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Adverse effects of progestin-primed ovarian stimulation: combination of clinical study and single-cell analysis

BIOGRAPHY

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KEY MESSAGE

Progestin-primed ovarian stimulation (PPOS) reduces the premature LH surge rate but also reduces the live birth rate compared with gonadotrophin-releasing hormone antagonist protocols. Single-cell RNA sequencing reveals elevated mitochondrial DNA gene expression in granulosa cells with PPOS, suggesting a potential decline in oocyte quality. Caution is recommended when employing PPOS.

ABSTRACT

Research question: Does progestin-primed ovarian stimulation (PPOS) have a negative effect on reproductive outcomes compared with a gonadotrophin-releasing hormone (GnRH) antagonist protocol?

Design: This retrospective cohort study included 907 patients aged <40 years with normal ovarian reserves undergoing either PPOS ($n = 299$) or a GnRH-antagonist protocol ($n = 608$) in their first IVF cycle between 2018 and 2020. An additional genetic analysis, single-cell RNA sequencing (scRNA-seq), was performed on the mural granulosa cells (mGC) of metaphase II oocyte follicles retrieved from 16 patients, with the above inclusion criteria, undergoing PPOS ($n = 8$) or a GnRH-antagonist protocol ($n = 8$) between 2021 and 2022. Inverse probability of treatment weighting (IPTW) was performed on the clinical data.

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KEY WORDS

Progestin-primed ovarian stimulation
Inverse probability weighting
Single-cell RNA sequencing
Mitochondrial DNA
Mural granulosa cell

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Declaration: The authors report no financial or commercial conflicts of interest.

Predetermined primary outcomes were the premature LH surge rate and the live birth rate of the first frozen embryo transfer cycle for the first and second IPTW analyses, respectively.

Results: The premature LH surge rate was lower in the PPOS group compared with the GnRH-antagonist group (3.1% versus 20.1%, OR 0.13, 95% CI 0.07−0.23; $P < 0.001$) in the first IPTW analysis. The good-quality cleavage embryo rate was lower in the PPOS group compared with the GnRH-antagonist group (37.2% versus 49.1%; $P < 0.001$). The live birth rate was lower in the PPOS group compared with the GnRH-antagonist group (31.5% versus 42.3%, OR 0.63, 95% CI 0.46−0.86; $P = 0.004$) in the second IPTW analysis. The scRNA-seq analysis demonstrated higher expression of 12 mitochondrial DNA (mtDNA) genes in the PPOS group compared with the GnRH-antagonist group.

Conclusion: PPOS suppressed the premature LH surge rate but was associated with a lower live birth rate compared with the GnRH-antagonist protocol. The elevated expression of mtDNA genes in mGC may also indicate a decline in oocyte quality with PPOS.

INTRODUCTION

Despite the increasing number of assisted reproductive technology (ART) cycles performed worldwide, the live birth rate following IVF remains relatively low (*Adamson et al., 2023*), highlighting the importance of selecting an appropriate ovarian stimulation protocol to retrieve competent oocytes. Progestin-primed ovarian stimulation (PPOS) is a widely used method in which oral progestin is used to suppress the LH surge during ovarian stimulation. Since the first report of its use in 2015 (*Kuang et al., 2015*), PPOS has been adopted internationally due to its simplicity and convenience. Randomized controlled trials have yielded inconsistent findings regarding reproductive outcomes, with some reporting that PPOS performs equivalently to GnRH-antagonist protocols (*Chen et al., 2024*; *Giles et al., 2021*), and others indicating that PPOS is inferior (*Beguería et al., 2019*). One meta-analysis demonstrated no difference in reproductive outcomes between PPOS and GnRH-antagonist protocols (*Ata et al., 2021*). Whereas randomized controlled trials and meta-analyses are invaluable for evaluating clinical outcomes, the complexity of ART treatment complicates the accurate assessment of ovarian stimulation efficacy from clinical trials alone. The difference in the effects caused by ovarian stimulation protocols might be mitigated when evaluations are confined to pregnancy outcomes after first embryo transfer in cases with multiple transferable embryos. A more precise evaluation of ovarian stimulation effects might be attained by incorporating biological assessments of individual oocytes.

For a comprehensive assessment of the efficacy of ovarian stimulation methods, it is imperative to incorporate evaluations from a basic scientific perspective. Single-cell RNA sequencing (scRNA-seq) provides a platform for non-biased gene expression evaluations through the analysis of individual cell transcriptomes (*Gong et al., 2022*). Furthermore, this approach enables the detection of variations in gene expression, which can easily be masked or overlooked when performing bulk RNA-seq. As granulosa cells are essential for oocyte development and maturation, the accurate analysis of gene expression patterns in these cells is vital to determining oocyte quality (*May-Panloup et al., 2016*). Although scRNA-seq has been utilized in various clinical studies, including those analysing the tumour microenvironment and determining the efficacy of cancer treatment (*Cosgrove et al., 2024*; *Zhang et al., 2021b*), its application for assessing ovarian stimulation methods remains largely unexplored (*Choi et al., 2023*; *Zhang et al., 2018*). As direct examinations of oocytes for clinical use are ethically challenging, scRNA-seq analyses of gene expression in granulosa cells could provide an alternative technique for evaluating the effects of ovarian stimulation methods on oocytes (*Gong et al., 2022*).

