oral administration of 600,000 IU in vitamin-D-deficient woman undergoing IVF. The dosage and supplementation methods of vitamin D varied between the studies. In a study in Iran in 2014, infertile patients with PCOS who underwent intrauterine insemination were given an intramuscular injection of vitamin D 300,000 IU, it was found that there was a significant difference in endometrial thickness between the vitamin D treatment group and the placebo group (*Asadi et al., 2014*). In another study, vitamin D supplementation had a significant effect on the concentrations of cardiovascular risk factors, blood lipids and other hormones in patients with PCOS. The dose of vitamin D supplementation in the experimental group was 3200 IU (*Asadi et al., 2014*). A clinical trial by *Khatiwada et al. (2021)* showed that substantial vitamin D supplementation (4400 IU/day) increases the 25-(OH)D3 concentration throughout pregnancy, and vitamin D supplementation before conception conceivably influences the immune-mediator response during pregnancy in healthy women. Based on the above clinical studies and the present findings, it is suggested that the use of high-dose vitamin D supplementation for 2 months followed by maintenance doses can be used as a new adjuvant therapy to improve infertility in vitamin-D-deficient patients with PCOS.

Patients with PCOS often have sex hormone disorders characterized by high concentrations of testosterone and LH, which greatly reduces the success rate of

ovulation induction for pregnancy. Most patients are pretreated with oral short-acting contraceptives, including Diane-35, before ovulation induction to improve sex hormone disorders. In the present prospective study, all patients used short-acting contraceptives before ovulation induction. The LH concentration and LH/FSH ratio in both groups diminished before and after drug intervention, which indicated that Diane-35 reduced the LH concentration and improved the LH/FSH ratio. The results showed that the LH concentration in the VDS group was significantly lower compared with the PBS group after drug intervention, which revealed that vitamin D supplementation exerted a positive effect on the high LH concentration characteristic of patients with PCOS. Hyperandrogenism is one of the first symptoms of patients with PCOS, and may be related to the interaction between insulin resistance and reproductive endocrine disorders. A systematic review found that vitamin D plays a regulatory role in PCOS hyperandrogenaemia, and is related to adverse fertility outcomes, but the correlation was insufficient to establish a causal relationship (*Trummer et al., 2018*). In addition, a meta-analysis revealed that vitamin D supplementation in patients with PCOS reduces insulin resistance and hyperandrogenaemia, and improves lipid metabolism (*Miao et al., 2020*). The present study ascertained that, following drug intervention, the testosterone concentration in the PBS group decreased significantly, indicating that Diane-35 reduced the serum concentration of testosterone. Moreover, after drug intervention, the serum concentration of testosterone was lower in the VDS group compared with the PBS group, suggesting that vitamin D supplementation reduced the excessive testosterone concentration and improved the hyperandrogenic state of patients with PCOS.

Compared with the normal metabolic situation, patients with PCOS are more likely to suffer from impaired glucose tolerance, type II diabetes, obesity and metabolic diseases (*Ng et al., 2019*). Insulin resistance is one of the most remarkable characteristics of the PCOS phenotype, and is one of the principal clinical manifestations of endocrine and metabolic disorders (*Mohan et al., 2023*). An observational study published by Saudi researchers in 2018 showed that low vitamin D concentrations in pregnant women were significantly associated with high risk of gestational diabetes mellitus (*Al-Ajlan et al., 2018*). Some studies have demonstrated that insulin resistance is directly related to vitamin D; thus, vitamin D therapy may be helpful in improving the insulin sensitivity of patients with PCOS (*Selimoglu et al., 2010*). For patients with PCOS, metformin is also critical to reduce insulin resistance and improve hyperandrogenism. In a randomized, double-blind, placebo-controlled trial, women with vitamin D deficiency and PCOS phenotype B (hyperandrogenism and ovarian dysfunction without PCOM) exhibited beneficial changes in the parameters of glucose homeostasis after 12 weeks of vitamin D supplementation (*Maktabi et al., 2017*). In the present prospective study, a slight (non-significant) decrease in FINS was noted between the VDS group and the PBS group before versus after drug intervention. This result may be due to the detection methods adopted, which cannot diagnose insulin resistance accurately. It would be more accurate to evaluate insulin resistance using the euglycaemic hyperinsulinaemic clamp technique.

Limitations

This study has several limitations. First, it is debatable whether healthy women and patients with PCOS have the same reproductive potential. Second, alterations in oocyte competence, which are considered potential causative factors for low fertility in patients with PCOS, were not compared. Third, lifestyle modifications and metformin may improve underlying endometrial dysfunction and pregnancy outcomes in obese and insulin-resistant patients. Bariatric surgery has been shown to be efficacious in severely obese patients with PCOS, but a careful evaluation of the benefit/risk ratio is warranted. Fourth, vitamin D concentration is affected by seasonal changes and sun exposure. This study spanned 2 years, and included patients across various seasons, which may have influenced the results. Finally, analyses of a larger sample and high-quality randomized controlled trials are necessary to fully understand the effects of vitamin D on improving the pregnancy rate in patients with PCOS.

CONCLUSION

In summary, vitamin D deficiency was found more often in patients with PCOS compared with healthy women of childbearing age. Vitamin D concentration was found to be correlated with the pregnancy rate in patients with PCOS. Vitamin D supplementation can thus improve the pregnancy rate and alleviate the basic hormonal disorders inherent in patients with PCOS. It is suggested that vitamin D supplementation can improve the pregnancy rate after ovulation induction in infertile patients with PCOS; however, further research is important to determine the mechanism of action. Therefore, it is crucial to monitor vitamin D supplementation and check vitamin D concentrations before and during intervention in patients with PCOS receiving ovulation induction in order to enhance their chance of becoming pregnant.

DATA AVAILABILITY

Data will be made available on request.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.103909.

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RBMO

ELSEVIER

ARTICLE

Extended balloon stent placement for reducing intrauterine adhesion recurrence: a retrospective cohort study

BIOGRAPHY

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KEY MESSAGE

This retrospective cohort study revealed that intrauterine balloon stent retention for 6 weeks rather than 4 weeks after hysteroscopic adhesiolysis further reduced post-operative adhesion reformation rates and adhesion severity without increasing intrauterine bacterial colonization.

ABSTRACT

Research question: What are the efficacy, safety and reproductive outcomes of intrauterine balloon stent placement for 4 or 6 weeks after hysteroscopic adhesiolysis?

Design: This retrospective cohort study was conducted at a university-affiliated hospital, and included 155 women with moderate to severe intrauterine adhesions who underwent hysteroscopic adhesiolysis between March 2016 and December 2019. Participants were divided according to whether the heart-shaped balloon stent was left in place for 4 (group 1) or 6 (group 2) weeks after surgery. Stents removed at the second-look hysteroscopy 4 or 6 weeks after surgery were sent for culturing of common bacteria. The incidence of adhesion reformation, adhesion score reduction, bacterial colonization of the intrauterine balloon stent, live birth rate and time to live birth were analysed.

Results: Group 2 had a significantly lower adhesion reformation rate (45.7% versus 28.2%, $P = 0.024$) and a more significant reduction in adhesion score (5.2 ± 2.1 versus 6.3 ± 2.2, $P = 0.003$) compared with group 1. However, no statistical difference was observed in the percentage of bacterial colonization of the intrauterine balloon stent (55.9% versus 66.7%, $P = 0.174$), live birth rate (52.4% versus 42.3%, $P = 0.331$) or time to live birth (hazard ratio 1.09, 95% confidence interval 0.60–1.96, $P = 0.778$) between the two groups.

Conclusions: Extending intrauterine balloon stent use from 4 to 6 weeks further reduces the adhesion reformation rate after hysteroscopic adhesiolysis in patients with moderate to severe intrauterine adhesion. No increase in bacterial colonization of the balloon stent was observed. Extending the duration of intrauterine balloon stent placement did not significantly affect live birth rates.

INTRODUCTION

Intrauterine adhesion (IUA) involves uterine wall adhesions caused by trauma or infection that damage the basal layer of the endometrium (*Yu et al., 2008*). The prevalence varies from 19% to 45%, depending on the study population and availability of diagnostic investigations (*Hooker et al., 2014*; *Khan and Goldberg, 2018*; *Shen et al., 2022*; *Yu et al., 2008*). IUA significantly affects reproductive health, with clinical manifestations including hypomenorrhoea, amenorrhoea, infertility and recurrent

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KEYWORDS

Asherman's syndrome
Bacterial colonization
Balloon stent
Intrauterine adhesion
Live birth

spontaneous abortion (*Yu et al., 2008*). Additionally, IUA can lead to obstetric complications such as preterm birth, a morbidly adherent placenta and post-partum haemorrhage (*Deans et al., 2018*; *Wang et al., 2021*).

Hysteroscopic adhesiolysis is the primary treatment for IUA, separating scar tissue and restoring the typical anatomical structure of the uterine cavity (*AAGL Elevating Gynecologic Surgery, 2017*; *Di Guardo et al., 2020*). However, high post-operative IUA recurrence rates remain an issue, particularly in patients with moderate to severe IUA (*AAGL Elevating Gynecologic Surgery, 2017*; *Yu et al., 2008*). Globally, many anti-adhesion treatments have been used to improve reproductive outcomes and reduce IUA reformation after surgery, yet a consensus regarding the best option is lacking (*Bosteels et al., 2017*; *Vitale et al., 2022*).

A heart-shaped balloon stent has been introduced as a solid barrier to prevent IUA recurrence following hysteroscopic adhesiolysis (*March, 2011*). For most conditions, intrauterine devices are positioned inside the uterine cavity for 7–10 days (*AAGL Elevating Gynecologic Surgery, 2017*). Complete endometrial healing after hysteroscopic surgery requires 1–3 months (*Yang et al., 2013*), and some clinicians have experimented with extending balloon stent placement to ≥30 days (*Huang et al., 2020*; *Lin et al., 2015*; *Wang et al., 2022*).

Based on the current authors' extensive experience, long-term observations and the existing literature (*Yang et al., 2020*), prolonging the duration of balloon stent placement to 4 weeks has been shown to be more efficacious in preventing the recurrence of adhesions in patients with moderate to severe IUA compared with leaving the balloon stent in place for only 1 or 2 weeks. Therefore, it is recommended that a minimum of 4 weeks of balloon stent application should be considered in patients with severe disease who meet specific criteria: involvement of over one-third of the cavity extent, fibrotic and dense adhesions, invisibility of at least one uterine horn, and minimal residual functional endometrium. Meanwhile, the final decision regarding the duration should also take into account patient preferences and scheduling constraints.

To further explore the potential benefits, some patients undergo an additional 2 weeks of balloon stent placement. Thus, a subset of patients underwent the 4-week protocol involving balloon stent insertion, while another subset opted for the extended regimen lasting for 6 weeks. This study aimed to compare the effect of balloon stent placement for 4 or 6 weeks on IUA reformation and live birth rate after hysteroscopic adhesiolysis, and to assess the bacterial colonization of the removed balloon stents to provide more information on managing IUA.

MATERIALS AND METHODS

Participants

A retrospective analysis was conducted on women with moderate to severe IUA who underwent hysteroscopic adhesiolysis at the Hysteroscopic Center of Fuxing Hospital, affiliated with Capital Medical University, between March 2016 and December 2019. Written informed consent was obtained from all patients. This study was approved by the Ethics Committee of Fuxing Hospital (IRB Review Approval Notice Number 2023FXHEC-KSP037, 30 June 2023).

The electronic medical records database of the authors' institution was searched to identify eligible patients. Patients who met the following conditions were enrolled in the study: (i) 20–40 years of age; (ii) moderate to severe IUA (as per an 1988 American Fertility Society [AFS] IUA score of ≥5); and (iii) insertion of a uterine balloon stent immediately after surgery and removed 4 or 6 weeks later at the second-look hysteroscopy. Patients with abnormal endocrine function, genital tract malformations, submucosal fibroids, severe endometriosis or adenomyosis were excluded.

The following information was recorded: age, body mass index (BMI), gravidity, parity and gynaecological status, including the number of selected abortions, spontaneous miscarriages, induced abortions and dilation and curettage procedures, number of gestational weeks at the time of abortion or dilation and curettage, menstrual history, surgery history, aetiology of IUA and previous treatment for IUA. Two experienced endoscopists reviewed the pre-operative and second-look hysteroscopic images or videos. In cases of disagreement, a senior endoscopist was consulted for a final verdict. All these endoscopists were blinded to the patient assignment and outcomes.

The extent of the cavity involved, types of adhesion and visibility of the tubal ostium pre- and post-operatively were recorded. Follow-up information — including patients' post-operative symptoms and reproductive outcomes — was obtained from outpatient medical records and telephone interviews. The follow-up period for the reproductive outcome was at least 3 years, with a range of 3–6 years after surgery. Live birth was defined as the delivery of a live newborn after 28 weeks of gestation, and the time to live birth (TTLB) referred to the time (in months) from the day of surgery to the day of delivery.

Hysteroscopic procedures

All patients underwent hysteroscopic adhesiolysis under general anaesthesia performed by an experienced endoscopist during the follicular phase of the menstrual cycle. For cervical preparation, 80 mg of phloroglucinol (*Xu et al., 2015*) was administered intravenously 15–30 min before surgery. After the vulva and vagina had been thoroughly disinfected, a rigid 8.5 mm resectoscope (Olympus Optical Co., Japan) was introduced into the uterine cavity to evaluate the anatomical structure of the cavity and confirm the presence of IUA. A bipolar electrode needle or loop (Olympus Optical, Tokyo, Japan) was used to resect adhesions. The cutting and coagulation powers were set to 310 W and 90 W, respectively. Uterine cavity distension was achieved with 0.9% saline at a pressure of 120 mmHg and a 320 ml/min flow rate. Following the procedure, a heart-shaped balloon stent (Cook Medical, USA, or Obgyn, China) filled with 3.0–3.5 ml of normal saline was immediately inserted into the uterine cavity.

All procedures were successfully performed under transabdominal ultrasonography guidance, ensuring that the uterine cavity returned to a normal morphology without adhesions, and ensuring bilateral uterine horn restoration and bilateral tubal openings — either visible or invisible (Supplementary Figure 1).

Post-operative treatments and sample collection

Following the centre's routine treatment protocol, all patients received prophylactic antibiotic therapy (Cefaclor 0.375 mg twice daily) for 5–7 days. Hormone therapy was initiated on the day of surgery

and consisted of 4 mg/day 17β-oestradiol for 21 days, with 20 mg/day dydrogesterone during the last 10 days of oestrogen therapy. An additional cycle of hormone therapy was repeated on day 5 of the withdrawal bleeding.

On post-operative day 1, the portion of the balloon tail protruding beyond the cervix was trimmed at the external os of the cervix. All patients returned to the hospital 4 or 6 weeks post-operatively for a second-look hysteroscopy. After the vulva, vagina and cervical canal had been thoroughly disinfected, the balloon stent was carefully removed without touching the vaginal wall. The balloon was cut from the shaft, placed in a sterile jar and immediately sent to the microbiology laboratory of Fuxing Hospital to be cultured for common aerobic microorganisms.

After the culture had been obtained, all patients underwent a second-look hysteroscopy to evaluate the uterine cavity. A 4.5 mm rigid hysteroscope (Olympus Optical, Japan) was used without cervical preparation or anaesthesia, and normal saline was administered to distend the uterine cavity at a distending pressure of 100 mmHg. The extent and severity of reformed IUA were re-evaluated at the second-look hysteroscopy. In cases of identified reformed IUA, blunt adhesiolysis was performed using the hysteroscope tip and repeated hysteroscopic evaluation was necessary until the endometrial wound had fully healed. If no IUA was detected during the second-look hysteroscopy, no further procedures were deemed to be performed.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS Statistics for Windows, version 26.0, IBM, USA). Two-sided tests with a *P*-value of <0.05 were considered statistically significant. The two groups were compared using Student's t-test, Mann–Whitney *U*-test, Pearson chi-squared test or Fisher's exact test, as appropriate. Multivariable logistic regression analysis was performed to assess IUA recurrence after hysteroscopic adhesiolysis. The final model only included variables with a trend of association with IUA recurrence in the univariable analysis ($P < 0.10$). IUA recurrence likelihood was presented as the odds ratio (OR) and 95% confidence interval (95% CI). Kaplan–Meier analyses and log-rank tests were used to compare the TTLB between groups. Hazard ratios (HR) and their 95% confidence intervals were calculated using Cox regression analysis.

RESULTS

Participant demographic and clinical characteristics

The study flow chart is illustrated in FIGURE 1. In this retrospective study, 155 patients with moderate to severe IUA meeting the screening criteria were included in either group 1 ($n = 70$) or group

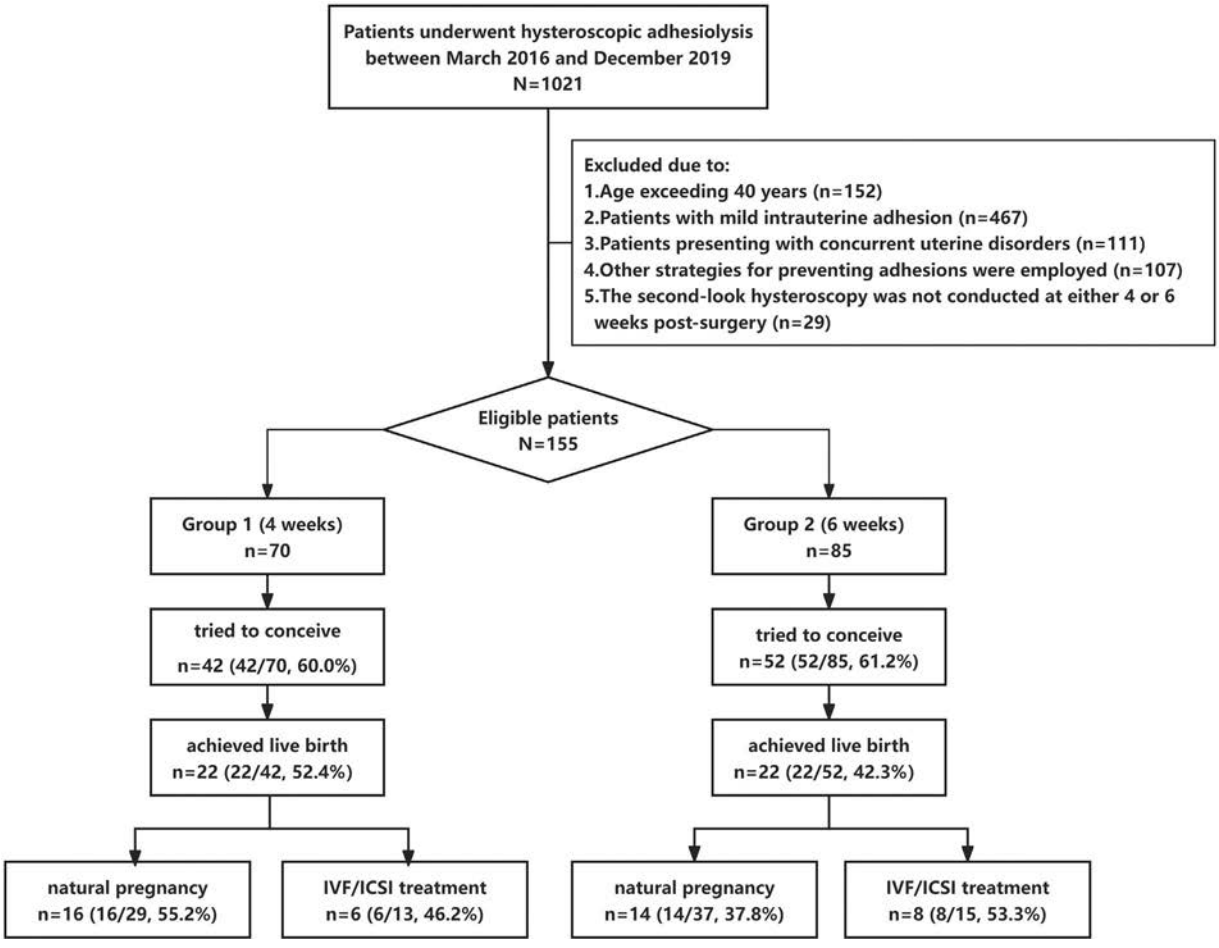

FIGURE 1 Flowchart of the study. ICSI, intrauterine sperm injection.

TABLE 1 DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF STUDY GROUPS 1 AND 2, WITH THE BALLOON STENT *IN SITU* FOR 4 AND 6 WEEKS, RESPECTIVELY

Variables	Group 1 (n = 70)	Group 2 (n = 85)	*P*-value
Age[a]	32.6 ± 4.5	32.3 ± 4.1	0.645
Body mass index (kg/m2)[a]	22.1 ± 2.8	21.9 ± 3.1	0.725
Gravidity[b]	2 (0–7)	2 (0–10)	0.232
Parity[b]	0 (0–2)	0 (0–2)	0.581
No. of curettage procedures[b]	2 (0–7)	2 (0–9)	0.071
No. of previous surgical adhesiolysis procedures[b]	1 (0–9)	0 (0–4)	0.420
Infertility[c]	14 (20.0)	12 (14.1)	0.329
IUA grade[c]			0.271
Moderate	22 (31.4)	20 (23.5)	
Severe	48 (68.6)	65 (76.5)	
Menstrual pattern[c]			0.041
Hypomenorrhoea	63 (90.0)	66 (77.7)	
Amenorrhoea	7 (10.0)	19 (22.4)	
Causes of IUA[c]			0.873
D&C during the first trimester	42 (60.0)	47 (55.3)	
D&C during the second or third trimester	10 (14.3)	11 (12.9)	
Pelvic tuberculosis and uterine embolization	5 (7.1)	8 (9.4)	
Secondary to surgical procedures	13 (18.6)	19 (22.4)	

[a] Values are mean ± standard deviation, Student's t-test.
[b] Values are median (range), Mann–Whitney *U*-test.
[c] Values are *n* (%), chi-squared test or Fisher's exact test.
D&C, dilation and curettage; IUA, intrauterine adhesion.

2 (n = 85), based on whether they kept the balloon stent for 4 or 6 weeks. Severe IUA accounted for 72.9% of the total cohort. The patients' mean age and BMI were 32.4 ± 4.2 years and 22.0 ± 3.0 kg/m^2. The primary cause of IUA was curettage for early abortion, accounting for 57.4% of cases. Pelvic tuberculosis and uterine artery embolization contributed to 8.4% of the cases. The demographic characteristics between the study populations were comparable, except for a higher proportion (10.0% versus 22.4%, P = 0.041) of participants with amenorrhoea in group 2 (TABLE 1).

Primary outcomes

At the second-look hysteroscopy, the IUA reformation rate was significantly lower in group 2 than in group 1 (45.7% versus 28.2%, P = 0.024). In addition, patients in group 2 experienced a more significant decrease in AFS scores (5.2 ± 2.1 versus 6.3 ± 2.2, P = 0.003) than those in group 1. However, the AFS score and tubal ostium visibility before and after surgery were comparable between the two groups (TABLE 2).

Confounders (identified a priori from the literature) and significant differences in baseline characteristics were related to age, gravidity, parity, number of curettages, amenorrhoea, tubal ostium visibility, causes of IUA, IUA grade, balloon stent placement duration (4 versus 6 weeks) and stent culture positivity. In this study, only the variables that exhibited a trend of association with IUA recurrence in the univariable analysis (P < 0.10) were included in the final model, including IUA grade, balloon stent placement duration, tubal ostium visibility before and after surgery, and causes of IUA. After adjusting for confounders, the multivariable analysis of IUA recurrence revealed that the duration of balloon stent placement still influenced IUA recurrence after hysteroscopic adhesiolysis (OR 0.31, 95% CI 0.13–0.78, P = 0.012) (TABLE 3).

The sub-group analysis among patients attempting conception was in agreement with the primary analysis. The AFS score reduction remained greater in group 2 than in group 1 (5.0 ± 2.0 versus 6.0 ± 2.3, P = 0.026). However, no significant difference was observed in the IUA reformation rate between groups in this sub-population (20/42, 47.6% versus 17/52, 32.6%, P = 0.141).

Secondary outcomes

Stent dislodgement was not reported. The culture results from the retrieved stents are illustrated in FIGURE 2. Nine common microbial species were cultured in group 1, whereas 13 were detected in group 2, including two mixed bacteria. *Escherichia coli* was the most frequently detected bacterium, accounting for 25% and 31% in groups 1 and 2, respectively. Other microbial species among the top five with the highest detection rate in group 1 included *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Candida* and *Streptococcus agalactiae*. In group 2, the microbial species among the top five in addition to *Escherichia coli* were *Candida*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Streptococcus agalactiae*.

The rate of positive cultures did not differ significantly between the two groups (55.9% versus 66.7%, P = 0.174), and the distribution of microorganisms was also comparable (P = 0.316). Notably, no patients with positive cultures exhibited signs of infection such as fever, abdominal pain, abnormal vaginal discharge or an elevated white blood cell count during the balloon stenting.

The mean duration of follow-up was comparable in the two groups: 49 (interquartile range [IQR] 36–60) months in group 1 and 44 (IQR 36–59) months in group 2 (P = 0.096). In group 1, 42 patients had attempted conception, 15 patients with a fertility desire were recorded as not having initiated a conception attempt, 1 patient had no fertility desire and 12 patients were lost to follow-up. In group 2, these numbers were 52, 19, 4 and 10, respectively. Reproductive information is presented in TABLE 2.

Approximately one-third of patients underwent IVF/intracytoplasmic sperm injection (ICSI) treatment. The proportion of patients attempting natural conception (69.0% versus 71.2%) or undergoing IVF/ICSI (31.0% versus 28.8%) was similar between groups 1 and 2 (P = 0.824). Comparisons of the live birth rates between the two groups revealed no significant differences, either in the overall study population (52.4% versus 42.3%, P = 0.331) or in the subgroup analysis in terms of conception method. When the

TABLE 2 COMPARISON OF THE ADHESION REFORMATION RATE, AFS SCORE BEFORE AND AFTER SURGERY, AFS SCORE REDUCTION AND REPRODUCTIVE PARAMETERS BETWEEN GROUPS 1 AND 2, WITH THE BALLOON STENT IN SITU FOR 4 AND 6 WEEKS, RESPECTIVELY

Variables	Group 1 (n = 70)	Group 2 (n = 85)	95% CI of difference	P-value
Pre-operative AFS score[a]	9.1 ± 2.1	9.7 ± 1.9	−1.24 to 0.03	0.060
Pre-operative visibility of tubal ostium[b]				0.079
Bilaterally visible[b]	21 (30.0)	19 (22.4)	−0.06 to 0.22	0.279
Unilaterally invisible[b]	15 (21.4)	10 (11.8)	−0.02 to 0.22	0.104
Bilaterally invisible[b]	34 (48.6)	56 (65.9)	−0.33 to −0.02	0.030
Post-operative AFS score[a]	3.9 ± 2.7	3.4 ± 2.2	−0.32 to 1.26	0.471
Post-operative visibility of tubal ostium[b]				0.183
Bilaterally visible[b]	45 (64.3)	62 (72.9)	−0.23 to 0.06	0.246
Unilaterally invisible[b]	16 (22.9)	10 (11.8)	−0.01 to 0.23	0.066
Bilaterally invisible[b]	9 (12.9)	13 (15.3)	−0.13 to 0.09	0.665
AFS score reduction[a]	5.2 ± 2.1	6.3 ± 2.2	−1.78 to −0.38	0.003
Adhesion reformation rate[b]	32 (45.7)	24 (28.2)	0.02 to 0.33	0.024
Mode of conception				
n	42	52	–	–
Natural pregnancy[b]	29 (69.0)	37 (71.2)	−0.21 to 0.17	0.824
IVF/ICSI[b]	13 (31.0)	15 (28.8)	−0.17 to 0.21	0.824
Live birth rate[b]	22/42 (52.4)	22/52 (42.3)	−0.10 to 0.30	0.331
Natural pregnancy[b]	16/29 (55.2)	14/37 (37.8)	−0.07 to 0.41	0.160
IVF/ICSI[b]	6/13 (46.2)	8/15 (53.3)	−0.44 to 0.30	0.705

[a] Values are mean ± standard deviation, Student's t-test.

[b] Values are n (%) or n/N (%),chi-squared test.

AFS, American Fertility Society; ICSI, intracytoplasmic sperm injection.

patients who were registered as 'no conception attempt' were considered as 'not pregnant' and the results were recalculated, the live birth rate comparison between the two groups remained statistically insignificant (22/57, 38.6% versus 22/71, 31.0%, $P = 0.368$).

FIGURE 3 illustrates a Kaplan–Meier curve of the cumulative probability of live birth over time in patients pursuing a pregnancy. The overall time to achieve a live birth was comparable between groups 1 and 2 (HR 1.09, 95% CI 0.60–1.96, $P = 0.778$). When adjusted for age and IUA grade (moderate/severe), the results did not change significantly (HR 0.76, 95% CI 0.42–1.39, $P = 0.376$).

DISCUSSION

This study evaluated the effect of different uterine balloon stent placement durations (4 versus 6 weeks) on IUA recurrence, analysed the bacterial colonization of the removed balloon stent and assessed live birth outcomes. The findings indicated that leaving the balloon stent for an additional 2 weeks, i.e. for 6 rather than 4 weeks, resulted in a significantly lower rate of IUA recurrence and a greater reduction in the AFS IUA score during the second-look hysteroscopy, without increasing intrauterine bacterial colonization. Nonetheless, the live birth rates and TTLB were similar between the 4-week and 6-week groups.

High-grade IUA predicted an increased risk of spontaneous IUA recurrence post-operatively (*Yang et al., 2016*). To prevent wound adhesion during endometrial healing, routine clinical practice involves inserting an intrauterine device for approximately 7 days (*Huang et al., 2020*). However, this intervention does not address the high post-operative recurrence rates of severe IUA. No consensus has been reached regarding barrier placement duration. At the study centre, follow-up hysteroscopy is routinely performed, scheduled 3–4 weeks after the initial hysteroscopic adhesiolysis. The procedures are repeated every 3–4 weeks until no reformed IUA is detected.

Recurrent IUA is almost always flimsy and loose at the early stage and can be easily separated with the tip of the hysteroscope. However, patients with severe and extensive IUA often require repeat procedures to achieve a satisfactory uterine cavity morphology (*Hanstede et al., 2015*; *Xiao et al., 2014*), especially if they have conical uterine cavities caused by bilateral uterine corner closure, or partial or total uterine cavity occlusion. The endometrial healing process in these patients requires a considerable amount of time. However, with time since the initial surgery, the adhesions tend to become denser and more difficult to separate. Two prospective randomized controlled trials (RCT) have confirmed the efficacy of using a balloon stent for approximately 30 days in preventing IUA reformation (*Wang et al., 2022*; *Yang et al., 2020*).

Most patients (72.9%) in the current study had severe IUA, including those with poor prognoses who underwent uterine embolization or had tuberculosis. Several factors, such as IUA severity and intrauterine procedures, influence the

TABLE 3 MULTIVARIABLE ANALYSIS FACTORS INFLUENCING INTRAUTERINE ADHESION (IUA) RECURRENCE.

Adhesion recurrence determinants	Recurrence (*n* = 56)	Non-recurrence (*n* = 99)	OR (95% CI)	*P*-value	*P*-trend
IUA grade[a]					0.214
Moderate	5 (8.9)	38 (38.4)	–		
Severe	51 (91.1)	61 (61.6)	0.81 (0.19–3.39)	0.774	
Preoperative visibility of tubal ostium[a]				0.030	0.048
Bilaterally visible	3 (5.4)	39 (39.4)	–		
Unilaterally invisible	11 (19.6)	14 (14.1)	8.36 (1.10–63.43)	0.040	
Bilaterally invisible	42 (75.0)	46 (46.5)	13.55 (1.97–93.23)	0.008	
Post-operative visibility of tubal ostium[a]				<0.001	0.010
Bilaterally visible	21 (37.5)	86 (86.9)	–		
Unilaterally invisible	23 (41.1)	3 (3.0)	18.77 (4.58–76.95)	<0.001	
Bilaterally invisible	12 (21.4)	10 (10.1)	2.73 (0.85–8.81)	0.093	
Time of balloon stent placement[a]					0.003
4 weeks	32 (57.1)	38 (38.4)	–		
6 weeks	24 (42.9)	61 (61.6)	0.31 (0.13–0.78)	0.012	
Causes of IUA[a]				0.024	0.340
D&C during the first trimester	25 (44.6)	64 (64.6)	–		
D&C during the second or third trimester	10 (17.9)	11 (11.1)	2.01 (0.59–6.82)	0.265	
Pelvic tuberculosis and uterine embolization	9 (16.1)	4 (4.0)	22.45 (2.91–173.31)	0.003	
Secondary to surgical procedures	12 (21.4)	20 (20.2)	1.53 (0.52–5.51)	0.438	

[a] Values are *n* (%), chi-squared test. Variables included in the univariable analysis were age, gravidity, parity, number of curettages, amenorrhoea, tubal ostium visibility, causes of IUA, IUA grade, time of balloon stent placement and stent culture positivity.

D&C, dilation and curettage; IUA, intrauterine adhesion.

post-operative recurrence rate in patients with IUA (*Hooker et al., 2014*; *Shen et al., 2022*; *Yang et al., 2016*). In this particular study, a higher proportion of patients with amenorrhoea was observed in group 2 than group 1. Moreover, although it was not statistically significant, group 2 exhibited slightly elevated pre-operative AFS scores compared with group 1. These findings suggest that patients in group 2 may have had more severe pre-operative adhesions and potentially worse prognoses with a higher recurrence rate.

However, despite these factors indicating poorer outcomes initially for group 2, a significant reduction in post-operative adhesion recurrence rates was discovered, along with a greater improvement in AFS scores among patients who underwent an extended duration of balloon stent placement (group 2). These findings underscore the importance of extending the duration of balloon stent by an additional 2 weeks, as this significantly contributes towards preventing adhesion reformation in patients suffering from severe disease.

Prolonged retention of an intrauterine balloon stent bears a risk of bacterial

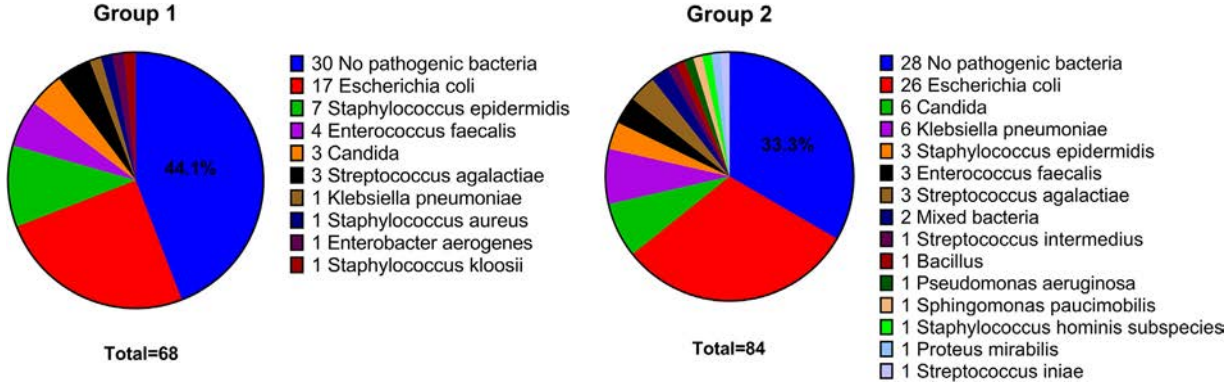

FIGURE 2 Culture results for the removed balloon stent for group 1 and group 2. The numbers in the key represent the frequency of each culture finding.

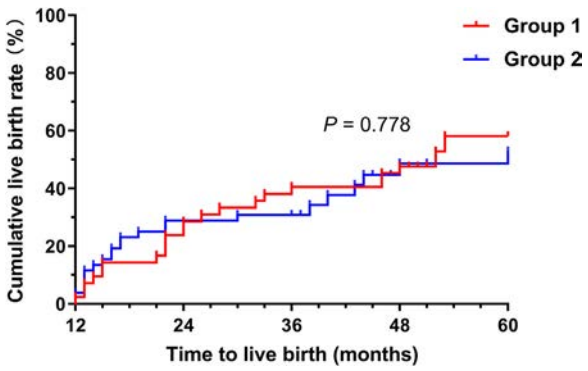

FIGURE 3 Kaplan–Meier curve for cumulative live birth rates. The live birth rates in groups 1 and 2 were compared using the log-rank (Mantel–Cox) test.

colonization or infection, which contributes to the development or recurrence of IUA. However, recent advances in sequencing techniques challenge the assumption that the uterus is sterile (*Sola-Leyva et al., 2021*). Furthermore, a previous prospective RCT revealed that the bacterial colonization rate of the uterine cavity 30 days after hysteroscopic surgery was increased compared with pre-operative cultures (*Lin et al., 2015*), although not significantly so. This trend was observed regardless of whether a balloon stent had been placed, indicating that leaving the balloon stent in the uterine cavity for up to 30 days post-operatively does not increase bacterial colonization or clinical infection. Results from other studies have also confirmed that it is safe to leave the balloon stent in the uterine cavity for approximately 30 days post-operatively (*Wang et al., 2022*; *Yang et al., 2020*).

Owing to the concern that prolonged antibiotic use may lead to dysbacteriosis and antibiotic resistance, prophylactic antibiotics were used for 5–7 days in all patients according to the routine treatment protocol at the current authors' centre. Additionally, the diameter of the balloon stent was reduced at the external os of the cervix to prevent upstream bacterial infection from the vagina. Consequently, no evidence of infection was observed in this study. No statistical difference was observed in the rate of positive bacterial cultures from the retrieval balloon in either group, indicating that leaving the balloon stent in the uterine cavity for an additional 2 weeks, i.e. for a total of 6 weeks, is as safe as leaving it for only 4 weeks. Considering this, extending the duration of balloon stent placement may have important practical implications for treating moderate to severe IUA.

Many adhesion prevention methods have been evaluated in comparison with controls in terms of IUA recurrence rate; however, data on live birth rates remain insufficient. Live birth after adhesiolysis varies from 16.7% to 67.4%, negatively correlating with the severity of IUA (*Bhandari et al., 2015*; *Chen et al., 2017*; *Deans et al., 2018*; *Hanstede et al., 2021*). In a case-control study of 51 women with infertility and IUA who underwent IVF treatment after surgery, the live birth rate was only 23.5%, much lower than in the women with infertility alone (56.8%). Meanwhile, women with infertility combined with IUA exhibited a significantly longer TTLB than controls. In that study, the proportion of patients with moderate-to-severe IUA accounted for 74.5% of the study group (*Fouks et al., 2022*). This study focused on patients with high-grade IUA with a poor prognosis. Live birth rates were 52.4% and 42.3% in groups 1 and 2, respectively, in accordance with previous studies. However, prolonging the duration of balloon stent placement by 2 weeks did not significantly impact the live birth rate or TTLB.

The current study has several strengths. It is the first to comprehensively analyse the effect of prolonged balloon stent placement (up to 6 weeks) in preventing post-operative IUA recurrence in patients with moderate to severe IUA, especially by considering both second-look hysteroscopy and bacterial cultures. Moreover, the live birth rates and TTLB of these patients were observed during long-term follow-up. Furthermore, the authors' centre is a national hysteroscopy training and tertiary referral centre with specialists who are experienced and highly qualified in the diagnosis and treatment of complex intrauterine disorders. Many publications focus on IUA, making the management of high-grade IUA reliable (*Guo et al., 2022*; *Yang et al., 2020*; *Yu et al., 2008*; *Zhou et al., 2021*).

Considering the inherent limitations of retrospective studies and the complexities associated with real-world clinical practice, potential confounding variables may have affected the study results, which is the primary limitation of this study. To address this issue, multivariate logistic regression was conducted to control for confounding factors such as amenorrhoea, tubal ostium visibility and IUA grade while investigating the impact of different durations of balloon stent placement on the recurrence of adhesions. Additionally, sub-group analysis was performed in patients attempting to conceive. These findings were consistent with the authors' primary analysis. Furthermore, it should be noted that the sample size of this study was insufficient to adequately assess differences in live birth rates; therefore, caution should be exercised when interpreting the reproductive outcomes. Well-designed RCT with large sample sizes are necessary to validate these findings.

Patients with severe disease often have a thin endometrium due to intrauterine scarring and require more time to pursue pregnancy (*Fouks et al., 2022*; *Movilla et al., 2020*; *Wang et al., 2021*); this poses a challenge for fertility management after adhesiolysis. Future research should continue to monitor live birth rates in patients with high-grade IUA and focus on strategies to optimize post-operative fertility.

In conclusion, extending intrauterine balloon stent use from 4 to 6 weeks further reduces the IUA reformation rate after hysteroscopic adhesiolysis in patients with moderate to severe IUA. No increase in bacterial colonization of the balloon stent was observed, and an extended intrauterine balloon stent placement duration did not significantly affect live birth rates.

DATA AVAILABILITY

Data will be made available on request.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

Yiyang Luo: conception and design of the study, acquisition of data, analysis and interpretation of data, drafting of the article, final approval of the version to be submitted; Yuhuan Liu: conception and design of the study, critical revision of the article, final approval of the version to be submitted; Wei Xie and Yan Guo: acquisition of data, final approval of the version to be submitted; Yu Xiao: critical revision of the article, final approval of the version to be submitted. All the authors contributed to this article and approved the version submitted for publication.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.103947.

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REVIEW

Fertility preservation parameters in patients with haematologic malignancy: a systematic review and meta-analysis

BIOGRAPHY

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KEY MESSAGE

The effect of haematologic malignancy on ovarian reserve and response to ovarian stimulation is unclear. Our meta-analysis demonstrated that women with haematologic malignancy have lower anti-Müllerian hormne serum levels. This effect can be overcome by the application of relevant IVF protocols and stimulation doses to achieve an adequate oocyte yield.

ABSTRACT

Patients with haematologic malignancies represent one of the most common groups referred for fertility preservation before gonadotoxic oncological treatment. The aim of this systematic review and meta-analysis was to evaluate the effect of haematologic cancer on ovarian reserve and response to ovarian stimulation compared with healthy controls. A total of eight observative studies were included in the final quantitative analysis. Despite a younger age (mean difference −4.17, 95% CI −6.20 to −2.14; $P < 0.0001$), patients with haematologic malignancy had lower serum anti-Müllerian hormone levels compared with the control group (MD −1.04, 95% CI −1.80 to −0.29; $P = 0.007$). The marginally higher total recombinant FSH dose (MD 632.32, 95% CI −187.60 to 1452.24; $P = 0.13$) and significantly lower peak oestradiol serum level (MD −994.05, 95% CI −1962.09 to −26.02; $P = 0.04$) were demonstrated in the study group compared with the healthy controls. A similar number of retrieved oocytes were achieved in both groups (MD 0.20, 95% CI −0.80 to 1.20; $P = 0.69$). In conclusion, haematologic malignancies may detrimentally affect ovarian function manifesting in decreased AMH serum levels despite a younger age compared with healthy controls. This effect can be overcome by the application of relevant IVF protocols and stimulation doses to achieve an adequate oocyte yield.

INTRODUCTION

Haematologic malignancy is one of the top five common cancers in reproductive-age women (*Miller et al., 2020*). Advances in anti-cancer treatment modalities have markedly increased the survival rate of reproductive-age females with haematologic cancer over the past decades, with a 5-year survival rate of about 70% (*Ohbiki et al., 2023*). Concurrently, the adverse effects of oncological treatment on ovarian function have become evident. Chemotherapy based on alkylating agents was found to deplete the primordial follicle pool and induce ovarian atrophy through apoptosis (*Fleischer et al., 2011*). These treatments consequently affect female long-term fertility (*Wo and Viswanathan, 2009*; *Jadoul et al., 2012*) and impair response to ovarian stimulation (*Meirow et al., 2010*; *Bedoschi et al., 2016*). Patients with

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KEYWORDS
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Hematologic malignancy
Oocyte cryopreservation

haematologic malignancies represent one of the most common groups referred for fertility preservation before gonadotoxic oncological treatment.

Because increased serum levels of cytokines and growth factors trigger a proinflammatory response (*Paradisi et al., 2016*), aggressive lymphoid neoplasms may cause systemic effects, such as paraneoplastic neurologic syndrome, fever, weight loss and night sweats (*Sharma et al., 2009*; *Graus et al., 2014*), along with distant organ impairment, including gonadal dysfunction even before oncological treatment (*Eghbali and Papaxanthos-Roche, 2010*; *Volodarsky-Perel et al., 2020*).

In men diagnosed with lymphoma, sperm quality assessment undertaken as part of the cryopreservation protocol has shown a significant decrease in sperm concentration and motility even before chemotherapy treatment. Reduction in spermatogenesis was found to be caused by multiple factors, including intercurrent fever, the general stress effect of the malignant disease and immunological disturbance, meaning the primary disease might have detrimental gonadal effects (*Rueffer et al., 2001*; *Shrem et al., 2022*).

In contrast, the fundamental question about the negative effect of haematologic cancer on ovarian function has not yet been answered (*Friedler et al., 2012*; *Dolinko et al., 2018*). Numerous hypotheses and theories have been proposed to elucidate this phenomenon, encompassing germline mutations in the FA–BRCA DNA repair pathway (*Sklavos et al., 2015*), unexplained gamete impairment (*Fabbri et al., 2011*) and cytokine higher concentration levels and their systemic influence (*Maggio et al., 2002*). Determining whether the primary disease affects the ovarian response is clinically significant and may prompt modifications to the ovarian stimulation protocol.

The present systematic review and meta-analysis aims to evaluate whether the ovarian reserve and response to ovarian stimulation is affected by haematologic cancer before anti-cancer treatment compared with the healthy control group.

MATERIALS AND METHODS

Literature search

A systematic review based on a literature search using *PubMed*, *Ovid*, *Embase*, *MEDLINE*, *Google Scholar*, *ClinicalTrials.gov* and the *Cochrane Central Register of Controlled Trials* was conducted. No language or publication period restrictions were imposed. The search strategy was based on the following medical subject heading terms and their combinations: 'fertility preservation', 'oocyte cryopreservation', 'ovarian reserve', 'antral follicle count', 'anti-Müllerian hormone', 'lymphoma', 'leukemia', 'myeloma', 'hematologic malignancy', 'hematologic cancer'. The reference lists of identified studies were manually searched.

Selection criteria

All randomized controlled studies, cohort studies, case-control studies and meeting abstracts on ovarian response to ovarian stimulation for fertility preservation in patients with haematologic malignancies before anti-cancer treatment compared with healthy controls were included. Systemic reviews, meta-analyses, case series and case reports were excluded. Two authors (TK and GS) selected and evaluated studies independently. Any disagreements were resolved by a third author (AVP). In cases of duplicate publication, the most recent and complete versions were selected.

Data extraction

The following data were obtained from all eligible studies: study design, year of publication, country, study period, age and number of patients, serum anti-Mullerian hormone (AMH) levels, baseline antral follicles count (AFC), total recombinant FSH stimulation dose, stimulation duration, peak oestrogen level and the number of retrieved oocytes. The corresponding authors were contacted in cases of insufficient data.

The studies evaluating the ovarian response of patients with haematologic cancer compared with that of healthy controls were included in the quantitative analysis. The primary outcome was the number of retrieved oocytes. Secondary outcomes included serum AMH level, baseline AFC, total recombinant FSH stimulation dose, peak oestradiol serum level and duration of stimulation. Age was also evaluated as the baseline parameter of ovarian stimulation cycles.

The systematic review and meta-analysis followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement (*Moher et al., 2009*) and the Meta-analysis of Observational Studies in Epidemiology guidelines (*Stroup et al., 2000*). As all studies were extracted from previously published data, Institutional Review Board approval was not requested. This study protocol was registered in the International Prospective Register of Systematic Reviews database (registration number CRD42022349016).

Data synthesis

The Cochrane Collaboration tool was applied for the risk of bias assessment of randomized controlled trials (*Higgins et al., 2011*). Non-randomized studies were assessed using the Risk of Bias in Non-Randomized Studies of Interventions tool (ROBINS-I) (*Sterne et al., 2016*) and the Newcastle–Ottawa scale (*Stroup et al., 2000*). The Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach was used for certainty of evidence assessment of randomized and observational studies (*Guyatt et al., 2011*).

Continuous variables were expressed as a mean difference, with a 95% confidence interval. Statistical heterogeneity was evaluated by using the I-squared statistic method and considered absent if $P > 0.05$. I squared values of 25%, 50% and 75% indicated low, moderate, and high heterogeneity, respectively. A fixed-effects model was used in cases of low heterogeneity. A random-effects model was used in cases with moderate and high heterogeneity. Review Manager 5.3 Software (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) was used for the meta-analysis conduction.