The aim of this retrospective cohort study was to compare the efficacy of PPOS, in terms of oocyte retrieval and embryo transfer outcomes, with that of a GnRH-antagonist protocol, using: (i) inverse probability of treatment weighting (IPTW), which adjusts for confounding variables, on clinical data; and (ii) scRNA-seq on mural granulosa cells (mGC) from metaphase II (MII) oocytes obtained through follicular aspiration to elucidate the mechanisms by which PPOS affects oocyte development.

MATERIALS AND METHODS

Ethical approval

This retrospective study was approved by the Ethical Review Board of Osaka University Hospital (No. 19197, 19 September 2019; No. 21113, 31 August, 2021). Clinical patient information was collected retrospectively between March 2018 and October 2020 under Approval No. 19197 for the IPTW analysis. Samples for scRNA analysis were obtained from patients who underwent ovarian stimulation cycles between November 2021 and January 2022 under Approval No. 21113.

Study population

This study, conducted at the Reproduction Clinic Tokyo/Osaka between March 2018 and October 2020, initially evaluated 1263 patients aged <40 years with anti-Müllerian hormone (AMH) concentration ≥1.1 ng/ml and who had their first IVF cycle at the study centre to determine their eligibility for inclusion in the study (FIGURE 1). The inclusion criteria aligned with the lower limits established by the Bologna criteria (*Ferraretti et al., 2011*). The exclusion criteria were: history of ovarian surgery; severe forms of male infertility requiring simple or micro-dissection testicular sperm extraction; oocyte cryopreservation; chronic diseases (such as cancer or diabetes); congenital uterine anomalies; chromosomal abnormalities in either member of couple; polycystic ovary syndrome; and had undergone other ovarian stimulation protocols. After excluding 356 patients, 907 patients were included in this study, with 299 undergoing PPOS with chlormadinone acetate (CMA) and 608 undergoing a GnRH-antagonist protocol with cetrorelix (FIGURE 1).

Between November 2021 and January 2022, a subset of 16 patients with normal ovarian reserves who underwent intracytoplasmic sperm injection (ICSI) was selected for scRNA-seq analysis. These patients received either PPOS with CMA ($n = 8$) or a GnRH-antagonist protocol with cetrorelix ($n = 8$), and were selected based on the same inclusion and exclusion

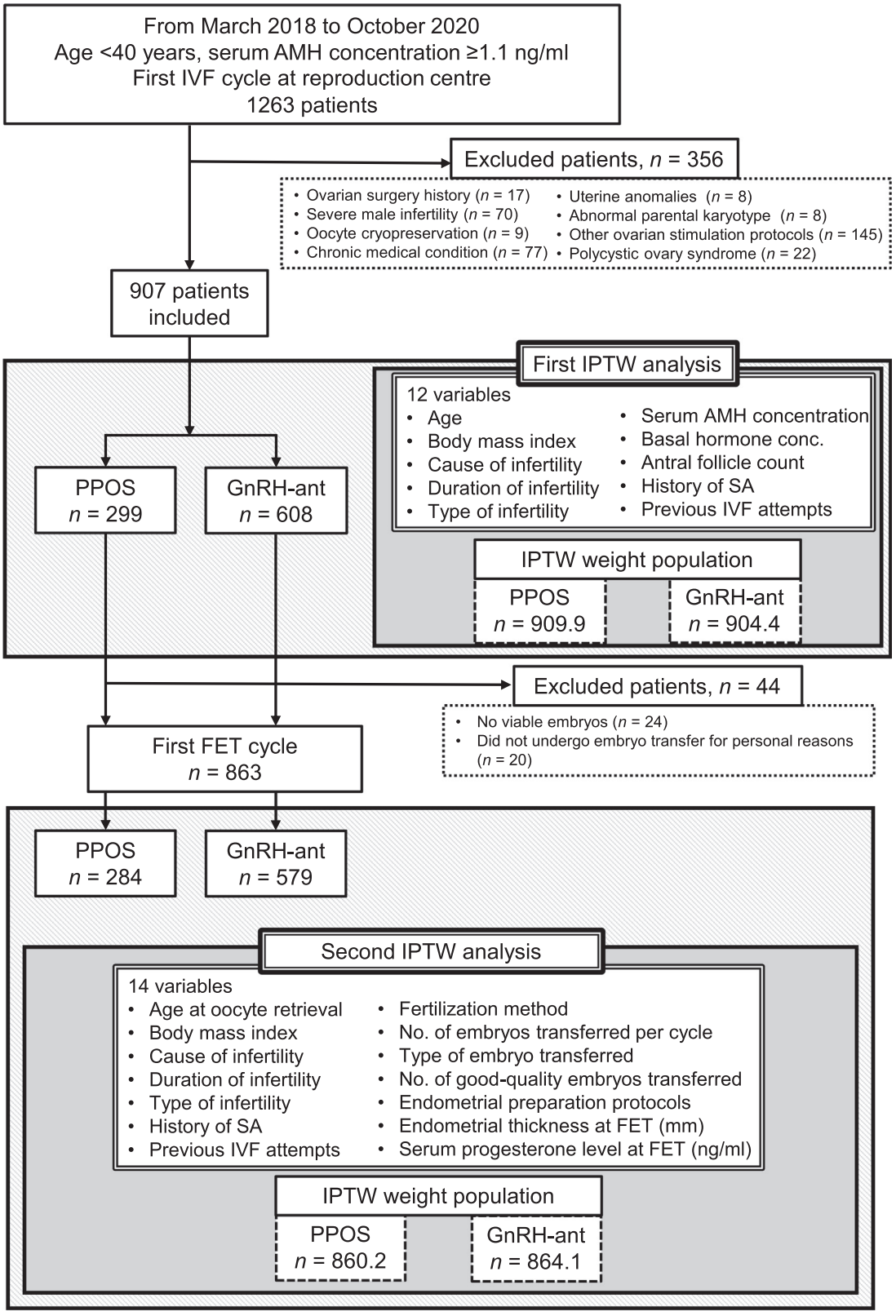

FIGURE 1 Flow chart of participant selection, and first and second inverse probability of treatment weighting (IPTW) analyses of progestin-primed (PPOS) and GnRH-antagonist (GnRH-ant) ovarian stimulation protocols and first frozen embryo transfer (FET) cycle, respectively. AMH, anti-Müllerian hormone; SA, spontaneous abortion; basal hormone conc., serum FSH, LH and oestradiol concentrations.

criteria as those used in the retrospective analysis described above.

Treatment protocol

Ovarian stimulation

Ovarian stimulation commenced on days 2–5 of the menstrual cycle. Considering the age, AMH concentration and body mass index (BMI) of the patient, the initial dose of human menopausal gonadotrophin (HMG Ferring; Ferring Pharmaceuticals, Japan; HMG Fuji; Fuji Pharma, Japan) or recombinant FSH (Gonal-f; Merck, Japan) ranged from 150 to 450 IU. Hormone concentrations, including LH, were monitored during the ovarian stimulation cycle using blood tests analysed with an AIA-CL1200 system (TOSOH Corporation, Japan).

For PPOS, 2 mg of CMA (Lutoral tablets; Fuji Pharma) was administered orally each day from days 2–5 of the menstrual cycle until the trigger day. If the serum LH concentration exceeded 5 mIU/ml on days 8–10, the dose of CMA was increased by 2 mg/day, and the patient was reassessed 2–3 days later. If the serum LH concentration still exceeded 5 mIU/ml, the dose of CMA was further increased by an additional 2 mg/day, up to a maximum of 6 mg/day.

For the GnRH-antagonist protocol, patients received cetrorelix (0.25 mg/ml; Merck) every other day, commencing between days 8 and 10 of the cycle or when the leading follicles reached ≥14 mm in diameter.

Patients at high risk of ovarian hyperstimulation syndrome (serum AMH concentration ≥5.0 ng/ml or antral follicle count ≥15 on transvaginal ultrasonography) were administered the aromatase inhibitor, letrozole (Femara; Novartis, Japan) at 2.5 mg/day for 2–5 days. Those with an AMH concentration <2.0 ng/ml were administered 50–100 mg of clomiphene citrate (Clomid; Fuji Pharma) daily throughout stimulation. The use of these agents – an aromatase inhibitor and clomiphene citrate – was determined by the physician's clinical experience.

Ovulation was induced with human chorionic gonadotrophin (HCG Mochida; Mochida Pharmaceutical Co., Japan), a GnRH-agonist (Buserecur; Fuji Pharma), or a dual trigger when leading follicles exceeded 18 mm in diameter, followed by oocyte retrieval after 36 ± 2 h. Oocytes were fertilized via conventional IVF, ICSI or split ICSI, based on semen parameters and the number of retrieved oocytes. Embryos were cultured using a one-step culture system with Sage 1-Step (CooperSurgical, USA). Cleavage embryos were evaluated on day 3 in accordance with established criteria (*Veeck, 1988*). Morphological blastocyst evaluation was performed following the Gardner and Schoolcraft grading system (*Gardner and Lane, 1997*). In most cases, after the cleavage embryos were graded, the third-ranked embryo was cryopreserved, and the remaining embryos were cultured to blastocyst stage. All embryos were preserved according to the freeze-all strategy using vitrification. Blastocysts were collapsed using an RI Saturn 5 Active laser (CooperSurgical) prior to vitrification. A vitrification VT507 kit and Cryotop (Kitazato, Japan) were used, with all steps performed in accordance with the manufacturer's protocol.