RESULTS

Study selection

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses scheme of the literature search and selection is presented in FIGURE 1. A total of 1373 articles fulfilled the search criteria. After removing duplicate records, and title and abstract screen, 35 records were

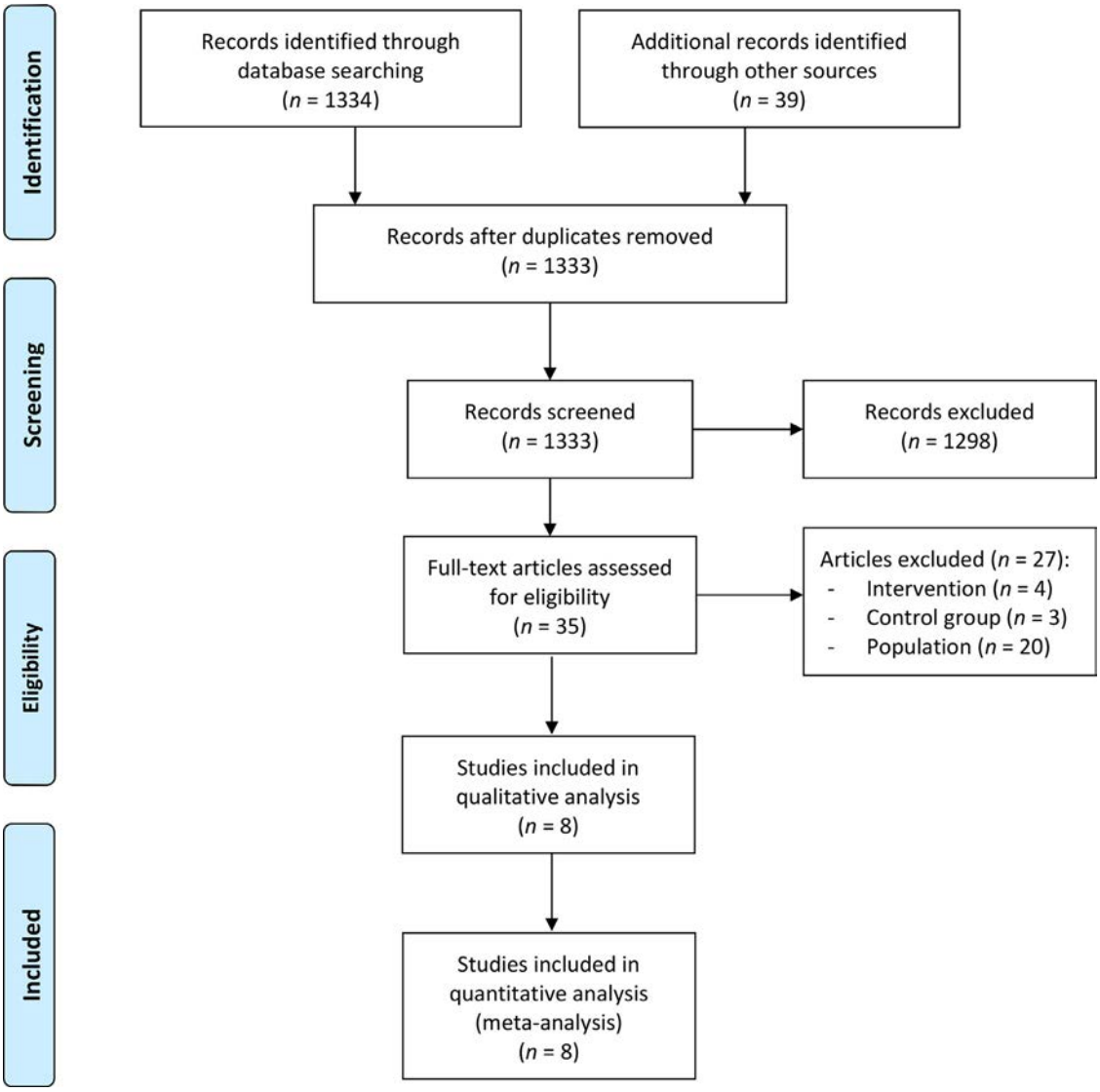

FIGURE 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow diagram of literature search and selection.

selected for full-text review (TABLE 1). Date of publication ranged from 2009 to 2022. The most common reason for exclusion from quantitative analysis was the absence of separate data for patients with haematologic malignancy (*Knopman et al., 2009*; *Klock et al., 2010*; *Michaan et al., 2010*; *Noyes et al., 2010*; *Quintero et al., 2010*; *Werner et al., 2010*; *Robertson et al., 2011*; *Domingo et al., 2012*; *Garcia-Velasco et al., 2013*; *Devesa et al., 2014*; *Cardozo et al., 2015*; *Kim et al., 2015*; *Nurudeen et al., 2016*; *Schon et al., 2017*; *Cobo et al., 2018*; *Harzif et al., 2019*; *Moraes et al., 2019*; *Kawwass et al., 2020*; *Virant-Klun et al., 2021*; *Fabiani et al., 2022*). In two studies, patients were exposed to radiotherapy or chemotherapy before fertility preservation treatment (*Pavone et al., 2014*; *Dolinko et al., 2018*). One study was excluded because patients underwent ovarian tissue cryopreservation before ovarian stimulation (*Dolmans et al., 2014*). Three studies evaluated cancer patients without comparing their outcomes with healthy controls (*Lawrenz et al., 2011*; *Lefebvre et al., 2018*; *Specchia et al., 2019*). One study evaluated the results of only in-vitro maturation between patients with haematologic cancer and healthy controls (*Moria et al., 2011*).

A total of eight observational studies comparing ovarian reserve parameters and fertility preservation outcomes in patients with haematologic malignancy with controls were included in the final qualitative and quantitative analyses (*Das et al., 2011*; *Almog et al., 2012*; *Lawrenz et al., 2012*; *Lekovich et al., 2016*; *Naasan et al., 2016*; *Paradisi et al., 2016*; *Decanter et al., 2018*; *Brun et al., 2021*). No randomized controlled trials were found to be eligible for the present analysis. The number of cases varied from 12 to 73 in the study group and from 25 to 5231 in the control group. The study group included patients diagnosed with myeloid leukaemia, lymphocytic leukaemia, Hodgkin's lymphoma and non-Hodgkin's lymphoma. The control group included age-matched healthy volunteers, patients referred to IVF treatment for elective fertility preservation, male-factor infertility treatment and egg donors.

Data on AMH level were insufficient in two studies (*Das et al., 2011*; *Almog et al., 2012*). Data on the mean number of retrieved oocytes were insufficient in three

TABLE 1 CHARACTERISTICS OF STUDIES SELECTED FOR FULL-TEXT REVIEW

Study, year of publication	Country	Period	Design	Study group, *n*		Control group, (*n*)	Outcome	Inclusion	Reason for exclusion
				Haematologic malignancy	Lymphoma				
Almog et al. *(2012)*	Israel	2000–2011	RCS	12	Not specified	Male factor (81)	Age, oocytes	Included	–
Brun et al. *(2021)*	France	2014–2019	RCS	2	39	Egg donor (117)	Age, AFC, AMH, FSH, duration of stimulation, oocytes	Included	–
Cardozo et al. *(2015)*	USA	1997–2014	RCS	5	0	Age matched (122)	Age, BMI, FSH, peak oestradiol, oocyte	Excluded	Population
Cobo et al. *(2018)*	Spain	2007–2018	RCS	180	180	Elective fertility preservation (5289)	Age, IVF protocols, oocyte, live births	Excluded	Population
Das et al. *(2011)*	Canada	2003–2010	RCCS	2	14	Male factor (48)	Age, AFC, FSH, duration of stimulation, peak oestradiol, oocytes	Included	–
Decanter et al. *(2018)*	France	2011–2014	RCS	25	Not specified	Male factor (180)	Oocytes, AMH, AFC, FSH	Included	–
Devesa et al. *(2014)*	Spain	2007–2012	RCS		6	Male factor (1536)	Age, AFC, FSH dose, duration of stimulation, peak oestradiol, oocytes	Excluded	Population
Dolinko et al. *(2018)*	USA	2007–2014	RCS	42	19	Male-factor infertility (644)	Age, AFC, AMH, oocyte, FSH, duration of stimulation, oocytes	Excluded	Intervention
Dolmans et al. *(2014)*	Belgium	Missing data	RCS		8	Male factor (100)	Age, FSH, oestradiol, oocytes duration of stimulation	Excluded	Intervention
Domingo et al. *(2012)*	Spain	2007–2011	RCS		37	Male factor (97)	Age, duration of stimulation, oestradiol level, oocytes, FSH, live birth	Excluded	Population
Fabiani et al. *(2022)*	Italy	2016–2019	RCS		15	Male factor (180)	Age, AMH, FSH, oestradiol, oocytes, duration of stimulation	Excluded	Population
Garcia-Velasco et al. *(2013)*	Spain	2007–2012	RCS		76	Non-oncological (560)	Duration of stimulation, FSH, peak E2, oocytes, pregnancy rate	Excluded	Population
Harzif et al. *(2019)*	Indonesia	2015–2017	RCS		8	Age matched (44)	Age, AMH	Excluded	Population
Kawwass et al. *(2020)*	USA	2012–2016	RCS		250	Control (26,916)	Age, FSH, cancellations, hyperstimulation, oocyte	Excluded	Intervention population
Kim et al. *(2015)*	Korea	2012–2013	RCS		2	Infertility (44)	Age, BMI, AMH, gonadotrophins, oocytes, duration of stimulation	Excluded	Intervention population
Klock et al. *(2010)*	USA	2005–2008	RCCS		20	Age matches (57)	Fertility-preservation techniques	Excluded	Intervention
Knopman et al. *(2009)*	USA	2001–2006	RCS		4	Male factor (135)	Age, oestradiol, FSH, number of oocytes	Excluded	Population
Lawrenz et al. *(2011)*	Germany	2007–2009	RCS		360	No control	Counselling indication, number of	Excluded	Intervention, no control

(*continued on next page*)

TABLE 1 (*Continued*)

Study, year of publication	Country	Period	Design	Study group, *n*		Control group, (*n*)	Outcome	Inclusion	Reason for exclusion
				Haematologic malignancy	Lymphoma				
							children, age, complications		
Lawrenz et al. *(2012)*	Germany	2007–2011	RCS		38	Healthy control (38)	Age, AMH	Included	–
Lefebvre et al. *(2018)*	France	2013–2016	RCS		25	No control	Age, AFC, AMH, FSH duration of stimulation, oocytes	Excluded	No control
Lekovich et al., *2016*	USA	2010–2013	RCS		64	Elective fertility preservation (365)	Age, AMH, FSH, duration of stimulation, oocytes	Included	–
Michaan et al., *2010*	Israel	2002–2007	RCS	2	0	Tubal indication (22)	Gonadotrophins, oestradiol, progesterone, duration of stimulation, oocytes, pregnancy rate	Excluded	Population
Moraes et al., *2019*	Brazil	2010–2017	RCS		4	Non-oncological (164)	Age, BMI, oocytes duration of stimulation	Excluded	Population
Moria et al., *2011*	Canda	2003–2009	RCS		16	Infertile control 79)	Age, FSH, oocytes	Excluded	Intervention
Naasan et al., *2016*	Ireland	2009–2013	RCS	7	22	Healthy control (5231)	Age, AMH	Included	–
Noyes et al., *2010*	USA	2005–2009	RCS		8	Other infertility (32)	Age, duration of stimulation, oestradiol, oocytes, FSH, delivery rate	Excluded	Population
Nurudeen et al., *2016*	USA	2005–2012	RCS		4	Age matched (52)	Age, AMH, oestradiol level, duration of stimulation, oocyte, FSH dose	Excluded	Intervention
Paradisi et al., *2016*	Italy	2011–2015	RCS	73		Healthy control (25)	Age, AMH	Included	–
Pavone et al., *2014*	USA	2005–2011	RCS	27		Male factor (176)	Age, duration of stimulation, oestradiol, oocytes	Excluded	Intervention, population
Quintero et al., *2010*	USA	1999–2007	RCS	11		Healthy control (50)	Oocytes duration of stimulation, gonadotrophins	Excluded	Population
Robertson et al., *2011*	USA	2001–2007	RCS	6		Male factor (921)	Age, FSH, oestradiol, duration of stimulations, oocytes	Excluded	Population
Schon et al., *2017*	USA	2008–2016	RCS	NS		Elective fertility preservation (210)	Age, BMI, AMH, AFC, oocytes, oestradiol	Excluded	Population
Specchia et al., *2019*	Italy	2001–2019	RCS		66	No control		Excluded	No control
Virant-Klun et al., *2021*	Slovenia	Not specified	RCS	8		Other infertility (21)	IVM, oocyte	Excluded	Intervention, population
Werner et al., *2010*	USA	2006–2010	RCS		6	Elective fertility preservation (73), oocyte donation (8)	Age, oestradiol, oocyte	Excluded	Population

AFC, antral follicle count; AMH, anti-Müllerian hormone; BMI, body mass index; IVM, in-vitro maturation; RCS, retrospective cohort study; RCCS, retrospective case-control study; –, not applicable.

studies (*Lawrenz et al., 2012*; *Naasan et al., 2016*; *Paradisi et al., 2016*). Data on the number of mature oocytes were available only in one study (*Decanter et al., 2018*). Five studies (*Almog et al., 2012*; *Lawrenz et al., 2012*; *Naasan et al., 2016*; *Paradisi et al., 2016*; *Decanter et al., 2018*) were missing data on baseline AFC. Data on total recombinant FSH dose and the duration of stimulation were insufficient in four studies (*Almog et al., 2012*; *Lawrenz et al., 2012*; *Naasan et al., 2016*; *Paradisi et al., 2016*). Data on peak oestradiol level were available in four studies (*Das et al., 2011*; *Almog et al., 2012*; *Decanter et al., 2018*; *Brun et al., 2021*). Although the ovarian reserve parameters in one study (*Lawrenz et al., 2012*) were compared between patients with haematologic malignancy and healthy controls, the stimulation parameters and outcomes were compared between patients with haematologic and breast cancer and were not included in the final quantitative analysis. The corresponding authors were contacted twice using an e-mail address provided in the articles.

Quality assessment

The studies included in the quantitative analysis were assessed by the ROBINS-I tool for risk of bias evaluation. There was a low risk of bias for selection of study participants, classification of interventions, deviations from intended interventions, missing data and selection of the reported results. As expected, there was a moderate to serious risk of bias for the measurement of outcomes owing to the study design. The ROBINS-I tool detailed guide indicates that if the study is judged to be at moderate or serious risk of bias in at least one domain, the study's risk of bias overall should be judged as moderate or serious risk respectively. Accordingly, seven studies scored an overall moderate risk of bias and one study was judged as having an overall serious risk of bias (FIGURE 2). Although the overall risk of bias was low for the studies included in the meta-analysis, the GRADE approach demonstrated low certainty of evidence due to the observational study design (**Supplementary Table 1**). The overall quality of the studies included in the meta-analysis was assessed using the Newcastle–Ottawa scale (**Supplementary Table 2**). All studies achieved scores of 6 to 8 out of a maximum 9 points; the main concerns were comparability by analysis and duration of follow-up.

Quantitative analysis

Age

The patient's age was evaluated in all eight included in the quantitative analysis studies (*Das et al., 2011*; *Lawrenz et al., 2012*; *Almog et al., 2012*; *Lekovich et al., 2016*; *Naasan et al., 2016*; *Paradisi et al., 2016*; *Decanter et al., 2018*; *Brun et al., 2021*). The meta-analysis showed that the patients with haematologic malignancy were significantly younger compared with the control group (mean difference −4.17, 95% CI −6.20 to −2.14; $P < 0.0001$) (FIGURE 3A).

Ovarian reserve parameters

The patient's AFC was evaluated in three studies (*Das et al., 2011*; *Lekovich et al., 2016*; *Brun et al., 2021*), and was found to

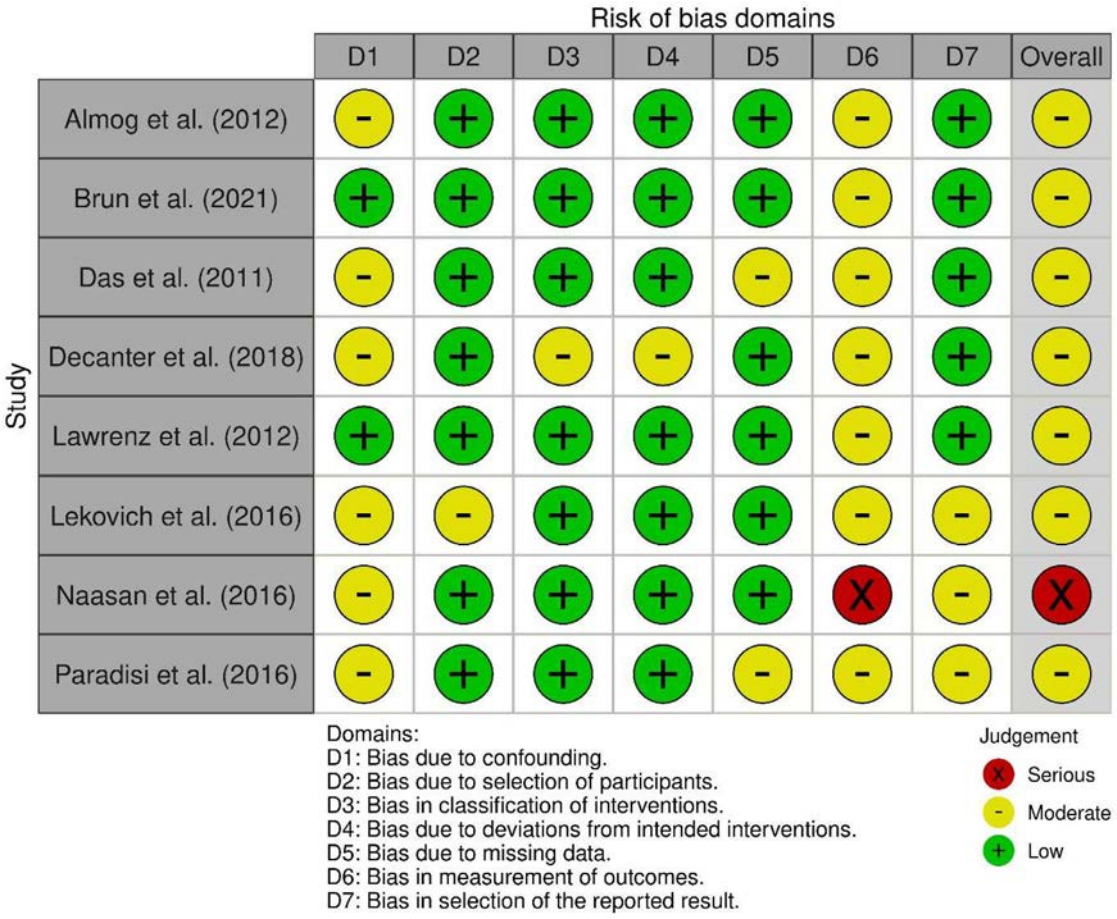

FIGURE 2 The Risk of Bias in Non-randomized Studies – of Interventions (ROBINS-I) assessment tool: application to the studies included in the review.

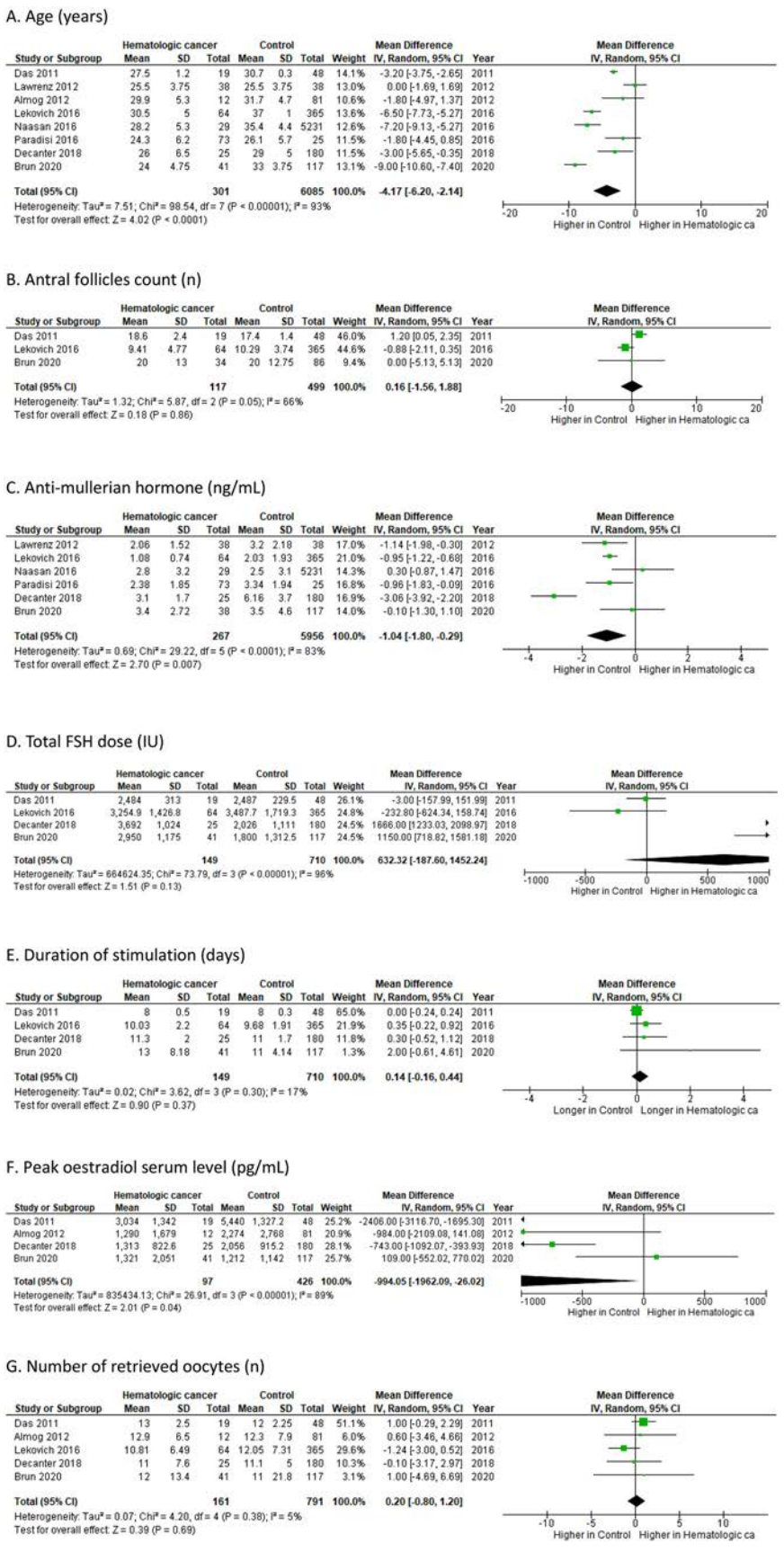

FIGURE 3 Quantitative analysis of data from the included studies relating to age and parameters of ovarian reserve, stimulation cycle and fertility preservation outcome.

be similar between the groups (MD 0.16, 95% CI −1.56 to 1.88; $P = 0.86$) (FIGURE 3B). The patient's AMH was evaluated in six studies (*Lawrenz et al., 2012*; *Lekovich et al., 2016*; *Naasan et al., 2016*; *Paradisi et al., 2016*; *Decanter et al., 2018*; *Brun et al., 2021*). The meta-analysis demonstrated a lower AMH serum level in the study group compared with the control group (MD −1.04, 95% CI −1.80 to −0.29; $P = 0.007$) (FIGURE 3C).

Stimulation cycle parameters

The total FSH dose evaluated in four studies (*Das et al., 2011*; *Lekovich et al., 2016*; *Decanter et al., 2018*; *Brun et al., 2021*) was found to be marginally higher in the study group. The difference, however, did not achieve statistical significance (MD 632.32, 95% CI −187.60 to 1452.24; $P = 0.13$) (FIGURE 3D).

Duration of stimulation was evaluated in four studies (*Das et al., 2011*; *Lekovich et al., 2016*; *Decanter et al., 2018*; *Brun et al., 2021*) (FIGURE 3E) was similar between the groups (MD 0.14, 95% CI −0.16 to 0.44, $P = 0.37$).

Peak oestradiol level was evaluated in four studies (*Das et al., 2011*; *Almog et al., 2012*; *Decanter et al., 2018*; *Brun et al., 2021*) (FIGURE 3F) was lower in the study group compared with the healthy controls (MD = −994.05, 95% CI −1962.09 to −26.02, $P = 0.04$).

Fertility preservation outcome

The total number of retrieved oocytes was evaluated in five studies (*Das et al., 2011*; *Almog et al., 2012*; *Lekovich et al., 2016*; *Decanter et al., 2018*; *Brun et al., 2021*;). Our meta-analysis showed no statistical difference in the number of retrieved oocytes between the haematologic malignancy and control group (MD = 0.20, 95% CI −0.80 to 1.20; $P = 0.69$) (FIGURE 3G).

DISCUSSION

This study systematically investigated the ovarian reserve parameters and results of ovarian stimulation in patients with haematologic malignancies undergoing fertility preservation before oncological treatment compared with healthy controls. Our quantitative analysis demonstrated a similar total number of retrieved oocytes between the groups. Despite being younger, patients with haematologic malignancy had lower serum AMH levels

compared with the control group. The marginally higher total recombinant FSH dose and significantly lower peak oestradiol serum level in the study group probably represent a dose adjustment to a lower AMH level and lead to a similar number of retrieved oocytes.

In recent years, there has been a notable rise in the incidence rate for all types of haematologic malignancies. Fortunately, advancements in oncological treatment have contributed to an improvement in life expectancy for affected individuals (*Zhang et al., 2023*). This emerging trend has prompted concerns surrounding the fertility of women undergoing oncological treatments. While previous research has established a negative effect of chemotherapy and radiation therapy on fertility (*Meirow et al., 2010*; *Fleischer et al., 2011*), a pertinent question remains: is ovarian function affected by the underlying disease even before the anti-cancer treatment starts? The response to this question is essential for appropriate consultation regarding a critical issue preoccupying the patients during a trembling period of oncological treatment — their future fertility.

Haematologic malignancies apply a systemic inflammatory response with increased serum levels of vascular endothelial growth factor, transforming growth factor (TGF)-b, cytokines (interleukin-6), and C-reactive protein (*Niitsu et al., 2002*; *Nakahata et al., 2010*). Studies in animals demonstrated that TGF-b up-regulation is associated with inhibition of folliculogenesis, oocyte maturation and ovulation (*Ingman and Robertson, 2002*; *Bilyk et al., 2017*). The association between haematological malignancy and germ-cell damage before oncological treatment was demonstrated when histological and ultrastructural analysis of fresh ovarian cortex of patients with Hodgkin's disease revealed a significantly higher rate of vacuolated follicles compared with the healthy controls (*Fabbri et al., 2011*). This may explain the decreased granulosa-cell release of AMH representing ovarian quantitative reserve in IVF patients, suggesting the probable ovarian follicle dysfunction in patients with haematologic malignancy despite their younger age (*Broekmans et al., 2009*).

It has previously been shown that prolonged oral contraceptive pills use can be associated with decreased AMH levels compared with non-users (*Bentzen et al., 2012*; *Nelson et al., 2023*). Healthy controls in four studies (*Lawrenz et al., 2012*; *Lekovich et al., 2016*; *Paradisi et al., 2016*; *Decanter et al., 2018*) had a higher proportion of oral contraceptive pills users than the study group yet demonstrated significantly higher mean AMH levels.

Our meta-analysis did not reveal a statistical difference in AFC between the groups. This discrepancy may be attributed to the detrimental effect of haematologic cancer on the developing primary to early antral follicles as a primary AMH source. Although a lower peak oestradiol serum level was demonstrated in the study group, after the administration of sufficient doses of ovarian stimulation agents, an adequate number of antral follicles and a similar number of retrieved oocytes can be achieved in both groups.

To the best of our knowledge, this is the first systematic review and meta-analysis evaluating the ovarian reserve parameters and fertility preservation outcome in patients with haematologic cancer compared with healthy controls. Our meta-analysis included eight observational studies that were analysed using the ROBINS-I tool, the GRADE approach and the Newcastle–Ottawa scale for risk of bias assessment. The moderate overall risk of bias by the ROBINS-I tool and the low certainty of evidence by the GRADE approach is explained by the limitations of the retrospective study design. All studies achieved scores of 6 to 8 out of a maximum 9 points using the Newcastle–Ottawa scale.

Because of the limited number of studies, with most of them involving a small cohort size (from 12 to 73 cases in the study group), we had to admit the generalizability limits of our results. This fact reflects an overall low prevalence of 6.7 haematologic malignancy cases per 100,000 standard population (*Zhang et al., 2023*).

As we were aware of the study limitations, we contacted the corresponding authors of each study included in the quantitative analysis to request additional data to conduct the meta-analysis based on individual patient data. Only one author, however, responded to our request (*Almog et al., 2012*), which makes individual patient data meta-analysis on this topic unreliable. Our results necessitate the confirmation by further well-designed prospective studies.

The publication period of the included studies in the qualitative analysis ranged from 2009 to 2022. This can be considered an additional limitation, as this period reflects significant changes in IVF treatment protocols. As both the study and control groups of each study were treated during the same period, the results of IVF outcomes analysed in the meta-analysis are still reliable.

In our quantitative analysis, a short flare-up protocol with gonadotrophin releasing hormone (GnRH) agonist downregulation from day 1 onwards and recombinant FSH from day 3 was applied in one included study (*Almog et al., 2012*). Ovarian stimulation using a GnRH-antagonist protocol and administration of recombinant FSH was used in three studies (*Das et al., 2011*; *Decanter et al., 2018*; *Brun et al., 2021*). One study reported the application of GnRH agonist and antagonist downregulation protocols for included patients (*Lekovich et al., 2016*). Random-start protocol was applied in the group of patients with haematological malignancies, which may cause an additional bias. Previously published studies, however, have shown similar fertility preservation results in conventional and random-start protocols (*Cakmak et al., 2013*; *Danis et al., 2017*).

High statistical heterogeneity was observed in the quantitative analysis of age, baseline AMH, total recombinant FSH dose and peak oestradiol level. To increase the confidence level of the results, we applied the random-effects meta-analysis across all evaluated parameters.

Gaining a comprehensive understanding of how haematologic cancer affects ovarian function is essential in devising effective fertility preservation strategies. This knowledge would enable us to identify optimal interventions and provide valuable guidance for clinical decision-making, ultimately enhancing reproductive outcomes and improving the quality of life for those affected by haematologic cancer.

In conclusion, haematologic malignancies may detrimentally affect ovarian function manifesting in decreased AMH serum levels despite a younger age compared with healthy controls undergoing fertility preservation. This effect can be overcome by the application of relevant IVF protocols and stimulation doses to achieve an adequate oocyte yield.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.103978.

DATA AVAILABILITY

Data will be made available on request.

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ARTICLE

Patterns of menstrual cycle, menstrual pain and medication usage in young women from high- and middle-income countries

BIOGRAPHY

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KEY MESSAGE

A family history of menstrual pain was associated with severe menstrual pain in women from high- and middle-income countries, and low body mass index and early onset of menarche were associated with severe menstrual pain in women from high-income countries. An educational programme may assist women to understand the consequences of intractable cyclic/acyclic pain, facilitating early detection and timely management.

ABSTRACT

Research question: Do patterns of the menstrual cycle, menstrual pain and the use of medication for menstrual pain differ between young women from high-income countries (HIC) and middle-income countries (MIC)?

Design: A multinational, multicentre, cross-sectional study using pen-and-paper questionnaires was conducted between 2016 and 2021 to assess patterns of the menstrual cycle, menstrual pain and the use of medication for menstrual pain. Various parameters were evaluated to identify high-risk factors for severe menstrual pain in women from two HIC ($n = 1550$) and nine MIC ($n = 7139$).

Results: From a total of 9114 young women, 4920 medical students (HIC $n = 696$, MIC $n = 4224$) and 3769 nursing students (HIC $n = 854$, MIC $n = 2915$) were included in this study. Compared with those from HIC, a significantly higher proportion of medical and nursing students from MIC reported cyclic pain (83.9% and 86.8%, respectively) and acyclic pain (33.8% and 31.9%, respectively) (both $P < 0.001$). Multivariate regression analysis revealed that low body mass index and early onset of menarche were independent risk factors for severe cyclic/acyclic pain among women from HIC, and a family history of menstrual pain was a risk factor for severe cyclic/acyclic pain among women from HIC and MIC.

Conclusions: Differential patterns of the menstrual cycle, menstrual pain and use of medication for menstrual pain were found between young women from HIC and MIC. A proper educational programme may be necessary for these women and healthcare providers to understand the consequences of intractable cyclic/acyclic pain, in order to facilitate early detection and timely management of menstrual pain and its negative consequences, such as endometriosis.

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KEY WORDS
Menstrual pain
Medication
Endometriosis
Young women
High-income countries
Middle-income countries

INTRODUCTION

Menstrual symptoms among women of reproductive age impose a heavy burden on affected women and their families that is often underestimated and inadequately investigated. Patterns of the menstrual cycle and menstrual pain vary considerably between woman, influenced by level of education, nutritional status, exposure to stress and cultural background (*Sharp et al., 2022*). The prevalence of menstrual pain, manifesting mainly as dysmenorrhoea, is reported to range from 25% among all women to 90% among adolescents (*Osuga et al., 2005*; *Zondervan et al., 2020*). Menstrual pain is the most common gynaecological complaint in women of reproductive age. Less than 15% of adolescent girls with dysmenorrhoea seek medical help, despite the fact that their symptoms interfere with their daily life. More than one-third do nothing about their condition (*Wong and Khoo, 2010*). A large survey-based research study of 4000 Japanese women of reproductive age revealed that only 12% sought medical help for their menstrual pain, and most felt that it was natural to experience pain during menstruation (*Taketani, 2001*; *Tanaka et al., 2014*). This perception may account for the low consultation rate for menstrual problems in Japan, and as a result, women may suffer from the consequences of inaction, such as endometriosis.

In many countries, adolescent girls, young women, the general public and many front-line healthcare providers are unaware that distressing and life-altering menstrual pain is anything but normal, leading to normalization and stigmatization of symptoms and significant diagnostic delay (*Zondervan et al., 2020*). Young women who could benefit from timely medical management of their symptoms are not always provided with options for intervention due to poor or limited knowledge and awareness of menstrual pain and its consequences, such as endometriosis, among primary healthcare providers. Basic understanding of menstrual pain and its consequences among young women in Japan and other Asian countries remains insufficient. Therefore, raising awareness of menstrual pain and endometriosis — two conditions that are often neglected — is important among young women in low-to-middle-income countries (LMIC) as well as high-income countries (HIC). Recently, the authors reported rudimentary knowledge about menstrual pain and endometriosis among young women, and the effects of a targeted educational programme to improve this knowledge, in young women from HIC and middle-income countries (MIC) (*Khan et al., 2022*). This educational programme may be useful to raise awareness and motivate these women to seek early medical intervention in order to reduce or eliminate unnecessary delays in treatment.

Endometriosis is one of the consequences or a hidden cause of constant cyclic/acyclic pain in women, especially those who are resistant to medical treatment (*Janssen et al., 2013*). According to the recent guideline of the European Society of Human Reproduction and Embryology, endometriosis can only be diagnosed with the presence of endometriosis-related pain (*Becker et al., 2022*). Endometriosis affects approximately 10% (190 million) of females of reproductive age globally (*Zondervan et al., 2020*). Similar to menstrual pain, endometriosis has significant social, public health and economic implications. It can have a negative impact on quality of life due to severe pain, fatigue, depression, anxiety and infertility. Menstrual pain and endometriosis-associated pain cause considerable absenteeism from work or school (*Culley et al., 2013*). In these situations, addressing menstrual pain in young women can reduce work/school absenteeism and increase an individual's ability to improve school performance or work productivity. A range of factors, such as short menstrual cycle, early onset of menarche, chronic cyclic/acyclic pain, family history of endometriosis and/or dysmenorrhoea in a first-degree relative, and resistance to over-the-counter medication, have been considered as possible determinants to identify individuals who are at high risk of development of endometriosis (*Culley et al., 2013*; *Janssen et al., 2013*). It is conceivable that social infrastructure, cultural/ethnical background, religious beliefs and influence of the internet/mass media in the country may affect the pattern of the menstrual cycle, self-identification of menstrual pain, and the decision to take proper medication for menstrual pain in young women from HIC and LMIC. However, data on these issues in young women from HIC and/or MIC are scant, or do not exist at all.

Based on the significance of these unresolved issues facing young women in HIC and MIC, the following questions were raised to form the core of the authors' current research: (1) Would analysis of menstrual pain and use of medication for menstrual pain help to identify high-risk individuals for early detection and timely management of endometriosis? (2) Would analysis of the pattern of the menstrual cycle and background profiles of young women in HIC and MIC give some clues to identify high-risk individuals who are susceptible to endometriosis? In order to address these issues, a self-reported, questionnaire-based study of female medical and nursing students was undertaken between 2016 and 2021. Demographic profiles, patterns of the menstrual cycle, cyclic/acyclic pain, and use of medication for menstrual pain by young women from HIC and MIC were analysed in order to understand the differences, if any, between individuals from HIC and MIC which might be helpful to identify high-risk individuals, and prompt them to see a gynaecologist for early diagnosis and management. Also, based on the finding that underweight or normal-weight women are more susceptible to endometriosis compared with overweight or obese women (*Farland et al., 2017*; *Hediger et al., 2005*; *Shah et al., 2013*), and that suffering from dysmenorrhoea and/or chronic pelvic pain could be an indirect parameter of endometriosis (*Bloski and Pierson, 2008*; *Fauconnier and Chapron, 2005*), the relationship between body mass index (BMI) and visual analogue scale (VAS) scores (0—10) in these young women was analysed. Finally, multiple logistic regression analyses with different confounding variables were undertaken to identify high-risk factors associated with severe cyclic/acyclic pain.

MATERIALS AND METHODS

Participants

This was a multinational, multicentre, cross-sectional, collaborative study of medical and nursing students conducted between September 2016 and December 2021. Before initiation of this self-reported

pen-and-paper questionnaire survey, the principal investigator (KNK) of the study visited and/or e-mailed different medical and nursing schools in two HIC (Japan and Lithuania) and nine MIC (Kazakhstan, Sri Lanka, Vietnam, Indonesia, Turkey, Thailand, Iran, Taiwan and Bangladesh) to discuss the study design with medical and nursing personnel. The definitions of HIC and MIC were based on the new World Bank classification (1 July 2022) of gross national income (GNI) per capita in US dollars (*Hamadeh et al., 2022*). GNI per capita is the value of a country's income per year divided by its population using Atlas methodology (http://data.worldbank.org/indicator/NY.GNP.PCAP.CD/countries). According to this classification, MIC have GNI per capita between $1046 and $12,695, and HIC have GNI per capita of > $12,695 (http://www.blogs.worldbank.org).

The Endometriosis Awareness Promotion Project (EAPP) has two parts. Part I was designed to evaluate the basic understanding of menstrual pain and endometriosis among young women, and the effect of education after group discussion and lectures. Part I was published recently (*Khan et al., 2022*). The current study (Part II of EAPP) was designed to identify high-risk individuals who are susceptible to endometriosis based on their particular patterns, if any, of the menstrual cycle, menstrual pain, use of medication for menstrual pain, and demographic profiles among medical and nursing students from HIC and MIC. After a full explanation of the purpose, methodology and impact of the study, the research coordinators of each university and/or institution collected survey data from medical or nursing students. All incomplete survey data were excluded. A letter was sent to the principal/dean/director of each participating institution and/or hospital explaining the purpose and nature of the study. Confidentiality was ensured and emphasized. All academic institutions obtained ethical approval for this study from their institutional review board (IRB), or institutional approval using the IRB approval letter of Kyoto Prefectural University of Medicine (IRB approval no. ERB-C-648-4, approval date 13 September 2016).

Questionnaire survey

The study was conducted using a self-reported pen-and-paper method based on original and structured questionnaires. All questions written in English were translated into Japanese and the native language of each participating country. After clarification of the purpose of the study to each category of students, all paper questionnaires were distributed to groups of students in the classroom before the attending lecturer started the lecture. Students were given adequate time to complete the questionnaires, which were collected from each student in the same sitting.

The questionnaires for Part II of EAPP for medical and nursing students had four sections, as follows. (i) Demographic profiles of students (age, BMI, age at menarche, age at cyclic/acyclic pain, family history of cyclic/acyclic pain, and vaginal discharge, if any). BMI was defined as weight in kilograms divided by height in meters squared (kg/m^2), and was categorized into four groups: underweight (BMI <18.5 kg/m^2), normal weight (BMI 18.5–24.9 kg/m^2), overweight (BMI 25–29.9 kg/m^2), and obese (BMI ≥30 kg/m^2), as described elsewhere (*Di Angelantonio et al., 2016*). (ii) Pattern of menstrual cycle (length, regularity, duration of bleeding per cycle). (iii) Pattern of menstrual pain (cyclic/acyclic pain, type of pain symptoms, multiplicity of pain, severity of cyclic/acyclic pain, school absenteeism due to cyclic pain). The severity of menstrual pain was self-measured using a VAS (0–10). A VAS score <7 was considered as mild, moderate or no pain, and a VAS score ≥7 was considered as severe pain. (iv) Pattern of medication usage for menstrual pain (medication received or not; if yes, type of medication, duration of medication, usefulness of medication received). All paper questionnaires were validated before use in an initial trial of female college students in Japan (*Akira, 2013*).

Statistical analysis

After completion of all four sections of the questionnaire survey, all data from each institution were sent, as Excel files (Microsoft Corp., USA), to the principal investigator (KNK) of Kyoto Prefectural University of Medicine in Japan, who reviewed and summarized all data in Excel files and tabulated them in Word files (Microsoft Corp.) with the help of a co-author (KO). All survey data were double-checked, and were excluded if there were any duplicated data or missing data (KO and HO). Continuous variables were compared between groups using Wilcoxon rank sum test, and categorical variables were compared using chi-squared test or Fisher's exact test. Any correlation between groups was analysed using Spearman's rank correlation coefficient. Multivariate logistic regression analyses were used to identify independent significant risk factors for the severity of cyclic/acyclic pain, and to estimate OR and 95% CI for each of the selected factors (age, BMI, age at menarche, family history of cyclic/acyclic pain, length of menstrual cycle, duration of blood loss per cycle, medication found to be useful, vaginal discharge). All reported *P*-values were two-sided. $P < 0.05$ was considered to indicate significance. All data analyses were conducted using SAS Version 9.4 (SAS Institute Inc., USA).

RESULTS

In total, 9114 young women were recruited from all HIC and MIC for the EAPP survey between June 2016 and December 2021. After careful double-checking and scrutiny of all data, a proportion of medical students (HIC $n = 33$, MIC $n = 155$) and nursing students (HIC $n = 235$) were excluded from the data analysis due to duplicated and/or missing data. Finally, 4920 female medical students (HIC $n = 696$, MIC $n = 4224$) and 3769 female nursing students (HIC $n = 854$, MIC $n = 2915$) who completed all four sections of the questionnaire survey were included in the data analysis. A flow chart describing the number of medical and nursing students from each HIC and MIC is shown in FIGURE 1. University-, institution- and/or hospital-based information on the number of medical and nursing students from each HIC and MIC is shown in Supplementary Table 1.

Demographic profiles of medical and nursing students from HIC and MIC

The demographic profiles of enrolled medical and nursing students are shown in TABLE 1. The median age of the medical students from HIC was significantly higher compared with those from MIC ($P < 0.001$), and the age of the nursing students from HIC was found to be significantly lower compared with those from MIC ($P < 0.001$). Regarding age at menarche, the onset of menarche was significantly earlier among medical and nursing students from HIC ($P < 0.001$ for both) compared with those from MIC. The majority of medical and nursing students from both HIC and MIC reported onset of menarche at the age of 12–14 years.

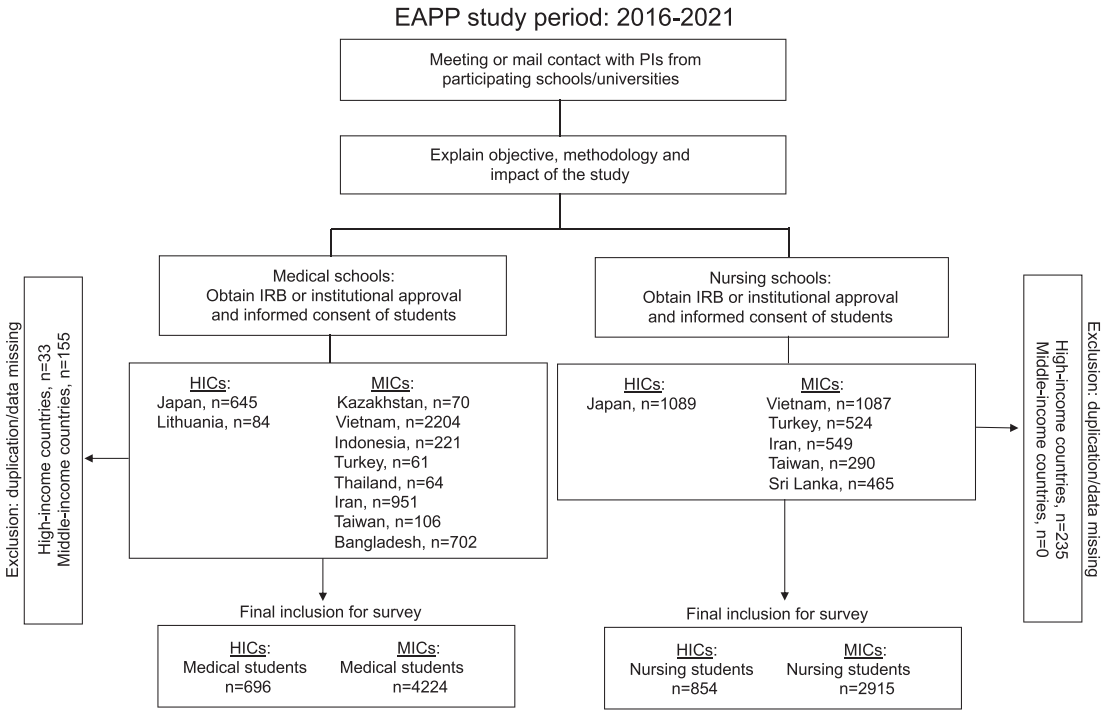

FIGURE 1 Flow chart of the study population from high-income countries (HIC) and middle-income countries (MIC). EAPP, Endometriosis Awareness Promotion Project; PI, principal investigator; IRB, institutional review board.

TABLE 1 DEMOGRAPHIC PROFILES OF MEDICAL AND NURSING STUDENTS FROM HIGH- AND MIDDLE-INCOME COUNTRIES

Demographic	Medical student		*P*-value	Nursing student		*P*-value
	HIC (*n* = 696)	MIC (*n* = 4224)		HIC (*n* = 854)	MIC (*n* = 2915)	
Age (years)	21.6 ± 2.4	21.2 ± 2.4		19.2 ± 1.3	21.0 ± 3.6	
Median (range)	21 (18-37)	21 (16–45)	<0.001	19 (18–38)	20 (20-24)	<0.001
No response, *n*	27	11		22	24	
Body height (cm)	159.8 ± 6.3	158.8 ± 6.7		157.9 ± 5.1	159.9 ± 6.6	
Median (range)	160 (143–179)	158 (120–197)		<0.001 158 (141–185)	160 (120–186)	<0.001
No response, *n*	32	14		24	13	
Body weight (kg)	51.6 ± 7.1	52.9 ± 8.7		50.3 ± 5.6	52.9 ± 9.7	
Median (range)	50 (37–90)	52 (35–112)	0.013	50 (37–83)	51 (32–120)	<0.001
No response, *n*	78	17		94	25	
BMI (kg/m^2)	20.2 ± 2.0	20.9 ± 3.0		20.2 ± 2.1	20.7 ± 3.2	
Median (range)	20 (15.0–30.9)	20.4 (13.9–37.4)	<0.001	20 (14.6–35.6)	20.1 (12.1–47.2)	0.192
Underweight, <18.5, *n* (%)	118 (19.1)	868 (20.7)		134 (17.6)	766 (26.6)	
Normal weight, 18.5–24.9, *n* (%)	484 (78.3)	2958 (70.5)		612 (80.5)	1841 (63.9)	
Overweight, 25–29.9, *n* (%)	15 (2.4)	318 (7.6)		12 (1.6)	234 (8.1)	
Obese, ≥30, *n* (%)	1 (0.2)	54 (1.3)		2 (0.3)	41 (1.4)	
No response, *n*	78	26		94	33	
Age at menarche (years)	12.3 ± 1.4	13.1 ± 1.5		12.4 ± 1.4	13.3 ± 1.4	
Median (range)	12 (7–17)	13 (7–20)	<0.001	12 (9–17)	13 (7–19)	<0.001
9–11, *n* (%)	177 (27.3)	558 (13.3)		219 (27.0)	243 (8.4)	
12–14, *n* (%)	423 (65.3)	2981 (70.9)		525 (64.7)	2133 (73.9)	

(*continued on next page*)

TABLE 1 (*Continued*)

Demographic	Medical student		*P*-value	Nursing student		*P*-value
	HIC (*n* = 696)	MIC (*n* = 4224)		HIC (*n* = 854)	MIC (*n* = 2915)	
>14, *n* (%)	48 (7.4)	666 (15.8)		67 (8.3)	510 (17.7)	
No response, *n*	48	19		43	29	
Age at cyclic pain (years)	15.5 ± 2.9	15.3 ± 2.6		15.1 ± 2.1	15.1 ± 3.0	
Median (range)	15 (10–28)	15 (9–25)	0.120	15 (9–22)	15 (9–33)	0.002
No response, *n*	193	877		209	933	
Age at acyclic pain (years)	17.0 ± 3.3	15.5 ± 2.3		16.0 ± 2.3	15.0 ± 2.2	
Median (range)	17 (10–26)	15 (9–24)	<0.001	16 (10–25)	15 (9–32)	<0.001
No response, *n*	564	1747		658	1788	
F/H of cyclic pain, yes, *n* (%)	270 (57.2)	1937 (54.8)	0.326	353 (60.1)	1318 (52.7)	0.001
No response, *n*	224	690		267	412	
If yes, mother/sister/both (*n*)	185/72/13	1247/406/187	0.009	NR/NR/NR	899/281/132	
No response, *n*	0	97		353	6	
F/H of acyclic pain, yes, *n* (%)	65 (44.2)	584 (42.4)	0.674	84 (49.7)	336 (37.9)	0.004
No response, *n*	549	2847		685	2029	
If yes, mother/sister/both (*n*)	43/20/2	401/122/51	0.088	NR/NR/NR	224/79/18	
No response, *n*	0	10	84	15		
Vaginal discharge, yes, *n* (%)	206 (31.0)	2474 (58.9)	<0.001	179 (22.8)	1815 (62.9)	<0.001
If yes, regular/irregular (*n*)	84/122	1003/1370	0.678	53/126	757/799	<0.001
No response, *n*	0	101		0	259	

Results are expressed as mean ± SD, except where otherwise indicated.