Frozen embryo transfer

All patients underwent the first frozen embryo transfer (FET) after oocyte retrieval, based on either a hormone replacement cycle (HRC) or a modified natural cycle. HRC-FET, involving the sequential administration of oestrogen and progesterone, was performed as described previously (*Ohara et al., 2022*). The administration of oestrogen and progestin supplements was continued until 10 weeks of gestation in cases of conception. The number of embryos transferred was determined based on patient age and their IVF history, with a maximum of two embryos per transfer.

Inverse probability of treatment weighting

Outcomes and variables in inverse probability of treatment weighting analysis

IPTW analysis was performed twice on the clinical data, as described in the statistical analysis section, in order to address confounding factors and to balance the baseline characteristics and enable comparisons of the two treatment types.

In the first IPTW analysis, the primary outcome was the incidence of a premature LH surge, which was predetermined based on the definition by *Olivennes et al. (2000)* as serum LH >10 mIU/ml. Secondary outcomes included: duration of ovarian stimulation (days); total gonadotrophin dose (IU); use of oral medications for ovarian stimulation (none, clomiphene citrate or letrozole); endocrine profiles on the day before oocyte retrieval (LH and peak oestradiol levels); fertilization method (conventional IVF, ICSI or split ICSI); incidence of moderate-to-severe ovarian hyperstimulation syndrome; ovulation findings before oocyte retrieval; number of oocytes retrieved; oocyte maturation rate; fertilization rate (defined as the number of normally fertilized oocytes divided by the total number inseminated); number of cryopreserved cleavage embryos and blastocysts; rate of good-quality cleavage embryos; and rate of good-quality blastocysts. The oocyte maturation rate was defined as the ratio of MII oocytes to total oocytes retrieved. The rates of good-quality cleavage embryos and blastocysts were defined as the number divided by the total cryopreserved in each category. Good-quality cleavage embryos (grade 1 or 2) had more than six cells by day 3 without morphological abnormalities (*Veeck, 1988*), and good blastocysts were graded as AA, AB, BA or BB, with an expansion grade ≥3 (*Gardner and Lane, 1997*).

In the second IPTW analysis, the primary outcome was the live birth rate (delivery of a viable infant after 24 weeks of gestation) per embryo transfer, which was predetermined. Secondary outcomes included: implantation rate; biochemical pregnancy rate; clinical pregnancy rate; early miscarriage rate; ongoing pregnancy rate; and multiple pregnancy rate. The implantation rate was defined as the number of gestational sacs, which was verified through transvaginal ultrasonography performed around 5–7 weeks of gestation, divided by the number of embryos transferred.

mGC collection from preovulatory follicles of MII oocytes

mGC were collected from 16 patients with normal ovarian reserves undergoing ICSI procedures and PPOS with CMA (n = 8) or a GnRH-antagonist protocol with cetrorelix (n = 8) from November 2021 to January 2022. Follicular fluid was aspirated separately from each follicle during oocyte retrieval and kept on ice. Oocyte maturity was assessed at the time of ICSI, and only the follicular fluid from follicles containing MII oocytes was selected for analysis. These selected fluid samples were centrifuged at 550 × g for 10 min (*Qu et al., 2010*). The sedimented cells from the patient were resuspended in phosphate-buffered saline. The resuspended cells

from MII follicles were then combined per patient for further processing. mGC isolation involved further centrifugation at 250 × *g* for 10 min using a lymphocyte separation solution with a density of 1.077 g/ml (Nacalai Tesque Inc., Japan) (*Lu et al., 2019*). Washed mGC were cryopreserved in Bambanker medium (Nippon Genetics, Japan) at −80°C. After thawing, cell counts were standardized across patients and normalized to those of the patient with the minimum cell count. Cells were pooled within each group to form the PPOS (PPOS_MII) and GnRH-antagonist (GnRH-antagonist_MII) groups. Dead cells were removed through magnetic cell sorting (Dead Cell Removal Kit; Miltenyi Biotec, Germany) with LS columns and pre-separation filters from the MidiMACS Starting Kit (Miltenyi Biotec), following DNase treatment. Procedures were performed in accordance with the manufacturer's instructions. Live cells were counted using a haemocytometer (Improved Neubauer; Sunlead Glass, Japan).

scRNA-seq analysis of mGC

Libraries were prepared based on a concentration of 1.0×10^3 cells/μl using Chromium Next GEM Single Cell V(D)J Reagent Kits (10x Genomics, USA) in accordance with the manufacturer's instructions, and libraries were sequenced using a DNBSEQ-G400 sequencer (MGI, China). The reads were subsequently processed through the following methods, and analysed using Cellxgene (Chan Zuckerberg Initiative, USA; https://cellxgene.cziscience.com/), an interactive data explorer designed specifically for visualizing single-cell transcriptomic data, and cellxgene-VIP (Biogen Inc., USA; https://doi.org/10.1101/2020.08.28.270652), an interactive visualization plugin of the cellxgene framework. scRNA-seq was performed on 2224 mGC from the MII oocyte follicles (56 from PPOS group and 39 from GnRH-antagonist group) of 16 patients. Fastq data were aligned to the hg38 dataset (refdata-gex-GRCh38-2020-A) with the cellranger count in Cell Ranger v6.1.2 (10x Genomics) and processed using Seurat v4.0.1 (*Butler et al., 2018*). Quality control was performed prior to downstream analyses. Initial clustering was performed using fastcluster in scDblFinder (*Germain et al., 2021*), excluding cells with more than four absolute deviations from the median for the unique molecular identifier (UMI) count, gene count and mitochondrial UMI percentage (up to a maximum of 20% mitochondrial UMI) for each cluster. Doublet detection was carried out using DoubletFinder v2.0.3 (Satija Lab, USA), an R package that uses artificial nearest neighbours to identify potential doublets (*McGinnis et al., 2019*). QC metrics such as nFeature_RNA and %mito were also used to identify and remove low-quality cells. Cells were annotated using CellO (*Bernstein et al., 2020*). To merge libraries, SelectIntegrationFeatures was used in Seurat to select common variable features. Libraries were combined, and these features were scaled to a mean of 0 unit variance for 20 principal components. Harmony v0.1 (*Korsunsky et al., 2019*) was applied for batch correction with default parameters. Clustering was performed with FindClusters using the Leiden algorithm (*Traag et al., 2019*) at a resolution of 1. RunTSNE and RunUMAP were also used with default parameters.

Validation via reverse transcription quantitative polymerase chain reaction

Differentially expressed genes (DEG) between the PPOS_MII and GnRH-antagonist_MII groups were validated via reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using an RNAqueous-Micro Total RNA Isolation Kit (Invitrogen, Thermo Fisher Scientific, USA), and reverse transcription was performed using the Super Script IV VILO Master Mix (Invitrogen) in accordance with the manufacturer's instructions in each case. RT-qPCR was performed using Step One Plus, with a total reaction volume of 10 μl/well in TaqMan Gene Expression Array Plates, using the predesigned TaqMan Assay 5′-FAM reporter and 3′-MGB quencher for the primers, and 10 ng of diluted cDNA per well. Reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 amplification cycles with 15 s of denaturation at 95°C and 1 min of annealing and extension at 60°C. *GAPDH* was used as the internal control. TaqMan primer sequences and reaction conditions are listed in Supplementary Table 1. Analysis of the relative gene expression data was conducted using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

A generalized linear model analysis was conducted with the propensity score (*Robins et al., 2000*). The standardized mean difference (SMD) was used to assess the covariate balance before and after applying IPTW. SMD <0.25 indicated a negligible imbalance between the two groups (*Austin, 2011*). OR and 95% CI, based on the generalized linear model with IPTW analysis with sandwich variance, were computed.

In the first part of the study, which focused on ovarian stimulation outcomes, IPTW was applied to all stimulated cycles based on baseline characteristics. Outcomes were analysed based on multivariate logistic regression, incorporating the following 12 variables: age; BMI; cause of infertility; duration of infertility; type of infertility; serum AMH concentration; basal hormone concentrations (FSH, LH and oestradiol); antral follicle count; history of spontaneous abortion; and previous IVF attempts. An additional analysis of the good-quality embryo rate was conducted by performing chi-squared test.

For the second part of the study, which focused on embryo transfer outcomes, IPTW was applied to the first FET cycles following oocyte retrieval. The analysis incorporated the following 14 variables: age at oocyte retrieval; BMI; cause of infertility; duration of infertility; type of infertility; history of spontaneous abortion; previous IVF attempts; fertilization method; number of embryos transferred per cycle; type of embryo transferred; number of good-quality embryos transferred; endometrial preparation protocols; endometrial thickness at FET; and serum progesterone concentration at FET.

Additional analyses of good-quality cleavage embryo rate, good-quality blastocyst rate and implantation rate were conducted using chi-squared test. Subgroup analyses for obstetric outcomes were conducted by performing Wilcoxon signed-rank and chi-squared tests. E-values were calculated to evaluate the robustness of the results against potential residual or unmeasured confounders, using an E-value calculator (*VanderWeele et al., 2017*). All tests were two-tailed, and $P < 0.05$ was considered to indicate significance for comparisons between two groups. All statistical analyses of clinical data were performed with R v4.2.2 (R Foundation for Statistical Computing, Austria; https://www.R-project.org/). scDblFinder (*Germain et al., 2021*) was used for doublet detection, and CellO (*Bernstein et al., 2020*) was used for cell type classification. For the scRNA-seq analysis, the conventional approach for identifying cell cluster/type-specific genes involved

the detection of DEG between clusters by employing the Wilcoxon rank-sum test and multiple testing corrections. Statistical analyses of the RT-qPCR validation assay were performed using a two-tailed unpaired Student's *t*-test. Statistical analyses were performed using GraphPad Prism v8.0 (GraphPad Software Inc., USA).