Continuous variables were compared between groups using Wilcoxon rank sum test, and categorical variables were compared using chi-squared test or Fisher's exact test.

HIC, high-income countries; MIC, middle-income countries; BMI, body mass index; F/H, family history, NR, no response.

A family history of cyclic/acyclic pain in a first-degree relative is considered as a high-risk factor for the development of endometriosis. This study found no difference in the proportion of medical students with a family history of cyclic/acyclic pain between HIC and MIC. However, compared with MIC, a significantly higher proportion of nursing students from HIC reported a family history of cyclic pain (60.1% versus 52.7%, $P = 0.001$) and acyclic pain (49.7% versus 37.9%, $P = 0.004$) (TABLE 1).

Patterns of menstrual cycle among medical and nursing students from HIC and MIC

The length of menstrual cycles was categorized as short (<24 days), normal (24–28 days and 29–32 days) or prolonged (>32 days) among the medical and nursing students from HIC and MIC (TABLE 2). The results showed significant differences between HIC and MIC for both medical and nursing students ($P = 0.002$ and $P < 0.001$, respectively). A considerable proportion of medical students from MIC reported a short menstrual cycle (<24 days, 4.8% versus 2.8%) compared with those from HIC. The proportions of medical and nursing students reporting a prolonged menstrual cycle (>32 days) were higher for HIC compared with MIC (TABLE 2).

Excessive and/or prolonged blood loss during the menstrual cycle among young women is a high-risk factor for the development of endometriosis (*Missmer et al., 2004*). This study found that the proportions of medical and nursing students from HIC with prolonged blood loss (≥5 days per cycle) were significantly higher compared with those from MIC (64.8% versus 37.1% for medical students, 65.2% versus 45.2% for nursing students) (TABLE 2).

Patterns of menstrual pain among medical and nursing students from HIC and MIC

The proportion of students reporting cyclic/acyclic pain was significantly higher among medical students (83.9% versus 70.3%, $P < 0.001$) and nursing students (86.8% versus 70.0%, $P < 0.001$) from MIC compared with those from HIC (TABLE 3). Both medical and nursing students from HIC and MIC reported variable menstrual pain symptoms (FIGURE 2). Abdominal pain and pelvic/back pain were the most common pain symptoms among these students from both HIC and MIC. A variable proportion of students from HIC and MIC reported dysuria (pain at urination), dyschezia (pain at defaecation), and other digestive and non-digestive symptoms during menstruation (TABLE 3).

Considering the severity of pain, both medical and nursing students from HIC reported severe cyclic/acyclic pain (VAS score ≥7) significantly more frequently compared with those from MIC ($P = 0.001$ for acyclic abdominal pain, $P < 0.001$ for others) (TABLE 3). FIGURE 3 shows the patterns of menstrual pain and severity of cyclic/acyclic pain among medical and nursing students from HIC and MIC.

In response to the question, 'How many days did you refrain from going to school due to cyclic pain', this study found that

TABLE 2 PATTERNS OF MENSTRUAL CYCLE AMONG MEDICAL AND NURSING STUDENTS FROM HIGH- AND MIDDLE-INCOME COUNTRIES

	Medical student		*P*-value	Nursing student		*P*-value
	HIC (*n* = 696)	MIC (*n* = 4224)		HIC (*n* = 854)	MIC (*n* = 2915)	
Length of menstrual cycle (days)[a]			0.002			<0.001
<24, *n* (%)	18 (2.8)	203 (4.8)		42 (5.0)	164 (5.7)	
24–28, *n* (%)	146 (22.5)	1130 (26.8)		223 (26.6)	943 (32.6)	
29–32, *n* (%)	354 (54.5)	2190 (51.9)		410 (48.9)	1396 (48.2)	
>32, *n* (%)	131 (20.2)	693 (16.4)		163 (19.5)	392 (13.5)	
No response, *n*	47	8		16	20	
Regularity of menstrual cycle (days difference)			0.246			0.001
≤2, *n* (%)	176 (27.5)	911 (24.4)		165 (19.6)	748 (26.1)	
3–4, *n* (%)	319 (49.8)	1937 (51.9)		448 (53.2)	1407 (49.1)	
≥5, *n* (%)	145 (22.7)	885 (23.7)		229 (27.2)	711 (24.8)	
No response, *n*	56	491		12	49	
Duration of blood loss per cycle (days)			<0.001			<0.001
<3, *n* (%)	7 (1.0)	183 (4.6)		7 (0.8)	99 (3.4)	
3–4, *n* (%)	235 (34.2)	2305 (58.3)		284 (33.9)	1483 (51.4)	
≥5, *n* (%)	445 (64.8)	1465 (37.1)		546 (65.2)	1303 (45.2)	
No response, *n*	9	271		17	30	

Categorical variables were compared using chi-squared test.

[a] <24 days, short menstrual cycle length; 24–28 days and 29–32 days, normal menstrual cycle length; >32 days, prolonged menstrual cycle length.

HIC, high-income countries; MIC, middle-income countries.

TABLE 3 PATTERNS OF MENSTRUAL PAIN AMONG MEDICAL AND NURSING STUDENTS FROM HIGH- AND MIDDLE-INCOME COUNTRIES

	Medical student		*P*-value	Nursing student		*P*-value
	HIC (*n* = 696)	MIC (*n* = 4224)		HIC (*n* = 854)	MIC (*n* = 2915)	
Pattern of menstrual pain						
Cyclic, yes, *n* (%)	474 (70.3)	3542 (83.9)	<0.001	597 (70.0)	2508 (86.8)	<0.001
No response, *n*	22	3		1	24	
Acyclic, yes, *n* (%)	160 (24.4)	1425 (33.8)	<0.001	178 (22.1)	910 (31.9)	<0.001
No response, *n*	39	3		50	60	
Pain symptom, *n* (%)						
Abdominal pain	547 (78.6)	2775 (65.7)		522 (61.1)	1832 (62.8)	
Pelvic/back pain	306 (44.0)	1866 (44.2)		399 (46.7)	1157 (39.7)	
Pain at urination	11 (1.6)	467 (11.1)		14 (1.6)	190 (6.5)	
Pain at defaecation	73 (10.5)	512 (12.1)		23 (2.7)	163 (5.6)	
Headache	73 (10.5)	450 (10.7)		186 (21.8)	138 (4.7)	
Ovulation pain	74 (10.6)	327 (7.7)		96 (11.2)	119 (4.1)	
Nausea/vomiting/bloat	113 (16.2)	709 (16.8)		99 (11.6)	230 (7.9)	
Constipation/diarrhoea	259 (37.2)	667 (15.8)		291 (34.1)	485 (16.6)	
Other (joint/muscle pain)	25 (3.6)	198 (4.7)		24 (2.8)	146 (5.0)	

(*continued on next page*)

TABLE 3 (*Continued*)

	Medical student		*P*-value	Nursing student		*P*-value
	HIC (*n* = 696)	MIC (*n* = 4224)		HIC (*n* = 854)	MIC (*n* = 2915)	
Multiplicity of pain, *n* (%)			<0.001			<0.001
Single type	326 (51.9)	1062 (37.9)		344 (88.9)	1028 (56.1)	
Multiple types	302 (48.1)	1743 (62.1)		43 (11.1)	804 (43.9)	
Severity of cyclic pain (VAS score, 0–10), *n* (%)						
Abdominal pain <7	294 (62.0)	2978 (84.1)	<0.001	353 (59.1)	2042 (83.7)	<0.001
≥7	180 (38.0)	564 (15.9)		244 (40.9)	399 (16.3)	
Pelvic/back pain <7	320 (67.5)	2817 (83.8)	<0.001	60 (30.5)	2117 (86.7)	<0.001
≥7	154 (32.5)	544 (16.2)		137 (69.5)	325 (13.3)	
Severity of acyclic pain (VAS score, 0–10), *n* (%)						
Abdominal pain <7	135 (84.4)	1304 (92.4)	0.001	142 (79.8)	764 (93.9)	<0.001
≥7	25 (15.6)	107 (7.6)		36 (20.2)	50 (6.1)	
Pelvic/back pain <7	121 (75.6)	1172 (95.0)	<0.001	154 (86.5)	756 (96.4)	<0.001
≥7	39 (24.4)	62 (5.0)		24 (13.5)	28 (3.6)	
School absence due to cyclic pain[a]						
Yes, *n* (%)	63 (13.3)	921 (26.6)	<0.001	67 (11.2)	417 (16.7)	0.001
If yes, <2 days, *n* (%)	58 (92.1)	802 (87.1)	0.249	61 (91.0)	380 (91.1)	0.983
≥2 days, *n* (%)	5 (7.9)	119 (12.9)		6 (9.0)	37 (8.9)	
No response, *n*	0	82		0	6	

Categorical variables were compared using chi-squared test or Fisher's exact test.

School absence due to cyclic pain only assessed if answered 'yes' to cyclic pain.

HIC, high-income countries; MIC, middle-income countries; VAS, visual analogue scale to self-measure severity of pain on a scale of 0–10 (<7, mild-to-moderate pain; ≥7, severe pain).

school absence due to cyclic pain was significantly more common among both medical and nursing students from MIC compared with those from HIC (26.6% versus 13.3%, $P < 0.001$ for medical students; 16.7% versus 11.2%, $P = 0.001$ for nursing students) (TABLE 3, FIGURE 3).

Correlation between BMI and VAS scores of menstrual pain among medical and nursing students from HIC and MIC

No significant relationship was found between BMI and VAS scores for abdominal pain and/or pelvic/back pain during menstruation for medical students or nursing students from HIC and MIC (Supplementary Figure 1).

Patterns of medication usage for menstrual pain among medical and nursing students from HIC and MIC

Patterns of medication usage for menstrual pain differ among young women depending on healthcare access, health insurance system, family structure, social/cultural background, and religious beliefs of their country. This analysis revealed that medication usage for menstrual pain was more common in medical students from MIC compared with those from HIC (67.8% versus 62.4%, $P < 0.001$), and in nursing students from HIC compared with those from MIC (54.7% versus 38.7%, $P < 0.001$) (TABLE 4). Non-steroidal anti-inflammatory drugs (NSAID) were the most commonly used type of medication by these students from HIC and MIC. The use of antispasmodic drugs, such as hyoscine butyl bromide (Buscopan), by these students was less common in HIC than MIC. There was no significant difference in the use of hormonal medication (combined oestrogen–progesterone pill or progestin compound) by these students between HIC and MIC. The duration of NSAID intake was significantly longer (six cycles or more) among medical and nursing students from HIC compared with those from MIC. The duration for the use of hormonal medication among medical students from HIC was significantly longer compared with those from MIC (46.2% versus 21.0%, $P = 0.001$) (TABLE 4).

In response to the question 'Was your medication useful to control your menstrual pain?', most students from HIC and MIC responded that either an NSAID or hormonal medication was useful to control their menstrual pain. This finding was more significant for medical ($P < 0.001$) and nursing ($P = 0.018$) students from MIC compared with those from HIC.

Risk factors associated with the severity of cyclic/acyclic pain

Several confounding factors were selected to identify risk factors, if any, for the severity of cyclic/acyclic pain (VAS score ≥7): age, BMI, age at menarche, family history of cyclic pain, family history of acyclic pain, length of menstrual cycle, duration of blood loss/cycle, usefulness of medication, and vaginal discharge. The results of multiple regression analyses are shown in Supplementary Table 2, and a detailed explanation of risk factors identified for cyclic/acyclic pain is summarized in the supplementary material.

Collectively, multiple regression analyses revealed that younger age was a risk factor for the occurrence of cyclic pelvic/back pain in HIC (OR 0.87, 95% CI 0.79–0.96,

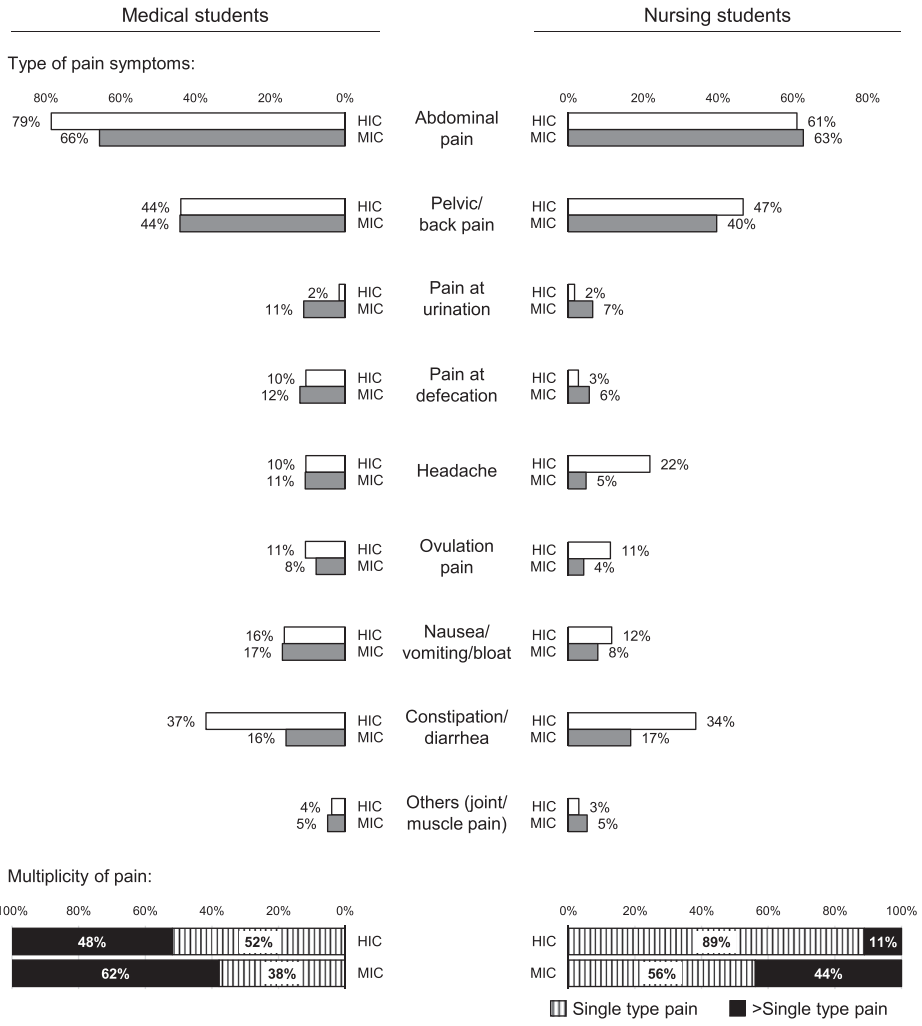

FIGURE 2 Different types of gynaecologic or non-gynaecologic pain symptoms, and their prevalence (%) among medical students (left panel) and nursing students (right panel) who were recruited from high-income countries (HIC, white bars) and middle-income countries (MIC, grey bars). Multiplicity of pain is shown as a single type of pain (striped bars) or multiple types of pain (black bars).

$P = 0.007$) and MIC (OR 0.93, 95% CI 0.90–0.96, $P < 0.001$). Low BMI (OR 0.74, 95% CI 0.56–0.97, $P = 0.03$) and early onset of menarche (middle age group versus youngest age group) (OR 0.64, 95% CI 0.43–0.96, $P = 0.030$) were independent risk factors associated with severe acyclic pelvic/back pain and cyclic pelvic/back pain, respectively, among women from HIC, and a family history of cyclic menstrual pain was a risk factor for severe cyclic pain among women from HIC ($P = 0.002$ for abdominal pain, $P = 0.011$ for pelvic/back pain) MIC ($P = <0.001$ for each of abdominal pain and pelvic/back pain (Supplementary Fig. 2A and Fig. 2B). In addition, a family history of acyclic pain was a risk factor for severe acyclic pain for women from HIC ($P = 0.001$ for abdominal pain, $P = 0.002$ for pelvic/back pain) MIC ($P = 0.001$ for abdominal pain, $P = 0.024$ for pelvic/back pain) (Supplementary Fig. 2C and Fig. 2D). Blood loss ≥5 days was also a key risk factor for cyclic pelvic/back pain in both HIC and MIC ($P = 0.031$ and 0.012, respectively), acyclic pelvic/back pain in HIC ($P = 0.005$), and cyclic/acyclic abdominal pain in MIC ($P < 0.001$ and 0.002, respectively).

DISCUSSION

This multinational, cross-sectional questionnaire-based study was conducted among young women between 2016 and 2021 in an attempt to identify those at high risk of severe cyclic/acyclic pain. Differences in demographic profile, and patterms of the menstrual cycle, menstrual pain and use of medication for menstrual pain were found between young women from HIC and MIC. These differences may be due to differences in the local environment, the knowledge gap on menstrual health hygiene and its management, exposure to stress/ frustration, basic understanding of menstrual pain and early medication attention, healthcare access and parents' view of free discussion and medical care, religious taboo, and social infrastructure between HIC and MIC. Multiple regression analyses revealed that low BMI and early age at onset of menarche (middle age group versus youngest age group) were independent risk factors associated with severe acyclic/cyclic pelvic/back pain among women from HIC, and a family history of menstrual pain was associated with severe cyclic/acyclic pain among women from HIC or MIC. In fact, persistent intractable cyclic/acyclic pain has been reported as a high-risk factor leading to the development of

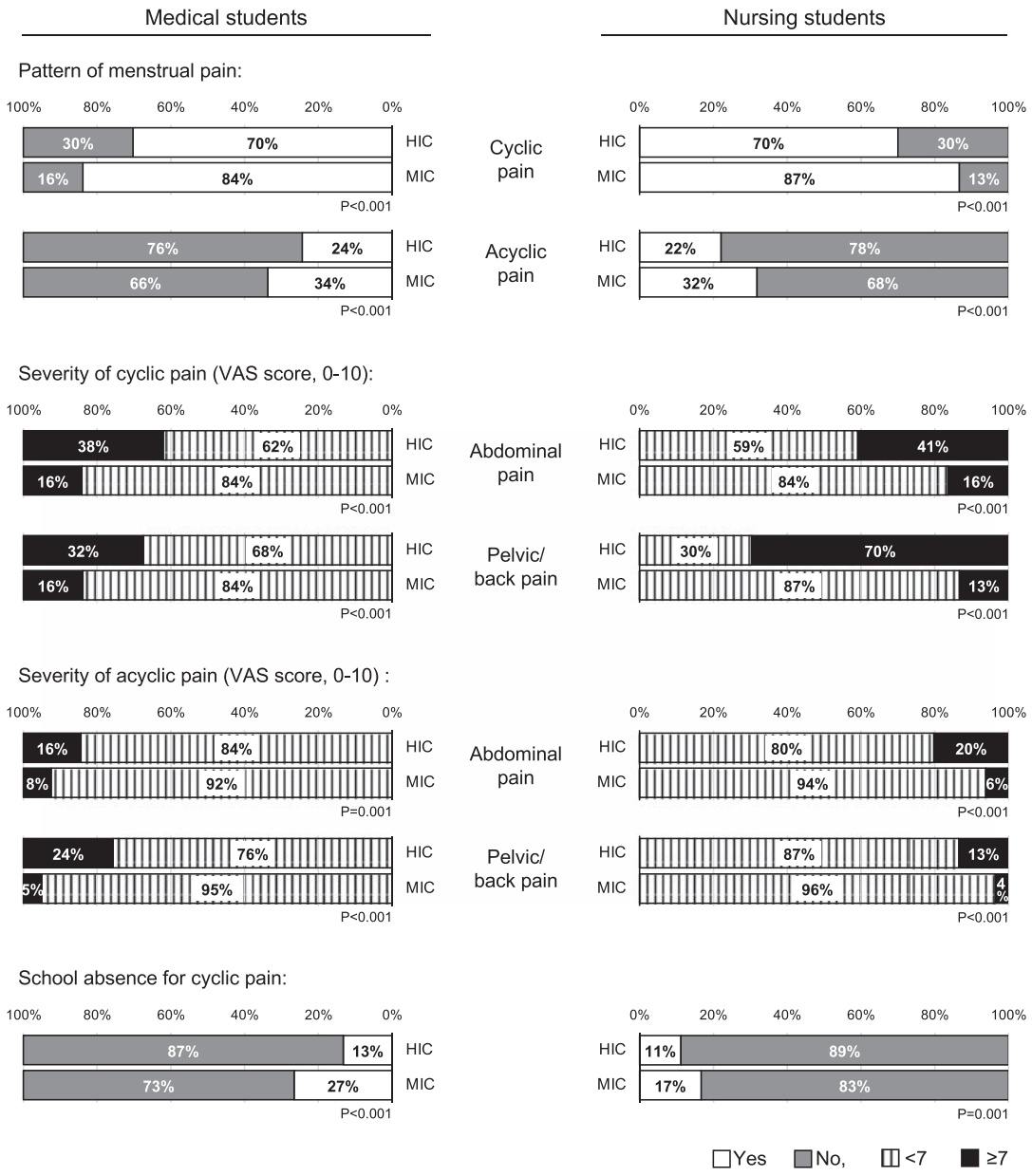

FIGURE 3 Pattern of menstrual pain (yes, white bars; no, grey bars), severity of cyclic/acyclic pain [visual analogue scale (VAS) score <7, striped bars; ≥7, black bars], and pattern of school absence for cyclic pain among medical students (left panel) and nursing students (right panel) who were recruited from high-income countries (HIC) and middle-income countries (MIC). Chi-squared test was used to assess significance.

endometriosis (*Culley et al., 2013*; *Janssen et al., 2013*).

A considerable proportion of medical students from MIC reported a short menstrual cycle compared with those from HIC. The proportions of medical and nursing students with prolonged menstrual cycles were significantly higher for those from HIC compared with those from MIC. Numerous epidemiological studies have indicated that nulliparous women and women with shorter menstrual cycles and heavy menstrual bleeding are at increased risk of endometriosis (*Eskenazi and Warner, 1997*; *Missmer and Cramer, 2003*; *Vigano et al., 2004*), and these results are consistent with young women from MIC in this study. Another meta-analysis suggested that a menstrual cycle length of ≤27 days is associated with increased risk of endometriosis, while a cycle length of ≥29 days decreases the risk of endometriosis (*Wei et al., 2016*). Together, a greater number and/or higher frequency of menstruations with excessive blood loss may expose these young women to more retrograde flow of menstrual blood, and may consequently elevate the risk of developing endometriosis. There is a need to raise awareness of these issues among local healthcare providers as well as young women in both HIC and MIC.

The proportions of students reporting cyclic/acyclic pain were significantly higher among medical and nursing students from MIC compared with those from HIC. This could be due to inadequate health care. Proper education is needed urgently for these young women from MIC in order to improve their knowledge about menstrual pain and its consequences, such as endometriosis. This analysis revealed that the use of medication for menstrual pain was

TABLE 4 USE OF MEDICATION FOR MENSTRUAL PAIN BY MEDICAL AND NURSING STUDENTS FROM HIGH- AND MIDDLE-INCOME COUNTRIES

	Medical student		*P*-value	Nursing student		*P*-value
	HIC (*n* = 696)	MIC (*n* = 4224)		HIC (*n* = 854)	MIC (*n* = 2915)	
Medication for pain						
Yes, *n* (%)	405 (62.4)	1319 (32.2)	<0.001	426 (54.7)	1128 (38.7)	<0.001
No response, *n*	47	125		75	0	
If yes, type of medication						
NSAID, *n* (%)	321 (82.3)	1025 (78.7)	0.124	351 (85.4)	898 (82.2)	0.135
No response, *n*	15	17		15	35	
Antispasmodic, *n* (%)	34 (8.6)	187 (14.4)	0.003	0 (0)	73 (7.1)	<0.001
No response, *n*	10	16		36	94	
Hormonal, *n* (%)[a]	39 (10.1)	162 (12.4)	0.203	29 (7.3)	73 (6.7)	0.660
No response, *n*	17	16		29	31	
TCM, *n* (%)	3 (0.8)	103 (7.9)	<0.001	0 (0)	68 (9.4)	<0.001
No response, *n*	10	15		35	90	
Duration of medication, cycles, *n* (%)						
NSAID <6	136 (42.4)	638 (62.2)	<0.001	140 (39.9)	486 (54.1)	<0.001
≥6	185 (57.6)	387 (37.8)		211 (60.1)	412 (45.9)	
Hormonal <6	21 (53.8)	128 (79.0)	0.001	20 (69.0)	45 (61.6)	0.488
≥6	18 (46.2)	34 (21.0)		9 (31.0)	28 (38.4)	
Was medication useful?						
NSAID or hormonal, yes, *n* (%)	193 (55.1)	992 (85.7)	<0.001	263 (72.1)	726 (78.2)	0.018
No response, *n*	10	30		15	43	

Categorical variables were compared using chi-squared test or Fisher's exact test.

HIC, high-income countries; MIC, middle-income countries; NSAID, non-steroidal anti-inflammatory drug; TCM, traditional Chinese medicine.

[a] Combined oestrogen–progesterone pill or progestin compound.

more common in medical students from MIC compared with those from HIC, and in nursing students from HIC compared with those from MIC. The reason for this differential uptake of medication between HIC and MIC is unclear.

Multiple regression analyses further revealed that younger age was an independent protective factor for the occurrence of cyclic pelvic/back pain in women from HIC and MIC. The fact that younger age was associated with less menstrual pain could be explained by more frequent anovulation or irregular ovulation with hormonal fluctuation among younger women. Alternatively, for a woman at a very young age, not enough menstruations have occurred to allow the development of endometriosis and/or the onset of dysmenorrhoea. Although not significant among the nursing students, medical students from HIC had lower BMI compared with MIC. Compared with MIC, the lean physique of young women from HIC in this study may be due to more educational information on health care or the personal health consciousness of these women. In the correlation analysis, no significant association was found between BMI and the severity of cyclic/acyclic pain in young women from HIC or MIC. On the other hand, multiple regression analyses indicated that low BMI was a significant risk factor associated with severe acyclic pelvic/back pain among women from HIC. These groups of women may be more susceptible to the development of endometriosis. These findings are consistent with two published reports. In one report, endometriosis was inversely associated with BMI at 18 years and with current adult BMI (*Shah et al., 2013*). In a laparoscopic cohort study, women reporting severe menstrual pain and diagnosed with endometriosis had a consistently lean physique during adolescence and young adulthood (*Hediger et al., 2005*). All these findings have been validated in an experimental mouse model, suggesting that altered metabolism mediated by the liver contributes to the clinically observed low BMI that is characteristic of women with endometriosis (*Goetz et al., 2016*).

Age at onset of menarche was significantly younger among medical and nursing students from HIC, and the proportion of nursing students from HIC with a family history of cyclic/acyclic pain was significantly higher compared with those from MIC. These findings were consistent with the findings of the multiple regression analyses showing that age at menarche and a family history of cyclic/acyclic pain were significant risk factors for severe cyclic/acyclic pain. In fact, early menarche (≤12 years), endometriosis and/or dysmenorrhoea in first-degree relatives, and a short menstrual cycle were considered as high-risk factors for the development of endometriosis in young women (*Missmer et al., 2004*; *Nnoaham et al., 2012*; Steenberg et al., 2013; *Treloar et al., 2010*). A study examining the hereditary aspects of endometriosis demonstrated an ≤7-fold higher risk of endometriosis in women with first-degree relatives with

endometriosis (*Nouri et al., 2010*). The present findings support this report.

Early menarche in young women from HIC can be explained by several factors, such as better nutrition, stressful family environments, different lifestyle and psychological factors, raised in urban environments (*Padez, 2003*; *Wronka and Pawlinska-Chmara, 2005*), and consumption of more animal proteins (*Berkey et al., 2000*). Despite the risk of endometriosis, a recent population-based study in Korea showed a strong association between early menarche and the risk of metabolic syndrome, diabetes, breast cancer and cardiovascular disease in adulthood (*Kim and Lim, 2021*). On the other hand, delayed menarche, as found in the present study among young women from MIC, has been shown to be associated with decreased bone mineral density, resulting in osteoporosis and increased risk of fracture later in life (*Fox et al., 1993*). Therefore, it is necessary to educate prepubertal girls and their parents, as well as young women, in both HIC and MIC on the progression of puberty, development of the menstrual cycle, and the risks of early and delayed onset of menarche.

A recent systematic and meta-analysis report demonstrated that the prevalence of dysmenorrhoea was high (71.1%) among school and university students, irrespective of the economic status of the country (*Armour et al., 2019*). The present findings suggest that the prevalence of cyclic/acyclic pain in university students differs between HIC and MIC. Both medical and nursing students from MIC reported cyclic/acyclic pain significantly more frequently compared with their counterparts from HIC. This may lead to more frequent absence of the women from MIC from school or work, as this study found that both nursing and medical students from MIC had higher absence rates. Therefore, it is crucial to identify these young women in order to manage their intractable menstrual pain.

The main strengths of this study are as follows: (i) large sample size; (ii) detailed analysis of demographic profiles, and patterns of the menstrual cycle, menstrual pain and use of medication for menstrual pain among all women; and (iii) multiple regression analyses with different confounding variables conducted to identify independent risk factors related to the occurrence of severe cyclic/acyclic pain.

This study also had a few limitations. Firstly, it was not possible to include participants from low-income countries (LIC) due to the lack of contact and/or academic interest, and insufficient study organizations in different institutions of LIC. A more motivational approach is needed for these countries. Next, only medical and nursing students were included in this study. The ongoing school/college-based EAPP study in HIC and LMIC may improve knowledge about these issues among adolescent girls. Finally, the presence of any obstructive anomalies of the reproductive tract, that may cause severe menstrual pain and elevate the risk of endometriosis, was not investigated among the study population.

In conclusion, this study identified some high-risk factors associated with severe cyclic/acyclic pain, with the consequent possibility of developing endometriosis, among young women from HIC and MIC. Multivariate analyses revealed that early menarche, low BMI and a family history of menstrual pain were high-risk factors, consistent with published reports (*Culley et al., 2013*; *Janssen et al., 2013*; *Zondervan et al. 2020*). While lower BMI and early onset of menarche were independent risk factors for severe acyclic pelvic/back pain and cyclic pelvic/back pain, respectively, among young women from HIC, a family history of menstrual pain was associated with severe menstrual pain among women from HIC and MIC. Differential demographic profiles, and patterns of the menstrual cycle, menstrual pain and use of medication for menstrual pain were found between women from HIC and MIC.

Based on the findings of the current study, there is a need for a targeted educational programme to improve basic understanding and raise awareness of menstrual pain and its consequences among young women from HIC and LMIC. This approach may motivate these young women to seek early medical intervention to relieve their intractable menstrual pain and to improve their quality of life. Addressing the consequences of menstrual pain, such as endometriosis, in the early life of young women will empower those affected, and may motivate them to visit a gynaecologist without any further delay. There is also a need to educate parents, teachers, healthcare providers and society leaders about this long-overlooked issue in HIC and LMIC. If prevention is still better than cure, the findings of this study may be useful to prevent the cascade of disease progression from early to advanced endometriosis, and to preserve fertility.

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DATA AVAILABILITY

Data will be made available on request.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.103942.

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RBMO

ARTICLE

Late selective termination in dichorionic twins: comparing late second and third trimester procedures

BIOGRAPHY

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KEY MESSAGE

Late second versus third trimester selective termination presents lower rates of Caesarean delivery and intrauterine growth retardation, higher birthweight centiles and similar rates of preterm birth. A cervical length of 35 mm or less and reduction of the presenting twin are independent risk factors for subsequent preterm birth (odds ratios 3.8 and 8.7).

ABSTRACT

Research question: Do perinatal outcomes of selective termination performed in the late second versus third trimester differ and what risk factors are associated with subsequent preterm birth?

Design: This is a retrospective cohort study of late selective terminations performed in dichorionic twins between 2009 and 2021. Perinatal outcomes were compared between two groups: group A, late second trimester (20.2 to 24.2 weeks, $n = 26$), and group B, third trimester ($\geq$28.2 weeks, $n = 55$) selective terminations. Univariate and multivariate analyses were conducted to identify factors associated with post-procedure preterm birth.

Results: In total, 81 dichorionic twin pregnancies were included. There were no pregnancy losses but 16% (13/81) of cases experienced complications. Group A had a higher median birthweight centile (36.5th versus 15th centile, $P = 0.002$) and lower rates of intrauterine growth restriction (IUGR) and Caesarean delivery (11.5% versus 32.7%, $P = 0.04$; and 26.9% versus 61.8%, $P = 0.003$) than group B. Preterm birth rates were similar (46.2% versus 63.6%, $P = 0.15$). Multiple regression revealed that reduction of the presenting twin and cervical length $\leq$35 mm were independently associated with post-procedure preterm birth (odds ratio [OR] 8.7, $P = 0.001$, 95% confidence interval [CI] 2.5–29.8; OR 3.8, $P = 0.015$, 95% CI 1.3–11).

Conclusions: Late second trimester selective termination is associated with a higher birthweight centile and lower rates of IUGR and Caesarean delivery, compared with third trimester selective termination. Cervical length 35 mm or less and reduction of the presenting twin are independent risk factors for post-procedural preterm birth. These findings may help determine the optimal time to perform a late selective termination.

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KEY WORDS
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Multiple pregnancy
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Twin pregnancy

INTRODUCTION

Selective termination in dichorionic twins is performed in pregnancies discordant for structural anomalies, genetic abnormalities, fetal infection or other abnormalities, in countries where it can be legally performed (*Alvarado et al., 2012*; *Bennasar et al., 2021*; *Bigelow et al., 2014*; *Dural et al., 2017*; *Eddleman et al., 2002*; *Evans et al., 1999*; *Greenberg et al., 2020*; *Hartoov et al., 1998*; *Lynch et al., 1996*; *Sorrenti et al., 2023*; *Weissbach et al., 2022*). In contrast to fetal reduction, conducted as a preventive measure for risk reduction in patients at risk of preterm delivery or placental insufficiency (*Sebghati and Khalil, 2021*; *van de Mheen et al., 2015*; *Vieira et al., 2019*; *Zemet et al., 2021*), selective termination is usually performed at a later stage in pregnancy, after an abnormality has been detected (*Bennasar et al., 2021*; *Dural et al., 2017*; *Greenberg et al., 2020*; *Hartoov et al., 1998*; *Sorrenti et al., 2023*; *Weissbach et al., 2022*).

In fetal reduction the non-presenting twin is usually chosen for reduction to decrease the risk of subsequent pregnancy loss (*Eddleman et al., 2002*; *Lynch et al., 1996*), while in selective termination the anomalous fetus is reduced, regardless of its order. These factors may contribute to an increased risk of pregnancy loss and preterm birth (PTB) in twin pregnancies undergoing selective termination (*Bigelow et al., 2014*; *Eddleman et al., 2002*; *Hartoov et al., 1998*; *Zemet et al., 2021*). Theoretically, postponing selective termination to the third trimester may help to avoid procedure-related pregnancy loss and reduce the risk of extreme PTB following the procedure. Therefore, investigating the outcomes of this procedure, performed at different gestational ages, may provide valuable data for the optimal timing of selective termination.

The aims of this study were: (i) to compare the outcome of late selective termination performed in the second and third trimesters in dichorionic pregnancies; and (ii) to identify possible risk factors for PTB following a late selective termination.

MATERIALS AND METHODS

Study population

This was a retrospective cohort study of dichorionic twin pregnancies undergoing a late selective termination (≥20 weeks of gestation) performed between 2009 and 2021 due to major anomaly, genetic abnormality or symptomatic fetal cytomegalovirus infection found in the co-twin. Included in the study were dichorionic twin pregnancies in which couples opted for a selective pregnancy termination, as registered with the local Ministry of Health ethical committee. Pregnancies were excluded if any of the following applied: (i) missing essential clinical data; (ii) higher order multiple pregnancies (more than two viable fetuses); (iii) selective termination performed before 20 weeks of gestation; (iv) termination of the whole pregnancy; or (v) monochorionic pregnancies (due to differences in the indication and method of selective termination).

Retrospective analysis showed two groups of patients who underwent selective termination at distinctly different gestational age ranges. In group A the termination was performed between 20.2 and 23.2 weeks, and in group B it was performed between 28.1 and 35.2 weeks of gestation (FIGURE 1). None of the selective terminations were performed between 24 and 28 weeks of gestation. Therefore, the comparison of perinatal outcome was performed according to this gestational age distribution.

A second comparison was conducted between preterm and term deliveries, to identify factors associated with PTB (<37 weeks). Hence, the cohort was

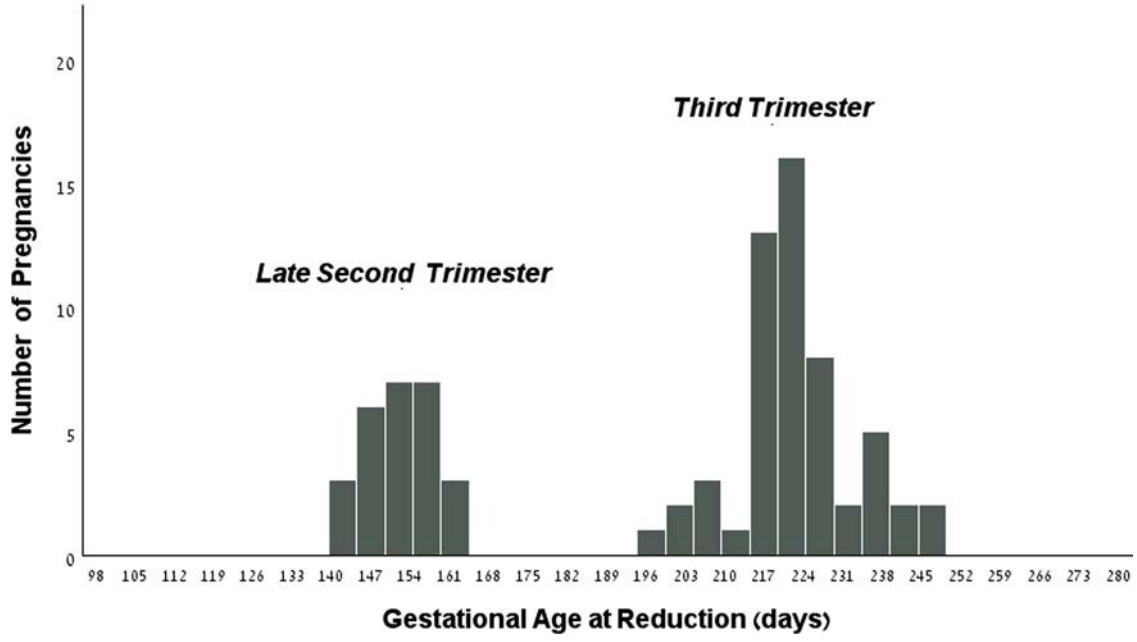

FIGURE 1 The dispersion of gestational age at the time of selective termination in the study groups.

divided according to the gestational age at time of delivery: <37 weeks or ≥37 weeks.

A previous case-control study addressing the risk of placental insufficiency in post-selective termination singleton pregnancies (*Weissbach et al., 2022*) shares some of the current study's cohort.

Local management of selective termination

According to Israeli legislation, termination of pregnancy is permitted at any stage of pregnancy for severe conditions, after receiving approval from the Ministry of Health's ethical committee. Once an indication for selective termination arises, the fetal medicine team discusses with the patients the option of an immediate or a postponed selective termination, scheduled for 30–32 weeks of gestation. In both scenarios, lung maturation with corticosteroids is prescribed beyond 23 weeks of gestation, prior to the procedure. Dichorionic pregnancies are selectively terminated by intracardiac potassium chloride injection. Asystole is confirmed twice, during the procedure and after an hour. All procedures are performed by experienced fetal medicine specialists under ultrasound guidance.

Outcome measures

Outcomes compared between the late second trimester selective termination group and the third trimester selective termination group included the following: rate of pregnancy loss, rate of PTB at less than 37, 35 and 32 weeks of gestation, gestational age at birth, rate of a latency (between selective termination and delivery) of over 3 weeks, birthweight and birthweight centile (*Hadlock et al., 1985*) and rates of intrauterine growth restriction (IUGR), Caesarean section and neonatal death.

Parameters compared between the preterm and term delivery groups included the following: rate of late second trimester selective termination, gestational age at the time of selective termination, rates of previous PTB, selective termination of the presenting twin, cervical length of 35 mm or less at the time of selective termination (*Pagani et al., 2016*), cervical length at the time of termination, maternal age, body mass index (BMI) and nulliparity.

Statistical analysis

Normality of the data was tested using a Shapiro–Wilk or Kolmogorov–Smirnov test. Data are presented as percentage and number, or as median and interquartile range, as appropriate. Comparisons between two unrelated variables were made using a Student's t-test or Mann–Whitney *U*-test, as appropriate. Chi-squared and Fisher's exact tests were used for comparisons between categorical variables. Pearson correlation was conducted to investigate the correlation of continuous variables. A multivariate logistic regression was conducted to identify factors independently associated with preterm delivery. Significance was determined at a level of $P < 0.05$. Statistical analyses were conducted using the IBM Statistical Package for the Social Sciences (IBM SPSS v.25; IBM Corporation Inc., USA).

The study protocol was approved by the Institutional Ethical Committee on 11 April 2019 (approval number SMC-6073-19).

RESULTS

Details of 127 twin pregnancies that were registered with the hospital's pregnancy termination committee were retrieved. Of these, 46 terminations involving monochorionic twins were excluded, resulting in 81 dichorionic twin pregnancies with data available for analysis.

The background and demographic details of the included terminations are presented in TABLE 1. The median gestational age at time of the procedure was 31 weeks (range 20.2–35.2 weeks) with two distinct gestational age groups: termination at 20–24 weeks (late second trimester, Group A), performed in 32.1% (26/81) of patients, and beyond 28 weeks of gestation (third trimester, Group B), performed in 67.9% (55/81) of cases (FIGURE 1). The leading indications for selective termination were structural anomalies (42.0%) and genetic abnormalities (49.4%). The median gestational age at the time of the indication (diagnosis of abnormality) was 20.5 weeks for group A (range 17–22.5 weeks) and 24.2 weeks for group B (range 14.3–33.4 weeks) (TABLE 2).

Of the 63 cases indicated in the second trimester, 37 (58.7%) were postponed to the third trimester, mostly for risk reduction (91.9%, 34/37) and some due to the couple's hesitation (8.1%, 3/37). In those postponing selective termination, the mean gestational age at the time of the indication for selective termination was significantly higher than in those performing a second trimester selective termination ($23^{5}/_{7}$ weeks versus $20^{3}/_{7}$ weeks, $P < 0.001$). There was no statistical difference in the rate of previous preterm labour (5.4% [2/37] versus 11.5% [3/26], $P = 0.64$) or in the rate of reduction of the presenting twin (40.5% [15/37] versus 34.6% [9/26], $P = 0.63$) between the group that postponed the selective termination and the group that performed a second trimester selective termination.

Half of the patients (50.6%) delivered by Caesarean section, mostly due to breech presentation of the presenting fetus (68%, FIGURE 2). There were no pregnancy losses among the group but 16.0% (13/81) of cases experienced a complication after the procedure, such as preterm premature rupture of membranes, PTB, bleeding or chorioamnionitis. There was one case of neonatal death, which occurred following an extremely early PTB at 24 weeks, 3.5 weeks after selective termination was performed on the presenting twin. The patient had a short cervical length of 8 mm at the time of the procedure and a history of PTB. The neonate suffered from severe pulmonary insufficiency and renal insufficiency and finally succumbed due to sepsis.

Comparison of late second (group A) versus third trimester (group B) selective termination

TABLE 2 displays the comparison of outcomes between the two groups. Compared with group B, group A had a significantly higher median birthweight centile (36.5th versus 15th centile, $P = 0.002$), less IUGR (11.5% versus 32.7%, $P = 0.04$) and a lower rate of breech presentation of the viable co-twin (15.4% versus 43.6%, $P = 0.013$), leading to a lower rate of Caesarean delivery (26.9% versus 61.8%, $P = 0.003$).

The median cervical length at time of the procedure was significantly shorter in the third trimester group (35 versus 40 mm, $P = 0.02$). There were no statistically significant differences in the rate of PTB below 37, 35 and 32 weeks of gestation between the groups. There was no statistically significant difference in the gestational age at the time of delivery between the groups ($37^{1}/_{7}$ versus $36^{3}/_{7}$ weeks, $P = 0.99$) or in the rate of induction of labour (23.1% versus 20.0%, $P = 0.75$). Altogether, there were 19.75% (16/81) labour inductions, of which 6 were preterm: 5 due to preterm premature

TABLE 1 DEMOGRAPHIC AND BACKGROUND CHARACTERISTICS

Parameter	Study group (*n* = 81)
Gestational age at selective termination Range (weeks)	31 (22.4–32) 20.2–35.2
Gestational age at time of indication	22.6 (21.2–26)
Trimester at selective termination	
Late second trimester	32.1% (26/81)
Third trimester	67.9% (55/81)
Postponed from second trimester	37/55 (67.3%)
Selective termination indicated in third trimester	18/55 (32.7%)
Previous preterm birth	7.4% (6/81)
Presenting twin reduced	37.0% (30/81)
Reason for selective termination	
Genetic abnormality	49.4% (40/81)
Structural anomalies	42.0% (34/81)
Severe IUGR	4.9% (4/81)
Fetal CMV[a]	1.2% (1/81)
Brain haemorrhage	1.2% (1/81)
Cervical length at selective termination (mm)	37 (32–41)
Maternal age (years)	36 (32–39)
Maternal BMI (kg/m^2)	22.86 (20.76–25.4)
Nulliparity	42.0% (34/81)
Gestational age at delivery (weeks)	36.4 (34.5–37.6)
Birthweight (g)	2600 (2120–3017)
Birthweight centile	27.5 (8.5–41.5)
PTB <37 weeks	58.0% (47/81)
PTB 35.1–36.6 weeks	29.6% (24/81)
PTB 32.1–35 weeks	23.5% (19/81)
PTB ≤32 weeks	4.9% (4/81)
Mode of delivery	
Vaginal	49.4% (40/81)
Caesarean section	50.6% (41/81)
Post-procedure pregnancy loss[b]	0%
Post-procedure complication[c]	16.0% (13/81)

Data are presented as median (interquartile range) or *n*/*N* (%).

[a] The reduced twin had symptomatic CMV infection (microcephaly, cystic brain lesions and brain calcifications) while the surviving twin had asymptomatic CMV infection.

[b] Defined as stillbirth or pre-viable birth.

[c] Preterm premature rupture of membranes, bleeding, cervical shortening or contractures leading to PTB, or chorioamnionitis.

BMI, body mass index; CMV, cytomegalovirus; IUGR, intrauterine growth restriction; PTB, preterm birth.

rupture of membranes and 1 due to suspected abruption. Ten patients were induced at term (3 for pre-eclampsia, 2 for spontaneous rupture of membranes, 1 for oligohydramnios, 1 for a non-reassuring fetal heart rate, 1 for gestational diabetes, 1 for suspected chorioamnionitis and 1 for maternal anxiety).

Comparison of preterm versus term delivery

Comparison of the background charcteristics for the term and PTB groups are presented in TABLE 3. The PTB group had statistically significant higher rates of previous PTB (12.8% versus 0%, $P = 0.037$) and of reduction of the presenting twin (55.3% versus 11.8%, $P < 0.001$). Furthermore, the PTB group had a significantly shorter cervix at the time of the procedure (34 versus 40 mm, $P < 0.001$) and a higher rate of cervical length of 35 mm or less (53.5% versus 23.5%, $P = 0.007$). In fact, a positive correlation coefficient of 0.52 ($P < 0.0001$) was found between the cervical length at the time of the procedure and the gestational age at the time of birth, using Pearson correlation.

No statistically significant differences were detected between the groups in the rate of second trimester procedures, gestational age at selective termination, rate of nulliparity and maternal age or BMI.

Multiple regression included parameters that were found to be significantly associated with the PTB group in the univariate analysis. Although there was a statistically significant difference in the rate of previous PTB between the groups, this parameter could not be incorporated in the regression analysis, as there were no occurrences in the term group. Both reduction of the presenting twin and a cervical length below 36 mm at the time of the procedure were found to be independently associated with PTB (odds ratio [OR] 8.7, $P = 0.001$, 95% confidence interval [CI] 2.5–29.8; and OR 3.8, $P = 0.015$, 95% CI 1.3–11, respectively).

DISCUSSION

This study compared the perinatal outcome of late second and third trimester selective terminations in dichorionic twins and assessed for risk factors of post-procedure PTB. Despite the common policy of postponing selective termination to avoid pregnancy loss/severe prematurity (*Sebghati and Khalil, 2021*), this study's findings show that a late second trimester selective termination appears to be as safe as a third trimester selective termination, while offering the advantages of less IUGR, higher birthweight centiles and a lower rate of Caesarean delivery. A second significant finding of this study was that reduction of the presenting twin and a cervical length of 35 mm or less were both found to be independently associated with post-procedure PTB.

These observations should be regarded with caution due to the retrospective nature of this study and the limited

TABLE 2 COMPARISON OF OUTCOMES ACCORDING TO TRIMESTER AT THE TIME OF THE PROCEDURE

Parameter	Group A Selective termination 20–24 weeks (*n* = 26)	Group B Selective termination ≥28 weeks (*n* = 55)	*P*-value
Gestational age at Indication	20.5 (19.4–21.4)	24.2 (22.6–27.4)	<0.0001
PTB ≤37 weeks	46.2% (12/26)	63.6% (35/55)	0.15
PTB ≤35 weeks	30.8% (8/26)	27.3% (15/55)	0.74
PTB ≤32 weeks	11.5% (3/26)	1.8% (1/55)	0.09
Latency ≥3 weeks	100%	63.6% (35/55)	<0.0001
Latency (weeks)	15.3 (13–16.6)	3.6 (2.2–5.4)	<0.0001
Procedure-related complications[a]	15.4% (4/26)	16.4% (9/55)	1
Labor induction	19.2% (5/26)	20.0% (11/55)	0.93
Gestational age at birth (weeks)	37.1 (34.4–38.3)	36.3 (34.4–37.3)	0.99
Birthweight (g)	2834 (2122–3250)	2444 (2105–2835)	0.12
Birthweight centile	36.5 (17–69)	15 (5–32)	0.002
IUGR	11.5% (3/26)	32.7% (18/55)	0.04
Caesarean section	26.9% (7/26)	61.8% (34/55)	0.003
Breech presentation	15.4% (4/26)	43.6% (24/55)	0.013
Neonatal death	3.8% (1/26)	0 %	0.15
Cervical length at selective termination (mm)	40 (37–43)	35 (28–40)	0.02

Data are presented as median (interquartile range) or *n*/*N* (%).