RESULTS

Patient baseline characteristics

The baseline characteristics of patients who underwent PPOS and the GnRH-antagonist protocol and FET are provided in Tables 1 and 2, respectively. Prior to IPTW adjustment, several baseline characteristics showed significant differences between the groups. In TABLE 1, age at oocyte retrieval, basal LH concentration and number of previous IVF attempts differed significantly, with SMD exceeding 0.25 for the latter two variables. Similarly, in TABLE 2, the number of previous IVF attempts and progesterone conccentration on the day of embryo transfer also showed significant differences ($P < 0.01$, SMD > 0.25). These imbalances highlight the need for IPTW adjustment to reduce confounding and to improve comparability between groups.

Oocyte retrieval outcomes after PPOS and GnRH-antagonist protocol (first IPTW analysis)

Following the initial IPTW analysis, the baseline clinical data for both groups were well balanced, with SMD <0.25 for each factor between the two hypothetical groups (TABLE 1). Under these analysis conditions, the premature LH surge rate, the primary outcome in the first IPTW, was significantly lower in the PPOS group (3.1%) compared with the GnRH-antagonist group (20.1%) (OR 0.13, 95% CI 0.07–0.23; $P < 0.001$; TABLE 3). The E-value for the observed association between PPOS and the reduced premature LH surge rate was 14.9, indicating that the results are unlikely to be explained by unmeasured confounders. The incidence of ovulation prior to oocyte retrieval was 0% and 0.5% in the PPOS and GnRH-antagonist groups, respectively. This difference was significant (OR 0.00, 95% CI 5.65E−09–5.55E−08; $P < 0.001$; TABLE 3). No significant differences in the number of oocytes retrieved, oocyte maturation rate and fertilization rate were observed (TABLE 3). However, a significant decrease in the good-quality cleavage embryo rate was observed in the PPOS group compared with the GnRH-antagonist group [128/344 (37.2%) versus 318/648 (49.1%), respectively; $P < 0.001$], with no significant difference in the good-quality blastocyst rate between the groups [1156/1605 (72.0%) versus 2317/3327 (69.6%); $P = 0.09$].

Pregnancy outcomes for the first FET cycle after PPOS and GnRH-antagonist protocol (second IPTW analysis)

An analysis of 863 autologous FET cycles in both groups revealed balanced baseline characteristics after the second IPTW analysis (TABLE 2). Under these analysis conditions, the live birth rate, the primary outcome in the second IPTW, was significantly lower in the PPOS group compared with the GnRH-antagonist group (31.5% versus 42.3%, respectively; OR 0.63, 95% CI 0.46–0.86; $P = 0.004$), despite the comparable rates of good-quality embryos transferred (TABLE 4). The E-value for the observed association between PPOS and the lower live birth rate was 2.55, suggesting that the results are unlikely to be explained by unmeasured confounders. The biochemical pregnancy rate (OR 0.66, 95% CI 0.49–0.90; $P = 0.009$) and ongoing pregnancy rate (OR 0.65, 95% CI 0.48–0.89; $P = 0.007$) were also significantly lower in the PPOS group, while there was no significant difference in the early miscarriage rate between the two groups (TABLE 4). In the additional analysis, the implantation rate was significantly lower in the PPOS group compared with the GnRH-antagonist group (43.4% versus 51.9%, respectively; $P = 0.02$). In the subgroup analysis, the rates of caesarean section, preterm birth, low birth weight, congenital malformation, and complications per delivery were comparable between the two groups (Supplemental Table 2).

Baseline characteristics of patients and overview of scRNA-seq data

The baseline characteristics of the 16 patients who underwent scRNA-seq analysis are presented, showing no significant differences between the groups (Supplemental Table 3). Two populations of mGC (PPOS and GnRH-antagonist groups) from MII oocytes were analysed. After quality control (nFeature, %mito, DoubletFinder), 1197 and 1027 cells from the PPOS and GnRH-antagonist groups, respectively, were selected and analysed (FIGURE 2A). Single-cell clustering based on the Seurat package was performed, followed by cell proportion analysis, resulting in comparable cluster distributions between the PPOS and GnRH-antagonist groups (Supplemental Figure 1), with the granulosa cell cluster identified successfully in both (FIGURE 2B-I). Granulosa cell annotation was confirmed based on the expression of marker genes (AMH, FSHR, DHCR24, TIMP1, CD99, HSD11B1, VTN and IGFBP2) (*Fan and Chuva de Sousa Lopes, 2021*) (Supplemental Figure 1). Focusing on granulosa cells from both groups, clustering was performed using fastcluster in scDblFinder, as described herein for each granulosa cell cluster (Supplemental Figure 2). The uniform manifold approximation and projection analysis showed a similar distribution of granulosa cells between both the PPOS_MII and GnRH-antagonist_MII groups (FIGURE 2B-II).