[a] Preterm premature rupture of membranes, bleeding, cervical shortening or contractures leading to PTB, or chorioamnionitis.

IUGR, intrauterine growth restriction; PTB, preterm birth.

number of patients. However, the data support the performance of a late second trimester selective termination when indicated. When selective termination of the presenting twin is required, it may be reasonable to discuss postponing the procedure to the third trimester to reduce the risk of extreme prematurity, as this was found to be the most significant risk factor for PTB (OR 8.7). If the patient has a cervix measuring less than 36 mm, there remains the dilemma of postponing the selective

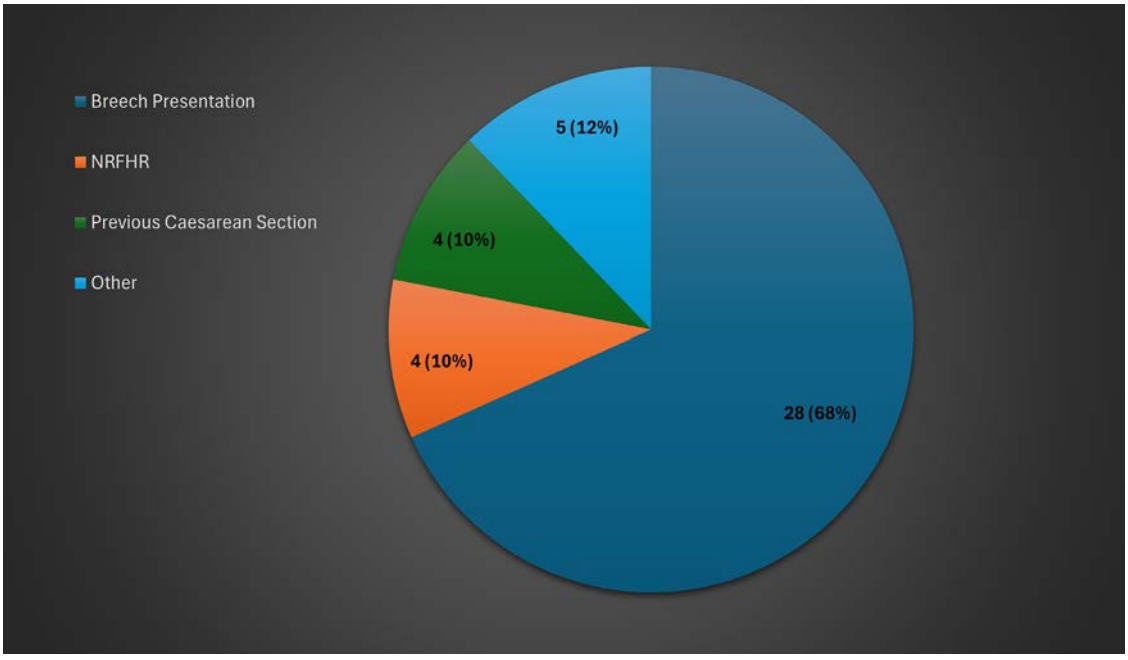

FIGURE 2 Caesarean section indication for the entire cohort. NRFHR, non-reassuring fetal heart rate.

TABLE 3 COMPARISON OF PARAMETERS OR CHARACTERISTICS ACCORDING TO PRETERM AND TERM DELIVERY FOLLOWING A LATE SELECTIVE TERMINATION

Parameter	Preterm birth <37 weeks	Term birth	*P*-value
Group assignment			0.15
Group A – late second trimester	25.5% (12/47)	41.2% (14/34)	
Group B – third trimester	74.5% (35/47)	58.8% (20/34)	
Previous preterm birth	12.8% (6/47)	0%	0.037
Presenting twin reduced	55.3% (26/47)	11.8% (4/34)	<0.001
Gestational age at selective termination	31.3 (23.1–32.1)	29.2 (21.5–31.6)	0.1
Cervical length at selective termination (mm)	34 (25–40)	40 (35.75–42.25)	<0.001
Cervical length ≤35 mm	53.2% (25/47)	23.5% (8/34)	0.007
Maternal age (years)	36 (32–39)	35.5 (31–39.5)	0.52
Maternal BMI (kg/m^2)	23.1 (21.5–27.3)	23.26 (19.7–26.4)	0.4
Nulliparity	34.0% (16/47)	52.9% (18/34)	0.1

Data are presented as median (interquartile range) or *n/N* (%).

BMI, body mass index.

termination, since both an ongoing twin pregnancy and performing selective termination carry a risk of PTB (*American College of Obstetricians and Gynecologists and Society for Maternal-Fetal Medicine, 2014*; *Cheong-See et al., 2016*; *Graham and Gaddipati, 2005*). It should be kept in mind that if selective termination is in fact postponed, the risk of delivering an anomalous fetus persists.

To the best of the authors' knowledge this is the first study to compare the perinatal outcome of selective termination performed in the late second and third trimesters. Previous studies have investigated selective termination performed before 24 weeks (*Alvarado et al., 2012*; *Bigelow et al., 2014*; *Eddleman et al., 2002*; *Zemet et al., 2021*) with only a few that have included a limited number of selective terminations beyond 24 weeks (*Dural et al., 2017*; *Evans et al., 1999*; *Hartoov et al., 1998*). Therefore the comparison of the current findings is limited to studies that included late second and third trimester selective terminations (*Dural et al., 2017*; *Evans et al., 1999*; *Greenberg et al., 2020*; *Hartoov et al., 1998*; *Sorrenti et al., 2023*). The sparsity of literature addressing selective termination in advanced pregnancy results from legal constraints limiting pregnancy termination beyond 24 weeks in numerous countries (*ESHRE Capri Workshop Group, 2017*; *Harvey et al., 2023*).

The safety of a late selective termination has to be considered. Contrary to the current study, which found 100% co-twin survival after selective termination, a meta-analysis by Sorrenti and colleagues reported a pooled pregnancy loss rate of 8% for selective terminations performed at around 18–24 weeks (*Sorrenti et al., 2023*). This difference in the rate of post-procedure pregnancy loss might stem from an earlier pre-viable gestational age at which the procedure was performed in the studies included in the meta-analysis, compared with the current study (performed beyond 20 weeks), resulting in a longer post-procedure pre-viable period during which progression to labour resulted in a late abortion. This observation is further supported by a previous study (*Dural et al., 2017*), in which pregnancy loss occurred exclusively in selective terminations performed between 15 and 19 weeks while none occurred in the 22 selective terminations performed beyond 20 weeks. Although there were no pregnancy losses in the current study, there was one neonatal death due to complications of extreme prematurity following selective termination at 20.5 weeks. This occurred in a patient who presented three risk factors for PTB – a short cervical length, a history of PTB and reduction of the presenting twin.

Furthermore, a late second trimester selective termination does not seem to increase the risk of PTB compared with ongoing twins. In a previous case-control study, the rate of PTB was similar between ongoing twins and pregnancies that underwent a late second trimester selective termination (41.9% versus 43%, $P = 0.7$). In addition, there was no statistically significant difference in the rate of PTB between pregnancies that underwent a third trimester selective termination and ongoing twins (61.7% versus 45.7%, $P = 0.07$) (*Weissbach et al., 2022*).

The current study found that there was less IUGR and higher birthweight centiles when performing selective termination in the second trimester. A similar observation was described by Sorrenti and colleagues, who found a higher mean birthweight when selective termination was performed earlier (*Sorrenti et al., 2023*). Reduced growth in twins is well described (*Hiersch et al., 2022*) and may explain the increased growth observed when earlier selective termination is performed, extending the period of time that the pregnancy continues as a singleton pregnancy.

One of the main concerns of invasive interventions in pregnancy is the risk of PTB (*Picone et al., 2008*), especially in high-order pregnancies (*Sebghati and Khalil, 2021*). Idenifying risk factors of PTB is essential for optimal patient management since on of the main concerns of invasive interventions in pregnancy is the risk of PTB, especially in high order pregnancies. A univariate analysis of background parameters identified three factors associated with PTB: a history of PTB and a cervix measuring 35 mm or less, which are also associated with PTB in ongoing twin pregnancies, and reduction of the presenting twin, which applies exclusively to selective termination. Of these, a cervix

of 35 mm or less and reducing the presenting twin were independently associated with post-procedure PTB. Likewise, a recent study by Miremberg and co-authors has also found the reduction of the presenting twin a significant risk factor for PTB (*Miremberg et al., 2023*).

The strengths and limitations of the current study should be considered. To the best of the authors' knowledge, this is the largest study to compare the perinatal outcome of late selective termination performed in the second and third trimesters, providing practical clinical insights to guide clinicians debating the optimal time to perform a late selective termination, when indicated. As a single-centre study, there was uniformity in medical management, increasing its internal validity. Moreover, medical charts were accessible for thorough examination and rigorous data retrieval, providing an informative database.

The limitations of the study should also be acknowledged. Despite being the largest existing study addressing late selective terminations, a larger cohort could increase the statistical certainty of the study findings and shed light on uncommon events, such as pregnancy loss and neonatal death. The disadvantage of single-centre studies is the limited extrapolation to other centres, which may differ in patient characteristics and medical management. Finally, the distribution of gestational age at the time of selective termination was skewed as it is the accepted management at the authors' centre to postpone selective termination to the third trimester when an indication arises between 24 and 28 weeks of gestation. This is intended to avoid provoking an extremely early PTB. Overall, there were 15 cases in which the procedure was postponed from 24–28 weeks to the third trimester.

To conclude, late second trimester selective termination displays better perinatal outcomes including higher birthweight centiles, fewer Caesarean deliveries and IUGR, without increasing PTB and pregnancy loss, compared with a third trimester selective termination. A cervical length of 35 mm or less and reduction of the presenting twin increase the risk of PTB, with odds ratios of 3.8 and 8.7, respectively. In cases presenting these risk factors, postponing the procedure to the third trimester should be discussed. These findings may be of clinical importance when determining the optimal gestational age at which to perform a late selective termination.

DATA AVAILABILITY

Data will be made available on request.

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ARTICLE

A comprehensive study of pre-eclampsia in IVF and natural conceptions: clinical phenotypes, perinatal outcomes and neonatal echocardiography

BIOGRAPHY

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KEY MESSAGE

There is a weak link between the severity of pre-eclampsia and newborn cardiac abnormalities in IVF pregnancies, hinting that this correlation is influenced by factors other than the method of conception. Comprehensive newborn echocardiographic screening has shown that pre-eclampsia may affect the newborn heart.

ABSTRACT

Research question: What differences exist in the phenotypes of pre-eclampsia, perinatal outcomes and neonatal echocardiography between pregnancies conceived naturally and through IVF?

Design: Six hundred and ten women diagnosed with pre-eclampsia between January 2002 and December 2022 were included in this study. This research was conducted within the IVF and Maternal-Fetal Medicine Department of Kaohsiung Chang Gung Memorial Hospital, Taiwan. Participants were divided into two groups: those who achieved pregnancy through IVF, and those who conceived naturally. The phenotypes of pre-eclampsia and perinatal outcomes were assessed using a propensity-matched sample (n = 218), along with neonatal echocardiography.

Results: After conducting propensity score matching, the natural conception group had a higher prevalence of early-onset pre-eclampsia (53.9% versus 37.7%, P = 0.04) and exhibited more severe features of pre-eclampsia (89.1% versus 69.8%, P = 0.01) compared with the IVF group. Regarding perinatal outcomes, neonates in the IVF group had higher placental weights compared with the natural conception group (580 versus 480 g, P = 0.031). The prevalence of abnormal findings on neonatal echocardiography was similar between the groups. Multivariate analysis showed that greater gestational age at delivery reduced the likelihood of abnormal findings on echocardiography [adjusted risk ratio (aRR) 0.950, P = 0.001], while pregestational diabetes mellitus increased the likelihood of abnormal findings (aRR 1.451, P = 0.044). Septal defects were the most common type of defect, occurring in 16.1% of infants.

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KEY WORDS
Pre-eclampsia
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Conclusion: The impact of IVF conception on the severity of pre-eclampsia is not as expected. Neonatal echocardiography revealed a higher prevalence of abnormalities in offspring of women with pre-eclampsia compared with the general population. However, these issues were not linked to the method of conception, suggesting the existence of undisclosed factors that could influence the clinical features and perinatal outcomes of pre-eclampsia.

INTRODUCTION

Pre-eclampsia is a challenging obstetric condition that affects 4.6% of pregnancies worldwide, making it a leading cause of maternal and fetal morbidity and mortality (*Abalos et al., 2013*). Following the widespread introduction and application of assisted reproductive technology (ART), the Taiwan Health Promotion Administration's statistics for 2019 show that six of every 100 newborns in Taiwan were conceived through IVF. Previous studies have indicated increased risk of pre-eclampsia in IVF pregnancies, with some demonstrating more severe clinical phenotypes. Possible mechanisms include problems with the uterine environment, in-vitro culture conditions and placental insufficiency during implantation (*Gui et al., 2020*; *Omani-Samani et al., 2020*). However, many confounding factors and underlying maternal medical conditions may introduce selection bias, leading to ongoing debate regarding the associations between severity of illness, IVF and preeclampsia.

The impact of pre-eclampsia on the offspring's cardiovascular system begins during embryonic development and persists after birth. There is growing evidence linking pre-eclampsia to congenital heart defects (CHD) due to an imbalance in associated angiogenic biomarkers. This linked pathophysiological pathway indicates that the pathology of pre-eclampsia starts at the time of fetal heart morphogenesis (*Boyd et al., 2017*). Cardiovascular sequelae in the offspring, including hypertension, altered vascular function and increased body mass index (BMI), have been reviewed extensively (*Herrera-Garcia and Contag, 2014*).

This study assessed the manifestations of pre-eclampsia, including onset time and severity, maternal–fetal complications and perinatal outcomes, based on different methods of conception, accounting for the increasing prevalence of IVF pregnancies in recent times. Additionally, neonatal echocardiography was used to assess infants born to mothers with pre-eclampsia, predisposing maternal risk factors.

MATERIALS AND METHODS

This retrospective study was conducted at Kaohsiung Chang Gung Memorial Hospital, and included 610 mothers with pre-eclampsia between 2002 and 2022. The study was approved by Chang Gung Memorial Hospital Ethics Committee (Institutional Review Board No. 202300811B0C502, approval date 6 September 2023). All data were sourced from the electronic delivery database established by the Maternal–Fetal Medicine Department, with pregnant women diagnosed with the keyword 'pre-eclampsia' included in the study. Additionally, supplementary information required for the study was collected by cross-referencing medical records. Participants were divided into two groups: those who achieved pregnancy through IVF, and those who conceived naturally. Women who conceived through intrauterine insemination were not included in this study. Cases without clinician intervention were included in the natural conception group. All pregnant women in the study met the criteria for pre-eclampsia according to the guidelines of the American College of Obstetricians and Gynecologists or the guidelines of the International Society for the Study of Hypertension in Pregnancy (*Anonymous, 2020*; *Brown et al., 2018*). The diagnostic criteria for pre-eclampsia included new-onset hypertension after 20 weeks of gestation, with systolic pressure/diastolic pressure ≥140/90 mmHg measured on two occasions at least 4 h apart, or systolic pressure/diastolic pressure ≥160/110 mmHg for a short interval (min), accompanied by any of the following: (i) presence of proteinuria; or (ii) other organ dysfunction, including acute kidney injury (creatinine >1.1 mg/dl), liver impairment (elevated transaminases exceeding twice the upper limits), pulmonary oedema, neurological complications (severe headache, visual disturbance, altered mental status) or platelet count <100,000/μl. Proteinuria was defined as ≥300 mg of protein collected in 24 h, protein/creatinine ratio ≥0.3 or a urine dipstick reading ≥2+. Blood pressure ≥160/110 mmHg or any systemic organ involvement was diagnosed as pre-eclampsia with severe features.

Maternal characteristics, manifestations of pre-eclampsia, maternal–fetal complications and perinatal outcomes were compared between the IVF and natural conception groups. Relevant literature (*Calhoun et al., 2011*; *Gui et al., 2020*) and factors known to influence the prognosis of pre-eclampsia, including maternal age, parity, BMI, gestational age at delivery, multiple gestations, history of pre-eclampsia, underlying medical diseases, smoking and use of aspirin, were reviewed to ensure parity between the two groups. In total, 13 variables were determined in the propensity score matching (PSM) analysis, which was used to minimize potential confounding factors. Subanalyses of the original group are presented in the tables.

Manifestations of pre-eclampsia were categorized as maternal or fetal complications. Maternal complications included onset time (early onset, defined as <34 weeks), pre-eclampsia with severe or severe features accompanied by systemic organ dysfunction, HELLP syndrome, eclampsia, placental abruption, postpartum haemorrhage, postpartum cardiomyopathy and cerebral thrombosis. Fetal complications included oligohydramnios, abnormal end-diastolic velocity (AEDV), reverse end-diastolic velocity (REDV), non-variable non-stress test and intrauterine fetal death.

Perinatal outcomes included delivery mode, fetal sex, birth weight, small for gestational age (defined as birth weight <10th percentile for gestational age according to national growth charts), placental weight, Apgar score, admission to neonatal intensive care unit (NICU), and first neonatal echocardiographic examination before 1 month of age. Each echocardiographic examination was conducted by a paediatric cardiologist using a Sonos 5500 Ultrasound System (Philips, The Netherlands). Patients with patent foramen ovale and patent ductus arteriosus were included in the study population, but these were not classified as abnormal findings because they may correct later in preterm infants, and are unlikely to be pathological. Maternal characteristics and possible factors related to pre-eclampsia were analysed to

determine their relationship with abnormal findings on neonatal echocardiography.

Statistical analysis

Descriptive data are presented as median [interquartile range (IQR)] or percentage. Chi-squared test or Fisher's exact test was used for categorical variables, and Mann–Whitney *U*-test was used for continuous variables. It was calculated that a sample size of 610 would have 98% power to detect an effect size of 0.1640 using a chi-squared test with one degree of freedom, with a significance level (alpha) of 0.05000.

To address potential confounding factors in the population, PSM analysis was employed to reduce the dimensionality of covariates and achieve balanced groups, thereby reducing bias in the assessment of outcomes. Thirteen independent variables (maternal age, parity, BMI, multiple gestations, gestational age at delivery, history of pre-eclampsia, chronic hypertension, pregestational diabetes mellitus, gestational diabetes mellitus, chronic kidney disease, autoimmune disease, smoking, use of low-dose aspirin) were used to determine the propensity scores. Subsequently, 53 IVF cases were matched in a 1:4 ratio with 165 natural conception cases. A standardized mean difference (SMD) <0.1 suggests a relatively small difference between two groups. The maternal characteristics of the initial population (n = 610) and the propensity-matched population (n = 218) are presented in TABLE 1. Furthermore, the manifestations of pre-eclampsia, maternal–fetal complications and perinatal outcomes were compared after the matching study.

To assess the association between maternal risk factors and abnormal findings on neonatal echocardiography, univariate and multivariate logistic regression analyses were performed on the initial study samples. Relative risk ratio and 95% CI were calculated, and $P < 0.05$ was considered to indicate significance. The analyses were conducted using SPSS Statistics Version 25.0 (IBM Corp., USA).

RESULTS

In total, 610 mothers diagnosed with pre-eclampsia were enrolled in this study, of whom 539 conceived naturally and 71 conceived through IVF. Of the 71 IVF patients, three received donor eggs and the remaining 68 used autologous eggs. A comparison of the maternal characteristics and possible pre-eclamptic factors is presented in TABLE 1. After PSM, the comparison between the natural conception group (n = 165) and the IVF group (n = 53) revealed smaller SMD values (<0.1) for the following factors, indicating less disparity between the two groups: BMI at delivery, multiple gestations, gestational age at delivery, history of pre-eclampsia, chronic hypertension, pregestational diabetes mellitus, chronic kidney disease, autoimmune disease and smoking. Maternal age, parity, gestational diabetes mellitus and use of low-dose aspirin had larger SMD values (>0.1). Although SMD values >0.1 were noted for four of the 13 variables after matching, significant reductions in SMD values were noted.

TABLE 2 includes information regarding the phenotypes of pre-eclampsia and maternal–fetal complications. After PSM, in comparison with the IVF group, the natural conception group showed a higher proportion of early-onset pre-eclampsia (53.9% versus 37.7%, P = 0.04), more cases with severe features (89.1% versus 69.8%, P = 0.01), higher systolic blood pressure [median 175 (IQR 164–190) versus 167 (IQR 155–179) mmHg, P = 0.002], higher diastolic blood pressure [median 106 (IQR 98–117) versus 104 (IQR 91–111) mmHg, P = 0.01], and higher incidence of AEDV or REDV (16.4% versus 5.7%, P = 0.049). There were no significant differences between the two groups in

TABLE 1 MATERNAL CHARACTERISTICS AND PRE-ECLAMPTIC RISK FACTORS FOR WOMEN WHO CONCEIVED NATURALLY OR THROUGH IVF

	Eligible study sample (n = 610)			PSM sample (n = 218)		
	Natural conception (n = 539)	IVF (n = 71)	SMD	Natural conception (n = 165)	IVF (n = 53)	SMD
Maternal age (years)	33 (30–37)	37 (34–39)	0.705	35 (33–39)	37 (34–40)	0.257
Primiparous	287 (53.2)	53 (74.6)	0.457	107 (64.8)	37 (69.8)	0.106
BMI at delivery (kg/m^2)	30.8 (27.1–35.0)	30.5 (26.7–34.5)	0.123	30.0 (27.0–34.5)	31.5 (26.2–35.1)	0.023
Multiple gestations	19 (3.5)	22 (31)	0.780	18 (10.9)	7 (13.2)	0.071
GA at delivery (weeks)	35 (33–37)	36 (34–37)	0.379	36 (34–37)	37 (33–38)	0.093
History of pre-eclampsia	62 (11.5)	2 (2.8)	0.342	5 (3)	2 (3.8)	0.041
Chronic hypertension	138 (25.6)	10 (14.1)	0.292	31 (18.8)	10 (18.9)	0.002
PGDM	43 (8.0)	6 (8.5)	0.017	13 (7.9)	5 (9.4)	0.055
GDM	60 (11.2)	12 16.9)	0.214	24 (14.5)	10 (18.9)	0.116
CKD	6 (1.1%)	1 (1.4%)	0.026	3 (1.8)	1 (1.9)	0.005
Autoimmune disease[a]	14 (2.6)	3 (4.2)	0.090	9 (5.5)	2 (3.8)	0.080
Smoking	7 (1.3)	0	0.162	0	0	<0.001
Use of LDA	87 (16.1)	20 (28.2)	0.293	33 (20)	14 (26.4)	0.152

Data are expressed as median (interquartile range) or n (%).

[a] Defined as systemic lupus erythematosus, antiphospholipid syndrome, IgA nephropathy and thyroid disease.

PSM, propensity score matched; BMI, body mass index; GA, gestational age; PGDM, pregestational diabetes mellitus; GDM, gestational diabetes mellitus; CKD, chronic kidney disease; LDA, low-dose aspirin; SMD, standardized mean difference.

TABLE 2 ANALYSIS OF SEVERITY OF PRE-ECLAMPSIA AND MATERNAL AND FETAL COMPLICATIONS IN THE NATURAL CONCEPTION AND IVF GROUPS

Complications	PSM sample (n = 218)		
	Natural conception (n = 165)	IVF (n = 53)	P-value[a]
Maternal complication			
Onset time (weeks)			0.040
Early (<34)	89 (53.9)	20 (37.7)	
Late (≥34)	76 (46.1)	33 (62.3)	
Severe features	147 (89.1)	37 (69.8)	0.010
SBP (mmHg)	175 (164–190)	167(155–179)	0.002
DBP (mmHg)	106 (98–117)	104 (91–111)	0.010
Urinary protein ≥2+	130 (78.8)	36(67.9)	0.484
Severe features with systemic involvement	63 (38.2)	20 (37.7)	0.954
Liver impairment (>2x upper limit)	27 (16.4)	0	0.916
Low platelet count (<10,000/μl)	19 (11.5)	9 (17.0)	0.301
AKI (>1.1 mg/dl)	14 (8.5)	2 (3.8)	0.368
Headache, blurred vision	25 (15.2)	11 (20.8)	0.339
Pulmonary oedema	8 (4.8)	0	0.204
HELLP syndrome	8 (4.8)	4 (7.5)	0.491
Eclampsia	5 (3.0)	0	0.339
Placental abruption	5 (3.0)	1 (1.9)	1.000
Postpartum haemorrhage	2 (1.2)	2 (3.8)	0.249
PPCM	1 (0.6)	1 (1.9)	0.395
Brain thrombosis	-	-	-
Fetal complication			
Oligohydramnios	7 (4.2)	3 (5.7)	0.708
AEDV/REDV	27 (16.4)	3 (5.7)	0.049
Non-variable NST	10 (6.1)	3 (5.7)	1.000
IUFD	7 (4.2)	1 (1.9)	0.683

Data are expressed as median (interquartile range) or n (%) per pregnancy.

Urinary protein was measured by dipstick test. Urinary protein 2+ represents ≥100 mg/dl.

[a] Chi-squared test or Fisher's exact test used for categorical variables, Mann–Whitney U-test used for continuous variables.

PSM, propensity score matched; SBP, systolic blood pressure; DBP, diastolic blood pressure; AKI, acute kidney injury; PPCM, postpartum cardiomyopathy; NST, non-stress test; AEDV, abnormal end-diastolic velocity; REDV, reverse end-diastolic velocity; IUFD, intrauterine fetal death.

terms of other severe features with systemic involvement, such as liver impairment, thrombocytopenia, acute kidney injury, neurological symptoms (headache or blurred vision) or pulmonary oedema. Additionally, no differences were observed in the occurrence of HELLP syndrome, eclampsia, placental abruption, postpartum haemorrhage, postpartum cardiomyopathy, brain thrombosis, oligohydramnios, non-variable non-stress test or stillbirth.

Perinatal outcomes are depicted per fetus in TABLE 3. After PSM, only placental weight was found to be significantly higher in the IVF group compared with the natural conception group [median 580 (IQR 400–720) versus 480 (IQR 360–620) g, P = 0.03], and no significant differences were observed in terms of delivery mode, fetal sex, birth weight, small for gestational age, Apgar score <7 at 1 or 5 min, NICU admission, or abnormal findings on neonatal echocardiography.

TABLE 4 shows potential maternal characteristics and risk factors associated with abnormal findings on neonatal echocardiography. Univariate analysis found that a greater gestational age at delivery was associated with lower likelihood of abnormal findings on neonatal echocardiography (relative risk 0.961, 95% CI 0.934–0.988, P = 0.006). Additionally, pregestational diabetes mellitus was found to increase the risk of abnormal findings on neonatal echocardiography (relative risk 1.453, 95% CI 1.071–1.972, P = 0.016). Multivariate regression analysis showed that a greater gestational age at delivery remained associated with reduced likelihood of abnormal findings on neonatal echocardiography (adjusted risk ratio 0.950, 95% CI 0.922–0.979, P = 0.001). Furthermore, pregestational diabetes mellitus remained a significant risk factor for abnormal findings on neonatal echocardiography (relative risk 1.451, 95% CI 1.011–2.082, P = 0.044). Maternal age, parity, BMI at delivery, method of conception, multiple gestations, history of pre-eclampsia, gestational diabetes mellitus, chronic kidney disease,

TABLE 3 PERINATAL OUTCOMES IN THE NATURAL CONCEPTION AND IVF GROUPS

Outcome	PSM sample (*n* = 243)		
	Natural conception (*n* = 183)	IVF (*n* = 60)	*P*-value[a]
Delivery mode			0.469
Vaginal	16 (8.7)	7 (11.7)	
Caesarean section	167 (91.3)	53 (88.3)	
Fetal sex			0.683
Male	89 (48.6)	31 (51.7)	
Female	94 (51.4)	29 (48.3)	
Birth weight (g)	2060 (1600–2600)	2285 (1662–2952)	0.067
Placental weight (g)	480 (360–620)	580 (400–720)	0.031
SGA	85 (46.4)	23 (38.3)	0.272
Apgar score at 1 min <7	36 (19.7)	16 (26.7)	0.216
Apgar score at 5 min <7	10 (5.5)	4 (6.7)	0.749
NICU	125 (68.3)	36 (60.0)	0.238
Abnormal echocardiography	74 (40.4)	16 (26.7)	0.055

Data are expressed as median (interquartile range) or *n* (%), given for individual fetuses.

[a] *P*-value <0.05 considered to indicate significance. Chi-squared test or Fisher's exact test used for categorical variables, Mann–Whitney *U*-test used for continuous variables.

PSM, propensity score matched; SGA, small for gestational age; NICU, neonatal intensive care unit.

autoimmune disease and use of low-dose aspirin were not significantly associated with abnormal findings on neonatal echocardiography.

TABLE 5 outlines the abnormal echocardiographic findings of 626 infants born to mothers with pre-eclampsia. These examinations were conducted within 1 month of birth. Among these infants, 101 (16.1%) had septal defects, including atrial septal defects (12.3%), ventricular septal defects (1.9%) and hypertrophic interventricular septum (1.9%). Valve defects were observed in 64 (10.2%) neonates, with occurrences of mitral or tricuspid regurgitation (8.3%), aortic regurgitation (0.8%) and pulmonary valve stenosis (1.1%). Ventricular problems were present in 14 (2.2%) neonates, including ventricular hypertrophy (1.1%) and chamber dilatation (1.1%). Vessel issues were detected in 72 neonates (11.5%), including peripheral pulmonary stenosis (10.5%), aortic coarctation (0.5%) and coronary dilatation (0.5%). Additionally, 51 (8.1%) neonates were diagnosed with functional complications, specifically pulmonary hypertension.

TABLE 4 POTENTIAL MATERNAL CHARACTERISTICS AND RISK FACTORS IN RELATION TO ABNORMAL NEWBORN ECHOCARDIOGRAPHY

Variables	Univariate regression analysis			Multivariate regression analysis		
	RR	95% CI	*P*-value	aRR	95% CI	*P*-value
Maternal age	1.009	0.987–1.030	0.429	1.012	0.989–1.035	0.299
Primigravida	0.892	0.720–1.105	0.295	0.963	0.757–1.225	0.758
BMI at delivery (kg/m^2)	1.015	0.997–1.033	0.107	1.011	0.991–1.032	0.265
IVF	0.811	0.557–1.182	0.276	0.816	0.540–1.234	0.335
Multiple gestations	0.955	0.616–1.482	0.838	1.201	0.744–1.941	0.454
GA at delivery	0.961	0.934–0.988	0.006	0.950	0.922–0.979	0.001
History of pre-eclampsia	1.108	0.800–1.537	0.537	1.008	0.703–1.445	0.965
PGDM	1.453	1.071–1.972	0.016	1.451	1.011–2.082	0.044
GDM	1.196	0.889–1.610	0.237	1.359	0.998–1.852	0.052
CKD	1.205	0.509–2.854	0.671	0.830	0.340–2.026	0.682
Autoimmune disease[a]	0.654	0.276–1.551	0.335	0.631	0.265–1.503	0.299
Smoking	1.615	0.842–3.095	0.149	1.688	0.874–3.260	0.119
Use of LDA	0.961	0.722–1.278	0.785	0.921	0.588–1.233	0.580

All listed variables were included as potential confounders.

[a] Defined as systemic lupus erythematosus, antiphospholipid syndrome, IgA nephropathy and thyroid disease.

BMI, body mass index; GA, gestational age; PGDM, pregestational diabetes mellitus; GDM, gestational diabetes mellitus; CKD, chronic kidney disease; LDA, low-dose aspirin, (a)RR (adjusted) risk ratio.

DISCUSSION

These findings show that patients with pre-eclampsia who conceived naturally exhibited a higher prevalence of early-onset pre-eclampsia and severe features compared with patients who conceived through IVF. While most previous studies have reported a higher rate of preterm birth in patients who conceived through IVF compared with those who conceived naturally (*Lang et al., 2023*; *Okby et al., 2018*), this has not been compared specifically within the pre-eclampsia population prior to the present study. Severe features primarily manifested as blood pressure >160/110 mmHg upon admission. However, no discernible differences were observed between the two groups in terms of systemic involvement, as indicated by abnormal laboratory results or organ dysfunction. During the prenatal period, the natural conception group demonstrated a higher incidence of waveform abnormalities, AEDV and REDV. Postnatally, apart from greater placental weight in the IVF group, there were no notable differences in perinatal outcomes. This analysis suggests that potential maternal factors contributing to abnormal findings on neonatal echocardiography include lower gestational age at delivery and pregestational diabetes mellitus. The most common findings on neonatal echocardiography were septal defects; followed by abnormalities in blood vessels, valves, pulmonary circulation and ventricles.

The development of pre-eclampsia is thought to be influenced by a combination of genetic, environmental, immune and metabolic factors. These factors can contribute to insufficient migration of

TABLE 5 ABNORMAL ECHOCARDIOGRAPHIC FINDINGS IN 626 INFANTS BORN TO MOTHERS WITH PRE-ECLAMPSIA

	All *n* = 626	Natural conception *n* = 535	IVF *n* = 91
Septum			
Atrial septal defect	77 (12.3)	70 (13.1)	7 (7.7)
Ventricular septal defect	12 (1.9)	11 (2.1)	1 (1.1)
Hypertrophy interventricular septum	12 (1.9)	12 (2.2)	0
Total	101 (16.1)	93 (17.4)	8 (8.8)
Valve			
Mitral/tricuspid regurgitation	52 (8.3)	43 (8.0)	9 (9.9)
Pulmonary valve stenosis	7 (1.1)	6 (1.1)	1 (1.1)
Aortic regurgitation	5 (0.8)	5 (0.9)	0
Total	64 (10.2)	54 (10.1)	10 (11.0)
Ventricle			
Ventricular hypertrophy	7 (1.1)	7 (1.3)	0
Chamber dilatation	7 (1.1)	7 (1.3)	0
Total	14 (2.2)	14 (2.6)	0
Vessel			
Peripheral pulmonary stenosis	66 (10.5)	61 (11.4)	5 (5.5)
Aortic coarctation	3 (0.5)	1 (0.2)	2 (2.2)
Coronary dilatation	3 (0.5)	3 (0.6)	0
Total	72 (11.5)	65 (12.1)	7 (7.7)
Functional			
Pulmonary hypertension	51 (8.1)	50 (9.3)	1 (1.1)

Data are expressed as *n* (%).

extravillous trophoblasts to spiral arteries, leading to the formation of vessels with limited capacity and increased resistance. Consequently, inadequate blood flow and oxygen supply in the placenta causes an imbalance in angiogenic factors. Ultimately, this leads to vascular endothelial cell dysfunction and subsequent complications affecting multiple organs (*Almaani, 2019*).

Some studies have shown that manifestations of pre-eclampsia are more severe in mothers who conceive through IVF (*Calhoun et al., 2011*; *Gui et al., 2020*; *Omani-Samani et al., 2020*). It has been observed that embryo transfer into the uterus through the cervix and an altered hormonal environment in the endometrium can disrupt development of the maternal–fetal interface, resulting in inadequate placentation. Additionally, as embryogenesis begins *in vitro*, various circumstances can increase the likelihood of abnormal placental attachment, compromised placental blood vessels, and insufficient circulation (*Thomopoulos et al., 2013*). *Calhoun et al. (2011)* included specific confounding variables such as age, race, BMI, parity, multiple gestations and different gestational ages at delivery, resulting in variations in the initial adjustments. Factors such as preventive use of aspirin, among others, were not mentioned explicitly but could influence the outcomes. Another study (*Gui et al., 2020*) implemented rigid, exclusive criteria during case selection. However, with its different statistical methods and patient inclusion criteria, it may have introduced self-selection bias. Previous studies are compared with the present study in Supplementary Table 1.

A cohort study examined both natural and IVF pregnancies in the same mother. The matched control group was compared with the patient herself, considering the characteristics that could potentially affect pregnancy outcomes. Obstetric and perinatal results were similar between natural and IVF pregnancies, with no significant differences in the rates of preterm labour, small for gestational age, or hypertensive disorders (*Ganer Herman et al., 2021*).

In line with a previous study, the present study found a weak link between IVF and pre-eclampsia using PSM analysis (*Watanabe et al., 2014*). However, it is worth noting that all the individuals included in the present study had pre-eclampsia, which constitutes a distinction. These findings suggest that the impact of IVF on the severity of pre-eclampsia may be less pronounced than that indicated in previous studies.

Echocardiological findings

The pathogenesis of pre-eclampsia may be initiated early, potentially even during the early stages of pregnancy, coinciding with fetal heart morphogenesis. Fetuses with CHD may display inherent alterations in angiogenesis that involve similar biomarkers implicated in the pathogenesis of pre-eclampsia. These biomarkers include proangiogenic signalling proteins such as vascular endothelial growth factor and placental growth factor, and antiangiogenic proteins such as soluble fms-like tyrosine kinase. Insufficient trophoblast invasion in the spiral arteries, possibly because of these biomarkers, can result in placental hypoxia, potentially leading to fetal hypoxia and associated abnormal heart development (*Aye et al., 2020*; *Llurba et al., 2014*). The estimated prevalence of CHD in the general population is approximately 8 per 1000 live births (*van der Linde et al., 2011*). However, *Ishikawa et al. (2011)* conducted echocardiographic screening and discovered a significantly higher prevalence of CHD (50 of 1000 live births). Septal defects accounted for most of these cases. This disparity in the prevalence of CHD could be attributed to variations in the diagnostic criteria and the age at which the examination was performed. Additionally, mild forms of asymptomatic CHD may have been overlooked. A population-based study revealed that the overall prevalence of CHD — specifically, non-critical heart defects such as endocardial cushion; heterotaxy syndrome; and septal, valve, aorta and pulmonary artery defects — was approximately 1.9 times higher among infants born to women with pre-eclampsia compared with those born to women without pre-eclampsia (16.7 versus 8.6 per 1000 live births) (*Auger et al., 2015*).

Of the 626 infants in the present study, 226 (34.7%) had abnormal findings on echocardiography, indicating a significantly higher prevalence than reported previously. At the study institution, based

on previous research on pre-eclampsia and its impact on the fetal heart (*Lin et al., 2021*), echocardiography is conducted routinely on newborns of mothers with pre-eclampsia; this has led to an increased detection rate. This approach contrasts with many other studies, where examinations are primarily based on clinical manifestations. It is important to note that the present study included all abnormal findings on echocardiography, even those that did not meet the diagnostic criteria for CHD. Many were minor structural or functional defects, and most affected infants did not show any symptoms. Through continuous monitoring with serial echocardiography, these problems may be found to resolve over time.

In this study, which was conducted among a population consisting exclusively of individuals with pre-eclampsia, a primary cause of congenital heart disease, multivariate analysis revealed that maternal pregestational diabetes mellitus and lower gestational age at delivery were associated with abnormal findings on infant echocardiography. Maternal diabetes mellitus during early pregnancy increases the risk of all subtypes of CHD in the offspring (*Hoang et al., 2017*). Maternal physiology and glucose metabolism are vital for fetal heart development. This involves disruptions in left–right cardiac embryogenesis patterning, increased apoptosis from oxidative stress, nitric oxide signalling deficiencies, impaired autophagy, and changes in neural crest cell formation and migration. However, considering the involvement of multiple cellular models and molecular mechanisms, the specific effects of the downstream metabolites on cardiac development are not well understood (*Helle and Priest, 2020*).

The precise mechanism linking fetal congenital heart disease to preterm delivery remains unclear and complex. It can vary according to specific heart defects and individual circumstances. Infants with cardiac malformations are more likely to be born preterm, and although these outcomes are sometimes referred to as 'risk factors', they are likely consequences of malformations. In this study, the gestational age at birth was relatively early for patients with abnormal findings on neonatal echocardiography. Haemodynamic changes in fetuses with heart defects can lead to a compromised oxygen supply, which may influence the timing of intervention and delivery (*Cedergren and Källén, 2006*). Among newborns with CHD, 13.5% were born preterm, which is approximately twice the rate in the general population. Furthermore, the risk varies depending on the specific heart defect (*Laas et al., 2012*).

Newborns born to mothers with pre-eclampsia undergo subtle changes in cardiac morphology and function. *In utero*, the fetus relies on right-ventricle-dominant circulation. One study reported an increase in right ventricular mass and a decrease in end-diastolic volume in infants between birth and 3 months of age, which correlated with the severity of hypertensive disorder (*Aye et al., 2020*). Significant changes were also observed in the left ventricular wall, interventricular septum, left ventricular internal diameter, and ventricular volume. These findings are likely caused by increased placental vascular resistance, leading to adaptation to the fetal vascular load and patterns of myocardial development. This can influence the systemic state of inflammation and oxidative stress, resulting in mild myocardial dysfunction during fetal life (*Vøgg et al., 2022*).

Furthermore, offspring of mothers with pre-eclampsia exhibit changes in the pulmonary and systemic circulation, with approximately 30% higher pulmonary pressure, possibly linked to pulmonary vascular wall remodelling (*Jayet et al., 2010*). The present study found that 5.7% of newborns had detectable pulmonary hypertension. However, it is important to note that pulmonary haemodynamics undergo a transition over the first few months postnatally, and may subside during future follow-up.

In a population-based study, pre-eclampsia, especially early-onset pre-eclampsia, was found to be associated with nearly all types of substructural heart defect. Late-onset pre-eclampsia was associated with more non-critical defects, with the majority being septal defects, likely due to the longer conformational maturation time of this structure. In line with the population of the present study, the median gestational ages at delivery for the natural conception and IVF groups were 35 and 36 weeks, respectively, and septal defects were the most common type of defect (*Auger et al., 2015*).

This study displayed all sites of cardiac structural and functional defect screening in the offspring of women with pre-eclampsia, and the prevalence appeared to be higher compared with the offspring of women in the general population, as reviewed previously. Nevertheless, it remains uncertain whether these subtle cardiac differences persist during follow-up, or have clinical significance. Women with pre-eclampsia have closer obstetric follow-up, leading to increased opportunities to detect heart defects compared with women with normotensive pregnancies. Detection would most likely be greater for non-critical defects, because even asymptomatic infants undergo echocardiography. However, no difference in the prevalence of cardiac anomalies was observed, regardless of whether conception was natural or through IVF.

Limitations

This was a retrospective study. As many pregnant women who develop pre-eclampsia in the late stages of pregnancy are referred to the study institution, it was not possible to conduct a detailed analysis of the IVF protocol. The IVF protocol used at the study institution is generally consistent with the current situation in Taiwan. A gonadotrophin-releasing hormone (GnRH) agonist protocol was used before 2010, and a GnRH antagonist protocol has been used since 2015. In the intervening years, both protocols were used (*Lin et al., 2017*; *Tsai et al., 2015*).

The effect of aspirin use in the two groups is shown in Supplementary Table 2. The results indicate a reduction in the proportion of severe features with systemic involvement in overall and naturally conceived populations. However, due to the smaller number of cases using prophylactic aspirin before 2013 (one of 190 pregnancies) compared with after 2013 (106 of 420 pregnancies), it was not possible to assess the true effect of aspirin objectively.

For neonatal echocardiography, the results were recorded within the first month, but some minor defects resolved after 6 months. Nonetheless, a strength of this study lies in the fact that it is the first study to thoroughly investigate the manifestations of pre-eclampsia and neonatal echocardiography in infants conceived by different methods. Moving forward, more patients should be recruited prospectively, and differences in the manifestation of pre-eclampsia between

various ART techniques should be delineated. Additionally, a series of follow-up assessments should be conducted regarding neonatal echocardiography, potentially extending to cardiovascular implications in adulthood.

CONCLUSION

Undisclosed factors may influence the clinical manifestations and outcomes of pre-eclampsia, regardless of the method of conception. Echocardiographic screening of the participants revealed a higher incidence of cardiac abnormalities in neonates born to women with pre-eclampsia compared with the general population. Nonetheless, the majority of these defects were minor, and may be rectified during subsequent follow-up. In future, comparison of differences in the phenotypes of pre-eclampsia and perinatal outcomes following different ART techniques, and tracking echocardiographic changes from birth to adolescence could enhance the comprehensiveness of research.

DATA AVAILABILITY

No data was used for the research described in the article.

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SUPPLEMENTARY MATERIALS

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RBMO

ELSEVIER

ARTICLE

Prospective reproductive outcomes according to sperm parameters, including DNA fragmentation, in recurrent pregnancy loss†

BIOGRAPHY

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KEY MESSAGE

Among couples experiencing recurrent pregnancy loss, baseline sperm DNA fragmentation, morphology and concentration could not identify those couples at risk of another pregnancy loss after referral. A high level of DNA fragmentation was associated with not achieving pregnancy, while increasing sperm morphology increased the odds of a live birth after referral.

ABSTRACT

Research question: Are the prospective reproductive outcomes in couples experiencing recurrent pregnancy loss (RPL) related to the sperm DNA fragmentation index (DFI), as measured by sperm chromatin structure assay, sperm morphology and sperm concentration at referral?

KEY WORDS

Recurrent pregnancy loss
Sperm DNA fragmentation
Sperm DNA integrity
Sperm morphology
Sperm parameters

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Design: This prospective cohort study included 95 couples seen between 1 April 2018 and 1 December 2019 at the tertiary Copenhagen RPL Unit, Copenhagen University Hospital, Rigshospitalet and Hvidovre Hospital, Denmark. The couples had experienced three or more unexplained consecutive pregnancy losses or two late pregnancy losses (>12 weeks gestation). Follow-up was 12–31 months.

Results: Eighty-one of 95 (85.3%) couples achieved pregnancy after referral. In the first pregnancy after referral, 46 (56.8%) couples achieved a live birth, and 35 (43.2%) couples experienced another pregnancy loss. There was no significant difference in baseline DFI between couples that experienced pregnancy loss [median 11.7, interquartile range (IQR) 9.1–17.3] and couples that achieved a live birth (median 12.5, IQR 9.3–16.5; $P = 0.971$). Improving sperm morphology increased the odds of a live birth after referral (adjusted OR 1.26, 95% CI 1.05–1.52; $P = 0.014$). DFI and sperm concentration were not associated with the outcome of the first pregnancy after referral. Overall, 35.9% of the men had DFI ≥15 at inclusion. Couples that failed to achieve pregnancy had a higher median DFI of 17.7 (IQR 7.7–27.2) compared with the rest of the cohort (median 12.0, IQR 9.3–16.5; $P = 0.041$).

Conclusions: At referral, sperm DFI, morphology and concentration cannot be used to identify RPL couples at risk of another pregnancy loss. Increased baseline DFI was associated with difficulty achieving another pregnancy, and improving sperm morphology was associated with increased odds of a live birth.

INTRODUCTION

Recurrent pregnancy loss (RPL) is defined differently between countries and reproductive societies, but is most often defined as two pregnancy losses or at least three consecutive pregnancy losses. RPL affects 2–3% of couples trying to conceive (*Bender Atik et al., 2023*; Larsen et al., 2013). Historically, it was believed that RPL was caused by non-viable fetuses (*Coomarasamy et al., 2021*; *Ogasawara et al., 2000*). At present, clinical evaluation of couples experiencing RPL focuses on the female rather than the male partner (*Bender Atik et al., 2023*; *Naglot et al., 2023*). A newly updated RPL guideline from the European Society of Human Reproduction and Embryology (ESHRE) recommends considering DNA fragmentation analysis for diagnostic purposes in evaluating RPL (*Bender Atik et al., 2023*). This represents a change in clinical practice, as the only previous recommendation on examination of the male partner was karyotyping after individual assessment (*Bender Atik et al., 2023*). The latest joint recommendation from the American Urological Association and the American Society for Reproductive Medicine from 2020 is to evaluate the male karyotype and consider DNA fragmentation testing (including sperm aneuploidy testing) in unexplained RPL (*Schlegel et al., 2020*).

Couples experiencing RPL are often able to achieve pregnancy but unable to carry the pregnancy to term. Accordingly, studies evaluating semen samples from men in couples experiencing RPL generally find normal sperm concentrations compared with fertile controls (*Bareh et al., 2016*; *Zhang et al., 2012*). Standard sperm analysis does not reflect the functional capacity and reproductive potential of the spermatozoa, and may be insufficient to detect sperm abnormalities linked to RPL. Even with sperm parameters within the normal range, the sperm cell can carry a high degree of DNA damage while retaining the ability to fertilize an oocyte. Conception by spermatozoa with a high level of DNA damage can potentially result in poor implantation or, ultimately, pregnancy loss (*Lin et al., 2008*). If the chromatin packaging is defective during spermatogenesis, sperm DNA strands are not well protected and have a higher risk of being damaged, especially when exposed to high levels of reactive oxygen species (ROS) (*Esteves et al., 2021*; *Marchesi and Feng, 2007*). This may result in single- or double-stranded DNA breaks or chromatin deformation (*Aitken et al., 2009*). DNA strand breaks may lead to impaired translation of the paternal genome, with detrimental consequences for either pregnancy outcome or the health of the children born (*Aitken et al., 2009*; *Evenson et al., 2020*; *Lewis and Kumar, 2015*; *Puri et al., 2010*; *Ribas-Maynou and Benet, 2019*). When the classical sperm parameters (concentration, morphology and motility) are not severely abnormal, morphology holds the most significant predictive value regarding fertility chances (*Coetzee et al., 1998*; *DeVilbiss et al., 2022*). However, the degree to which the sperm DNA fragmentation index (DFI) and morphology are independent remains the subject of debate. Nevertheless, if the DNA is packed incorrectly, the morphology may be abnormal and the DFI is likely to be high (*Wang et al., 2019*).

While several studies have documented that men in couples experiencing RPL have a higher level of sperm DNA fragmentation than fertile sperm donors using various assays (*Bareh et al., 2016*; *Carlini et al., 2017*; *Haddock et al., 2021*; *Kumar et al., 2012*; *Leach et al., 2015*; *Yuan et al., 2019*; *Zhu et al., 2020*; *Zidi-Jrah et al., 2016*), investigations of the association between sperm DNA fragmentation and future pregnancy outcome is warranted, as called for in systematic reviews and meta-analyses (*McQueen et al., 2019*; *Tan et al., 2019*).

A recent study on sperm DFI and reproductive outcome in the RPL population (defined as three consecutive pregnancy losses) showed no association between DFI (sperm chromatin dispersion test) and prognosis of future live birth (*Peuranpää et al., 2022*).

This study aimed to determine whether sperm DFI, morphology and concentration at referral are associated with reproductive clinical outcomes (primarily the chance of a live birth and risk of another pregnancy loss) in couples experiencing RPL.

MATERIALS AND METHODS

This study is part of the Microbiome in RPL (MiRPL) study (*Krog et al., 2022*) conducted at the tertiary RPL Unit at Copenhagen University Hospitals, Rigshospitalet and Hvidovre Hospital, Denmark. Couples were referred after three consecutive pregnancy losses (including biochemical pregnancies) or two late pregnancy losses (defined as a loss after a normal first-trimester screening). All couples were invited to participate at their first consultation between 1 April 2018 and 1 December 2019, and followed for at least 1 year after inclusion. The exclusion criteria were: female age >41 years; more than one shared child; known parental chromosomal aberrations (standard

karyotyping of the couple is performed routinely at the clinic); use of donor spermatozoa or donor oocytes; major uterine malformations; and use of antibiotics, antifungals or antivirals within the preceding 2 weeks (by either female or male partner).

All couples were informed about the study at a mandatory information meeting, and received a container for semen collection and instructions on delivering the sample to the laboratory. A few weeks before their appointment, the couples were invited again to participate, informed about the study by the study coordinator, and received written information. Ninety-five couples met the inclusion criteria, and the men provided a semen sample at the initial consultation (baseline) in the RPL unit. If the woman became pregnant and came for an early ultrasound scan (gestational age 5 + 0 to 8 + 0), the men were asked to bring a second semen sample.

Semen samples

The men were instructed to abstain from ejaculation for 2–3 days before semen collection, and to keep the semen sample at body temperature during transportation. They were asked to produce the sample a maximum of 1 h before the appointment at home or at the clinic facility. Eight men reported having fever (>38°C) during the 3 months preceding the first semen collection, and none reported fever around the time of semen collection.

After liquefication, the volume of the ejaculate was measured by pipetting, and an aliquot was stored for DNA fragmentation analysis (see below). Sperm concentration and morphology were analysed at the Department of Growth and Reproduction, Copenhagen University Hospital, Denmark. Sperm concentration was measured twice for each sample, as described previously (*Egeberg et al., 2013*; *Egeberg Palme et al., 2017*), using an image cytometer (NucleoCounter NC-3000; Chemometec, Denmark) and NucleoView software (ChemoMetec). Morphology was evaluated by trained laboratory technicians using Papanicolaou-stained smears and the World Health Organization's (WHO) 'stricter criteria', as reported previously (*Menkveld et al., 1990*; *WHO, 2021*). For DNA fragmentation analysis, 200 μl of the semen sample was diluted, mixed in 200 μl of TNE buffer (Tris-Cl-Natrium-EDTA), and frozen directly at -80°C. The DNA fragmentation index (DFI) was determined by the sperm DNA integrity test (SDI test) at SPZ Laboratory, Copenhagen, Denmark. After thawing, the samples were incubated on ice for 5 min, diluted to a concentration of 1.2 million sperm/ml, and processed according to the sperm chromatin structure assay (SCSA) protocol (*Evenson, 2013*). Samples were analysed using a FACSCalibur (BD Biosciences, USA) flow cytometer with an argon laser operating at 488 nm at 15 mW of power. Data were analysed using CellQuest (version 3.2) (BD Biosciences). Each analysis was run in replicate, and recording was stopped after 5000 events. The results were accepted if the variation (SD) between replicates was <2.5%. Seven samples showed excessive variation between replicates, likely due to debris or turbulence in the cytometer, and the measurements were repeated. After re-analysis, three of the seven samples could not be accepted. Sperm DFI was divided into three categories: <15, 15–25, and >25.

Questionnaires

The men answered an extensive questionnaire about background demographics, lifestyle factors (smoking, alcohol, diet and exercise) and their reproductive health. After each semen sample, the men answered a questionnaire regarding the period of abstinence from ejaculation, current urogenital symptoms, current illness (e.g. fever >38°C), and episodes of fever within the preceding 3 months.

Follow-up

The couples attempted to conceive after study inclusion and were followed until 1 December 2020; as such, the minimum follow-up time was 12 months (range 12–31 months).

Statistics

Continuous data are presented as median and interquartile range (IQR), and categorical data are presented as number and percentage. The Mann-Whitney *U*-test, Kruskal–Wallis test, Wilcoxon signed-rank test for paired samples, and Fisher's exact test were used to evaluate differences, as appropriate. The sperm concentration was transformed by square root and DNA fragmentation level by $\log_{10}$ to approximate a normal distribution, and compared using either one-way analysis of variance, Student's independent *t*-test or paired samples *t*-test, as appropriate.

This study investigated whether there was an association between sperm DFI, morphology and concentration and the chance of achieving pregnancy within 12 months, and whether that pregnancy resulted in pregnancy loss or live birth. Estimates of adjusted OR (aOR) with 95% CI were obtained in logistic regression models. *P*-values <0.05 were considered to indicate significance. Analyses were adjusted for paternal and maternal age, sperm concentration, primary and secondary RPL, number of previous pregnancy losses, and period of abstinence from ejaculation before collecting the semen sample. The study investigated if using a restricted cubic spline for DFI and sperm morphology improved the model. Models were compared using the log-likelihood ratio test. Restricted cubic splines can capture non-linear patterns. Potential collinearity issues were examined by calculating variance inflation factors (VIF). Collinearity can bias the results of a regression analysis. Firstly, it can inflate the standard errors of the coefficients, leading to reduced statistical power and making it challenging to ascertain the effect of each individual predictor. This is because it becomes difficult to isolate the unique contribution of each predictor, as the predictors are overlapping in the information they provide. Furthermore, in the presence of collinearity, the regression model becomes highly sensitive to changes in the model specification, such as the addition or removal of predictor variables. This can result in significant changes in the estimated coefficients, undermining the reliability of the model. Also, it can lead to counterintuitive signs of the regression coefficients. VIF >5 was considered as evidence of collinearity. The effects from restricted cubic splines were summarized using partial effects, in which all other predictor variables were set to the mean.

No formal power calculation was performed in this prospective study of pregnancy outcome in relation to sperm parameters in an RPL population.

Analyses were performed using SPSS 2017 (IBM Corp., USA), GraphPad Prism (version 9.3) and R (version 4.2). The R packages tidyverse (version 1.3.2) and rms (version 6.3) were used for illustration and statistical analysis.

Ethics

The Regional Ethics Committee (Ref. No. H-17017580, 12 September 2017) and the Data Protection Agency (Ref. No. 2012-58-

0004, RH-2017-280, I-Suite 055825, 7 September 2017) approved the study in the Capital Region of Denmark. All patients received oral and written information about the study before signing informed consent forms in accordance with the Declaration of Helsinki.

RESULTS

In total, 95 couples were included in this study, divided into three groups according to the outcome of the first pregnancy after referral; no pregnancy achieved during the follow-up period (n = 14); pregnancy loss (n = 35) (including biochemical losses); or live birth (n = 46) (FIGURE 1). Demographic and descriptive data are presented in TABLE 1. Men from couples that did not achieve pregnancy after referral were significantly older [median age 39.5 (IQR 32.8–42.3) years] compared with the rest of the cohort (pregnancy loss and live birth combined) [median age 35.0 (IQR 32.0–38.0) years; P = 0.032], whereas there was no difference in the median age of the female partner in these two groups [35.0 (IQR 32.5–37.3) years versus 34.0 (IQR 30.5–36.0) years; P = 0.372]. Also, more couples that failed to achieve pregnancy were receiving assisted reproductive technology (ART) treatment (without use of donor oocytes or donor spermatozoa) compared with couples that did achieve pregnancy and experienced pregnancy loss or live birth during follow-up (P = 0.099) (for details, see TABLE 1). Notably, no demographic differences were found between couples experiencing pregnancy loss and couples experiencing live birth.

Sperm DNA fragmentation

Overall, 35.9% (33/92) of the couples had DFI ≥15 at baseline (data available from 92 men at baseline) but had a normal sperm concentration (Supplementary Table 1). Among the men in couples that did achieve pregnancy, baseline DFI did not differ significantly between those resulting in pregnancy loss [median DFI 11.7 (IQR 9.1–17.3)] and those resulting in a live birth [median 12.5 (IQR 9.3–16.5); P = 0.971]. The semen parameters in the eight men who reported fever within the preceding 3 months were normally distributed and were not associated with reproductive outcome.

The men in couples that did not achieve pregnancy had significantly higher DFI values [median 17.7 (IQR 7.7–27.2)] compared with the rest of the cohort [median DFI 12.0 (IQR 9.3–16.5); P = 0.041]. Additionally, more than one-third of the men in couples that did not achieve pregnancy during follow-up (35.7%) had very high DFI values (>25) (TABLE 1), compared with 6.3% of the men in couples that experienced pregnancy loss and 2.2% of the men in couples that achieved a live birth. DFI correlated with sperm morphology (Pearson correlation -0.222; P = 0.039) (Supplementary Figure 1), period of abstinence from ejaculation (Pearson correlation 0.256; P = 0.018) and semen volume (Pearson correlation 0.266; P = 0.013), but did not correlate with any other sperm parameters, male age or any lifestyle parameters.

Sperm concentration and morphology

The outcome of the first pregnancy after referral was not associated with median sperm concentration at baseline. In all outcome groups, the median sperm concentration was above the lower reference limit of 15 million/ml as defined by *WHO (2021)* (TABLE 1). Men in couples that achieved a live birth in their first pregnancy after referral had a higher ejaculate volume compared with men in couples that experienced pregnancy loss or failed to achieve pregnancy (TABLE 1). Additionally, men in couples that achieved a live birth had a significantly higher percentage of sperm cells with normal morphology [median 9.8% (IQR 6.6–12.3)] compared with the rest of the cohort (i.e. pregnancy loss and no pregnancy) [median 6.8% (IQR 4.4–11.13); P = 0.027]. Sixty of

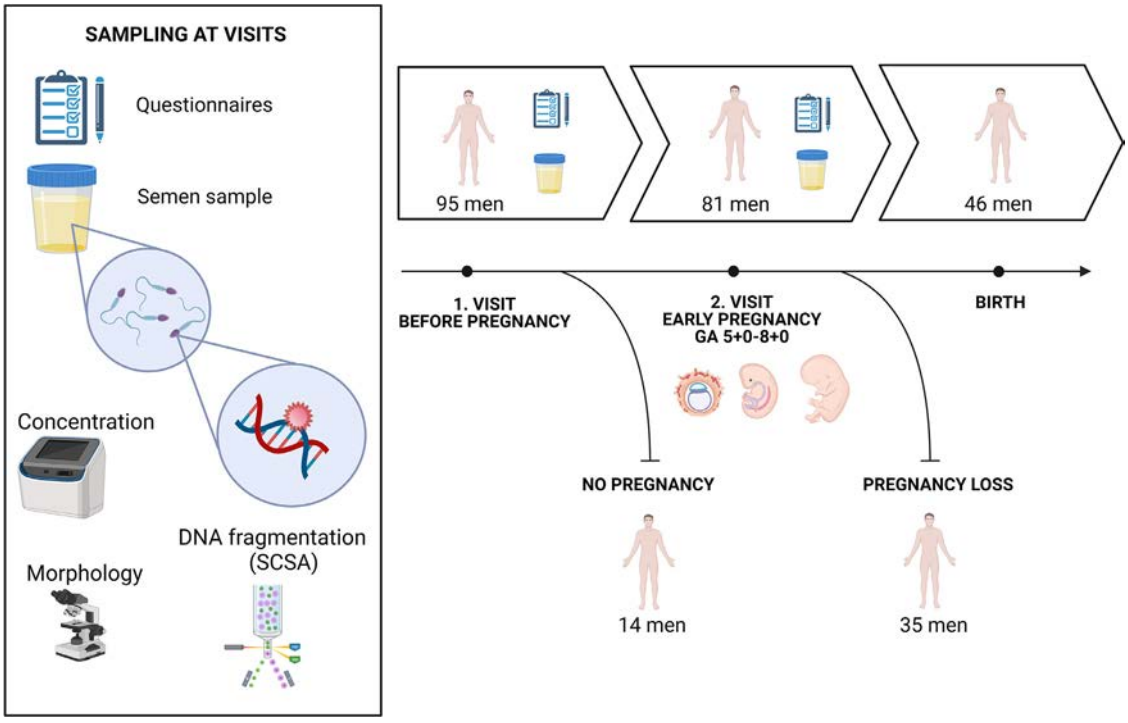

FIGURE 1 Study design: overview of visits, samples and reproductive outcome. SCSA, sperm chromatin structure assay.

TABLE 1 DEMOGRAPHIC DATA AND SPERM PARAMETERS ACCORDING TO OUTCOME OF FIRST PREGNANCY AFTER REFERRAL

	Outcome of first pregnancy after referral			
Male partner characteristic *n* = 95	**Not pregnant *n* = 14**	**Pregnancy loss *n* = 35[a]**	**Live birth *n* = 46**	***P*-value**
Age, years, median (IQR)	39.5 (32.8–42.3)	34.0 (32.0–38.0)	35.0 (32.0–39.0)	0.067[b]
BMI, kg/m^2, median (IQR)	26.1 (23.8–30.6)	25.1 (23.0–29.5)	24.3 (22.2–26.3)	0.138[b]
Higher education (university level)	35.7% (5/14)	45.7% (16/35)	50.0% (23/46)	0.670[c]
Male lifestyle				
Smoking	15.4% (2/13)	17.6% (6/34)	16.3% (7/43)	1.000[c]
Alcohol consumption, items/week				
<7	100.0% (12/12)	87.1% (27/31)	85.4% (35/41)	0.578[c]
≥7	0.0% (0/12)	12.9% (4/31)	14.6% (6/41)	
Current cannabis smoking	15.4% (2/13)	10.0% (3/30)	14.6% (6/41)	0.828[c]
Other narcotics in past 6 months	0.0% (0/13)	3.3% (1/30)	2.4% (1/41)	0.938[c]
Previous use of anabolic steroids	0.0% (0/13)	0.0% (0/30)	2.5% (1/40)	0.961[c]
Exercise				
Hard exercise several times/week	58.3% (7/12)	57.6% (19/33)	51.3% (20/39)	0.804[b]
Medium exercise ≥4 h/week	33.3% (4/12)	39.4% (13/33)	38.5% (15/39)	
No exercise	8.3% (1/12)	3.0% (1/33)	10.3% (4/39)	
Urogenital health				
Sexually transmitted diseases				
Past 3 months	7.1% (1/14)	8.6% (3/35)	0.0% (0/45)	0.104[c]
Lifetime	28.6% (4/14)	28.6% (10/35)	31.1% (14/45)	0.593[c]
Varicocele	7.7% (1/13)	3.3% (1/30)	7.5% (3/40)	0.907[c]
Cryptorchidism	0% (0/13)	12.9% (4/31)	0.0% (0/40)	0.164[c]
Surgery on reproductive organs	23.1% (3/13)	28.1% (9/32)	7.5% (3/40)	0.151[c]
Semen parameters at first consultation				
Sperm concentration (x10^6/ml), median (IQR)	64.3 (30.2–123.1)	61.2 (36.2–93.9)	67.6 (41.3–100.0)	0.676[d]
Semen volume, ml, median (IQR)	2.7 (2.3–4.2)	2.9 (2.1–3.7)	3.2 (2.9–4.6)	**0.038**[b]
Total sperm count (x10^6), median (IQR)	172.2 (113.3–405.0)	174.3 (112.9–318.3)	245.0 (143.0–398.0)	0.118[d]
Normal sperm morphology, %, median (IQR)	8.0 (2.8–12.0)	6.5 (4.8–10.8)	9.8 (6.6–12.3)	0.082[e]
Sperm DFI, median (IQR)	17.7 (7.7–27.2)	11.7 (9.1–17.3)	12.5 (9.3–16.5)	0.125[f]
Period of abstinence from ejaculation, days, median (IQR)	4.0 (2.25–6.0)	3.0 (2.8–4.0)	3.0 (3.0–5.0)	0.405[b]
Sperm DFI				
<15	35.7% (5/14)	71.9% (23/32)	67.4% (31/46)	**0.008**[b]
15–25	28.6% (4/14)	21.9% (7/32)	30.4% (14/46)	
>25	35.7% (5/14)	6.3% (2/32)	2.2% (1/46)	
Sperm morphology (WHO 5th centile)				
<4% normal	28.6% (4/14)	14.3% (5/35)	8.7% (4/46)	0.133[b]
≥4% normal	71.4% (10/14)	85.7% (30/35)	91.3% (42/46)	
Sperm concentration, million/ml (WHO 5th centile)				
<15	14.3% (2/14)	14.3% (5/35)	0% (0/46)	**0.012**[b]
≥15	85.7% (12/14)	85.7% (30/35)	100% (46/46)	
Female partner *n* = 95				
Age, years, median (IQR)	35.0 (32.5–37.3)	34.0 (30.0–36.0)	34.0 (31.0–36.0)	0.969[b]
Risk factors for RPL				
Thyroid disease	0% (0/14)	2.9% (1/35)	4.3% (2/46)	1.000[b]

(*continued on next page*)

TABLE 1 (*Continued*)

	Outcome of first pregnancy after referral			
Male partner characteristic *n* = 95	**Not pregnant *n* = 14**	**Pregnancy loss *n* = 35[a]**	**Live birth *n* = 46**	***P*-value**
Lupus positive	7.1% (1/14)	0.0% (0/33)	4.3% (2/46)	
Reproductive demographics				
Primary RPL	16.7% (10/60)	35.0% (21/60)	48.3% (29/60)	0.772[b]
Secondary RPL	11.4% (4/35)	40.0% (14/35)	48.6% (17/35)	
Number of previous pregnancy losses at referral, median (range)	4 (2–11)	3 (3–7)	3 (2–6)	0.067[b]
3	42.9% (6/14)	71.4% (25/35)	69.6% (32/46)	0.175[b]
≥4	57.1% (8/14)	31.4% (11/35)	30.4% (14/46)	
Time to positive pregnancy test, days, median (IQR)	NA	97.0 (38.0–152.0)	89.5 (36.3–159.3)	0.474[b]
Fertility treatment after referral[g]	57.1% (8/14)	37.1% (13/35)	26.1% (12/46)	0.099[b]
Insemination with partner	7.1% (1/14)	5.7% (2/35)	15.2% (7/46)	**0.013**[c]
IVF with partner	42.9% (6/14)	31.4% (11/35)	8.7% (4/46)	
ICSI with partner	7.1% (1/14)	0.0% (0/46)	2.2% (1/46)	

Data are number and percentage unless otherwise indicated. The denominator indicates the number of individuals who responded to the question.

P-values <0.05 are indicated in bold text.

[a] One couple experienced a still birth and the pregnancy outcome was categorized as pregnancy loss.

[b] Kruskal–Wallis test.

[c] Fisher's exact test.

[d] The difference between distributions was analysed with one-way ANOVA on square root transformed data.

[e] One-way ANOVA.

[f] The difference between distributions was analysed with one-way ANOVA on $\log_{10}$ transformed data.

[g] No use of donor spermatozoa or donor oocytes.

BMI, body mass index; IQR, interquartile range; DFI, DNA fragmentation index; WHO, World Health Organization; RPL, recurrent pregnancy loss; ICSI, intracytoplasmic sperm injection; ANOVA, analysis of variance.

the 81 men in couples that achieved pregnancy provided a second semen sample in early pregnancy. There was no significant variability when comparing sperm parameters between the pre-pregnancy visit and the visit in early pregnancy (Supplementary Table 2).

Achieving pregnancy and outcome of first pregnancy after referral

The cumulative percentage of couples that achieved pregnancy according to the baseline sperm parameters is depicted in FIGURE 2. A model that included a restricted cubic spline term for the DFI fit significantly better than a linear term ($P = 0.02$). Summarizing the effect from the spline as a partial effect, there were increased odds of achieving pregnancy for DFI values <27 (FIGURE 3a). Comparing a DFI of 9 with a DFI of 15, there was no change in the odds of achieving pregnancy within 12 months (aOR 1.41, 95% CI 0.48–4.18; $P = 0.52$). However, if the DFI increased to 30, there was a reduced chance of achieving pregnancy (aOR 0.05, 95% CI 0.005–0.51; $P = 0.01$) (FIGURE 3b). Neither sperm morphology nor sperm concentration affected the chance of achieving pregnancy ($P = 0.87$ and 0.81, respectively).

For the outcome of the first pregnancy after referral, improved sperm morphology significantly increased the odds of achieving a live birth (aOR 1.26, 95% CI 1.05–1.52; $P = 0.014$) (FIGURE 3c). A model with a restricted cubic spline for sperm morphology did not improve the model ($P = 0.79$). DFI (aOR 1.06, 95% CI 0.95–1.17; $P = 0.28$) and sperm concentration ($P = 0.99$) were not associated with the outcome of the first pregnancy after referral. There were no indications of collinearity in any models (all VIF <3).

Additional follow-up

After the follow-up period (minimum 12 months), 14.7% (14/95) of couples never achieved pregnancy, 72.6% (69/95) achieved a live birth, and 12.6% (12/95)

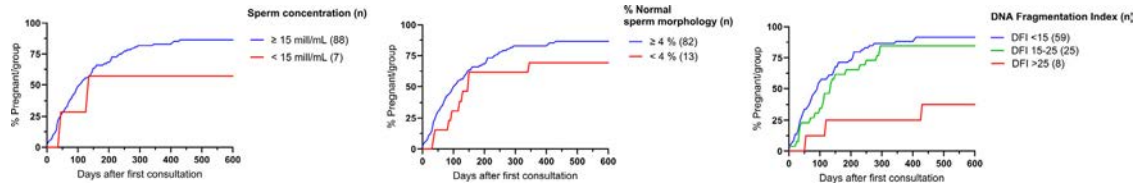

FIGURE 2 Cumulative pregnancy rate plotted against sperm concentration (left), percentage of normal sperm morphology (centre) and DNA fragmentation index (DFI) (right).

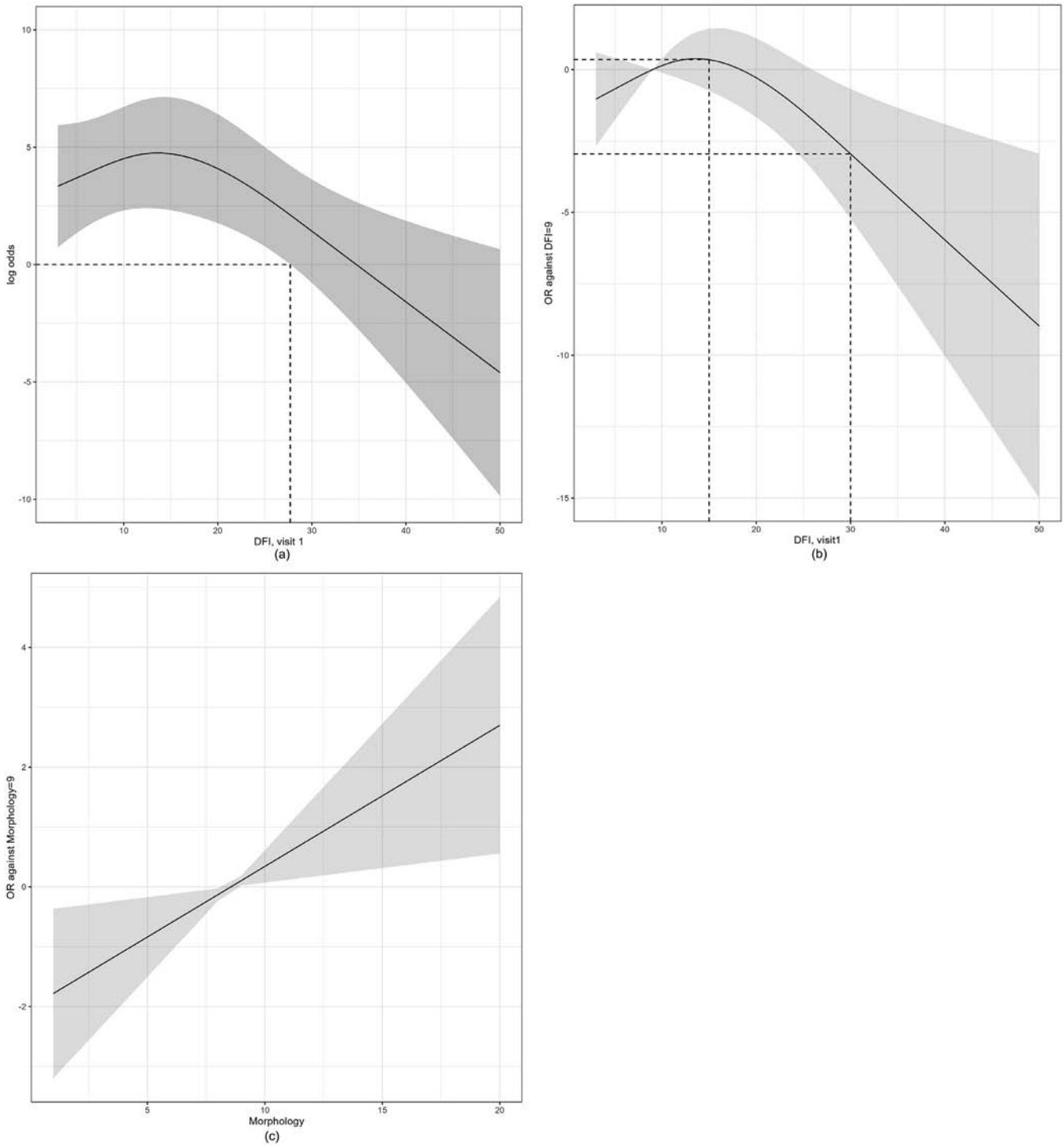

FIGURE 3 Odds of achieving pregnancy within 12 months according to baseline DNA fragmentation index (DFI) (a); baseline DFI compared with DFI of 9 (b); and odds of pregnancy resulting in live birth within 12 months with improved sperm morphology compared with reference sperm morphology of 9 (c).

experienced pregnancy loss(es), with one couple experiencing a stillbirth. In this study, 42.1% (40/95) of couples achieved pregnancy after 3 months, 66.3% (63/95) after 6 months, 78.9% (75/95) after 9 months, 82.1% (78/95) after 12 months, and 85.3% (81/95) at the end of follow-up. Men in couples that failed to achieve pregnancy after 9 months from inclusion had a significantly higher baseline DFI measured at first consultation compared with men in couples that achieved pregnancy [median DFI 16.7 (IQR 8.4–26.8) versus 11.9 (IQR 9.2–16.3); $P = 0.014$], but with no significant difference in sperm morphology [median percentage of normal sperm 9.0% (IQR 3–13) versus 9.0% (IQR 5.6–11.5); $P = 0.759$] or sperm concentration [median 64.8 mill/ml (IQR 45–131.8)

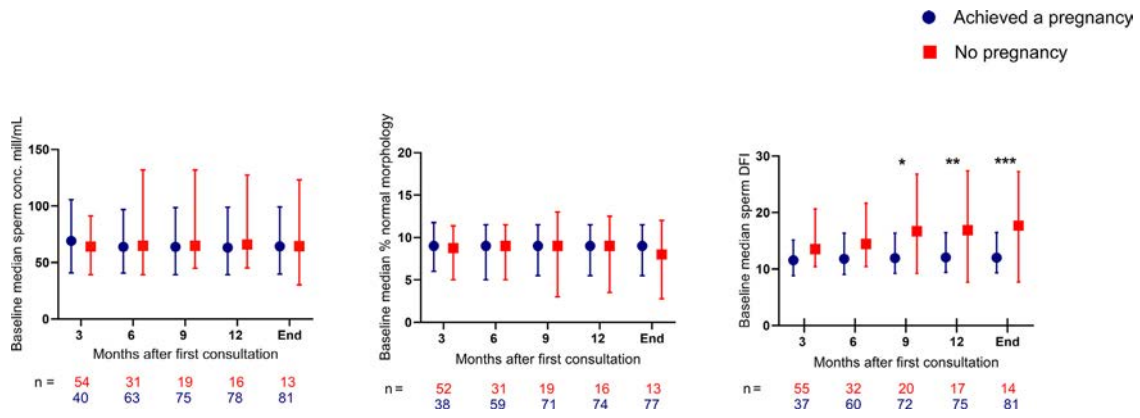

FIGURE 4 Cumulative numbers of couples that achieved pregnancy and did not achieve pregnancy, irrespective of outcome, at different time points after referral plotted against baseline median sperm concentration (left), baseline median percentage of normal sperm morphology (centre), and baseline median DNA fragmentation index (DFI) (right). *P = 0.014, **P = 0.023 ***P = 0.041. Error bars indicate interquartile range.

versus 64.0 mill/ml (IQR 39.5–98.2); P = 0.697] (FIGURE 4).

DISCUSSION

Men with high sperm DFI, who were referred for RPL with their partners, had no increased risk of experiencing another pregnancy loss, corroborating recent findings (*Coomarasamy et al., 2021*; *Ogasawara et al., 2000*; *Peuranpää et al., 2022*). At present, clinical evaluation of couples experiencing RPL focuses on the female rather than the male partner (*Bender Atik et al., 2023*; *Naglot et al., 2023*). A newly updated RPL guideline from ESHRE recommends considering DNA fragmentation analysis for diagnostic purposes in evaluating RPL (*Bender Atik et al., 2023*), which represents a change in clinical practice. These findings in the RPL population differ from those in the infertile population, including patients with high DFI following IVF/intracytoplasmic sperm injection treatment having a higher risk of pregnancy loss (*Zhao et al., 2014*). The present study of couples experiencing RPL found a high prevalence of increased DFI, with 35.9% of the men having DFI ≥15 at referral; this finding is in accordance with previous studies of the RPL population (*Kumar et al., 2012*; *Leach et al., 2015*; *Peuranpää et al., 2022*; *Yuan et al., 2019*). A novel finding in this study was that men in couples that did not achieve pregnancy during follow-up had higher DFI values compared with men in couples that did achieve pregnancy. This raises the clinical question of why men who have previously fathered pregnancy losses have difficulties achieving pregnancy. It is unknown whether these couples also have increased risk of pregnancy loss if they succeed in becoming pregnant after ART treatment. The main findings of this study may imply that a high DFI does not play a role in the pathogenesis of most pregnancy losses in RPL couples that are able to conceive.

Zhang et al. (2012) investigated whether sperm concentration and morphology are associated with prospective pregnancy outcomes in the RPL population in couples with two and three pregnancy losses. Couples that experienced another pregnancy loss were found to have significantly poorer sperm morphology, and couples that did not achieve pregnancy had a lower sperm concentration in comparison with fertile controls. This finding is in contrast to another study of patients experiencing RPL, selected retrospectively from a larger group of patients experiencing RPL, which found no association between sperm morphology and future pregnancy loss (*Sbracia et al., 1996*), and also found that men in infertile couples experiencing RPL had lower sperm concentration and motility, and higher abnormal sperm morphology compared with men in other couples experiencing RPL. The present study found that men in couples that achieved a live birth had a higher proportion of sperm with normal morphology and a higher ejaculate volume. However, poor sperm morphology was not associated with the risk of another pregnancy loss or not achieving pregnancy. In addition, the present study found no association between sperm concentration and reproductive outcome. There is a need for future, larger studies to determine whether sperm DNA fragmentation and morphology are risk factors for future pregnancy loss in couples experiencing RPL, or if the current negative results are due to a lack of statistical power.

Several paternal factors, including age, have been associated with high levels of sperm DNA fragmentation in the literature (*Esteves et al., 2021*; *Lewis and Kumar, 2015*). The present study found that men in couples that failed to achieve pregnancy during follow-up were significantly older than men in couples that achieved pregnancy, and it has been suggested that increasing male age impairs spermatogenesis with increasing double-stranded breaks (*Murugesu et al., 2022*; *Singh et al., 2003*). This study found no direct correlation between increasing male age and DFI, which contrasts with previous studies (*Evenson, 2013*; *Gonzalez et al., 2022*). This is surprising, and could be because the SDI test does not quantify DNA strand breaks directly, but rather represents an indirect measure of chromatin stability.

Varicocele is documented to increase the production of reactive oxygen species (ROS) in the testes, and a recent systematic review showed that a varicocele repair decreased DFI (*Roque and Esteves, 2018*). Only five men were reported to have a varicocele in the present study, although some cases may have been undiagnosed. A recent clinical practice guideline strongly recommends referring men with a high DFI for complete male evaluation by a reproductive urologist/andrologist to identify and possibly treat male factors (*Esteves et al., 2021*). Apart from varicocele repair, several potential treatments to reduce ROS production in semen have been suggested, such as antioxidant supplementation, the effectiveness of which remains to be

proven (*Chen et al., 2013*; *Gharagozloo and Aitken, 2011*; *Stenqvist et al., 2018*).

One of the aims of this study was to identify couples at risk of another pregnancy loss. Based on the present findings, it may be reasonable to consider semen analysis as a screening tool at first consultation or after 9 months without achieving pregnancy to identify an unrecognized male factor and refer the patient to an andrologist. This study conveys a novel awareness that a group of patients experiencing RPL, despite previous pregnancies, cannot even achieve pregnancy. These patients are as reproductively challenged as those couples that experience another pregnancy loss, with neither group having a live birth. This knowledge warrants careful consideration as to whether a more progressive clinical approach could be favourable to identify those couples that do not achieve pregnancy after referral to an RPL unit.

The SCSA is one of the most commonly used assays for evaluating DNA fragmentation. It is a robust test based on flow cytometry, with high intra- and interassay precision and low bias (*Evenson, 2013*). However, the assay does not measure DNA strand breaks directly, and instead measures the susceptibility of the DNA to acid denaturation. The SCSA cannot discriminate between single- and double-stranded DNA breaks. Other assays, such as the Comet and TUNEL assays, measure DNA breaks more directly (*Henkel et al., 2010*; *Ribas-Maynou and Benet, 2019*). It has been speculated whether single- and double-stranded breaks have different repercussions, and some researchers have suggested that double-stranded breaks could be causative in pregnancy loss (*Ribas-Maynou and Benet, 2019*). Thus, differences in single- versus double-stranded breaks could blur the findings of the present study, which is a limitation. Another limitation is not knowing the ploidy status of the lost fetuses. In future studies, grouping the patients by conception method should be considered, if the study design allows.

In conclusion, this study found high prevalence of increased DFI measured by SCSA in men in couples experiencing RPL, but high DFI was not associated with increased risk of another pregnancy loss. Instead, high DFI significantly increased the likelihood that an RPL couple did not conceive after referral. The men in these couples often had sperm concentration and morphology within the normal range, which suggests an unrecognized male factor contributing to reproductive failure.

DATA AVAILABILITY

Data will be made available on request.

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AUTHOR CONTRIBUTIONS

MCK, HSN and KA conceptualized and designed this study. MCK, JRN, AS and KVH included patients, collected samples and performed semen analyses. MCK analysed the data and wrote the first draft of the manuscript. DW is a bioinformatician and aided with statistical analyses of the data. HSN, AMK and SB collected samples and critically revised the manuscript. All authors critically discussed and revised the manuscript, and all authors approved the submitted version.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2023.103773.

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RBMO

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ARTICLE

Successful live birth in women with partial 17α-hydroxylase deficiency: report of two cases

BIOGRAPHY

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KEY MESSAGE

Partial 17α-hydroxylase deficiency is a rare type of congenital adrenal hyperplasia. Information regarding infertility care and conception in women with this disorder is extremely limited. This study provides evidence that such patients can conceive naturally.

ABSTRACT

Research question: Can women with partial 17α-hydroxylase deficiency (17-OHD) conceive naturally with adequate hormonal control and endometrial preparation?

Design: This report presents two cases of women with partial 17-OHD who achieved successful pregnancies. The first case involved a 27-year-old Chinese woman with recurrent cysts and infertility, and the second case involved a 32-year-old Chinese woman with a complex disorder requiring IVF. Both cases were treated with oral prednisone to control hormone concentrations and underwent endometrial preparation.

Results: In the first case, the patient resumed spontaneous ovulation, conceived naturally, and gave birth to a healthy baby. In the second case, after cryopreserving embryos due to a thin endometrium, the patient underwent frozen embryo transfer and achieved a singleton pregnancy.

Conclusion: This study suggests that women with partial 17-OHD can conceive naturally with appropriate hormonal management and endometrial preparation. These findings provide valuable insights into the reproductive potential of women with this disorder, and highlight the importance of further research in this area.

INTRODUCTION

Congenital adrenal hyperplasia (CAH) refers to a series of autosomal-recessive disorders in which patients are deficient in enzymes that mediate the synthesis of steroid hormones. Affecting approximately one in 50,000–100,000 live births, 17α-hydroxylase deficiency (17-OHD) is rare and comprises approximately 1% of cases of CAH (*Costa-Santos et al., 2004*; *Turcu and Auchus, 2015*). This condition results from mutations in the *CYP17A1* gene that encodes 17α-hydroxylase, and ultimately results in impaired synthesis of gonadal steroids, adrenal androgens and cortisol (*Bose et al., 1996*; *Speiser and White, 2003*). Several *CYP17A1* mutations have been linked to reductions in the function of this enzyme, and associated clinical consequences thereof.

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KEY WORDS

Congenital adrenal hyperplasia
Partial 17α-hydroxylase deficiency
CYP17A1
Infertility
Natural pregnancy

In 46,XX patients, complete 17-OHD results in symptoms including hypokalaemia, hypertension, primary amenorrhoea and the lack of secondary sexual characteristics. Relative to complete 17-OHD, partial disruption of the function of this enzyme is far rarer (*Tian et al., 2008*). Adolescent girls usually seek help for delayed puberty and primary amenorrhoea (*Beştaş et al., 2022*), and women of reproductive age often suffer from infertility. Some females diagnosed with partial 17-OHD may not exhibit the development of certain secondary sexual characteristics, such as pubarche, but others experience spontaneous menarche and regular menstrual cycles (*Bolu et al., 2020*; *Wu et al., 2017*).

As these patients generally suffer from poor endometrial growth and ovarian insufficiency, there have been few reports describing fertility treatment outcomes in females with 17-OHD. To date, only four cases of successful pregnancy have been reported in patients with 17-OHD (*Ben-Nun et al., 1995*; *Bianchi et al., 2016*; *Falhammar, 2018*; *Kitajima et al., 2018*), all of which were achieved through IVF/frozen embryo transer. To the authors' knowledge, no patients with 17-OHD have achieved pregnancy through natural conception.

This paper describes the clinical findings, laboratory test results, sequencing outcomes and fertility treatment efforts for two females diagnosed with partial 17-OHD, with the goal of providing valuable insights to aid the diagnosis and treatment of this disease.

This study was approved by the Medical Ethics Committee of the Third Affiliated Hospital of Zhengzhou University (Reference No. 2024-002-01, 17 January 2024). Written informed consent was obtained from individuals for the publication of any data included in this article.

CASES

Case 1

A 27-year-old female initially presented to the study department in 2019 for recurrent cysts and the inability to become pregnant over a 5-year period. She had experienced menarche at 16 years of age, followed by irregular menstrual cycles. Without any further investigations, she was started on oral contraceptives, masking her symptoms. A salpingogram was performed, which revealed that both fallopian tubes were patent. Ultrasound-based monitoring of follicular development and measurement of hormone concentrations revealed ovulation disorder and an unusual rise in progesterone to 19 nmol/l prior to ovulation, prompting further investigation. Her height was 158 cm, weight was 61 kg, body mass index (BMI) was 24.4 kg/m^2 and arterial blood pressure was 115/69 mmHg. Physical examination showed Tanner stage IV–V breast development, sparse pubic hair, a feminine vulva and a normal clitoris. Vaginal examination revealed a slightly smaller than average uterus, ovarian follicular cysts, and no palpable lumps in the labia or groin. Given the patient's history and presentation, hormone concentrations were examined, with chemiluminescent immunoassays used to measure concentrations of FSH, LH, oestradiol, progesterone, testosterone, pituitary prolactin, dehydroepiandrosterone sulfate (DHEA), androstenedione and 24-h urinary free cortisol. In addition, radioimmunoassays were used to measure plasma renin activity, aldosterone, adrenocorticotropic hormone (ACTH), cortisol, 17α-hydroxyprogesterone (17-OHP), and 24-h urinary adrenaline and norepinephrine. These analyses revealed abnormally decreased concentrations of androstenedione, testosterone, 17-OHP and cortisol, together with elevated concentrations of progesterone and prolactin. No abnormalities in the concentrations of FSH; LH; oestradiol; DHEA; and 24-h urinary free cortisol, aldosterone, adrenaline, norepinephrine and dopamine were detected. The results are presented in TABLE 1. No abnormalities were detected on pelvic ultrasound, and left adrenal hyperplasia was detected via adrenal computed tomography imaging. The patient's karyotype was 46,XX, and genetic diagnosis revealed the presence of the compound heterozygous missense variants c.887T>C(p.I296T) and c.1304T>C(p.F435S) in the *CYP17A1* gene, consistent with a potential diagnosis of CAH as a consequence of partial 17-OHD. Her partner was also investigated and was found to have normal semen analysis. After learning about the condition, she wanted to conceive naturally. In preparation for conception, prednisone 5 mg was administered in the morning and 2.5 mg in the evening, aiming to reduce the concentrations of ACTH and progesterone. She resumed regular menstruation and normal follicular development 2 months after the prednisone dose was up-titrated. On the fourth day of her menstrual cycle, she began follicular monitoring. On day 13, she had a dominant follicle in the right ovary, followed by further enlargement of the follicle. On day 18, she had a mature follicle, and on day 19, the mature follicle had collapsed, consistent with ovulation (TABLE 2). Intercourse was performed during the periovulation period. The patient ultimately achieved a natural pregnancy, giving birth to a live female newborn (3250 g, 47 cm in length) at a gestational age of 39 weeks and 4 days.

Case 2

In 2019, a 32-year-old woman (46,XX) presented to the study hospital due to menstrual disorders and the inability to conceive. This patient initiated puberty spontaneously and then developed oligomenorrhoea. She was treated intermittently with oestrogen–progesterone. Her height was 157 cm, weight was 58 kg, BMI was 23.5 kg/m^2 and arterial blood pressure was 127/81 mmHg. She exhibited relatively sparse axillary and pubic hair for her age, but gynaecological examinations did not reveal any abnormalities of the vagina or external genitalia. Enlarged polycystic ovaries were detected through transvaginal ultrasonography. As salpinography revealed obstruction of the bilateral fallopian tubes, the patient was referred to undergo IVF as a form of assisted reproductive technology (ART). She underwent standard ovarian stimulation with a down-regulation protocol using a gonadotrophin-releasing hormone agonist. Serum hormone concentrations and transvaginal ultrasonography were used to monitor the development of ovarian follicles. The patient continued to receive daily injections of recombinant FSH (Gonal-F; Merck Serono, Switzerland), human menopausal gonadotrophin (Menopur; Ferring Pharmaceuticals, Switzerland) and/or recombinant LH (Luveris; Merck Serono Australia, Australia), based on follicular development, until at least three follicles >18 mm in diameter were evident, at which time she was administered 250 mg of recombinant human chorionic gonadotrophin (Ovidrel; Merck Serono). Transvaginal oocyte retrieval was conducted 36 h post-injection. The hormone findings in the context of this ART treatment regimen are shown in TABLE 3. This approach led to the retrieval of 17 oocytes, of which 14 were fertilized successfully, with two high-quality

TABLE 1 CLINICAL CHARACTERISTICS AND HORMONAL PROFILES FOR THE TWO CASE PATIENTS WITH PARTIAL 17α-HYDROXYLASE DEFICIENCY

Parameter	Case 1	Case 2	Normal range
Age (years)	27	32	
Blood pressure (mmHg)	115/69	127/81	
FSH (IU/l)	6.54	4.69	3.5–12.5
LH (IU/l)	9.69	4.02	2.4–12.6
Oestradiol (pmol/l)	224.8	408.2	45.4–854.8
Progesterone (nmol/l)	19.18	16.74	0.18–2.84
Testosterone (nmol/l)	<0.09	<0.09	0.29–1.67
Prolactin (mIU/l)	1272	741.4	102–636
17-OHP (ng/ml)	0.4	0.33	0.4–1.02
DHEA (μg/dl)	47.9	25.7	35–430
Androstenedione (ng/ml)	<0.3	<0.3	0.3–3.3
Cortisol (8 am) (ng/ml)	85.1	132	171–536
Cortisol (4 pm) (ng/ml)	61.3	90.7	64–327
ACTH (8 am) (pg/ml)	141	76.5	7.2–63.3
ACTH (4 pm) (pg/ml)	56.3	10.4	4–32
PRA (lying) (ng/ml.h)	/	0.34	1.5–6.9
PRA (standing) (ng/ml.h)	/	2.46	0.2–1.9
Angiotensin II (lying) (pg/ml)	/	46.3	28.2–52.2
Angiotensin II (standing) (pg/ml)	/	56.46	55.3–115.3
Aldosterone (lying) (pmol/l)	/	129.30	28–138
Aldosterone (standing) (pmol/l)	/	159.90	138–415
24-h U-aldosterone (μg/day)	1.8	2.4	1–8
24-h U-FC (nmol/day)	200	226	73–372
24-h U-NRE (μg/day)	42	34	0–50
24-h U-AD (μg/day)	13	10	0–20
24-h U-DA (μg/day)	304	240	0–500
24-h urine volume (l)		1.63	
24-h U-K (mmol/24 h)		38.72	25–100
24-h U-Cl (mmol/24 h)		129.08	110–250
24-h U-Ca (mmol/24 h)		2.22	2.7–7.5
24-h U-Na (mmol/24 h)		130.88	130–260
24-h U-P (mmol/24 h)		13.30	9.7–42

17-OHP, 17-hydroxyprogesterone; DHEA, dehydroepiandrosterone sulfate; ACTH, adrenocorticotrophic hormone; PRA, plasma renin activity; U-aldosterone, urinary aldosterone; U-FC, urinary free cortisol; U-NRE, urinary norepinephrine; U-AD, urinary adrenaline; U-DA, urinary dopamine; U-K, urinary potassium; U-Cl, urinary chloride; U-Ca, urinary calcium; U-Na, urinary sodium; U-P, urinary phosphorus.

cleavage-stage embryos undergoing cryopreservation. The remaining embryos were cultured to blastocyst stage, yielding five blastocytes. Persistent increases in serum progesterone concentration were observed throughout the process of ovarian stimulation (11.99–32.19 nmol/l). An abnormally high concentration of progesterone has been associated previously with a poorer implantation rate; as a result, all embryos were cryopreserved, and the patient underwent further diagnosis and treatment in the Endocrinology Department for diagnosis and treatment. Hormone tests revealed reduced concentrations of testosterone, 17-OHP, androstenedione, cortisol and DHEA, and elevated concentrations of progesterone and prolactin. No abnormalities in the concentrations of FSH; LH; oestradiol; ACTH; and 24-h urinary free cortisol, aldosterone, adrenaline, norepinephrine and dopamine were detected. The results are presented in TABLE 1, together with specific results for biochemical parameters, including 24-h urine output and urinary potassium, calcium, chloride, sodium and phosphorus. Genetic analysis revealed the presence of the compound heterozygous c.1396G>A(p.E466K) and c.1459-1467del (p.Asp487-Phe489del) variants in the *CYP17A1* gene. The former was identified for the first time, and predicted to be pathogenic by both Mutation Taster and PolyPhen-2 analyses, while the latter has been confirmed to be pathogenic in prior literature reports (*Jiang et al., 2022*). These genetic results were consistent with the diagnosis of CAH as a result of partial 17-OHD. Following definitive diagnosis, adrenal progesterone production was suppressed via the administration of oral prednisone (2.5 mg in the morning and 5 mg in the evening), with the serum progesterone concentration decreasing to 3.34 nmol/l after 3 months. Endometrial preparation was then initiated using oral oestradiol valerate 6 mg/day (Progynova; Delpharm, France). When transvaginal ultrasonography on day 19 of the preparation process revealed that the endometrium had achieved a thickness of 7 mm, luteal phase support was added with daily vaginal progesterone gel 90 mg (Crinone; Merck Serono) and twice-daily oral dydrogesterone 10 mg (Duphaston; Abbott, Netherlands). The clinical data during hormone replacement therapy are shown in TABLE 4. Five days later, a cryopreserved blastocyst was transferred into the uterus. On day 14 post-transfer, positive β-human chorionic gonadotrophin test results were obtained, with transvaginal ultrasonography confirming a singleton pregnancy at the gestational age of 6 weeks. The patient ultimately gave birth to a normal female newborn (3400 g, 45 cm in length) via caesarean section at a gestational age of 40 weeks and 3 days. The puerperium was uneventful, and the newborn was discharged home in good condition.