Differential gene expression patterns in granulosa cells of both populations

Among the top 20 DEG in mGC – ranked by adjusted *P*-value – 12 mitochondrial DNA (mtDNA) genes were expressed more highly in the PPOS_MII group than in the GnRH-antagonist_MII group (Supplemental Table 4). These mtDNA genes encode components of the mitochondrial respiratory chain complexes I, III and IV, and are functionally associated with the oxidative phosphorylation (OXPHOS) system (*Fernandez-Vizarra and Zeviani, 2021*). Expression of the top seven mtDNA DEG is presented as violin plots based on scRNA-seq data (FIGURE 2B-III). Cell type proportion analysis of mGC from both the PPOS_MII and GnRH-antagonist_MII groups in each cluster revealed no significant differences in the distribution of cells expressing mtDNA (Supplemental Figure 3). Moreover, RT-qPCR results confirmed the increased mRNA expression of mtDNA genes, specifically MT-ND5, MT-CYB, MT-ND4 and MT-ND2, in the PPOS_MII group (FIGURE 2C).

DISCUSSION

The findings indicated that although PPOS suppressed the premature LH surge rate significantly, it also resulted in a lower pregnancy rate, concomitant with a lower rate of good-quality cleavage embryos, in patients with normal ovarian reserves compared with the GnRH-antagonist protocol. Concurrently, increased mtDNA gene expression in mGC was observed in the PPOS group.

TABLE 1 PATIENT BASELINE CHARACTERISTICS FOR PROGESTIN-PRIMED OVARIAN STIMULATION AND GONADOTROPHIN-RELEASING HORMONE ANTAGONIST PROTOCOLS, AND FOLLOWING FIRST INVERSE PROBABILITY OF TREATMENT WEIGHTING ANALYSIS

Characteristic	Category	Original (unadjusted)				IPTW			
		PPOS	GnRH-antagonist	*P*-value[a]	SMD[a]	PPOS	GnRH-antagonist	*P*-value[a]	SMD[a]
		***n* = 299**	***n* = 608**			**weight = 909.9**	**weight = 904.4**		
Age at oocyte retrieval (years): median (IQR)		34.0 (32.0–37.0)	35.0 (32.0–37.0)	<**0.018**	0.18	35.0 (32.0–37.0)	35.0 (32.0–37.0)	0.76	0.03
Body mass index (kg/m^2): median (IQR)		20.4 (19.1–22.1)	20.3 (19.1–21.9)	0.41	0.07	20.4 (19.1–21.6)	20.3 (19.1–21.9)	0.96	<0.01
Cause of infertility: *n* (%)	Tubal factor	19 (6.4)	26 (4.3)	0.14	0.16	50.6 (5.6)	47.8 (5.3)	0.98	0.04
	Male factor	97 (32.4)	165 (27.1)			248.6 (27.3)	256.5 (28.4)		
	Endometriosis	10 (3.3)	26 (4.3)			42.0 (4.6)	36.8 (4.1)		
	Unknown	173 (57.9)	391 (64.3)			568.7 (62.5)	563.4 (62.3)		
Duration of infertility (months): median (IQR)		24.0 (14.0–41.0)	24.0 (15.0–40.0)	0.39	0.05	26.0 (15.0–42.0)	24.0 (14.5–40.0)	0.54	0.02
Type of infertility: *n* (%)	Primary	200 (66.9)	397 (65.3)	0.69	0.03	611.4 (67.2)	598.5 (66.2)	0.77	0.02
	Secondary	99 (33.1)	211 (34.7)			298.5 (32.8)	305.9 (33.8)		
Serum AMH concentration (ng/ml): median (IQR)		3.90 (2.50–5.67)	3.54 (2.20–5.76)	0.06	0.09	3.81 (2.41–5.91)	3.65 (2.26–5.88)	0.39	0.04
Basal hormone concentrations: median (IQR)	FSH (mIU/ml)	8.30 (7.20–9.75)	8.7 (7.5–10.0)	0.08	0.11	8.4 (7.2–9.8)	8.6 (7.5–9.9)	0.28	0.02
	LH (mIU/ml)	4.80 (3.70–6.20)	4.2 (3.0–5.5)	<**0.001**	**0.38**	4.3 (3.3–5.7)	4.3 (3.2–5.8)	0.95	0.02
	Oestradiol (pg/ml)	33.9 (26.1–45.0)	32.8 (24.3–44.7)	0.24	0.07	32.9 (25.4–44.5)	33.1 (24.4–45.0)	0.92	0.03
Antral follicle count: median (IQR)		11.0 (7.0–15.0)	10.0 (7.0–15.0)	0.23	0.08	11.0 (7.0–15.0)	10.0 (7.0–15.0)	0.57	0.04
History of spontaneous abortion: *n* (%)	0	245 (81.9)	479 (78.8)	0.54	0.16	733.1 (80.6)	726.4 (80.3)	0.69	0.12
	1	44 (14.7)	95 (15.6)			133.4 (14.7)	134.2 (14.8)		
	2	9 (3.0)	23 (3.8)			39.3 (4.3)	30.6 (3.4)		
	3	1 (0.3)	9 (1.5)			4.2 (0.5)	10.9 (1.2)		
	4	0 (0)	1 (0.2)			0 (0)	1.2 (0.1)		
	5	0 (0)	0 (0)			0 (0)	0 (0)		
	6	0 (0)	1 (0.2)			0 (0)	1.2 (0.1)		
Previous IVF attempts: *n* (%)	0	163 (54.5)	244 (40.1)	<**0.001**	**0.29**	400.3 (44.0)	403.4 (44.6)	0.95	0.03
	1–2	81 (27.1)	214 (35.2)			292.0 (32.1)	294.4 (32.5)		
	≥3	55 (18.4)	150 (24.7)			217.6 (23.9)	206.7 (22.9)		