DISCUSSION

Clinical characteristics

At present, 17-OHD remains a rare subtype of CAH (*Fernández-Cancio et al., 2017*). The *CYP17A1* enzyme complex exhibits 17,20-lyase activity and 17-hydoxylase catalytic activity such that mutations in *CYP17A1* can result in the loss of such activity. The actual degree of altered

TABLE 2 TRANSVAGINAL SONOGRAPHIC FINDINGS, SERUM HORMONE CONCENTRATIONS AND TREATMENTS PERFORMED IN CASE 1

Menstrual cycle (day)	Follicle size (mm)		Hormone			Treatment
	Right	Left	LH (IU/l)	Oestradiol (pmol/l)	Progesterone (nmol/l)	
4	7	5				
13	11	7			2.19	
16	14	7				
17	16	7				
18	18	7				Intercourse
19	—	—	19.63	272.8	6.94	Intercourse

Reference ranges: LH (IU/l): fp 2.4–12.6, mc 14–95.6, lp 1.0–11.4; oestradiol (pmol/l): fp 45.4–854.8, mc 151–1461, lp 81.9–1251; progesterone (nmol/l): fp 0.18–2.84, mc 0.38–38.1, lp 5.82–75.9.

fp, follicular phase; mc, mid-cycle; lp, luteal phase (in premenopausal women).

enzymatic activity varies based on the particular location and type of mutation within the *CYP17A1* gene, resulting in different clinical manifestations in affected patients (*Kim et al., 2014*). To date, most descriptions of this disease pertain to cases of complete 17-OHD, whereas descriptions of cases of partial 17-OHD remain relatively rare. As partial 17-OHD has certain degrees of oestrogenic and androgenic functions, this disease is more difficult to diagnose. Partial 17-OHD can exhibit an insidious clinical course, as exemplified in the two cases presented herein in which breasts and pubic hair developed naturally to varying degrees. The absence of pubic hair can serve as an indication of adrenal involvement, providing a direction for the diagnosis of the disease. The primary clinical findings associated with this condition include menstrual disorders and oligomenorrhoea (*Carvalho et al., 2016*; *Wu et al., 2017*; *Xu et al., 2017*). Ultrasonographic evaluation of patients with partial 17-OHD revealed bilateral polycystic ovarian changes, which are likely to be misdiagnosed as polycystic ovary syndrome. Ultrasonography has also shown recurrent multiple ovarian cysts in some patients. Additionally, not all patients with partial 17-OHD have hypertension and hypokalaemia (*Van Den Akker et al., 2002*; *Wu et al., 2017*).

The diagnosis of partial 17-OHD presents certain challenges and requires differentiation from other types of CAH. Among them, 21-hydroxylase deficiency (21-OHD) is the most common, accounting for 90–95% of cases of CAH. 21-OHD can lead to a series of clinical manifestations, such as adrenal hyperplasia and clinical symptoms related to high androgens. In addition, it is also necessary to distinguish between 46,XX patients with 17-OHD and 46,XY patients with 17-OHD. Although the external genitalia of both are female, 46,XX patients have a uterus and ovaries, while 46,XY patients do not. In 46, XX patients, the original follicles in the ovaries repeatedly appear like luteinized cysts under high gonadotrophin concentrations, and sometimes misdiagnosed as ovarian tumours. In addition, the increase in FSH and multiple ovarian cysts in patients need to be differentiated from pituitary FSH tumours. Furthermore, this disease needs to be differentiated from ovarian insufficiency. Patients with a diminished ovarian reserve will show increased concentrations of FSH and LH, a decreased number of follicles, and a normal concentration of progesterone, and will not have hypertension, hypokalaemia or other metabolic disorders. In summary, the diagnosis of partial 17-OHD needs to

TABLE 3 OVARIAN STIMULATION FOR IVF AND HORMONE CHANGES DETECTED IN CASE 2

Cycle day	3	33	37	40	42	44	45
Prednisone (mg/day)	7.5						
Long-acting triptorelin (mg)	3.75	/	/	/	/	/	/
rFSH (IU/day)		150	150	150	150	75	
HMG (IU/day)			37.5	75	75	150	
rLH (IU/day)				75	75	75	
rHCG (μg)							250
HCG (IU)							2000
Follicles (mm), (*n*)	5 (3), 4 (4), 3 (14)	5 (2), 4 (2), 3 (8), 2 (10)	8 (2), 7 (3), 6 (6), 5 (6)	12 (2), 10 (5), 9 (2), 7 (7)	15 (4), 14 (4), 12 (5),10 (2)	18 (5), 16 (4), 14 (5), 12 (1)	21 (2), 19 (2), 16 (5), 14 (6), 12 (5)
Endometrial thickness (mm)	4.9	3	3.1	5.1	5.3	6	6.3
FSH (IU/l)	4.96	0.89					
LH (IU/l)	4.02	2.59		<0.1	1.11	1.54	1.5
Oestradiol (pmol/l)	408.2	18.70	106.7	205.8	761.4	1817	2361
Progesterone (nmol/l)	16.74	18.36			11.99	32.19	19.78

rFSH, recombinant FSH; HMG, human menopausal gonadotrophin; rHCG, recombinant human chorionic gonadotrophin; rLH, recombinant LH.

TABLE 4 CLINICAL DATA DURING HORMONE REPLACEMENT THERAPY FOR CASE 2

Cycle day	3	10	15	18	21	22
Endometrial thickness (mm)	5	5.6	6.2	6.5	6.5	7
Follicles (mm)	4	4	4	5	5	5
Oestradiol (pmol/l)	146.1	/	651.1	/	963.1	/
Progesterone (nmol/l)	3.34	4.42	0.66	/	1.47	/
Medication						
Ethinyl oestradiol (mg/day)	6	6	6	6	6	6
Natural micronized progesterone (mg/day)	/	/	/	/	/	600
Prednisone (mg/day)	7.5	7.5	7.5	7.5	7.5	7.5

consider clinical manifestations, changes in hormone concentations and genetic factors in order to avoid misdiagnosis, and ensure that patients receive appropriate treatment and management.

Molecular mechanism of pathogenesis

Both cases discussed in this report carried compound mutations in the *CYP17A1* gene. These mutations (p.I296T, p.F435S, p.E466K and p.Asp487_Phe489del) can result in total or partial disruption of enzymatic activity, contributing to the phenotypic outcomes in these patients. Of these mutations, p.Asp487_Phe489del is the most frequently reported pathogenic variant in Chinese patients with 17-OHD, with an estimated carrier rate of one in 10,000, and it has been found to be homozygous or compound heterozygous in many patients, leading to 17-OHD (*Qiao et al., 2010*). High frequencies of p.Asp487_Phe489del mutation associated with a founder effect have also been reported in China (*Zhou et al., 2012*). In-vitro functional tests have shown that the cells transfected with this variant do not have 17α-hydroxylase activity or 17,20-lyase activity. In addition, the present study identified the p.E466K mutation for the first time, and it was evaluated as 'pathogenic' according to both Mutation Taster and PolyPhen-2 analyses. It was postulated that the novel p.E466K variant did not abolish enzymatic activity completely. While homozygous mutations can result in complete 17-OHD, the two heterozygous compound mutations observed in this study only resulted in partial loss of enzymatic activity, such that these patients were able to achieve pregnancies with appropriate interventions. The second heterozygous mutation p.F435S is in a compound hybrid form in patients with 17-OHD. In-vitro functional studies have shown that p.F435S variants can abolish 17α-hydroxylase and 17,20-lyase activity completely (*Chen et al., 2019*). In addition, the heterozygous mutation p.I296T has been reported to cause partial 17-OHD in a woman (*Xia et al., 2021*). Mutation Taster and SIFT predicted that p.I296T was a disease-causing variant, but these computer programs could not predict how much enzyme activity remains *in vivo*. However, in this study, the heterozygote of p.F435S and p.I296T in Case 1 resulted in partial 17-OHD. The mechanism is not clear, which may be related to the post-translational modifications. Further work is needed to explore the relationship between 17-OHD genotypes and phenotypes.

Endocrine characteristics

The elevated concentration of progresterone in serum was an important diagnostic clue in both patients described in this report. The concentration of oestradiol was found to increase slowly during the induction of ovulation, while the concentration of androstenedione remained below the normal range. These findings were inconsistent with the increased number and size of follicles observed, revealing that these patients were only able to produce a limited amount of oestradiol. This finding, together with the abnormally high concentration of progesterone during the early follicular stage, was consistent with a possible diagnosis of 17-OHD. Patients diagnosed with 17-OHD often suffer from infertility or related issues due to abnormal steroidogenic cascades that cause impaired androstenedione, 17-OHP and oestradiol production, together with progesterone and 11-deoxycorticosterone accumulation (*Yanase et al., 1991*). Reduced oestradiol and increased progesterone concentrations in these women result in abnormal endometrial maturation and follicular development (*He et al., 2016*; *Young, 2013*). Weakened negative feedback of oestrogen to the pituitary gland may lead to slightly elevated FSH and LH concentrations, predisposing patients to ovarian cysts, most of which are multiple luteinizing ovarian cysts, which may be caused by elevated serum progesterone or gonadotrophin concentrations. At the same time, the exact mechanism for an elevated concentration of prolactin is not fully understood. It may be due to the disrupted sex hormone production in these patients, possibly leading the pituitary gland to secrete more prolactin in an attempt to stimulate sex hormone production. Additionally, the pituitary gland in patients with 17-OHD may have a diminished response to prolactin inhibiting factor, resulting in increased prolactin secretion. Other unknown factors, such as idiopathic hyperprolactinaemia, could also be involved. However, for patients with 17-OHD, ovarian cystectomy is not required. Meanwhile, feedback can also result in excessive release of ACTH from the pituitary, contributing to bilateral adrenocorticohyperplasia. Progesterone is chronically elevated in women with 17-OHD; oestradiol production may be limited but is normally maintained in women with partial 17-OHD due to gradual elevation of the gonadotrophin concentration. Another common manifestation in women with partial 17-OHD is a reduced concentration of testosterone. While it is known that testosterone plays a role in promoting the maturation and ovulation of oocytes, as well as participating in female libido and sexual response, the impact of testosterone concentration on a woman's fertility may not be significant. Research has shown that, even in women with low androgen concentrations, provided that ovarian function is normal, fertility may not be greatly affected (*Demers, 2010*). Another study found that androgens do not play a very significant role in the treatment of infertility (*Gleicher et al., 2011*). Nevertheless, this feature can still highlight the need to pay attention to the diagnosis of 17-OHD if pubic hair is absent or sparse on gynaecological examination. Overall, partial 17-OHD is associated with endocrine manifestations that are tied to the actual degree to which enzymatic activity is disrupted, often resulting in lower concentrations of oestradiol, testosterone and DHEA, together with an elevated concentration of progesterone, and a somewhat limited increase in the

concentration of ACTH, with or without a reduction in the concentration of 17-OHP.

Treatment and management of infertility

Ovulation cannot be initiated if concentrations of oestradiol and testosterone are very low. This study found that the concentration of oestradiol in these two patients with partial 17-OHD was low but was still within the normal range, which can be explained by the partial loss of 17-hydroxylase and/or 17,20-lyase activity in less-extreme phenotypes. Low-dose glucocorticoids were used to suppress the overproduction of ACTH in these patients with partial 17-OHD, and this was fairly effective to reduce the elevated concentration of progesterone. Thus, as shown in Case 1, the use of glucocorticoids can improve the decline in endometrial receptivity caused by an elevated concentration of progesterone, allowing the menstrual cycle to return to normal and follicles to develop normally, thus providing the conditions for a natural pregnancy. On the other hand, as shown in Case 2, recent advances in ART strategies can enable patients to become pregnant even when natural pregnancy is not an option (*Blumenfeld and Koren, 2021*; *Levran et al., 2003*). These treatments consist of an initial round of ovarian stimulation, such that oocytes can be harvested and cryopreserved following fertilization. Then, clinical efforts can be made to reduce the concentration of progesterone during the early follicular stage, such that the endometrium can develop normally under the influence of oestrogen and progesterone, allowing for appropriate implantation upon subsequent embryo transfer. A decrease in the concentration of oestradiol and an increase in the concentration of progesterone during ovarian stimulation did not have an adverse impact on the quality of the harvested oocytes in Case 2, and this was also verified for Case 1. Consistently, prior reports have described appropriate oocyte maturation and the development of viable embryos even when low follicular oestradiol concentrations are evident (*Fauser, 1997*). Progesterone does not impair the growth of follicles or the viability of oocytes. While progesterone concentrations that are prematurely elevated can lower IVF-associated live birth rates in the context of fresh embryo transfer cycles, the same has not been observed when cryopreserved embryos produced in the context of an elevated concentration of progesterone are used for subsequent embryo transfer (*Kuang et al., 2014*; *Venetis et al., 2013*). As such, conventional hormone replacement therapy under glucocorticoid supplementation can be used to achieve successful endometrial preparation in the context of freeze–thaw cycles. Consequently, standardized diagnostic and treatment strategies are essential for patients with partial 17-OHD to achieve pregnancy.

CONCLUSION

To the authors' knowledge, this is the first study to report live birth through natural pregnancy in a woman with partial 17-OHD. A novel mutation in *CYP17A1* was found in the other case. Some of the findings are very interesting compared with previous reports. Different combinations of mutations may have various effects on the activities of 17-hydroxylase and/or 17,20-lyase. Moreover, the appropriate treatment of patients diagnosed with partial 17-OHD can ultimately lead to satisfactory fertility outcomes. Natural or ART approaches combined with appropriate endocrine therapy can allow these patients to attain pregnancy. The timely diagnosis and standardized treatment of these women can thus lead to reproductive success.

DATA AVAILABILITY

No data was used for the research described in the article.

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ARTICLE

Metabolic profile of follicular fluid in patients with ovarian endometriosis undergoing IVF: a pilot study

BIOGRAPHY

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KEY MESSAGE

This study analysed the changes in follicular fluid metabolism in patients with ovarian endometriosis using untargeted metabolomics. The potential of metabolomic analyses for diagnosing and treating ovarian endometriosis should be investigated.

ABSTRACT

Research question: What are the metabolic characteristics of follicular fluid in patients with ovarian endometriosis undergoing IVF?

Design: This was an exploratory cohort study on endometriosis. In total, 19 infertile patients with ovarian endometriosis diagnosed by laparoscopy, and 23 controls matched in terms of age and body mass index (women with infertility due to male or tubal factors) were enrolled in this study. All patients underwent IVF treatment with a gonadotrophin-releasing hormone antagonist protocol, and follicular fluid was collected at oocyte retrieval. The metabolomics of follicular fluid samples was analysed using an ultra-high-performance liquid chromatography Orbitrap Exploris mass spectrometer (UHPLC-OE-MS). The best combination of biomarkers was selected by performing stepwise logistic regression analysis with backward elimination.

Results: Fifteen metabolites were identified as biomarkers associated with endometriosis. A final model containing 8-hydroxy-2-deoxyguanosine, biotin, n-acetyl-L-methionine and n-methylnicotinamide was constructed. Receiver operating characteristic analysis confirmed the value of these parameters in diagnosing endometriosis, with sensitivity of 94.7% and specificity of 95.7%. Enrichment analysis via the Kyoto Encyclopedia of Genes and Genome showed that 15 metabolites were enriched in eight metabolic pathways.

Conclusion: Metabolomics based on UHPLC-OE-MS effectively characterized the metabolomics analysis of follicular fluid in patients with ovarian endometriosis. These findings may provide a new basis for better understanding of how diseases progress, and for the discovery of new biomarkers.

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KEY WORDS
Endometriosis
Metabolomics
LC-MS/MS
Biomarkers
Follicular fluid
IVF

INTRODUCTION

Endometriosis is a chronic inflammatory disease characterized by the presence of functional endometrial glands and stroma in various locations such as the peritoneal cavity, ovary, rectovaginal septum and uterosacral ligament. Dysmenorrhoea, dyspareunia, chronic pelvic pain and infertility are the main clinical manifestations of endometriosis. It has been reported that approximately 10% of women of childbearing age have endometriosis, and 50% of these patients are infertile (*Shafrir et al., 2018*). However, the aetiology and pathogenesis of endometriosis remain unclear, and studies have suggested that it may be related to genetic, environmental and immune-inflammatory factors (*Saunders et al., 2021*). The serum marker carbohydrate antigen 125 (CA125) is often used in the clinical diagnosis of endometriosis. CA125 is usually elevated in the later stages of the disease, but is difficult to detect at early stages, limiting its sensitivity and specificity (*Hirsch et al., 2016*; *Kitawaki et al., 2005*; *Wang et al., 2008*). Therefore, there is a need for further study of the pathogenesis of endometriosis, and to find reliable biomarkers for its diagnosis. Endometriomas of the ovary, also called endometriotic ovarian cysts, are very common among women with endometriosis (*Nisolle et al., 1997*). Surgery is the most effective treatment for endometriomas, but this treatment can lead to follicular loss, which can lead to reduced ovarian reserve (*Loo et al., 2005*; *Shi et al., 2011*).

Follicular fluid is produced during folliculogenesis, and contains a variety of proteins that are important for follicular development and oocyte maturation (*Revelli et al., 2009*). The aetiology of endometriosis-related infertility is not fully understood. Previous studies have found that endometriosis may affect folliculogenesis, resulting in poor oocyte quality, low fertilization rate and poor embryo quality (*Garrido et al., 2000*; *Pellicer et al., 2000*). However, there is a lack of consensus in the literature regarding the results of assisted reproductive technology in patients with endometriosis.

Metabolomics helps to understand interactions within biological systems through the study of small molecules in biological fluids or tissues (*Muthubharathi et al., 2021*), and is considered a powerful tool to elucidate disease pathogenesis and identify disease biomarkers. At present, the most commonly used spectrometric techniques for metabolomics are nuclear magnetic resonance and mass spectrometry. Mass spectrometry has higher sensitivity than nuclear magnetic resonance (*Yan et al., 2018*). In recent years, many metabolomic studies have been conducted in the body fluids and tissues of patients with endometriosis, all reflecting significant changes in the metabolome of these patients (*Ortiz et al., 2021*). However, the pathogenesis and potential biomarkers of endometriosis remain unknown. Therefore, liquid chromatography-tandem mass spectrometry (LC-MS) was used in the present study to identify the metabolomic characteristics of follicular fluid in patients with endometriosis. Differences in the relative abundance of small molecules in follicular fluid samples were analysed by non-targeted metabolomics, and multivariate and univariate statistics were used to identify potential biomarkers.

The aim of this study is to improve understanding of ovarian endometriosis through follicular-fluid-derived metabolites, and identify potential biomarkers associated with the evolution of ovarian endometriosis.

MATERIALS AND METHODS

Sample collection

This study was a pilot study for a large cohort study of patients with endometriosis in China aiming to improve understanding of endometriosis. The main study has been recruiting women with endometriosis prospectively since June 2021 at the First Affiliated Hospital of Anhui Medical University, China. The aim is to recruit at least 2000 women over 2 years. At present, more than 2,000 women have been successfully recruited. The intention is to undertake annual Q2 follow-up of the cohort.

Participants were asked to complete detailed, validated questionnaires on their medical and family history, diet, mental health, quality of life and other clinical parameters. Clinical data including anthropometry, fitness and biological samples (endometriotic tissue, blood, peritoneal fluid, follicular fluid, granular cells, placenta and umbilical cord blood) were collected. Patients with surgically confirmed or high clinical suspicion of endometriosis were enrolled in the cohort. All women underwent IVF or intracytoplasmic sperm injection (ICSI). Infertility factors were determined clinically and in the laboratory for all patients, and follicular fluid samples were taken at oocyte aspiration followed by IVF/ICSI. Patients with endometriosis were diagnosed laparoscopically, and had endometrioma on the surface of the ovaries. Ovarian endometriosis was graded according to the revised system of the American Society for Reproductive Medicine, where Stage III is moderate (n = 16) and Stage IV is severe (n = 3) (*Guzick et al., 1997*). The control group consisted of infertile patients with tubal factors (i.e. ligation, excision and obstruction) and male factors (i.e. azoospermia). The control group did not have pelvic pain, pelvic inflammatory disease, polycystic ovary syndrome, cancer, premature ovarian failure, hydrosalpinx or other gynaecological factors leading to infertility. The inclusion criteria for both groups were: age 25–39 years; body mass index <30 kg/m^2; and no use of hormone therapy in the preceding 3 months. The exclusion criteria were: hydrosalpinx; chronic systemic disease (e.g. chronic hypertension, diabetes, thyroid disease); and history of smoking. All patients underwent IVF/ICSI therapy with an antagonist protocol so that sufficient follicular fluid could be extracted. Following strict screening, 19 patients with infertility due to surgically confirmed ovarian endometriosis and 23 patients with infertility due to male or tubal factors were selected for the pilot study.

The Institutional Ethics Review Committee of Anhui Medical University approved the study on 22 April 2021 (Reference No. PJ2021-05-16). All enrolled patients provided informed consent. This study was registered with the China Clinical Trial Registration Centre (Reference No. ChiCTR2100046249).

Ovarian stimulation protocol

All patients underwent ovarian stimulation using a gonadotrophin-releasing hormone (GnRH) antagonist protocol for IVF treatment. From day 2 or 3 of the menstrual cycle, gonadotrophin (Gonal F; Merck Serono, Italy) induced ovulation. A GnRH antagonist (0.25 mg/day; Cetrotide; Fareva Pau, France) was used to inhibit the release of endogenous LH on day 6 after treatment, or when the dominant follicle reached 14 mm in diameter and combined

with hormone levels. At each visit, serum oestradiol, FSH and LH concentrations were measured, and ultrasound scanning was undertaken to determine the ovarian response to drugs and adjust the doses of ovarian stimulation drugs. When transvaginal ultrasound showed two or more follicles ≥18 mm in diameter, ovarian stimulation drugs were stopped, and 250 mg of human chorionic gonadotrophin (HCG) (Ovidrel; Merck Serono, Italy) was injected the same day to promote ovum maturation. Oocytes were extracted 36 h after HCG injection. Oocytes were collected by transvaginal-ultrasound-guided needle aspiration of follicles. Embryo quality was assessed according to the criteria described in the Istanbul Consensus Workshop on Embryo Evaluation (*ASRM and ESHRE Special Interest Group of Embryology, 2011*).

Sample preparation for metabolomics

Follicular fluid was obtained from the follicles in both ovaries for all patients. Follicular fluid from both ovaries of each patient was mixed to form a sample, and kept in a 15-ml sterile tube. All follicular fluid samples were visually examined microscopically, and confirmed to be free of blood contamination before they were used for follow-up studies. Follicular fluid was centrifuged at 1006 *g* for 15 min to remove erythrocytes and leukocytes, and the supernatant was stored at -80°C until LC-MS analysis.

Metabolite extraction

First, 100 μl of the sample was transferred to an Eppendorf tube. Next, 400 μl of a solution of methanol (CNW Technologies, Germany) and acetonitrile (CNW Technologies), containing a mixture of isotopically-labelled internal standards, was added in a 1:1 ratio. The samples were vortexed for 30 s and then sonicated for 10 min in an ice-water bath. Next, the samples were incubated for 1 h at -40°C to precipitate proteins, and then centrifuged at 13,800 *g* for 15 min at 4°C. The supernatant was transferred into a fresh glass vial for analysis. Matching volumes of the supernatants from all samples were pooled to generate a quality control sample.

LC-MS/MS analysis

LC-MS/MS analyses were conducted using an ultra-high-performance liquid chromatography (UHPLC) system (Vanquish; Thermo Fisher Scientific, USA) with a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm) coupled to an Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific). Upon first use, technical terms were explained. The mobile phase contained 25 mmol/l of ammonium acetate and 25 mmol/l of ammonium hydroxide in water (pH 9.75) and acetonitrile. The temperature of the auto-sampler was set at 4°C, and the injection volume was 2 μl.

The Orbitrap Exploris 120 mass spectrometer was used to obtain MS/MS spectra in the information-dependent acquisition mode using Xcalibur software (Thermo Fisher Scientific). This mode ensures that the mass spectrometry spectrum is evaluated consistently throughout the scan using the acquisition software. The ESI source conditions were set as follows: capillary temperature of 320°C, MS/MS resolution of 15000, full MS resolution of 60,000, Aux gas flow rate of 15 Arb, sheath gas flow rate of 50 Arb, and collision energy of 10/30/60 in NCE mode. The spray voltage was set at 3.8 kV (positive) or -3.4 kV (negative).

Data preprocessing and statistical analysis

The mzXML format was used to convert the raw data after employing ProteoWizard. An in-house program, developed using the R language and based on XCMS, was employed for peak detection, extraction, alignment and integration. Deviation value filtering, missing value filtering, missing value filling, and internal standard normalization processing were applied to further refine the data. Subsequently, metabolite annotation was performed using the BiotreeDB MS2 database, and further classified by the Human Metabolome Database. The threshold for annotation was set at 0.3. Data underwent log conversion and UV formatting using SIMCA Version 16.0.2 (Sartorius Stedim Data Analytics, Sweden). The analysis involved principal component analysis and orthogonal projections to latent structures-discriminant analysis (OPLS-DA). The variable importance in projection (VIP) of the first principal component of the OPLS-DA model was employed to screen for different metabolites. Univariate analysis was conducted using *P*-values to screen for different metabolites. Metabolite accumulation patterns were subjected to hierarchical clustering analysis across various samples using R Version 3.3.5. The identified metabolites were annotated and enriched using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Additionally, a receiver operating characteristic (ROC) curve was implemented using SPSS Version 19.0 (IBM, USA) to evaluate the correlation between different metabolites in follicular fluid and endometriosis. The reliability of the model was confirmed by calculating the area under the curve (AUC). Clinical data are reported as mean ± SD. Quantitative variables were analysed using Student's *t*-test for comparison between two groups.

RESULTS

Clinical data

LH was found to be significantly higher in the control group compared with the endometriosis group (P=0.014; TABLE 1). No significant differences were found between the groups in terms of body mass index, FSH, oestradiol, progesterone, testosterone, prolactin, total gonadotrophin dose, number of follicles >14 mm, number of metaphase II oocytes, number of retrieved oocytes, fertilization rate, number of cleaved embryos, cleavage rate, rate of good-quality embryos on day 3, and total number of embryos produced.

Metabolism analysis by LC-MS/MS

In this study, 10,151 peaks were retained after original data processing, 4384 peaks using the positive ion model and 5767 peaks using the negative ion model. All metabolites were retrieved and sorted in the internal MS2 database (BiotreeDB), and were further classified using the Human Metabolome Database. In total, 433 metabolites were divided into 13 classes: two alkaloids and derivatives; 20 benzenoids; two hydrocarbons; 194 lipids and lipid-like molecules; 16 nucleosides, nucleotides and analogues; 76 organic acids and derivatives; 13 organic nitrogen compounds; 26 organic oxygen compounds; one organohalogen compound; 55 organoheterocyclic compounds; one organosulphur compound; 20 phenylpropanoids and polyketides; and seven others (FIGURE 1a).

As principal component analysis is an unsupervised classification model, data are affected by many variables unrelated to grouping information, and as the sample size was small, the differences between samples of different groups are not well described (FIGURE 1b). A classification model established by supervised OPLS-DA shows good separation (FIGURE 1c). The horizontal coordinate shows the differences between sample groups, and the vertical coordinate

TABLE 1 CLINICAL CHARACTERISTICS OF PATIENTS

	Ovarian endometriosis group (*n* = 19)	Control group (*n* = 23)	*P*-value
Stage			
III (moderate)	16	-	-
IV (severe)	3	-	-
Age (years)	31 ± 2.67	31.13 ± 3.82	0.901
Body mass index (kg/m^2)	21.66 ± 3.16	22.33 ± 3.44	0.515
FSH (mIU/ml)	8.11 ± 2.32	7.58 ± 1.68	0.393
LH (mIU/ml)	3.76 ± 1.79	5.36 ± 2.16	**0.014**
Oestradiol (pg/ml)	181.59 ± 103.91	169.93 ± 94.82	0.706
Progesterone (ng/ml)	1.72 ± 1	2.05 ± 1.19	0.338
Testosterone (ng/dl)	1.36 ± 0.77	1.36 ± 0.67	0.979
Prolactin (ng/ml)	17.14 ± 9.29	14.63 ± 6.17	0.302
Total gonadotropin dose (IU)	1964.47 ± 788.87	1873.91 ± 682.18	0.692
Follicles >14 mm (*n*)	8.84 ± 5.38	9.74 ± 5.82	0.610
Oocytes retrieved (*n*)	10.95 ± 6.9	11.83 ± 7.19	0.690
Metaphase II oocytes (*n*)	7 ± 5.22	8.04 ± 5.32	0.527
Fertilized oocytes (*n*)	5.26 ± 4.36	6.78 ± 4.32	0.265
Fertilization rate[a]	0.82 ± 0.24	0.88 ± 0.17	0.323
Cleaved embryos (*n*)	4.74 ± 4.45	6.7 ± 4.38	0.160
Cleavage rate[b]	0.86 ± 0.3	0.98 ± 0.8	0.085
Rate of good-quality embryos on day 3 (*n*)	3.37 ± 4	3.39 ± 3.31	0.984
Rate of good-quality embryos on day 5 (*n*)	2.16 ± 3.18	2.22 ± 2.65	0.948
Total embryos produced (*n*)	4.05 ± 4.17	4.83 ± 3.68	0.527

Data are reported as mean ± standard deviation.

Quantitative variables were compared using Student's *t*-test.

Bold text indicates significance.

[a] Number of fertilized oocytes/number of metaphase II oocytes.

[b] Number of cleaved embryos/number of fertilized oocytes.

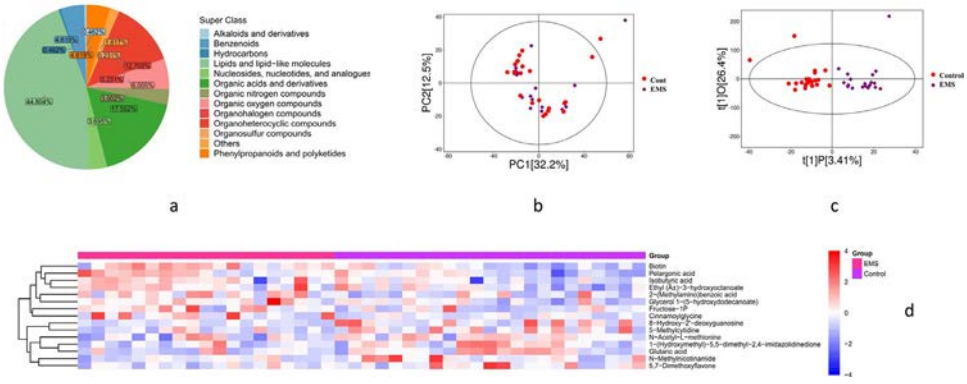

FIGURE 1 Principal component analysis (PCA) plots and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) plots describing the separation trend between the two groups of samples. (a) Classification of the 433 metabolites of follicular fluid samples. (b) Score scatterplot of PCA model for endometriosis (EMS) group versus control group. (c) Score scatterplot of OPLS-DA model for EMS group versus control group. (d) Heatmap of hierarchical clustering analysis.

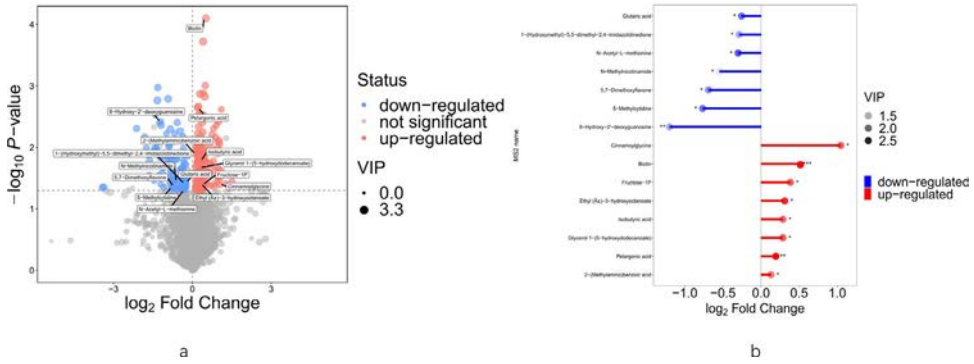

FIGURE 2 (a) Volcano plots showing differences in the metabolites in follicular fluid between patients with endometriosis and controls. Significantly up-regulated metabolites are shown in red, significantly down-regulated metabolites are shown in blue, and non-significantly different metabolites are shown in gray. (b) Matchstick plot analysis for endometriosis (EMS) group versus control group. The shade of the dot represents the variable importance of projection (VIP) value. *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$P < 0.001$.

shows the differences within sample groups. The two groups had good consistency within the sample groups, and the differences between the two groups are obvious. The *P*-value and VIP value were combined to screen the differentially expressed metabolites. Among the differentially expressed metabolites, those with a *P*-value <0.05 and VIP value >1 were considered significant. The accumulation pattern of metabolites in the two groups of samples can be visualized through hierarchical clustering analysis of a heat map, which can intuitively indicate the differences in metabolites between the two groups of samples (FIGURE 1d).

Differences in relative abundances of metabolites between groups

The volcano and matchstick plots (FIGURE 2a,b) show the results of screening out significantly different metabolites. Detailed information is summarized in TABLE 2. The 15 metabolites were considered to have significant differences. Concentrations of cinnamoylglycine ($P = 0.04$), biotin ($P < 0.001$), fructose-1P ($P = 0.04$), ethyl (A ±)-3-hydroxyoctanoate ($P = 0.04$), isobutyric acid ($P = 0.016$), glycerol 1-(5-hydroxydodecanoate) ($P = 0.021$), pelargonic acid ($P = 0.002$) and 2-(methylamino) benzoic acid ($P = 0.013$) were higher in the endometriosis group compared with the control group, whereas concentrations of 8-hydroxy-2′-deoxyguanosine ($P = 0.004$), 5-methylcytidine ($P = 0.045$), 5,7-dimethoxyflavone ($P = 0.041$), n-methylnicotinamide ($P = 0.034$, n-acetyl-L-methionine ($P = 0.048$, 1-(hydroxymethyl)-5,5-dimethyl-2,4-imidazolidinedione ($P = 0.038$) and glutaric acid ($P = 0.038$) were higher in the control group (FIGURE 3).

TABLE 2 SUMMARY OF 15 METABOLITES IN FOLLICULAR FLUID WITH SIGNIFICANT DIFFERENCES BETWEEN PATIENTS WITH ENDOMETRIOSIS AND HEALTHY CONTROLS

m/z[a]	RT (s)	Metabolite	*P*-value	VIP[b]	Fold change[c]
284.0988	280.761	8-hydroxy-2′-deoxyguanosine	0.004	1.49	0.44
179.0559	236.831	Fructose-1P	0.040	2.02	1.31
190.0516	236.835	N-acetyl-L-methionine	0.048	2.11	0.81
152.0707	250.066	2-(methylamino)benzoic acid	0.013	1.85	1.09
258.1082	264.349	5-methylcytidine	0.045	1.89	0.59
159.0761	173.902	1-(hydroxymethyl)-5,5-dimethyl-2,4-imidazolidinedione	0.038	1.52	0.82
137.0706	100.3635	N-methylnicotinamide	0.034	1.08	0.69
281.089	153.1655	5,7-dimethoxyflavone	0.041	1.69	0.62
204.0665	201.6285	Cinnamoylglycine	0.040	2.03	2.06
89.0595	35.73465	Isobutyric acid	0.016	1.92	1.22
245.0997	261.774	Biotin	<0.001	2.78	1.43
131.0348	82.7291	Glutaric acid	0.038	1.97	0.84
189.1482	45.63055	Ethyl (A ±)-3-hydroxyoctanoate	0.040	2.43	1.24
157.1232	52.7225	Pelargonic acid	0.002	2.75	1.14
291.2163	33.24735	Glycerol 1-(5-hydroxydodecanoate)	0.021	2.08	1.22

[a] Mass/charge ratio detected during liquid chromatography-tandem mass spectrometry/mass spectrometry runs.
[b] From orthogonal projections to latent structures-discriminant analysis model.
[c] Fold change in the metabolite ratio between the endometriosis group and the control group (higher value indicates higher level of metabolite expression in the endometriosis group).
m/z, mass/charge ratio; RT, retention time; VIP, variable importance in projection.

ROC analysis provides guidance for potential markers

ROC curves were plotted for each well-defined metabolite, and AUC were calculated. The AUC for all 15 potential biomarkers was between 0.62 and 0.84 (Table S1, see online supplementary material). After stepwise multiple logistic regression analysis and backward elimination, a model with four predictive factors was established, including 8-hydroxy-2′-deoxyguanosine, biotin, n-acetyl-L-methionine and n-methylnicotinamide, with sensitivity of 94.7% and specificity of 95.7%. The apparent AUC of the ROC curve for the model predicting endometriosis was 0.961 (95% CI 0.893–1.029) (FIGURE 4a). According to KEGG annotation and enrichment results, these 15 metabolites were mainly concentrated in eight metabolic pathways, and biotin metabolism ($P = 0.019$), vitamin digestion and absorption ($P = 0.0268$), protein digestion and absorption ($P = 0.032$), fatty acid degradation ($P = 0.034$) and lysine

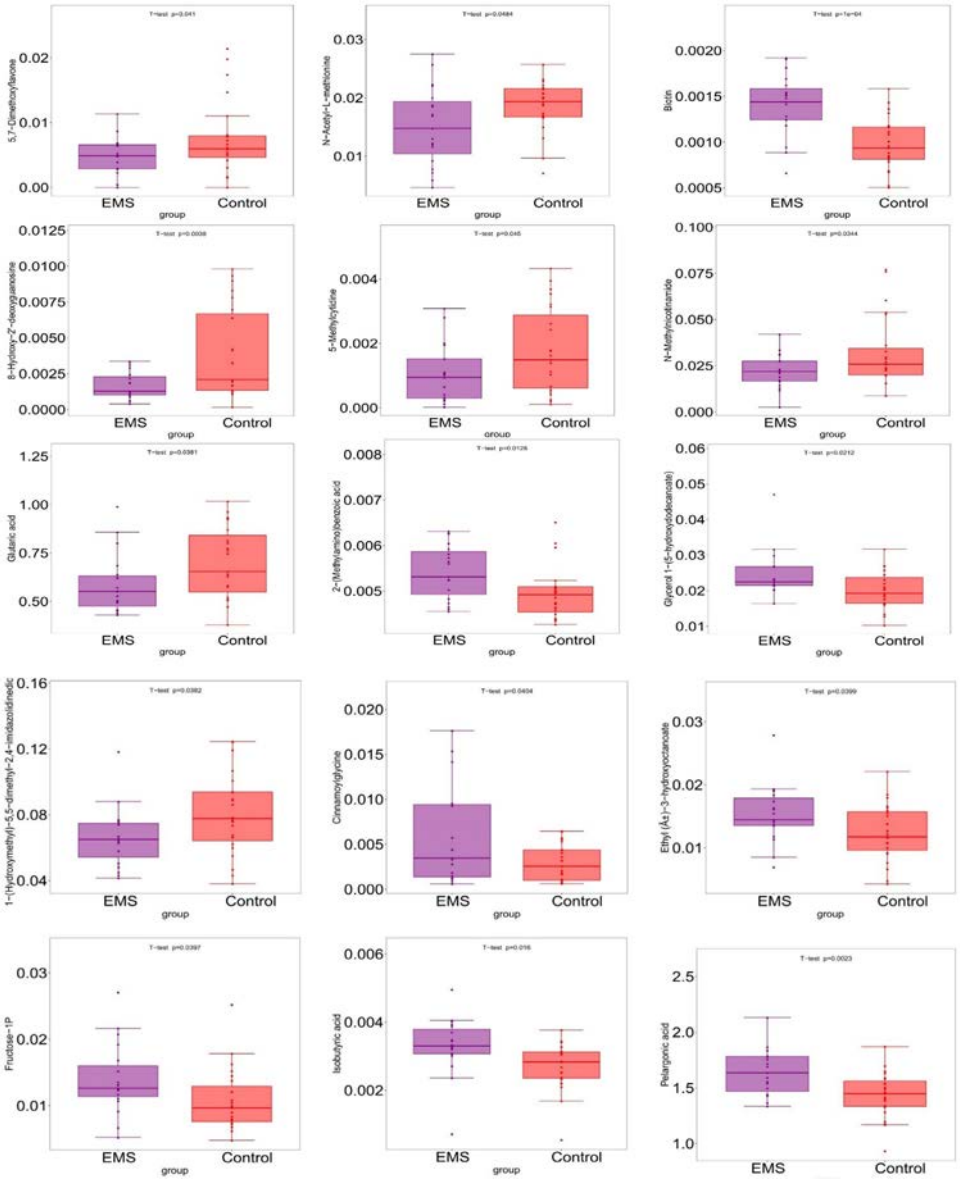

FIGURE 3 Boxplot analysis for the endometriosis (EMS) group versus the control group, showing median, interquartile range, maximum, minimum and outliers.

degradation ($P = 0.034$) were the most significant (FIG. 4b).

DISCUSSION

In this study, metabolic changes in follicular fluid samples from patients with endometriosis were studied using LC-MS/MS-based metabolome analysis. Significant metabolic differences in the follicular fluid were found between the two groups. After screening and comparison with the BiotreeDB database based on *P*-values and VIP values, 15 metabolites were determined. Concentrations of cinnamoylglycine, biotin, fructose-1P, ethyl (A ±)-3 hydroxyoctanoate, 1-(5-hydroxydodecanoate), pelargonic acid and isobutyric acid were higher in the endometriosis group compared with the control group. In contrast, 8-hydroxy-2'-deoxyguanosine, 5-methylcytidine, 5,7-dimethoxyflavone, n-methylnicotinamide, n-acetyl-L-methionine, 1-(hydroxymethyl)-5, 5-dimethyl-2, 4-imidazolidinedione and glutaric acid were lower in the follicular fluid of the endometriosis group compared with the control group. Among these 15 metabolites, cinnamoylglycine and 8-hydroxy-2'-deoxyguanosine had the most significant fold changes (2.06 and 0.44, respectively).

KEGG pathway enrichment analysis was performed to investigate the metabolic pathways involved in ESI-positive and -negative models. This showed that 15 metabolites were enriched in eight metabolic pathways. Further establishment of the prediction model with the combination of four variables could distinguish between patients with endometriosis and the control group. In epidemiology, ROC is an important indicator to evaluate the prediction model. However, the lack of a validation group was a limitation of this study, and further studies in serum are needed to verify the prediction model.

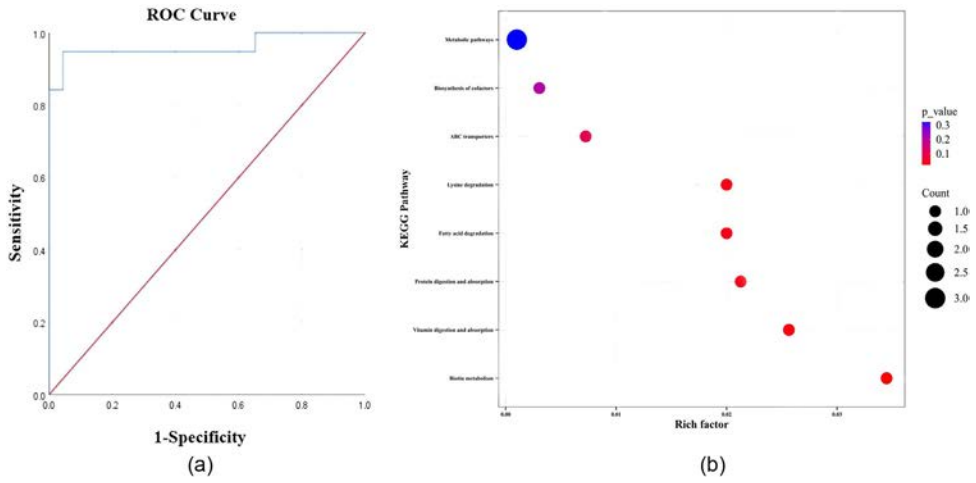

FIGURE 4 (a) Receiver operating characteristic (ROC) curve of endometriosis constructed with four metabolites: 8-hydroxy-2′-deoxyguanosine, biotin, n-acetyl-L-methionine and n-methylnicotinamide. (b) Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation of 15 metabolites, and enrichment of differentially expressed metabolites. The abscissa represents the rich factor corresponding to each pathway, and the ordinate is the name of the KEGG metabolic pathway. The size of the dots indicates the number of different metabolites enriched in this pathway. The colour represents the size of the *P*-value; the smaller the *P*-value, the redder the colour, indicating greater significance of the degree of enrichment.

Loy et al. (2021) used LC-MS to analyse the peritoneal fluid of patients with peritoneal endometriosis, and found that the concentration of phenylalanine-isoleucine increased in the peritoneal fluid of these patients. The same results were obtained in serum, and the concentrations of phenylalanine-isoleucine in peritoneal fluid (AUC = 0.77, 95% CI 0.61–0.92; $P = 0.006$) and serum (AUC = 0.81, 95% CI 0.64–0.99; $P = 0.004$) can better predict patients with peritoneal endometriosis. In the present study, a good prediction model was constructed using 8-hydroxy-2′-deoxyguanosine, biotin, n-acetyl-L-methionine and n-methylnicotinamide. Although follicular fluid is not tested routinely in clinical practice, some markers in the diagnostic model have also been reported to be significantly different in blood in previous studies (*Da Broi et al., 2016*; *Yanagihara et al., 2022*). The present study provides a theoretical basis for further detection of changes of these metabolites in peripheral blood, and relationships with the pathophysiology of ovarian endometriosis. A study with a larger sample size is needed to obtain more substantial evidence.

This study found no significant differences in the number of oocytes retrieved, number of metaphase II oocytes, number of fertilized oocytes, fertilization rate, number of cleaved embryos, cleavage rate, rate of good-quality embryos and total number of embryos produced between the two groups. Previous studies have shown significant reductions in oocyte maturation and fertilization rates, blastocyst rates, oocyte recovery and available embryos in patients with ovarian endometrioma treated with IVF/ICSI (*Alshehre et al., 2021*; *Wu et al., 2021*). However, *Opøien et al. (2012)* found no significant difference in the number of retrieved oocytes in patients with ovarian endometrioma. A meta-analysis of 33 studies showed no significant differences in the live birth rate and spontaneous abortion rate in patients with endometriosis (*Alshehre et al., 2021*). However, *Opøien et al. (2012)* and *Wu et al. (2021)* showed that the live birth rate was significantly lower in patients with ovarian endometriosis cysts compared with healthy women. There is a lack of consensus in the literature on the outcome of IVF/ICSI treatment in patients with ovarian endometrioma, and reliable conclusions may require studies with larger samples.

Currently, endometriosis affects approximately 10% of women of childbearing age and approximately 5–50% of infertile women (*Shafrir et al., 2018*). The 2022 guidelines of the European Society of Human Reproduction and Embryology recommend that clinicians should perform gynaecological examinations, including transvaginal examinations, in all patients with suspected endometriosis (*Becker et al., 2022*). The finding of painful induration or nodules in the rectovaginal wall during the examination should lead to consideration of a diagnosis of deep invasive endometriosis, and the finding of an adnexal mass should lead to consideration of a diagnosis of ovarian endometriosis. For patients with suspected endometriosis, if the gynaecological examination is normal, further imaging examinations should be considered; however, laparoscopy is only recommended for patients with negative imaging results, or unsuccessful or inappropriate empirical treatment. Gynaecological and ultrasound examinations are very dependent on the clinical experience of doctors, and the sensitivity and specificity of CA125, a commonly used serum marker in clinical diagnosis of endometriosis, are low (*Hirsch et al., 2016*; *Kitawaki et al., 2005*; *Wang et al., 2008*). Some patients have atypical symptoms or are asymptomatic, which makes the early diagnosis of endometriosis more difficult. Meanwhile, the pathogenesis of endometriosis-related infertility is poorly understood.

Oxidative stress is widely considered as an imbalance between reactive oxygen species (ROS) and antioxidants, and is related to the development of endometriosis (*Scutiero et al., 2017*). The imbalance between ROS and antioxidant systems in follicular fluid may be responsible for endometriosis-associated infertility and abnormal oocyte development (*Nasiri et al., 2017*). Many studies have demonstrated that 8-hydroxy-2′-deoxyguanosine is a biomarker of endogenous oxidative DNA damage and a risk factor for many diseases, including cancer (*Valavanidis et al., 2009*). It has

been reported that 8-hydroxy-2′-deoxyguanosine showed inhibitory properties on ROS in various in-vitro tests, suggesting that 8-hydroxy-2′-deoxyguanosine could be implicated in attenuation of oxidative stress (*Ock et al., 2011*). The present study found a significant reduction in the 8-hydroxy-2′-deoxyguanosine concentration in patients with endometriosis compared with the control group. As such, it is postulated that the decrease in 8-hydroxy-2′-deoxyguanosine concentration in follicular fluid was related to its inhibition of oxidative stress.

In contrast to this study, *Da Broi et al. (2016)* found elevated concentrations of 8-hydroxy-2′-deoxyguanosine in serum and follicular fluid in patients with endometriosis. They performed IVF/ICSI using a long protocol. GnRH analogues have been used as treatment for endometriosis, and their use may be a key reason for the different concentrations of 8-hydroxy-2′-deoxyguanosine in follicular fluid. The increase in the concentration of 8-hydroxy-2′-deoxyguanosine may be a marker to predict the good prognosis of endometriosis. *Tamura et al. (2008)* suggested that the concentration of 8-hydroxy-2′-deoxyguanosine in the follicular fluid of women with a high oocyte degeneration rate was significantly higher compared with women with a low oocyte degeneration rate, further suggesting that it plays a vital role in the follicular fluid micro-environment.

Biotin is a water-soluble vitamin. It has been reported that specific vitamins play a vital role in the development of mature oocytes and embryos (*Ciepiela et al., 2018*; *Firouzabadi et al., 2014*). *Yanagihara et al. (2022)* found that serum concentrations of biotin were higher than concentrations in follicular fluid in patients treated with IVF/ICSI, suggesting that biotin in follicular fluid may be derived from blood, and found that biotin did not contribute to the maintenance of oocyte quality or the fertilization rate. The latter findings are consistent with the present study, which found a significant increase in the concentration of biotin in follicular fluid of patients with endometriosis, and did not find differences in oocyte quality or fertilization rate between the endometriosis group and the control group. The present study found that biotin alone has great potential to differentiate between patients with endometriosis and healthy individuals. Based on the study by *Yanagihara et al. (2022)*, the present authors postulated that the diagnostic potential of biotin is more pronounced in blood. Biotin may be more reliable than CA125 as a serum marker for endometriosis, and further studies are needed to investigate this hypothesis.

Many studies have reported that endometriosis has similar pathological and histological features to malignant tumours (*Guidozzi, 2021*; *Munksgaard et al., 2011*). In previous studies, almost all amino acids were found to be elevated in cancerous tissues (*Denkert et al., 2008*). This finding was supported by the present study, as concentrations of cinnamoylglycine and n-acetyl-L-methionine were found to be significantly higher in the follicular fluid of patients with endometriosis compared with the control group. However, the specific mechanism of action needs to be investigated further. Notably, the present study also found a significant reduction in the concentration of 5,7-dimethoxyflavone, the antitumour effects of which have been reported in various cancer types (*Walle, 2007*; *Yang et al., 2012*). In addition, the potential of 5,7-dimethoxyflavone to treat oestrogen-dependent cancers has been investigated due to its demonstrated activity as an aromatase inhibitor (*Ta et al., 2007*). *Park et al. (2020)* found that 5,7-dimethoxyflavone induces apoptosis in endometriosis cell lines through DNA fragmentation and cell cycle arrest. In the present study, the reduced concentration of 5,7-dimethoxyflavone in the follicular fluid of patients with endometriosis could explain the carcinoid characteristics of endometriosis. The study findings suggest that the use of 5,7-dimethoxyflavone as a potential alternative therapy for hormonal and surgical treatment of endometriosis should be investigated.

This study had various strengths. First, to the authors' knowledge, it is the first study to comprehensively investigate the changes in follicular fluid metabolism in patients with endometriosis using LC-MS/MS. Second, compared with genomics and proteomics, metabolomics is closest to a phenotype, and can provide immediate information on the functional status of biological endometriosis and cells.

In terms of limitations, as this was a pilot study, the sample size was small. Further studies with large samples are needed to confirm the findings.

CONCLUSIONS

This study analysed the changes in follicular fluid metabolism in patients with ovarian endometriosis using untargeted metabolomics. Significant changes have been identified in metabolites in the follicular fluid of patients with ovarian endometriosis. The potential for metabolomic analyses to diagnose and treat ovarian endometriosis should be investigated. However, studies with large sample sizes are needed to verify the significance of individual metabolites in the occurrence and progression of endometriosis, and to confirm the present findings.

DATA AVAILABILITY

Data will be made available on request.

FUNDING

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AUTHOR CONTRIBUTIONS

Ting Luo, Zhaolian Wei, Yunxia Cao and Bin Liu conceived and designed the experiments. Ting Luo wrote the manuscript. Ye He and Mengyuan Zhang revised and polished the manuscript. Ting Luo performed all experiments with assistance as follows: Mengyao Wang, Youyan Fang and Wanlu Wang contributed to the clinical sample collection; and Wanqing Li and Yunyu Xu supported the clinical epidemiological information collection and analysis. All work was carried out under the supervision of Zhaolian Wei. All authors read, edited and approved the manuscript.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.103912.

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RBMO

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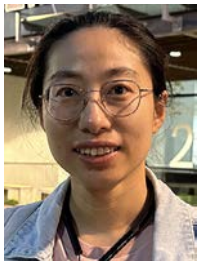
ARTICLE

Progesterone and 17-hydroxy-progesterone concentrations in follicular fluid and serum reflect their production in granulosa and theca cells



BIOGRAPHY

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KEY MESSAGE

Progesterone is mainly synthesized by granulosa cells during the follicular phase and by the corpus luteum in the luteal phase, while 17-hydroxy-progesterone is produced consistently by theca cells.

ABSTRACT

Research question: How is the production of progesterone (P_4) and 17-hydroxy-P_4 (17-OH-P_4) regulated between theca cells and granulosa cells during the follicular phase, during ovulation and after transformation into a corpus luteum?

Design: Three cohorts were examined: (i) 31 women undergoing natural and stimulated cycles, with serum hormone measurements taken every 3 days; (ii) 50 women undergoing ovarian stimulation, with hormone concentrations in serum and follicular fluid assessed at five time points during final follicle maturation; and (iii) 12 women undergoing fertility preservation, with hormone concentrations evaluated via the follicular fluid of small antral follicles.

Results: In the early follicular phase, theca cells primarily synthesized 17-OH-P_4 while granulosa cells produced limited P_4, maintaining the P_4:17-OH-P_4 ratio <1. As follicles reached follicle selection at a diameter of approximately 10 mm, P_4 synthesis in granulosa cells was up-regulated, but P_4 was mainly accumulated in follicular fluid. During final maturation, enhanced activity of the enzyme HSD3B2 in granulosa cells enhanced P_4 production, with the P_4:17-OH-P_4 ratio increasing to >1. The concentration of 17-OH-P_4 in the luteal phase was similar to that in the follicular phase, but P_4 production increased in the luteal phase, yielding a P_4:17-OH-P_4 ratio significantly >1.

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KEY WORDS

Human ovarian steroidogenesis
Progesterone
17-OH-progesterone
Theca cells
Granulosa cells
Ovarian stimulation

Conclusions: The P_4:17-OH-P_4 ratio reflects the activity of granulosa cells and theca cells during the follicular phase and following luteinization in the corpus luteum. Managing the function of granulosa cells is key for reducing the concentration of P_4 during ovarian stimulation, but the concerted action of FSH and LH on granulosa cells during the second half of the follicular phase makes this complex.

INTRODUCTION

Proper regulation of the production of ovarian sex steroids is mandatory for successful reproduction. The main location for the production of sex steroids is the ovaries, with follicles being the primary site of synthesis. Sex steroids perform many physiological tasks to secure an appropriate environment for the oocyte and the developing embryo, including appropriate endometrial receptivity.

Production of steroids is controlled by FSH and LH, with FSH receptors (FSHR) expressed exclusively on granulosa cells, and LH receptors (LHR) expressed constitutively on theca cells and expressed on granulosa cells when follicles exceed approximately 10 mm in diameter (*Jeppesen et al., 2012*). The regulation of ovarian steroidogenesis differs between mammalian species, but human ovarian steroidogenesis is distinct by having three terminal products: progesterone (P_4), 17-hydroxy-progesterone (17-OH-P_4) and oestradiol (FIGURE 1). Whereas synthesis of oestradiol is confined to granulosa cells, which are the only ovarian cells to express cytochrome P450 family 19 subfamily A member 1 (CYP19A1) which converts androgens into oestrogens, P_4 is synthesized by granulosa cells and theca cells, both of which express hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2 (HSD3B2), catalysing the conversion of steroids from the Δ5 pathway to the Δ4 pathway (FIGURE 1). The synthesis of P_4 requires the action of HSD3B2 alone to convert pregnenolone to P_4, while the production of 17-OH-P_4 requires the action of both HSD3B2 and cytochrome P450 family 17 subfamily A member 1 (CYP17A1). In essence, HSD3B2 is a unidirectional enzyme that supports the conversion of steroids from the Δ5 pathway to the Δ4 pathway (*Aedo et al., 1976*; *Andersen and Ezcurra, 2014*; *Miller, 2008*). Therefore, synthesis of 17-OH-P_4 can be achieved by the action of HSD3B2 followed by the action of CYP17A1 (i.e. via P_4), or the other way around (i.e. via 17-OH-pregnenolone). CYP17A1 is not expressed in granulosa cells but is expressed specifically in theca cells; therefore, synthesis of 17-OH-P_4 only takes place in theca cells. Furthermore, CYP17A1 in humans only supports the Δ4 lyase reaction to a very limited extent (i.e. the conversion of 17-OH-P_4 to androstenedione, which — in essence — makes 17-OH-P_4 a terminal product of theca cells), while P_4 is a terminal product of granulosa cells (*Miller, 2008*). In contrast, the Δ5 pathway in theca cells is open, with synthesis of 17-OH-pregnenolone, dehydroepiandrosterone and androstenedione, the latter serving as the substrate for oestradiol synthesis in granulosa cells (FIGURE 1).

Activity of the key enzymes in ovarian steroidogenesis is modulated by various co-factors, such as cytochrome b5 (CYB5A) and cytochrome P450 reductase (POR) (*Storbeck et al., 2015*). Moreover, substrate availability of cholesterol for further metabolism can impact the synthesis of ovarian sex steroids, including the expression of key enzymes such as

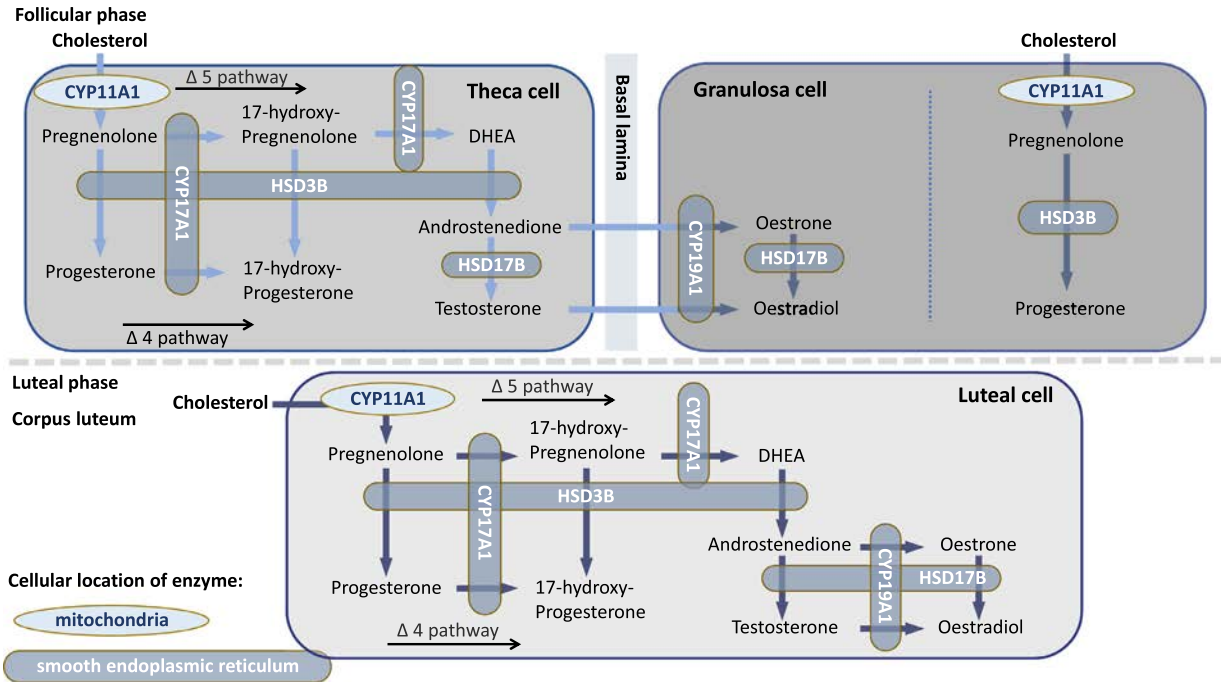

FIGURE 1 Overview of the enzymes involved in regulation of human ovarian steroidogenesis during the follicular and luteal phases. The Δ5 pathway is depicted in the upper panel, and the Δ4 pathway is depicted in the lower panel. CYP11A1, cytochrome P450, family 11, subfamily A, member 1; DHEA, dehydroepiandrosterone; HSD17B, 17β-hydroxysteroid dehydrogenase type 13; CYP19A1, cytochrome P450 family 19 subfamily A member 1; HSD3B, 3β-hydroxysteroid dehydrogenase.

3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), which serves as the rate-controlling enzyme in the mevalonate pathway leading to the production of cholesterol. Additionally, 24-dehydrocholesterol reductase (DHCR24) plays a crucial role in the synthesis of cholesterol, while low-density lipoprotein receptor (LDLR) is central for the uptake of cholesterol from the circulation.

The adrenal glands also have the capacity to produce both P_4 and 17-OH-P_4, and a potential adrenal contribution to the circulating concentrations of these steroids needs to be considered. However, circulating concentrations of both steroids are unaffected by dexamethasone suppression, which suggests that the ovaries are the major source for the concentrations measured in blood (*Abraham, 1974*).

There are several unanswered questions regarding the regulation of human ovarian steroidogenesis. It is unclear which cell type (i.e. granulosa cells or theca cells) is primarily responsible for the production of P_4 during the follicular phase. Moreover, it is unclear whether theca cells preferentially secrete P_4 or whether P_4 is converted to 17-OH-P_4 prior to release. Further, it is not known if P_4 is secreted by granulosa cells for conversion to 17-OH-P_4 in theca cells. Over recent years, the relative contributions of granulosa cells and theca cells has attracted massive reasearch interest due to the potentially negative consequences on the success of IVF in connection with ovarian stimulation; if the circulatory concentration of P_4 in the late follicular phase exceeds a critical level, the endometrium is potentially less receptive at the time of implantation (*Adda-Herzog et al., 2018*; *Bosch et al., 2010*; *Kalakota et al., 2022*; *Lawrenz et al., 2018a*,*b*). Furthermore, a recent study suggested that ovarian stimulation using recombinant FSH (with no LH activity present) resulted in higher concentrations of P_4 compared with the use of a gonadotrophin preparation with both FSH- and LH-like activity (*Bosch et al., 2023*). In order to approach these issues clinically, there is a need to improve understanding of the precise regulation of P_4 production during the human menstrual cycle.

The aim of this study was to evaluate the concentrations of P_4 and 17-OH-P_4 in serum and follicular fluid in three groups of women to investigate whether the relative concentrations of P_4 and 17-OH-P_4 can specify which cell type undertakes P_4 production during the follicular phase of the menstrual cycle in women. Increased understanding may enable interventions to reduce P_4 production in the follicular phase in connection with IVF treatment.

MATERIALS AND METHODS

Patients and sample collection

This study included three groups of women (Groups 1–3), described in previous studies (*Andersen, 2002*; *Poulsen et al., 2019a*,*b*, *2020*, *2022*).

Group 1: Women monitored during a natural menstrual cycle and during an ovarian stimulation cycle

The 31 women in Group 1 had serum hormone concentrations measured during both a natural menstrual cycle and a subsequent stimulated IVF or intracytoplasmic sperm injection (ICSI) cycle, randomized to receive either additional letrozole (5 mg) or placebo (*Poulsen et al., 2022*), as described previously (*Poulsen et al., 2022*) and in the supplementary material.

Group 2: Women monitored during final maturation of follicles

Group 2 included 50 women under the age of 36 years undergoing IVF/ICSI who donated the contents of two follicles, as described previously (*Poulsen et al., 2019a*, *b*, *2020*) and in the supplementary material.

Group 3: Small antral follicles from women who underwent fertility preservation

Group 3 included 12 women aged 18–34 years who underwent fertility preservation prior to gonadotoxic treatment, and gave consent for the use of medulla tissue containing growing follicles for research purposes. The indications for fertility preservation were breast cancer ($n = 6$), Hodgkin lymphoma ($n = 2$), non-Hodgkin lymphoma ($n = 1$), cervical cancer ($n = 1$), acute lymphoblastic leukaemia ($n = 1$) and systemic lupus erythematosus ($n = 1$).

Small antral follicles with diameter <10 mm were aspirated prior to procurement of the cortex. Follicular fluid and granulosa cells were separated by centrifugation at 300 *g* for 2 min, and after the cells had been washed twice in phosphate-buffered saline, follicular fluid and granulosa cells were stored at -80°C for further analysis.

Ethical approval

The use of follicular fluid from women undergoing natural and stimulated cycles was approved by the Scientific Ethical Committee of the Capital Region (H-15021852) and the Danish Health and Medical Agency (2015-005683-41). In addition, registration was completed on clinicaltrials.gov before inclusion (NCT02939898). The use of granulosa cells, follicular fluid and serum samples from women in Group 2 was approved by the Scientific Ethical Committee of Region Zealand, Denmark (SJ-530, approval date 12 May 2016) and the Danish Data Protection Agency. The use of surplus ovarian material was approved by the Scientific Ethical Committee of the Capital Region (H-2-2011-044, approval date 14 June 2020). All participants provided informed consent prior to their inclusion in the study.

Hormone measurement

Group 1

Serum P_4 and 17-OH-P_4 concentrations were measured using an automated Elecsys assay (Roche Diagnostics, Germany) and the UPLC-TQS LC-MSMS system (Waters Corp., USA), respectively, at the Clinical Biochemical Department, University Hospital of Copenhagen, Denmark. The coefficient of variation for P_4 was ≤6% for concentrations >1.9 nmol/l and ≤21% for concentrations ≤1.9 nmol/l. The coefficient of variation for 17-OH-P_4 was ≤8%.

Groups 2 and 3

P_4 and 17-OH-P_4 concentrations in follicular fluid and serum were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits: DNOV006 (NovaTec Immundiagnostica, Germany) for P_4 and RE52071 (IBL International, Germany) for 17-OH-P_4. The coefficients of variation were ≤7% for P_4 and ≤8% for 17-OH-P_4.

Organization of serum hormone measurements

Hormone measurements during the natural cycle were centred around the day of peak serum LH rather than the day of a positive LH urine test, as this resulted in more uniform alignment of the hormonal measurements of the study participants. The length of the menstrual cycle varied between women, so data were grouped as

follows to make them comparable: cycle day 3 (first measurement), cycle day 6 (second measurement), cycle day 9 (third measurement or excluded in the case of a short follicular phase), 3db-LH (2–3 days before LH peak) and LH peak (day of highest recorded LH concentration). In a few women with a follicular phase >15 days, one data point was omitted in the middle of the follicular phase. The luteal phase was categorized by the approximate number of days after the LH peak: LH + 3, LH + 6, etc.

In the ovarian stimulation cycle, the interval preceding ovulation triggering varied between women, resulting in the following data groupings: SD1 (stimulation day 1), SD5 (stimulation day 5–6), 2dbTrigger (2–3 days before ovulation induction) and trigger day (day of ovulation triggering or day before). During the luteal phase, data were grouped based on the time since oocyte retrieval (OCR): OCR, OOCR + 3, OCR + 6, etc.

Microarray analysis of genes from granulosa cells (Group 2)

Microarray data (Gene Expression Omnibus, No. GSE133868) from Group 2 granulosa cells were obtained as described previously (*Poulsen et al., 2020*) and in the supplementary material.

Raw intensity values from the .cel files in the dataset were normalized using the Robust Multi-array Average method. The background subtraction and normalization procedure were executed using the oligo package (version 1.64.1) (*Carvalho and Irizarry, 2010*) in R (version 4.3.1) (*R Core Team*; *RStudio Team*). For gene analysis, the limma package (version 3.56.2) (*Ritchie et al., 2015*) was employed to fit the expression profiles of the samples to linear models, and empirical Bayes methods were used for statistical analysis. Results with an adjusted *P*-value <0.05 and absolute logarithmic fold change (log FC) >1 were considered to be significant. Gene expression was visualized using the ggplot2 package (version 3.4.3) (*Wickham, 2016*).

The leukocyte-specific marker *PTPRC* (CD45) was used to check for possible blood contamination in granulosa cell samples and found to be very low, as were the theca cell marker genes insulin-like 3 (*INSL3*) and actin 2 gamma (*ACTG2*). All adjusted *P*-values were >0.05 and absolute log FC values were <1.

Statistics

Group 1

Comparisons of the P_4:17-OH-P_4 ratio between different time points were made using a linear mixed model fit by Restricted Maximum Likelihood due to the presence of five missing values. The model included a fixed effect of time and a random effect for each participant. Tukey's multiple comparisons test was used for pairwise comparison. The analyses were implemented in R (version 4.2.2), using the *lme4* (version 1.1-31) and *emmeans* (version 1.8.4) packages. Concentrations of P_4 and 17-OH-P_4 were log-transformed prior to analysis. Data are presented as mean (SEM) and median [interquartile range (IQR] in Tables 1 and 2, and Supplementary Tables 2 and 3.

The concentrations of P_4 and 17-OH-P_4 were compared by paired *t*-test between the natural and stimulated cycles by calculating the area under the curve (AUC) (*Pruessner et al., 2003*) for each hormone in the follicular and luteal phases, separated by LH peak or ovulation triggering, thereby assessing their pooled concentration (TABLE 3).

Group 2

All follicular fluid and serum concentrations were log-transformed prior to statistical modelling to ensure a normal distribution. Differences in concentrations between time points were analysed by a mixed model with 'patient ID' as a random factor and 'time' as a repeated, fixed factor using the first-order autoregressive [AR(1)] covariance matrix. A post-hoc test with Bonferroni's correction was used to test differences between the resulting estimated marginal means at each time point. The results of this test have been published previously (*Poulsen et al., 2020*).

The P_4:17-OH-P_4 ratio was compared between serum and follicular fluid with a paired *t*-test at each time point, and compared in groups with and without letrozole co-treatment using two-way mixed analysis of variance. A *P*-value <0.05 was considered significant.

RESULTS

Group 1: Women monitored during a natural menstrual cycle and during an ovarian stimulation cycle

While the concentration of P_4 remained low during the follicular phase and showed the expected rise during the luteal phase, the concentration of 17-OH-P_4 remained constant throughout the natural cycle (TABLE 1). This picture did not change with ovarian stimulation (TABLE 2), although 17-OH-P_4 appeared to become elevated around mid-cycle and in the early luteal phase, especially during ovarian stimulation. When comparing P_4 or 17-OH-P_4 concentrations between the natural and stimulated cycles using AUC, no differences were observed in the follicular phase, while both hormones showed significantly higher secretion during the luteal phase in the stimulated cycle (both *P* <0.001, TABLE 3). It should be noted that the mean antral follicle count at cycle day 2–3 was similar in the natural and stimulated cycles (approximately *n* = 21) (*P* = 0.69)

The P_4:17-OH-P_4 ratios during the natural and stimulated cycles are shown in Tables 1 and 2, Supplementary Tables 2 and 3, and Figures 2 and 3. In both the natural and stimulated cycles, the P_4:17-OH-P_4 ratio changed during the cycle, with the concentration of 17-OH-P_4 being 4–23 times higher than the concentration of P_4 during the entire follicular phase, resulting in a ratio <1. A pronounced shift took place following the mid-cycle surge of gonadotrophins (natural cycle) or the human chorionic gonadotrophin (HCG) trigger (stimulated cycle), where concentrations of P_4 reached much higher levels, consequently increasing the P_4:17-OH-P_4 ratio during the luteal phase (Tables 1 and 2, Supplementary Tables 2 and 3, and Figures 2 and 3). In the luteal phase, however, the P_4:17-OH-$_4$ ratio was much higher in the stimulated cycle (approximately 20–54 fold) compared with the natural cycle (4–12 fold) (FIGURE 3), reflecting the presence of multiple follicles and corpora lutea during ovarian stimulation, and the administration of a bolus trigger of HCG in the stimulated cycle. The P_4:17-OH-P_4 ratio was similar throughout the follicular phase of the natural and stimulated cycles (Tables 1 and 2, Supplementary Tables 2 and 3, and Figures 2 and 3).

Ovarian stimulation – with or without letrozole co-treatment

The concentration of 17-OH-P_4 remained relatively constant throughout the stimulated cycle, with a non-significant increase in the early luteal phase irrespective of whether or not letrozole was administered. However, the concentration of 17-OH-P_4 was higher in

TABLE 1 MEAN AND MEDIAN SERUM CONCENTRATIONS OF PROGESTERONE AND 17-HYDROXY PROGESTERONE, AND PROGESTERONE:17-HYDROXY PROGESTERONE RATIO DURING THE NATURAL MENSTRUAL CYCLE

Serum measurement		Cycle day 3	Cycle day 6	Cycle day 9	3 days before LH peak	LH peak	LH + 3	LH + 6	LH + 9	LH + 12	LH + 15
P_4 (nmol/l)	Mean ± SEM	0.9 ± 0.2	0.7 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	3.5 ± 0.6	29.7 ± 2.7	50.3 ± 2.9	47.1 ± 3.2	27.0 ± 3.9	7.3 ± 2.0
	Median	0.7	0.3	0.3	0.3	3.0	28.5	48.3	47.0	22.1	4.9
	(IQR)	(0.3–1.0)	(0.3–0.6)	(0.3–0.3)	(0.3–0.7)	(1.4–3.9)	(19.5–34.9)	(39.4–59.8)	(33.9–59.0)	(10.7–43.7)	(2.1–9.1)
17-OH-P_4 (nmol/l)	Mean ± SEM	7.2 ± 3.2	10.5 ± 2.7	6.5 ± 2.1	11.1 ± 3.5	9.7 ± 4.6	7.3 ± 2.3	7.8 ± 2.8	3.9 ± 1.0	9.1 ± 3.1	12.9 ± 6.3
	Median	1.4	5.1	3.1	4.6	4.4	2.7	3.1	1.3	2.5	1.2
	(IQR)	(0.9–5.1)	(1.1–12.6)	(1.2–7.0)	(1.4–12.7)	(1.5–6.6)	(1.2–7.1)	(1.2–5.3)	(0.8–6.2)	(1.2–5.4)	(0.8–9.9)
P_4:17-OH-P_4 ratio	Mean ± SEM	0.6 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	1.6 ± 0.3	17.2 ± 3.5	31.2 ± 7.9	34.2 ± 5.5	21.9 ± 6.7	6.5 ± 2.8
	Median	0.3	0.1	0.2	0.1	0.6	12.9	16.3	21.9	8.9	1.9
	(IQR)	(0.1–0.9)	(0.0–0.3)	(0.0–0.3)	(0.0–0.4)	(0.4–3.0)	(4.1–21.9)	(8.4–39.1)	(8.7–51.7)	(1.7–24.1)	(0.3–6.4)

N = 31.

P_4, progesterone; 17-OH-P_4, 17-hydroxy progesterone; IQR, interquartile range.

eight of 10 measurements with letrozole co-treatment. The concentration of P_4 was increased during the luteal phase in both groups, but as the P_4 concentration was higher in the women co-treated with letrozole, the P_4:17-OH-P_4 ratio was almost two-fold higher during the mid-luteal phase (OCR + 6) in the letrozole group compared with the placebo group (Supplementary Tables 2 and 3, and Supplementary Figure 1). The difference in the P_4:17-OH-P_4 ratio did not reach significance between the letrozole group and the placebo group on any single day in the stimulation cycle, as tested by two-way mixed analysis of variance ($P = 0.27$).

No difference in the total number of follicles during the follicular phase was noted between the letrozole group and the placebo group, but there was an increased number of large follicles (>16 mm) in the letrozole group, as described previously by *Poulsen et al. (2022)*.

Group 2: Concentrations of P_4 and 17-OH-P_4 during final maturation of follicles

The concentrations of P_4 and 17-OH-P_4 in follicular fluid and serum are shown in FIGURE 4. Just before induction of the final maturation of follicles (T0), the concentrations of P_4 and 17-OH-P_4 in follicular fluid were both relatively low. During the 36 h of final follicle maturation, the concentration of P_4 in follicular fluid showed a dramatic 25-fold increase ($P < 0.001$), while the concentration of 17-OH-P_4 only increased slightly (less than 50% compared with T0), although this was significant ($P = 0.038$). In contrast, the increase in the serum concentration of P_4 was moderate, around six- to seven-fold higher than at T0 ($P < 0.001$), while the serum concentration of 17-OH-P_4 increased significantly from T0 compared with the other time points ($P < 0.001$), with approximately a three-fold increase from T0 to T12, with levels remaining constant thereafter until T36/OCR (FIGURE 4). This was also reflected in the P_4:17-OH-P_4 ratio, which peaked at T17 in follicular fluid with a 10-fold increase compared with T0 ($P < 0.001$), while the P_4:17-OH-P_4 ratio in serum increased by a factor of 2 ($P < 0.001$). The P_4:17-OH-P_4 ratios in follicular fluid and serum differed significantly at all time points from T12 to T36 (all $P \leq 0.003$).

TABLE 2 MEAN AND MEDIAN SERUM CONCENTRATIONS OF PROGESTERONE AND 17-HYDROXY PROGESTERONE, AND PROGESTERONE:17-HYDROXY PROGESTERONE RATIO DURING A CYCLE WITH OVARIAN STIMULATION, IRRESPECTIVE OF RECEIPT OF LETROZOLE

Serum measurement		SD1	SD5	2 days before trigger	Trigger day	OCR	OCR + 3	OCR + 6	OCR + 10	OCR + 13	OCR + 16
P_4 (nmol/l)	Mean ± SEM	1.1 ± 0.3	0.5 ± 0.1	0.9 ± 0.2	1.7 ± 0.3	26.4 ± 2.6	364 ± 35.5	366 ± 42.4	118 ± 20.9	177 ± 36.9	203 ± 56.1
	Median	0.8	0.3	0.7	1.3	24.7	328	375	72.2	70.9	38.4
	(IQR)	(0.3–0.9)	(0.3–0.7)	(0.3–1.3)	(0.7–2.3)	(19.5–29.6)	(234–385)	(167–528)	(55.4–120)	(45.6–209)	(4.1–377)
17-OH-P_4 (nmol/l)	Mean ± SEM	4.1 ± 1.1	9.9 ± 3.7	11.7 ± 4	12.2 ± 2.9	9.3 ± 4.1	18.6 ± 5.3	6.8 ± 1.8	5.4 ± 1.7	9.2 ± 2.9	12.9 ± 4.8
	Median	1.7	3.4	2.9	5.3	2.5	5.0	2.2	2.1	3.1	1.8
	(IQR)	(1.0–4.8)	(2.3–5)	(1.4–8.7)	(3.5–15.9)	(1.2–6.4)	(1.9–25.2)	(1.1–6.9)	(1.2–6.0)	(1.0–10.4)	(1.1–4.3)
P_4:17-OH-P_4 ratio	Mean ± SEM	0.6 ± 0.1	0.2 ± 0.0	0.6 ± 0.2	0.7 ± 0.3	17.4 ± 3.7	127 ± 29.7	288 ± 70.5	82.7 ± 26.4	105 ± 38.8	64.0 ± 22.3
	Median	0.4	0.1	0.1	0.3	9.5	53.3	97.8	34.7	41.4	6.3
	(IQR)	(0.2–0.8)	(0.1–0.2)	(0.1–0.8)	(0.1–0.5)	(2.6–24)	(11.4–179)	(29.3–361)	(13.9–90.2)	(8.5–88.3)	(2.5–41.8)

n = 31.

P_4, progesterone; 17-OH-P_4, 17-hydroxy progesterone; SD, stimulation day; OCR, oocyte retrieval; IQR, interquartile range.

TABLE 3 COMPARISON OF HORMONE CONCENTRATIONS BETWEEN NATURAL AND STIMULATED CYCLES BY AREA UNDER THE CURVE

Comparison			Mean (SEM) (nmol/l*days)	*P*-value
P_4 AUC follicular phase		Natural cycle	13.3 (2.0)	0.34
		Stimulated cycle	10.8 (1.6)	
P_4 AUC luteal phase		Natural cycle	477.6 (21.5)	<0.001
		Stimulated cycle	3630.1 (338.6)	
17-OH-P_4 follicular phase		Natural cycle	99.6 (15.1)	0.39
		Stimulated cycle	81.7 (13.4)	
17-OH-P_4 luteal phase		Natural cycle	120.8 (19.1)	<0.001
		Stimulated cycle	179.2 (20.0)	

n = 31.

[a]Paired *t*-test.

P_4, progesterone; 17-OH-P_4, 17-hydroxy progesterone; AUC, area under the curve.

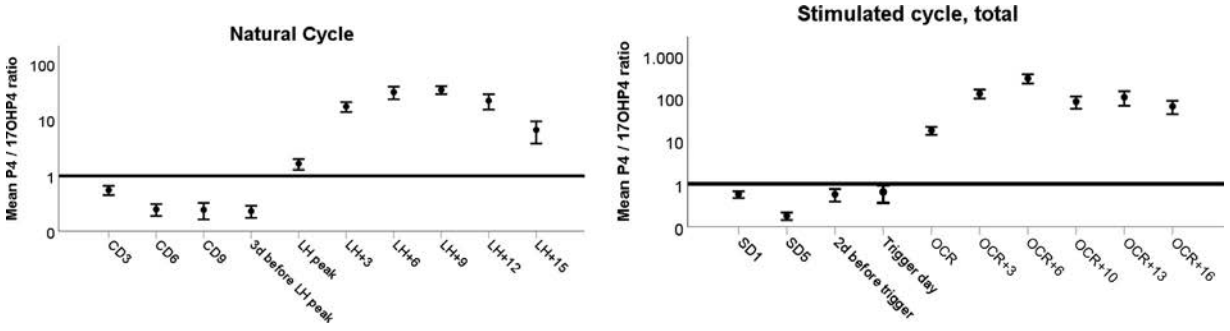

FIGURE 2 Progesterone:17-hydroxy progesterone (P_4:17-OH-P_4) ratio measured during the natural menstrual cycle (*n* = 31) and during an ovarian stimulation cycle (*n* = 31) with exogenous hormones with or without co-administration of an aromatase inhibitor, letrozole. Data are presented as mean ± SEM. CD, cycle day; SD, stimulation day; OCR, oocyte retrieval.

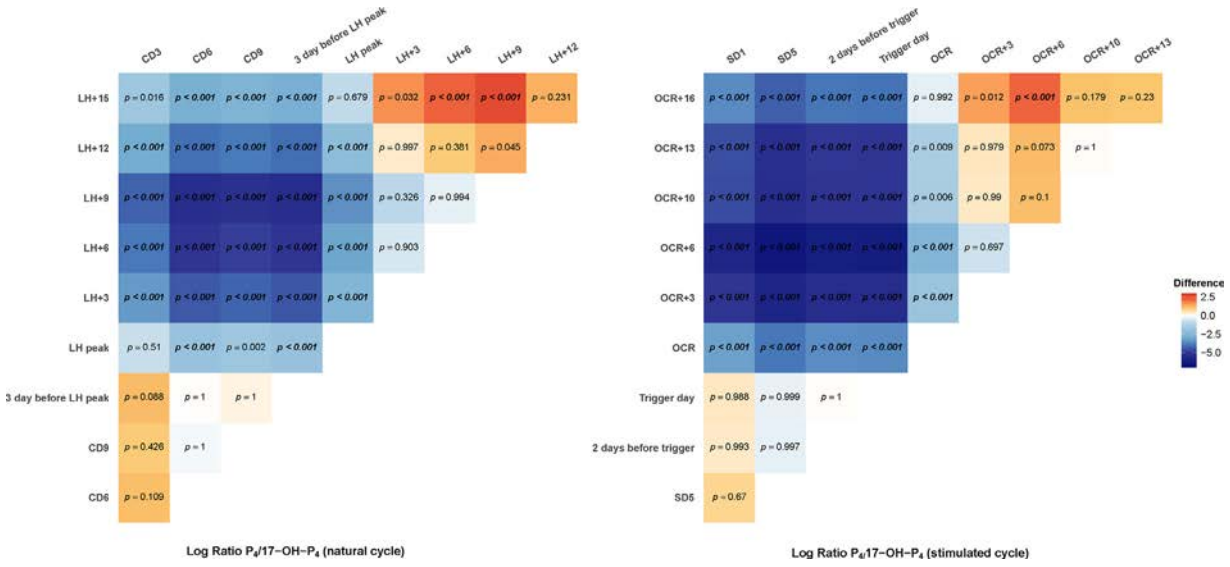

FIGURE 3 Heatmaps of time effects on hormone levels during the natural menstrual cycle and during an ovarian stimulation cycle (log-transformed). The heatmaps visualizing the pairwise differences in marginal means of hormone concentrations between time points are based on a linear mixed effects model. The magnitude of the values is indicated by the intensity of the colours, with darker colours showing larger differences. CD, cycle day; LH peak, mid-cycle surge of gonadotrophins; OCR, oocyte retrieval; SD, stimulation day; trigger day, day of induction of final maturation of follicles.

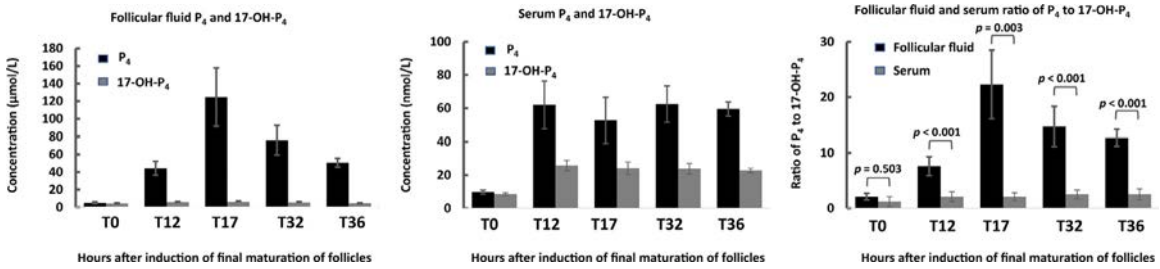

FIGURE 4 Concentrations of progesterone (P_4) and 17-hydroxy-progesterone (17-OH-P_4) (nmol/l in serum, μmol/l in follicular fluid), and the P_4:17-OH-P_4 ratio in serum and follicular fluid in relation to time since induction of final maturation of follicles. $n = 50$. The P_4:17-OH-P_4 ratio was compared between serum and follicular fluid with a paired t-test at each time point. Data are presented as mean ± SEM. T0, T12, T17, T32 and T36 indicate hours since induction of final maturation of follicles. Analysis of variance was used to compare concentrations over time points (17-OH-P_4 in follicular fluid $P = 0.038$, all other $P < 0.001$). Further analysis in Supplementary Table 4.

No significant differences in the measured concentrations of P_4 ($P = 0.132$) and 17-OH-P_4 ($P = 0.602$) were noted between women who received either recombinant FSH or human menopausal gonadotrophin. However, a significant positive effect was noted for HCG triggering compared with agonist triggering for P_4 ($P = 0.001$) and 17-OH-P_4 ($P = 0.028$) in follicular fluid. The significant regulation across time was unaffected by adjustment for the triggering effect. In addition, the choice of trigger did not affect the P_4/17-OH-P_4 ratio significantly in either follicular fluid or serum, leaving the shift in production unaffected. No difference in the number of mature follicles ≥14 mm at the last control visit was observed between the different groups of women, ranging from 12 to 14 ($P = 0.19$).

Gene expression of *HSD3B2* in granulosa cells followed the increase in P_4 in follicular fluid and serum, with peak expression after 12–17 h with a fold-change rise of approximately 2 (absolute Log FC = 1.184, adjusted $P = 0.006$). Expression returned to preovulatory levels after 32 and 36 h (FIGURE 5). The expression of *CYB5A* was significantly up-regulated (almost four-fold) during the ovulatory process, while the expression of *POR*, *LDLR* and *HMGCR* was significantly down-regulated and the expression of *DHCR24* was unaffected compared with T0 (Supplementary Tables 5 and 6).

Group 3: Concentrations of P_4 and 17-OH-P_4 in small antral follicles

The concentrations of P_4 and 17-OH-P_4 were measured in follicles with various diameters <10 mm. These follicles had not been exposed to ovarian stimulation with exogenous hormones. From a total of 23 follicles with diameters of 4–9 mm, the mean concentration of P_4 was 0.22 ± 0.03 μmol/l (median 0.18 μmol/l, IQR 0.06–0.10) and the mean concentration of 17-OH-P_4 was 0.33 ± 0.06 μmol/l (median 0.32 μmol/l, IQR 0.03–1.22) (Supplementary Table 7).

Concentrations in follicular fluid during the follicular phase

Data from Groups 2 and 3 were combined in order to compare the P_4 and 17-OH-P_4 concentrations and the P_4:17-OH-P_4 ratio in follicular fluid during the follicular phase (FIGURE 6).

Although the concentrations of both P_4 and 17-OH-P_4 increased in small antral follicles (approximately 0.22 μmol/l) and preovulatory follicles before ovulation triggering (i.e. T0) (approximately 4.9 μmol/l), the P_4:17-OH-P_4 ratio was low and fairly similar in both groups, except for two outliers in the preovulatory group that likely represented follicles that had already advanced in the ovulatory process (FIGURE 6 and Supplementary Table 7). In contrast, follicles aspirated at OCR, 36 h after ovulation triggering, showed a greater increase in the P_4:17-OH-P_4 ratio, confirming that the shift takes place because of the massive LH-like stimulation that transforms granulosa cells into lutein cells.

DISCUSSION

This study demonstrated a pronounced difference in the concentrations of P_4 and 17-OH-P_4 in follicular fluid and serum during the menstrual cycle, especially in the follicular phase, in relation to synthesis by granulosa cells and theca cells. The relative production of P_4 and 17-OH-P_4 was reflected by the low P_4:17-OH-P_4 ratio in the follicular phase of the menstrual cycle, which changed to a massive surplus of P_4 during the mid-cycle surge of gonadotrophins or HCG trigger, and a resulting high P_4:17-OH-P_4 ratio that continued into the luteal phase, irrespective of whether natural or stimulated cycles were evaluated. The data from both natural and stimulated cycles clearly show that the concentration of P_4 was very low in the first half of the follicular phase, and relatively high CYP17A1 activity in theca cells appears to convert P_4 into 17-OH-P_4 prior to release. This notion is supported by a recent study on the immunohistochemical expression of HSD3B2 and CYP17A1 in human small antral follicles, which co-localize to a minor subset of theca cells (*Zheng et al., 2023*). During the second half of the follicular phase, granulosa cells gradually start to synthesize P_4, as shown by the increase in P_4 concentration in follicular fluid in 10-mm antral follicles and preovulatory follicles (*Kristensen et al., 2017*). It is noticeable that P_4 production, measured as AUC, during the follicular phase is similar irrespective of whether or not ovarian stimulation is performed (Group 1), demonstrating that P_4 produced by granulosa cells in the second half of the follicular phase is mainly accumulated within the follicular fluid, and is only released to the circulation to a limited extent. Following the mid-cycle surge of gonadotrophins, there is pronounced further up-regulation of the HSD3B2 enzyme in granulosa cells, concomitant with strong accumulation of P_4 in follicular fluid, while 17-OH-P_4 is mainly secreted to the circulation. In fact, gene expression of *HSD3B2* in granulosa cells closely mirrors the intrafollicular concentration of P_4 during this period, although *CYB5B* is also up-regulated significantly, enhancing the conversion of pregnenolone to progesterone, while substrate availability in the form of *HMGCR*, *DHCR24*, *LDLR* and *POR* appears to be of less importance.

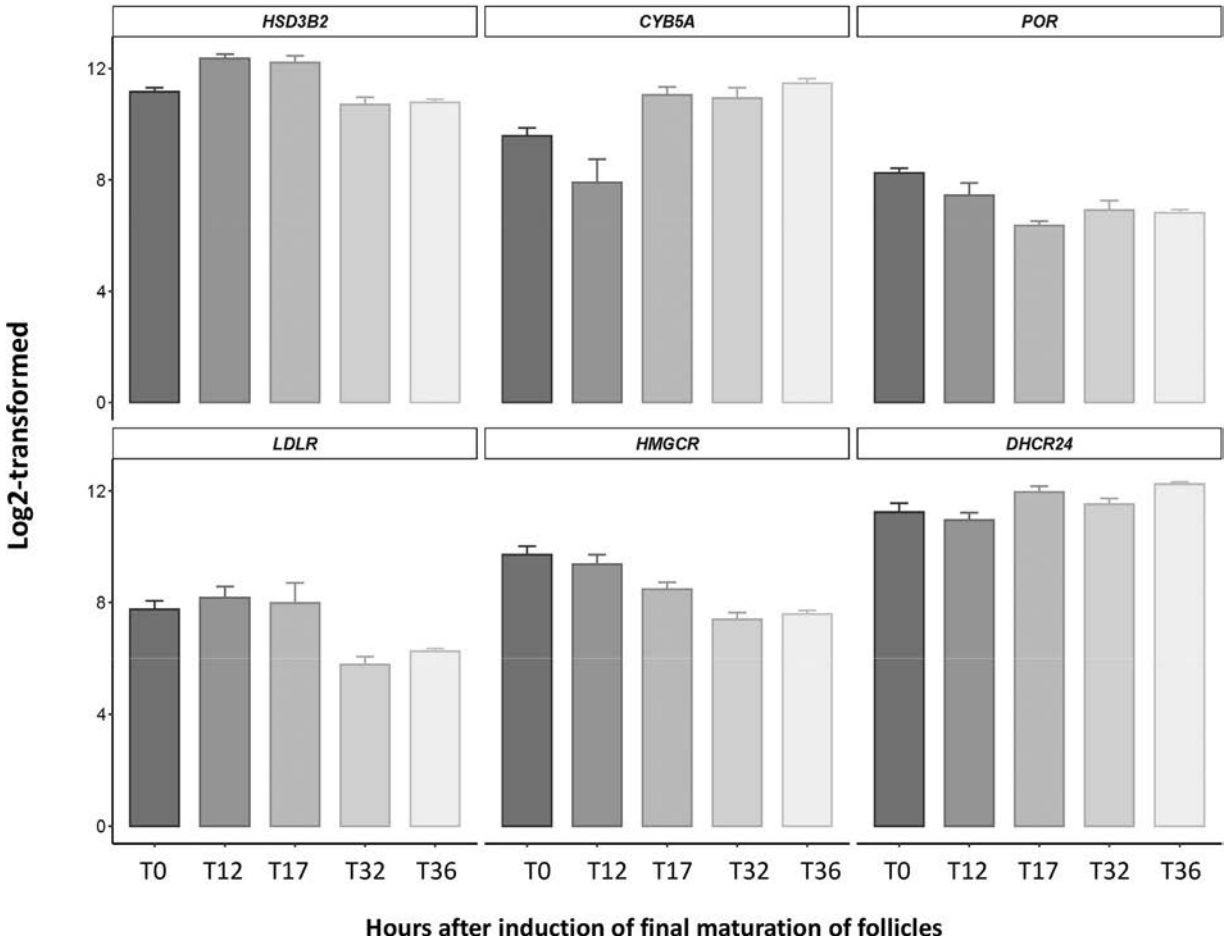

FIGURE 5 Gene expression levels of selected genes in granulosa cells in relation to hours since induction of final maturation of follicles. The mean expression levels of six selected genes at different time points are depicted. *n* = 82. Error bars indicate SEM values. Supplementary Table 5 shows levels of significance, and Supplementary Table 6 shows fold change data. HSD3B2, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2; CYB5A, cytochrome b5; POR, cytochrome P450 reductase; LDLR, low-density lipoprotein receptor; HMGCR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; DHCR24, 24-dehydrocholesterol reductase.

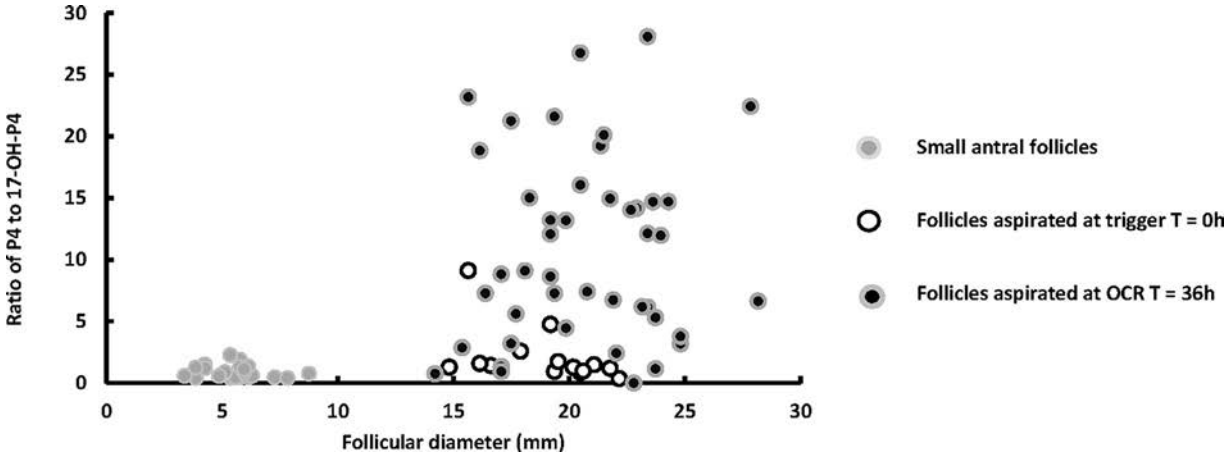

FIGURE 6 Concentrations of progesterone (P_4) and 17-hydroxy-progesterone (17-OH-P_4), and the P_4:17-OH-P_4 ratio in follicular fluid in relation to follicle diameter (mm) from three cohorts of follicles: follicles from small antral follicles aspirated from human ovaries without ovarian stimulation, obtained in connection with a procedure for fertility preservation (*n* = 22); follicles aspirated at the time of administration of the trigger for final maturation of follicles in women undergoing ovarian stimulation (T0) (*n* = 14); and follicles aspirated at the time of oocyte retrieval (OCR) in women undergoing ovarian stimulation (T36) (*n* = 44).

17-OH-P_4 found in follicular fluid must derive from theca cells, as CYP17A1 is not expressed in granulosa cells, entering the follicles by diffusion across the basal lamina. In follicles with diameter <9–10 mm, the concentration of 17-OH-P_4 surpassed that of P_4, suggesting that the production of P_4 by granulosa cells at this point of follicular development is less than that of 17-OH-P_4 accumulated from theca cells. The average serum and follicular fluid concentrations of P_4 and 17-OH-P_4 were similar at T0, in connection with the final maturation of follicles showing a gradient from follicular fluid to serum of approximately 1:500 for both hormones. This is surprising and suggests an equally pronounced increase in the synthesis of 17-OH-P_4 in theca cells and the synthesis of P_4 in granulosa cells. As FSHR are expressed exclusively in granulosa cells, it is postulated that LH acting via LHR, expressed on both cell types after follicle selection, is the main determinant for stimulation of both P_4 and 17-OH-P_4.

A pronounced shift in the P_4:17-OH-P_4 ratio takes place following induction of the final maturation of follicles, where P_4 becomes dominant (FIGURE 4). In the 36 h following ovulation induction, the concentrations of P_4 and 17-OH-P_4 increased significantly in serum, while the increase in the concentration of 17-OH-P_4 in follicular fluid was less pronounced. However, while the P_4:17-OH-P_4 ratio in serum increased from approximately 1 to approximately 2 during the mid-cycle surge of gonadotrophins, the ratio in follicular fluid increased more than 20-fold at 17 h (FIGURE 4), which demonstrates that the massive increase in P_4 secreted by granulosa cells is mainly accumulated in follicular fluid, while 17-OH-P_4 produced by theca cells is secreted into the circulation more readily, likely reflecting the fact that theca cells are vascularized whereas granulosa cells are not.

Collectively, the present data and a recent immunohistochemical study on human follicles (*Zheng et al., 2023*) indicate that theca cells are the main cells producing Δ4 metabolites (i.e. 17-OH-P_4) during the early follicular phase of the menstrual cycle, and there is only minor production of P_4 by either theca cells or granulosa cells. During the second half of the follicular phase, the production of P_4 by granulosa cells increases gradually, and this is mainly accumulated within follicular fluid.

In general, the concentration of 17-OH-P_4 fluctuates considerably during the natural menstrual cycle (*van der Veen et al., 2019*), and can become very low as a result of menopause or the use of oral contraceptives (*Bae et al., 2019*). Earlier studies reported an increase in connection with the mid-cycle surge of gonadotrophins and the early luteal phase (*Aedo et al., 1976*; *Landgren et al., 1977*). A close association between the concentrations of LH-like activity and 17-OH-P_4 has also been reported (*Landgren et al., 1977*). The present data confirm and extend these earlier studies by showing a significant increase in production of 17-OH-P_4 as a result of using HCG to induce supraphysiological concentrations of LH-like activity (Group 1). Furthermore, variability in the concentration of 17-OH-P_4 in women of reproductive age was confirmed in the present study, with a tendency to increase around ovulation and in the early luteal phase.

Letrozole specifically inactivates the aromatase enzyme and down-regulates the production of oestradiol. Previous work using the same samples as those from the present study (Group 1) showed reduced serum concentrations of oestradiol during the follicular phase in women co-treated with letrozole, while concentrations of 17-OH-P_4, androstenedione and testosterone were significantly elevated compared with placebo (*Poulsen et al., 2022*). This may be explained by upstream accumulation of androgens due to reduced production of oestradiol, and/or an increased circulatory concentration of letrozole affecting CYP17A1 activity. However, in contrast, the concentration of P_4 during the follicular phase remained similar regardless of whether or not letrozole was used (*Poulsen et al., 2022*).

During the luteal phase in the stimulated cycle, the multiple corpora lutea and the administration of exogenous HCG and luteal phase support caused concentrations of P_4 to increase as expected, and this is reflected in the P_4:17-OH-P_4 ratio, which was higher for the stimulated cycle compared with the natural cycle. The production of 17-OH-P_4 remained relatively constant. This suggests that luteinized theca cells maintain their production of 17-OH-P_4 during the luteal phase, as CYP17A1 is still not expressed in luteinized granulosa cells (*Zheng et al., 2023*). In addition, it appears that CYP17A1 activation is less sensitive to stimulation with LH-like activity compared with P_4 synthesis in luteinized granulosa cells, irrespective of whether or not ovarian stimulation is performed. However, in the letrozole group, the P_4:17-OH-P_4 ratio was almost twice as high in the mid-luteal phase compared with the placebo group (although this was only of borderline significance). This can be ascribed to a higher concentration of P_4 in this group, probably reflecting a higher circulating concentration of LH in the letrozole group.

The notion of theca cells being more active than granulosa cells in producing Δ4 metabolites in human follicles is supported by a recent observation (*Zheng et al., 2023*) confirming that HSD3B2 is expressed at both gene and protein level just above background levels in granulosa cells from human small antral follicles, showing that steroidogenesis is barely active in these follicles. In contrast, there is strong up-regulation of ovarian-steroidogenesis-related enzymes as the follicle enters the preovulatory stage and becomes selected (*Zheng et al., 2023*). Furthermore, this study showed that small antral follicles express HSD3B2 and CYP17A1 in a subset of theca cells, suggesting the release of 17-OH-P_4 rather than P_4 (*Zheng et al., 2023*), confirming and extending the results of earlier studies (*Breitenecker et al., 1978*; *Dupont et al., 1992*; *Friedrich et al., 1974*; *Kristensen et al., 2017*; *Sanyal et al., 1974*; *Sasano et al., 1990*; *Welt and Schneyer, 2001*). Collectively, these data suggest that the synthesis of P_4 by granulosa cells is relatively modest (or lacking) due to the lack of HSD3B2 expression until a diameter of approximately 10–13 mm, when expression of HSD3B2 starts to take place in granulosa cells, and the production of P_4 by granulosa cells commences (*Zheng et al., 2023*).

The importance of a premature increase in P_4 during the late follicular phase for pregnancy outcomes in women undergoing assisted reproductive technology has been debated intensively over recent years (*Adda-Herzog et al., 2018*; *Bosch et al., 2010*; *Griesinger et al., 2013*; *Kalakota et al., 2022*; *Lawrenz et al., 2018a*,*b*; *Yding Andersen et al., 2011*). It has been postulated that an increasing serum concentration of P_4 in the late follicular phase is associated with negative effects on the endometrium, reducing the implantation potential. To the authors' knowledge, the present study is the first to suggest that the production of P_4 in the late follicular phase is mainly by granulosa cells rather than theca cells. This implies that regulation of P_4 in the late follicular

phase should focus on the activity of granulosa cells. In the late follicular phase, granulosa cells express both FSHR and LHR, and the augmented expression of HSD3B may be a result of the concerted action of both gonadotrophins. Data suggest that the expression of HSD3B is stimulated by LH-like activity (*Thuesen et al., 2012*, *2013*), but the circulatory level of LH is usually low during ovarian stimulation with FSH preparations compared with the natural cycle, whereas the FSH concentration is supraphysiological high and provides strong stimulation of granulosa cells. Although FSH itself causes up-regulation of HSD3B (*Oktem et al., 2017*), a likely contributing factor is that LHR expression induced by FSH makes granulosa cells more sensitive to LH activity, even at low concentrations. However, the similar P_4:17-OH-P_4 ratio during the follicular phase in both natural and stimulated cycles suggests that the supraphysiological concentration of FSH may not be that important. Indeed, receptor expression and the concentrations of ligands may reach ceiling values above which only limited additional synthesis will take place. Rather, the significant increase in the amount of P_4 (and 17-OH-P_4), as measured by AUC, during the luteal phase (**TABLE 3**) may have a negative effect on endometrial–embryo synchrony.

The current study has some limitations. The conclusions are based on correlations between P_4 and 17-OH-P_4, and despite new information on the expression of enzymes active in human ovarian steroidogenesis (*Zheng et al., 2023*), in-vitro experiments with isolated cultures of granulosa cells and theca cells are needed to establish causative relationships. Different assays were used to measure concentrations of P_4 and 17-OH-P_4 in the different groups of patients. This resulted in higher concentrations of P_4 and 17-OH-P_4 measured with ELISA compared with liquid chromatography/mass spectrometry at comparable time points. However, the concentrations of P_4 and 17-OH-P_4 and the P_4:17-OH-P_4 ratio were only compared within groups, with no between-group comparisons; for instance, serum measurements were not compared directly between women in Groups 1 and 2. Further, it would be interesting to follow the expression of HSD3B2 in follicles with diameter from 10 mm to the preovulatory stage, and the corresponding concentrations of P_4 and 17-OH-P_4 in follicular fluid, during natural and stimulated cycles to study how hormonal gradients change between follicles and the circulation.

CONCLUSION

During the menstrual cycle, P_4 is mainly produced by granulosa cells in the second half of the follicular phase and during the luteal phase, while 17-OH-P_4 is produced by theca cells at a relatively constant rate throughout the cycle.

During the follicular phase, (i) theca cells mainly synthesize 17-OH-P_4, and the P_4:17-OH-P_4 ratio is substantially <1; (ii) the secretion of P_4 by theca cells is likely to be low and of limited importance for levels observed in the circulation; and (iii) granulosa cells synthesize P_4 in limited amounts until a follicular diameter of approximately 10–13 mm, where the expression of HSD3B2 becomes up-regulated, increasing P_4 production. P_4 appears to be mainly accumulated in follicular fluid.

A reduction in P_4 output in stimulated cycles should focus on regulation of the function of granulosa cells, but the concerted action of both FSH and LH on granulosa cells likely makes this difficult.

This study has clarified new aspects of human ovarian steroidogenesis during natural and stimulated cycles, and provided new information on how P_4 production is controlled during ovarian stimulation. This study suggests that the P_4:17-OH-P_4 ratio may be used to show the relative activity of theca cells and granulosa cells in the production of Δ4 metabolites.

DATA AVAILABILITY

Data will be made available on request.

AUTHOR CONTRIBUTIONS

CYA conceived the idea and planned the study. MXZ, LCP and CYA performed data analyses, interpreted data and drafted the manuscript. NFW, LSM, MLJ, BS, MLG, KL, ALME and SOS were involved in the interpretation of the results. All authors critically revised the manuscript and approved the final version.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.103853.

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RBMO

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ARTICLE

Living in a low socioeconomic status neighbourhood is associated with lower cumulative ongoing pregnancy rate after IVF treatment

BIOGRAPHY

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KEY MESSAGE

Low neighbourhood socioeconomic status (SES) is associated with reduced odds of achieving an ongoing pregnancy within 2.5 years of IVF treatment compared with high neighbourhood SES. This underscores that, even when fertility care access is equitable, individuals residing in a low SES neighbourhood face disadvantages.

ABSTRACT

Research question: Does an association exist between neighbourhood socioeconomic status (SES) and the cumulative rate of ongoing pregnancies after 2.5 years of IVF treatment?

Design: A retrospective observational study involving 2669 couples who underwent IVF or IVF and intracytoplasmic sperm injection treatment between 2006 and 2020. Neighbourhood SES for each couple was determined based on their residential postal code. Subsequently, SES was categorized into low (<p20), medium (p20–p80), and high (>p80). Multivariable binary logistic regression analyses were conducted, with the cumulative ongoing pregnancy within 2.5 years as the outcome variable. The SES category (reference category: high), female age (reference category: 32–36 years), body mass index (reference category: 23–25 kg/m^2), smoking status (yes/no), number of oocytes after the first ovarian stimulation, embryos usable for transfer or cryopreservation after the first cycle, duration of subfertility before treatment and insemination type were used as covariates.

Results: A variation in ongoing pregnancy rates was observed among SES groups after the first fresh embryo transfer. No difference was found in the median number of IVF treatment cycles carried out. The cumulative ongoing pregnancy rates differed

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KEYWORDS

fertilization in vitro
health services accessibility
infertility
low socioeconomic status
pregnancy outcome
social class

significantly between SES groups (low: 44%; medium: 51%; high: 56%; $P < 0.001$). Low neighbourhood SES was associated with significantly lower odds for achieving an ongoing pregnancy within 2.5 years (OR 0.66, 95% CI 0.52 to 0.84, $P < 0.001$).

Conclusion: Low neighbourhood SES compared with high neighbourhood SES is associated with reducing odds of achieving an ongoing pregnancy within 2.5 years of IVF treatment.

INTRODUCTION

Subfertility is defined as the inability to achieve a clinical pregnancy after 12 months of regular unprotected intercourse (*Zegers-Hochschild et al., 2009*). IVF is a well-established treatment for subfertility. Subfertility is experienced by one in six couples, making it a substantial public health concern (*Boivin et al., 2007*). Despite years of research and experience in the field of IVF, relatively modest results have been reported, with a global reported pregnancy rate of 34.6% after embryo transfer (*Wyns et al., 2021*).

Alongside established factors affecting subfertility and IVF outcomes, such as female age and body mass index (BMI) (*Rittenberg et al., 2011*), interest in exploring the potential influence of socioeconomic status (SES) is growing (*Imrie et al., 2021*). It has been suggested that lifestyle factors can affect subfertility and the success rate of IVF (*Rooney and Domar, 2014*). Within published research, variables such as educational attainment, occupation and ethnic background are often used either individually or collectively as indicators for SES (*Imrie et al., 2021*). This categorization allows for a better understanding of the profile of subfertile patients and provides a framework for investigating its potential effect on IVF outcomes.

One approach to characterize the SES of a subfertile patient is by using Neighbourhood SES, which classifies patients based on the average SES factors of their residential neighbourhoods (*CBS-Netherlands, 2016*). This can be used as an indicator for the living environment and conditions (*Burgos Ochoa et al.,2021*). Low neighbourhood SES exerts a detrimental effect on overall health and wellbeing, a phenomenon evident during pregnancy and in the initial stages of life. It is shown that maternal and perinatal mortality and morbidity are associated with neighbourhood SES (*de Graaf et al., 2013*; *Bertens et al., 2020*; *Burgos Ochoa et al., 2021*). Additionally, suboptimal fetal growth and development are associated with low SES (*Silva et al., 2010*; *Ball et al., 2013*; *Gootjes et al., 2019*; *Lu et al., 2021*). Moreover, specific adverse pregnancy outcomes, such as spontaneous miscarriage, preeclampsia, preterm delivery, large for gestational age infants, low birth weight, caesarean delivery and obstetrical haemorrhage, have been reported more frequently in woman with a low individual SES (*Silva et al., 2008*; *Kim et al., 2018*; *Amjad et al., 2019*, *Keenan-Devlin et al., 2022*).

The possible association between SES and IVF outcomes has received limited attention. A systematic review identified six studies specifically addressing the potential effects of SES on fertility treatment outcome (*Imrie et al., 2021*). Recent investigations have produced findings that contradict any substantial influence of SES on IVF outcomes (*Liu et al., 2021*; *Veira et al., 2022*). Nevertheless, an independent role for ethnicity in determining IVF and intracytoplasmic sperm injection success was suggested by two studies (*Patel et al., 2016*; *Andre et al., 2023*). Another recent study reported a higher risk of spontaneous miscarriage outcomes among pregnancies after embryo transfer for unemployed woman and couples living in non-capital areas (*Kim et al., 2023*). In addition, a large study showed that, among socioeconomically advantaged women in high-income countries, all forms of medically assisted reproduction (MAR) more commonly result in live births (*Goisis et al., 2023*).

IVF treatment can be considered costly; therefore, financial resources may account for some of the found differences in IVF outcomes between SES categories. Since the start of IVF treatment in the Netherlands, however, three cycles of IVF treatment have been reimbursed by the mandatory health insurance. This enables every couple to partake in IVF treatment, irrespective of their ability to pay for it. This makes the Netherlands a unique setting to further study the association between SES and IVF outcomes. Therefore, our aim was to investigate the association between neighbourhood SES and cumulative ongoing pregnancy within 2.5 years after the first IVF treatment. Neighbourhood SES was categorized into high (>p80), medium (p20–p80) and low SES (<p20) groups for analysis.

MATERIALS AND METHODS

Study design, participants and setting

In this retrospective cohort study, clinical data from IVF treatments carried out between 2006 and 2020 at the IVF centre of Erasmus MC, University Medical Centre, Rotterdam, were used (FIGURE 1). Individual electronic patient records were used for data on fertility treatments. Neighbourhood SES was used as an indicator for living environment and conditions of the couples undergoing IVF treatment.

Neighbourhood SES scores are calculated by the Netherlands Institute for Social Research (*CBS-Netherlands, 2016*) per four digit postal-code area (comprising around 4000 inhabitants or covering an area of approximately 5.3 km^2). These scores are based on household income, type of employment and educational attainment (*CBS-Netherlands, 2016*; *Burgos Ochoa et al., 2021*). The SES scores were grouped into quintiles, and categorized as follows: low SES (first quintile of neighbourhood SES score), medium SES (second to fourth quintile of neighbourhood SES score) and high SES (fifth quintile of neighbourhood SES score). These neighbourhood SES scores are updated and published every 4 years, and the SES score published closest to the start date of the couple's first IVF cycle was used.

It is worth noting that some couples might have changed residences during their treatment, potentially exposing them to different levels of neighbourhood SES. Unfortunately, these couples could not be identified, as only the most recent postal code was available to us. Research has shown that people typically move within a similar area (*Bilal et al., 2019*). In a previous study using the same neighbourhood SES data, a sensitivity analysis was conducted, omitting individuals who had moved, and it revealed no effect on the results (*Burgos Ochoa et al.,2021*). Therefore, the results of our study are unlikely to be influenced

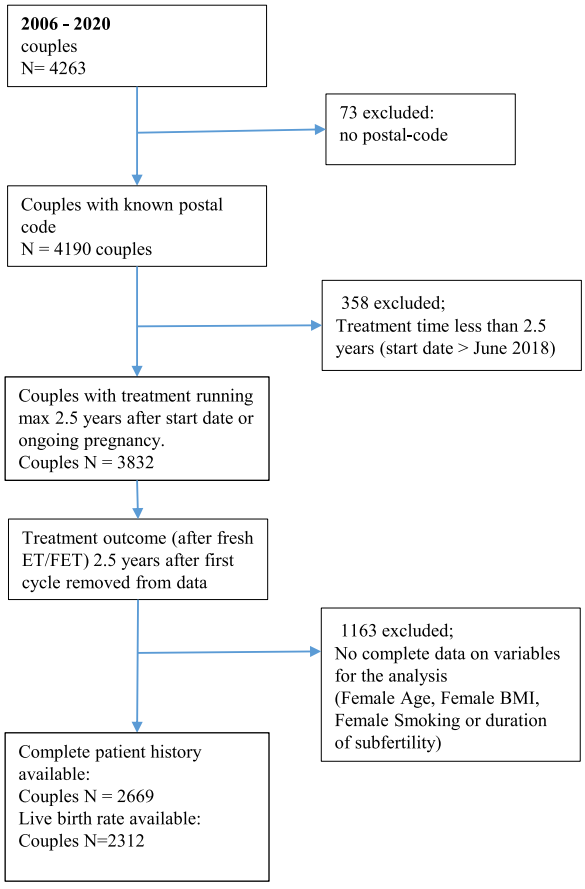

FIGURE 1 Included and excluded cycles. BMI, body mass index; ET, embryo transfer; FET, frozen embryo transfer; ICSI, intracytoplasmic sperm injection.

by patients who relocated to another SES area during the study period.

At the start of IVF treatment, information about smoking, BMI and subfertility was registered in the patient file by the gynaecologist. Treatment outcomes were proactively collected by health professionals to minimize the risk of under-reporting. Therefore, missing data are more likely to be linked to the responsible physician than to SES category.

IVF procedure

Women underwent routine ovarian stimulation using either a gonadotrophin-releasing hormone agonist or antagonist co-treatment protocol with recombinant FSH: Puregon (Organon, Oss, the Netherlands); Gonal-F (Merck Serono, Aubonne, Switzerland); Menupur (Ferring, St Prex, Switzerland); Bemfola (Gedeon Richter, Dilbeek, Belgium); or Rekovelle® (Ferring, St Prex, Switzerland) (*Eijkemans et al., 2006*; *Nyboe Andersen et al., 2017*) or highly purified urinary FSH (Menopur, Ferring, St Prex, Switzerland). The final follicular maturation was triggered using human recombinant HCG: Ovitrelle® (Merck Serono, Aubonne Switzerland); Pregnyl® (Organon, Oss, the Netherlands) or decapeptyl (Ferring, Parsippany, NJ, USA). After oocyte retrieval, IVF was carried out at the IVF laboratory of the Erasmus MC, University Medical Centre, Rotterdam. Standardized procedures were followed for the insemination of oocytes and embryo culture, as previously described (*van Marion et al., 2022*). Embryo selection for fresh transfer and cryopreservation was based on morphological assessment (*Alpha Scientists in Reproductive and Embryology, 2011*; *van Marion et al., 2021*). Single embryo transfer (SET) was the standard practice with the possibility of double embryo transfer (DET) (13.7% DET in the first cycle, with a declining trend from 2006 (29.4%) to 2018 (10.5%).

For this study, consecutive fresh and frozen embryo transfers resulting from a single ovarian stimulation were defined as one IVF cycle. Multiple IVF cycles within their IVF treatment could be undergone by couples.

Outcome variable

The primary outcome variable, cumulative ongoing pregnancy, was defined as the first ongoing pregnancy resulting from consecutive cycles within a maximum time frame of 2.5 years after the start of the first IVF cycle. Ongoing pregnancy was confirmed by a detected fetal heartbeat on ultrasound at 12 weeks of gestation. Couples with a confirmed ongoing pregnancy were censored from the dataset if they had undergone a subsequent IVF cycle or frozen embryo transfer.

A 1-year period is sufficient for assessing cumulative pregnancy rates (*Collins et al., 1995*; *Taylor, 2003*); however, a substantial number of couples had extended intervals without treatment for reasons unrelated to medical factors. After 1 year, 32% of the non-pregnant group remained in treatment, and this proportion decreased to 9.4% after 2.5 years. Therefore, a 2.5-year duration was chosen to determine the cumulative ongoing pregnancy rate within the database.

Live birth rates were not available for all treatments and were not considered as main outcome. They were, however, used in an additional analysis involving a subset of the data (n = 2312 couples). Maternal age, body mass index (BMI) and smoking status are potential confounding factors affecting the exposure and outcome variables, so they were added as covariates in the multivariable binary logistic regression model. Other covariates included the number of oocytes retrieved after the initial ovarian stimulation, the number of embryos suitable for transfer or cryopreservation after the first cycle, the insemination method (IVF or ICSI), and the duration of subfertility before treatment. These factors are likely to influence the success of IVF treatment and, consequently, the outcome variable; however, they cannot be considered confounders as their association with the exposure, e.g. neighbourhood SES, has not been researched and is not present in the dataset. Couples with incomplete or incorrect data on any of these covariates were excluded.

Female age and BMI were initially reported as continuous variables but were included in the model as categorical variables. Body mass index was rounded up to two decimal places. Smoking status, originally reported as the number of cigarettes smoked per day, was coded as either smoking (yes) or non-smoking (no).

In descriptive analyses, results from the first cycle were presented per SES group, including fertilization rate (rate of normally fertilized zygotes/oocyte retrieved), sperm concentration, motile sperm count (defined as the concentration progressive motile sperm cells x semen volume), number of oocytes obtained after the first ovarian stimulation, number of embryos usable for embryo transfer or cryopreservation, biochemical pregnancy after the first fresh transfer (defined as a positive urinary β-HCG test 10–12 days after the embryo transfer) and ongoing pregnancy after the first fresh transfer (defined as a fetal heartbeat on ultrasound at 12 weeks of gestation).

Missing data

From the entire dataset of couples (n = 4263), some were excluded from the analysis (FIGURE 1). First, 73 couples without a known postal code were excluded (1.7%). Next, couples with a first cycle after June 2018 were also excluded (n = 358 [8.4%]) to ensure that every couple had sufficient time to complete 2.5 years of treatment within the study period. Finally, couples with incomplete data on the variables were excluded (n = 1163 [27%]). This resulted in 2669 couples with complete data for our analysis. For the repeated analysis involving live births, an additional 357 couples were excluded owing to missing data on the outcome variable.

Individual SES characteristics, such as education and ethnicity, were only available in a limited sample. The main infertility diagnosis was not available in the data. Therefore, these variables could not be included in any analysis.

Statistical analysis

Baseline data were presented using descriptive analyses, including mean and SD or median and interquartile range for continuous variables, and number and percentages for categorical data. Continuous data were compared using a Mann–Whitney U test. Chi-squared test and Fisher's exact test were used to analyse the categorical data. Multivariable binary logistic regression analysis was conducted with cumulative ongoing pregnancy as outcome variable and SES categories as the main determinant. High SES served as reference category. In addition to SES categories, potential confounders included female age, female BMI, number of oocytes after the first ovarian stimulation, usable embryos after the first cycle, duration of subfertility before treatment, insemination method (IVF or ICSI) and female smoking. Female age and female BMI had a non-linear relationship with the outcome, particularly in the lower and upper ends of the distribution. Therefore, they were included as categorical covariates. The chosen BMI categories are narrower than that typically used, but they were selected to better represent the variation present in our data (BMI category: <22, 23–25, 26–28, >29) with BMI category 23–25 as a reference category. For age, the categories were defined as younger than 31 years, 32–36 years and older than 37 years, with the age group 32–36 years as the reference category.

Only couples with complete data for the variables in the model were included in this analysis.

In addition, the analysis was repeated in a subset of the data (n = 2312 couples), with cumulative live birth as the outcome variable. IBM SPSS Statistics 28.0.1.0. software (IBM, Almonk, NY, USA) was used for statistical analyses.

Ethical approval

The Medical Ethical Committee of the Erasmus MC examined the study protocol and issued a waiver for the Medical Research Act (MEC-2022-0434, dated 15 July 2022), so no formal consent was needed.

RESULTS

Participants

Between 2006 and 2020, a total of 4263 couples underwent IVF or IVF/ICSI treatment. A total of 2669 couples with complete postal-code information and patient characteristics (female age, BMI, smoking status and duration of subfertility) were included. These couples had all undergone treatment for up to 2.5 years (FIGURE 1). Throughout this period, the ratio between high and low SES couples remained stable, with about 23% of couples residing in low SES neighbourhoods. Baseline characteristics of the included treatments are presented in TABLE 1. Female BMI, female smoking and the duration of subfertility were significantly higher in the low neighbourhood SES group ($P < 0.001$, $P = 0.01$ and $P < 0.001$, respectively). Sperm parameters did not differ between SES groups, as well as the median number of started IVF cycles (TABLE 2).

Treatment outcome after the first cycle

After the first fresh IVF cycle, no significant differences were observed in fertilization rate, the number of embryos available for fresh transfer or cryopreservation after the first cycle, biochemical pregnancy, or miscarriages among the three SES groups. A small difference was noted in the mean number of oocytes retrieved (low SES: 8.42 ± 0.14; high SES: 8.58 ± 1.33; $P = 0.03$, pairwise value for low vs high).

TABLE 1 BASELINE CHARACTERISTICS OF INCLUDED COUPLES

Start of treatment	SES low	SES medium	SES high	Total	*P*-value
Total included	632 (23.7)	1434 (53.7)	603 (22.6)	2669 (100)	
Female age, years	34.1 (± 5.2)	33.9 (± 5.0)	34.1 (± 4.7)	34.0 (± 5.0)	0.56
Female body mass index, kg/m^2	24.8 (± 4.5)	24.2 (± 4.2)	23.8 (± 3.9)	24.3 (± 4.2)	<0.001
Female smoking	164 (25.9 %)	319 (22.2)	113 (18.7)	596 (22.0)	0.01
Duration of subfertility, months	47 (24–60)	41 (23–51)	39 (23–49)	42 (23–53)	<0.001

Each treatment is derived from a unique patient couple.

Data are presented as number (%), mean (± SD) or median (interquartile range). Continuous data were compared using a Mann–Whitney U test. Chi-squared test and Fisher's exact test were used to analyse the categorical data.

SES, socioeconomic status.

TABLE 2 OUTCOME CHARACTERISTICS OF THE FIRST IVF CYCLE AND CUMULATIVE OUTCOME AFTER 2.5 YEARS OF TREATMENT

Characteristics	SES low	SES medium	SES high	Total	*P*-value
Total included	632 (23.7)	1434 (53.7)	603 (22.6)	2669 (100)	
Started cycles per treatment	2 (1–3)	2 (1–3)	2 (1–3)	2 (1–3)	0.23
Sperm concentration in ejaculate during first cycle, 10^6/ml					
IVF	45 (23–75)	44 (24–75)	50 (26–81)	45 (24–77)	0.22
ICSI	2.3 (0.7–10)	2.0 (0.4–7.4)	2.0 (0.4–8.0)	2.1 (0.4–8.0)	0.21
Motile sperm count in ejaculate (VCM) during first cycle					
IVF	58 (29–120)	63 (26–125)	64 (25–140)	60 (27–126)	0.67
ICSI	1.2 (0.2–4)	0.9 (0.1–4)	0.5 (0.1–3)	0.5 (0.1–3)	0.18
Number of oocytes retrieved after first ovarian stimulation	7 (4–11)	7 (4–11)	7 (4–11)	7(4–11)	0.03
Fertilization rate of first cycle (number 2PN per oocyte), %					0.64
0–25	93 (17.7)	230(19.4)	106 (20.4)	429 (19.2)	
25–50	151 (28.8)	309(26.1)	125 (24.1)	585 (26.3)	
50–75	157 (30.0)	369(31.2)	155 (29.9)	681 (30.6)	
75–100	123 (23.5)	275(23.2)	132 (25.5)	530 (23.8)	
Number of embryos (for embryo transfer or cryopreservation after the first cycle)	1 (0–3)	1(0–3)	1 (1–3)	1 (0–3)	0.19
Biochemical pregnancy (HCG) first cycle (fresh embryo transfer)					
Yes	150 (23.7)	360 (25.1)	164 (27.2)	674 (25.3)	0.08
Ongoing pregnancy first cycle					
Yes	108 (17.1)	262 (18.3)	126 (20.9)	496 (18.6)	0.03
12-week pregnancy loss first cycle					
Yes	42 (28.0)	98 (27.2)	38 (23.2)	178 (26.4)	0.51
Cumulative ongoing pregnancy within 2.5 years					
Yes	275 (43.5)	734 (51.2)	337 (55.9)	1346 (50.4)	<0.001
Cumulative live birth rate within 2.5 years	194 (36.5)	560 (44.6)	267 (50.8)	1021 (44.2)	<0.001
Missing	101 (16.0)	179 (12.5)	77 (12.8)	357 (13.4)	

Each treatment is derived from a unique patient couple.

Data are presented as number (%) or median (interquartile range). Mann–Whitney U test was used to compare continuous data. Chi-squared test and Fisher's exact test were used to analyse categorical data.

ICSI, intracytoplasmic sperm injection; SES, socioeconomic status; VCM, volume–concentration–motility (amount of progressive motile sperm in whole ejaculate); 2PN, two pronuclei.

Also, the ongoing pregnancy outcome (low SES: 17%; medium SES: 18%; high SES: 21%; $P = 0.03$) varied between the SES groups (TABLE 2).

Cumulative treatment outcome

The cumulative ongoing pregnancy rate had significant variations among the three SES groups (SES low 44% versus SES medium 51% versus SES high 56%; $P < 0.001$).

The main analysis showed a significantly lower odds ratio for cumulative ongoing pregnancy for the low SES groups (OR 0.66, 95% CI 0.52 to 0.84, $P < 0.001$) compared with the high SES group. Notably, the inverse association of cumulative ongoing pregnancy rate with low SES was independent of the covariates female age, BMI and smoking and the number of oocytes after the first ovarian stimulation or number of embryos transferred or cryopreserved after the first cycle, duration of subfertility and insemination method (TABLE 3).

Additional analysis with cumulative live birth rates as outcome ($n = 2312$ couples) consistently demonstrated a significant association ($P < 0.001$) between low neighbourhood SES and lower live birth rate (TABLE 4). Missing data for live birth was slightly higher for the low neighbourhood SES group (low SES 16%, high SES 13%)

DISCUSSION

The study findings suggest that residing in low SES neighbourhoods is associated with a reduced likelihood of achieving an ongoing pregnancy within 2.5 years after starting IVF treatment. Furthermore, this association extends to live birth rates, as demonstrated in a subset of patients.

Existing research on the association between SES in general and IVF outcome has yielded conflicting results. Two recent studies (*Liu et al., 2021*; *Veira et al., 2022*) found no effect of SES on ongoing pregnancy rates, whereas a systematic review on the topic revealed contradictory findings among individual studies (*Imrie et*

TABLE 3 OUTCOME OF THE MULTIVARIABLE BINARY LOGISTIC REGRESSION ANALYSIS IN 2669 COUPLES, WITH CUMULATIVE ONGOING PREGNANCY AS THE OUTCOME VARIABLE AND SOCIOECONOMIC STATUS CATEGORIES AS MAIN DETERMINANT

			95% CI for OR	
Variable	**OR**	***P*-Value**	**Lower**	**Upper**
SES				
High	Reference			
Low	0.66	<0.001	0.52	0.84
Medium	0.87	0.18	0.71	1.07
Female age, years				
32–36	Reference			
<31	1.14	0.19	0.94	1.40
>37	0.43	<0.001	0.35	0.53
Female BMI, years				
23–25	Reference			
<22	1.18	0.11	0.96	1.44
26–28	0.87	0.27	0.67	1.12
>29	0.73	0.02	0.57	0.94
Female smoking	0.79	0.02	0.65	0.96
Duration of subfertility, quartile	Reference			
2	0.99	0.98	0.79	1.25
3	1.03	0.80	0.82	1.30
4	0.85	0.18	0.68	1.08
Number of oocytes (first stimulation)	1.07	0.44	0.99	1.03
Number of embryos used for embryo transfer/ cryopreservation (first cycle)	1.23	<0.001	1.17	1.30
Insemination method (ICSI/IVF)	1.25	0.01	1.05	1.49

Female age, female BMI, female smoking, duration of infertility, number of oocytes after the first ovarian stimulation, number of usable embryos after the first cycle and fertilization method were included as potential confounders.

$P < 0.05$ was considered significant.

BMI, body mass index; ICSI, intracytoplasmic sperm injection; SES, socioeconomic status.

al., 2021). In the present study, a difference in ongoing pregnancies between the neighbourhood SES groups was found after the first cycle. When using cumulative ongoing pregnancy within a time frame of 2.5 years, the disparities became more pronounced. Research shows that, in both fertile and subfertile populations, conception can take up to several months or longer (*Collins et al., 1995*; *Taylor, 2003*; *Garrido et al., 2011*). Furthermore, cumulative IVF outcomes are the preferred measures when reporting on IVF success (*Tiitinen et al., 2004*; *Maheshwari et al., 2015*; *Rienzi et al., 2021*). Altogether, in our view, pregnancy outcomes should be assessed after a longer period instead of just looking at one cycle. Conflicting results with earlier studies might be explained by the different approach. This approach showed that the association between neighbourhood SES and IVF outcome becomes clearer when using cumulative IVF outcomes.

Financial status is frequently cited as a contributory factor to diminished MAR, and specifically IVF treatment outcomes within low SES groups, with the assumption that couples with low financial status may not be able to afford as many IVF cycles (*Chambers et al., 2014*). A recently published study has challenged this assumption by revealing that household income and IVF insurance coverage are not associated with pregnancy, pregnancy loss or live birth (*Chung et al., 2022*). In the present study, the financial argument is essentially non-existent because, in the Netherlands, health insurance is mandatory, and this insurance includes the reimbursement of three IVF cycles for every patient. In the present study, we found no difference in the average number of cycles completed within 2.5 years between the different SES groups. A small portion (7.4%) of our patients, however, continued treatment after the last reimbursed treatment. When reanalysing our data with only reimbursed cycles, the odds ratio is similar (data not shown), confirming that the number of IVF cycles had no effect on the association found. Altogether, this implies that financial barriers to IVF access are less likely to explain the observed differences in outcome in the present study.

Financial status, however, may indirectly affect lifestyle behaviours that affect health. Lifestyle involves health risk behaviours, such as smoking and alcohol consumption, and also health promoting behaviours, such as physical exercise, eating healthy food and stress management (*Walker et al., 1987*; *Pronk et al., 2004*; *Morawa and Erim, 2018*). The range of possible choices can be determined by an individual's SES status (*Cockerham, 2010*; *Cockerham WC, 2016*). In the present study, only smoking as a health risk behaviour and BMI as a proxy of health risk behaviour could be included. Both were significantly higher for the low SES group. Our analyses, however, showed that the association of neighbourhood SES and IVF outcome persisted independent of female BMI, smoking and age. We cannot rule out that lifestyle factors have influenced our observations, as we only had information about smoking status and BMI.

The duration of subfertility is a known factor influencing pregnancy chance (*van Loendersloot et al., 2010*). A longer pre-treatment time might allow selection of a more severe subfertile population. It was found in this study that a longer duration of subfertility did not significantly affect the odds ratios of low neighbourhood SES on cumulative ongoing pregnancy. Interestingly, a significantly longer duration of subfertility was observed in the low SES group compared with the high SES group. This suggests that, even with equal financial access to reproductive care, patients within the low SES group may experience a delay in starting their treatment. This delay could be due to other life stressors or a lack of awareness about fertility and available treatment options.

Mechanisms explaining the association between neighbourhood SES and IVF outcomes are likely multifactorial. One

TABLE 4 OUTCOME OF MULTIVARIABLE BINARY LOGISTIC REGRESSION ANALYSIS OF 2312 COUPLES, WITH CUMULATIVE LIVE BIRTH AS THE OUTCOME VARIABLE AND SOCIOECONOMIC CATEGORIES AS MAIN DETERMINANT

			95% CI for OR	
Variable	**OR**	***P*-value**	**Lower**	**Upper**
SES				
High	Reference			
Low	0.63	<0.001	0.48	0.82
Medium	0.81	0.74	0.66	1.02
Female age, years				
32–36	Reference			
<31	1.22	0.07	0.98	1.51
>37	0.39	<0.001	0.31	0.49
Female BMI, years				
23–25	Reference			
<22	1.17	0.15	0.95	1.46
26–28	0.74	0.04	0.56	0.98
>29	0.65	0.03	0.49	0.87
Female smoking	0.70	0.001	0.56	0.87
Duration of subfertility, quartile	Reference			
2	1.00	0.98	0.78	1.28
3	0.94	0.94	0.73	1.21
4	0.73	0.02	0.57	0.95
Number of oocytes (first stimulation)	1.00	0.87	0.98	1.02
Number of embryos used for embryo transfer/ cryopreservation (first cycle)	1.22	<0.001	1.16	1.29
Insemination method (ICSI/IVF)	1.33	0.003	1.10	1.61

Female age, female body mass index (BMI), female smoking, duration of subfertility, number of oocytes after the first ovarian stimulation, number of usable embryos after the first cycle and fertilization method were included as potential confounders.

$P < 0.05$ was considered significant.

ICSI, intracytoplasmic sperm injection; SES, socioeconomic status.

explanation may be offered by chronic exposure to negative environmental factors. Individuals in a low SES environment may experience higher levels of perceived stress (*Algren et al., 2018*). Numerous stress factors are known to affect health, i.e. overcrowding, noise pollution, bad housing quality and high crime rates, which affects mental and physical health (*Middleton, 1998*; *Fabio et al., 2011*; *Barbaresco et al., 2019*; *Nkosi et al. 2019*; *Singh et al., 2019*). An effect of stress on implantation has been reported (*deCatanzaro, 2011*); however, our data do not directly support this. Several studies have reported a lower pregnancy rate in patients with higher stress levels (*Smeenk et al., 2005*; *Turner et al., 2013*; *Aimagambetova et al., 2020*). There are indications that social stress during the prenatal period and in childhood results in higher inflammation in later life (*Pedersen et al., 2018*). Pollution is another negative environmental factor that can affect health and pregnancy outcomes. An association between industrial air pollution and health in general, and more specifically pregnancy, is reported (*Frutos et al., 2015*; *Choe et al., 2018*; *Gaskins et al., 2019*; *Bergstra et al., 2022*). Certain low SES communities experience worse air pollution (*Hajat et al., 2015*). The Rotterdam area has a big industrial area (Europoort), and air pollution is not evenly distributed (*Bergstra et al., 2018*; *Arcadis, 2022*). Chronic exposure to stressors and pollution in early life may lead to epigenetic changes that affect biological responses in adulthood (*Lam et al., 2012*).

Proximity to the fertility clinic may be a factor affecting the likelihood of undergoing MAR treatment (*Lazzari et al., 2022*). Whether this affects the low SES group in the present study, and therefore our results, is unclear. It is known, however, that low SES neighbourhoods are more often in urbanized areas, which are closer to hospitals.

Most research into differences in IVF outcome (and also pregnancy) focus on maternal characteristics. Paternal characteristics, however, do also play a role, and this could be true for the association between SES and IVF outcome. Altered epigenetics in spermatozoa might play a role in the lower IVF success rate we found in the low SES group. Accumulating evidence suggests that environmental factors can influence epigenetics in spermatozoa (*Donkin and Barres, 2018*). There are indications that sperm RNA transmits environmental information to the offspring (*Vojtech et al., 2014*; *Zhang et al., 2019*). It remains unclear, however, if this RNA plays and role during early embryo development after fertilization, and, if so, what kind of role this RNA plays.

Second, chronic high overall stress might affect oocyte quality and, therefore, IVF success rate. Some small studies suggest a negative effect of low SES or individual level exposures, such as ethnicity, on ovarian reserve (*Iglesias et al., 2014*; *Barut et al., 2016*; *Bleil et al., 2018*). In addition, low SES is found to be associated with earlier menopause (*Gold et al., 2001*; *Castelo-Branco et al., 2005*; *Kapur et al., 2009*; *Velez et al., 2010*; *Richardson et al., 2014*). A systematic review on the association between educational level and age at menopause found no conclusive evidence, only a weak association between lower educational level and earlier age of menopause (*Canavez et al., 2011*). If oocytes of women exposed to low SES circumstances are affected by stress, this might manifest itself in oocyte quality. Interestingly, however, we did not find any significant differences in fertilization rate or embryo usage rate between the SES groups.

The large number of couples with known treatment outcome is a major strength of this study. During the long study period, the patient characteristics remained stable, and the neighbourhood SES was recalculated every 4 years. Furthermore, we adapted a longitudinal approach over a long study period between 2006 and 2020, making it possible to analyse

cumulative treatment outcomes instead of only first cycle treatment outcomes. Furthermore, SES neighbourhood scores are dynamic and may change over time. This was considered by using the most recently published score for each individual IVF cycle. Another major strength is that the design of the Dutch health system excludes the financial health seeking bias from our study in contrast to earlier published studies.

One limitation of our study is that data on smoking, BMI, duration of subfertility and live birth rate were not available for all couples. We were still able to include 2669 couples with complete data in a multivariable binary logistic regression analysis on cumulative ongoing pregnancy. A difference in missing data on live births was seen between the high SES and low SES group. This may give a small bias in our secondary outcome. As the treating physician was responsible for the collection of these data, it is unlikely that these differences result from selection bias by SES. Another limitation is that pregnant couples that conceived naturally could be unjustly labelled as not pregnant in our data. In our clinic, an IVF treatment starts on average at 3.5 years of subfertility (TABLE 1) and is often preceded by intrauterine insemination. Therefore, the chances of naturally conceived pregnancies are expected to be small and may account for a small proportion of our data. Unfortunately, additional analysis on individual characteristics, such as educational level, ethnicity, main infertility diagnosis or language proficiency could not be conducted owing to a lack of data. This study covers 14 years of IVF data, and IVF results may have improved over this period. To investigate the effect of changes over time, we separately analysed the data for 2014–2018 and 2006–2013, and found similar odds ratios for both time periods compared with the whole cohort (results not shown). Last, our study findings have low generalizability to countries that have other policies and facilities regarding IVF treatment.

As the observation of our IVF population will continue, it will be interesting to explore in the future whether other factors, such as severe acute respiratory syndrome coronavirus 2 and vaccination status, may affect outcomes. The question whether there might be an interaction between educational level and parity, or delayed infertility, are equally interesting to research further. The results suggest a potential direction for future research into a causal relationship between neighbourhood SES and IVF outcome.

Our results cautiously suggest that mechanisms through which neighbourhood SES influences IVF outcome may originate in the environment in which the embryo is transferred. During the in-vitro stages of the treatment, no significant differences between SES groups were found, except a minor variation in oocyte yield. In the analysis, the odds ratio on cumulative ongoing pregnancy rate for low neighbourhood SES group remained independent of the number of oocytes or embryos obtained. This suggests that the disparity may emerge from early in pregnancy, as the number of embryos obtained after follicle stimulation is linked to the oocyte reserve. There are some indications in small studies that the intestinal microbiome is associated with socioeconomic status (*Miller et al., 2016*; *Lapidot et al., 2021*), hypothesizing that an altered endometrial microbiome in women originating from low SES may influence implantation. For now, these are just speculations, and more research is needed on this subject.

In conclusion, the pr esent study suggests that residing in low SES neighbourhood is associated with a reduced probability of achieving an ongoing pregnancy within 2.5 years of IVF treatment. These disparities persist even with equal access to care, suggesting that factors beyond financial status play a significant role. The mechanism that explains our results is likely multifactorial. High overall stress (unmeasured) lifestyle factors and environmental factors are potential mechanisms behind the found associations. Further research is needed to uncover the underlying causes of this association and explore potential biological mechanisms.

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AUTHOR'S ROLES

JS, ES, and EB designed the clinical study; JS, EM and LB designed the statistical analysis; JS collected data; JS, EM and LB analysed the data and all authors interpreted the data; JS and EM drafted the manuscript; EB, ES, JL and LB substantively revised the manuscript; all authors have given approval for publication of the present version of this manuscript.

STATEMENT

During the preparation of this work the author(s) used ChatGPT to check the original grammar and word choice. After using this tool/service, the author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

DATA AVAILABILITY

The data underlying this article cannot be shared publicly due to the privacy of individuals that participated in the study. The data will be shared on reasonable request to the corresponding author.

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CORRIGENDUM

Corrigendum to 'ART in Latin America: the Latin American Registry, 2020' [Reproductive BioMedicine Online (2023) Volume 47, Issue 2] 103195

Fernando Zegers-Hochschild[1,2,*], Javier A. Crosby[1,3], Carolina Musri[1,3], Fanny Petermann-Rocha[3], Maria de Carmo de Souza[4], A. Gustavo Martinez[5], Ricardo Azambuja[6], Armando Roque[7], Gustavo Estofan[8], Mario Croker[9], on behalf of the Latin American Network of Assisted Reproduction [10]

The authors regret that in Supplementary Table 7 (Effect of peritoneal endometriosis in ART outcome) several cases were wrongly allocated as peritoneal endometriosis. After correcting the data to include all cases where the primary or secondary diagnosis was endometriosis, as diagnosed by direct observation (laparoscopy), re-analysis revealed that the differences in delivery rate were not significant. Correct supplementary table and revised results and discussions have been provided below in order to reflect the new analysis:

REVISED RESULTS TEXT

Endometriosis, as diagnosed by direct observation (laparoscopy), was present, either as a primary or secondary diagnosis, in 753 out of 39,418 initiated fresh cycles (1.9%). Laparoscopic observation included 67% of peritoneal fulguration, 22% of surgery for ovarian cysts, deep infiltration, or a combination of both, and 11% cases where no specific description was registered. A comparison was made between the outcome of cases where endometriosis was diagnosed, and a 'control group' (n=2839) of tubal and endocrine factors excluding premature ovarian insufficiency (Supplementary Table 7). In this 'control group', cases with a secondary diagnosis of endometriosis were also ruled out. Supplementary Table 7 provides information on the numbers and the mean number of oocytes collected, as well as the delivery rates in these two groups of women, stratified by age categories. Although the mean number of oocytes collected at all ages was significantly lower in the presence of endometriosis (<35: 9.51 [5.57] versus 10.50 [6.86]: P < 0.0001 (95% CI 0.6661 to 1.3139); 35–39: 6.87 [5.49] versus 10.28 [6.87]: P < 0.0001 (95% CI 3.1253 to 3.6947); >39: 5.59 [5.11] versus 6.12 [5.44]: P = 0.0053 (95% CI 0.1578 to 0.9022), the delivery rate per embryo transfer was not significantly different between the endometriosis and control groups (<35: 37.3% versus 35.2%: P = 0.6370 (95% CI -6.3307% to 11.0585%); 35–39: 29.3% versus 24.0%: P = 0.1027 (95% CI -1.0005% to 12.1257%); >39: 15.2% versus 12.6%: P = 0.5579 (95% CI -4.9503% to 13.5113%).

REVISED DISCUSSION TEXT

In 2020, for the first time, collaborating institutions were asked to describe the type of endometriosis when this was part of

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[10] Latin American Network of Assisted Reproduction (REDLARA), Montevideo, Uruguay
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a primary or secondary diagnosis. This included how the diagnosis was reached, and when reached surgically (mostly laparoscopic), centers were asked to describe the type of surgery performed, classified into five categories: peritoneal fulguration, cystectomy, or drainage of endometrioma, deep infiltration, partial oophorectomy and a combination of the above. Endometriosis was diagnosed by direct visualization in 753 out of 39,418 initiated cycles (1.9%). The number of oocytes collected as well as the delivery rate, stratified by age, were compared in women having endometriosis either as a primary or secondary diagnosis, excluding freeze-all cycles, and women having tubal and/or endocrine factors, excluding ovarian insufficiency. As seen in Supplementary Table 7, in spite of the fact that the number of eggs recovered in cases with endometriosis was significantly lower at all ages the delivery rates appeared to be higher in women with endometriosis compared with women with tubal or endocrine factors, although the difference was not statistically significant. These findings follow a similar direction of a study by Opøien et al. (2011) who showed better ART outcomes in minimal or mild endometriosis after surgical removal of endometriotic tissue; and a review by Senapati et al. (2016), using the SART database, showing, that in the absence of comorbidity, endometriosis yields fewer oocytes but higher pregnancy and delivery rates.

The authors would like to apologise for any inconvenience caused.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.104272.

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