[a] Bold type indicates statistical significance: *P*-value <0.05 and SMD >0.25. Weights applied without rounding; case numbers after weighting rounded to two decimal places for presentation purposes. *P*-values for continuous and categorical variables calculated using Wilcoxon rank sum and chi-squared tests, respectively. SMD were computed as difference in means divided by pooled SD. Statistical tests did not account for the estimation error associated with IPTW.
PPOS, progestin-primed ovarian stimulation; GnRH, gonadotrophin-releasing hormone; IPTW, inverse probability of treatment weighting; SMD, standardized mean difference; AMH, anti-Müllerian hormone.

TABLE 2 PATIENT BASELINE CHARACTERISTICS FOR PROGESTIN-PRIMED AND GONADOTROPHIN-RELEASING HORMONE ANTAGONIST OVARIAN STIMULATION AND FIRST FROZEN EMBRYO TRANSFER PROTOCOLS, AND FOLLOWING SECOND INVERSE PROBABILITY OF TREATMENT WEIGHTING ANALYSIS

Characteristic	Category	Original (unadjusted)				IPTW			
		PPOS	GnRH-antagonist	*P*-value[a]	SMD[a]	PPOS	GnRH-antagonist	*P*-value[a]	SMD[a]
		***n* = 284**	***n* = 579**			**weight = 860.2**	**weight = 864.1**		
Age at oocyte retrieval (years): median (IQR)		34.0 (32.0–37.0)	35.0 (32.0–37.0)	**0.02**	0.19	35.0 (32.0–37.0)	34.0 (32.0–37.0)	0.99	<0.01
Body mass index (kg/m^2): median (IQR)		20.4 (19.1–22.1)	20.3 (19.0–21.9)	0.46	0.06	20.4 (19.1–21.8)	20.3 (19.0–22.0)	0.87	0.01
Cause of infertility: *n* (%)	Tubal factor	17 (6.0)	26 (4.5)	0.17	0.16	46.2 (5.4)	44.4 (5.1)	1.00	0.02
	Male factor	94 (33.1)	157 (27.1)			243.1 (28.3)	248.2 (28.7)		
	Endometriosis	9 (3.2)	25 (4.3)			31.3 (3.6)	33.7 (3.9)		
	Unknown	164 (57.7)	371 (64.1)			539.5 (62.7)	537.7 (62.2)		
Duration of infertility (months): median (IQR)		24.5 (14.0–41.0)	24.0 (15.0–40.0)	0.39	0.06	25.0 (14.0–41.0)	24.0 (15.0–40.0)	0.96	<0.01
Type of infertility: *n* (%)	Primary	191 (67.3)	379 (65.5)	0.66	0.04	571.0 (66.4)	571.6 (66.1)	0.95	0.01
	Secondary	93 (32.7)	200 (34.5)			289.2 (33.6)	292.5 (33.9)		
History of spontaneous abortion: *n* (%)	0	235 (82.7)	454 (78.4)	0.46	0.17	689.6 (80.2)	689.1 (79.8)	0.94	0.07
	1	39 (13.7)	91 (15.7)			126.0 (14.7)	129.1 (14.9)		
	2	9 (3.2)	23 (4.0)			34.7 (4.0)	33.3 (3.9)		
	3	1 (0.4)	9 (1.6)			9.2 (1.1)	10.0 (1.2)		
	4	0 (0)	1 (0.2)			0 (0)	1.0 (0.1)		
	5	0 (0)	0 (0)			0 (0)	0 (0)		
	6	0 (0)	1 (0.2)			0 (0)	1.0 (0.1)		
Previous IVF attempts: *n* (%)	0	154 (54.2)	234 (40.4)	**<0.001**	**0.28**	391.6 (45.5)	390.0 (45.1)	0.98	0.02
	1–2	78 (27.5)	205 (35.4)			284.2 (33.0)	282.5 (32.7)		
	≥3	52 (18.3)	140 (24.2)			184.5 (21.4)	191.6 (22.2)		
Fertilization method: *n* (%)	cIVF	30 (10.6)	86 (14.9)	0.05	0.18	105.4 (12.3)	115.2 (13.3)	0.90	0.04
	ICSI	90 (31.7)	144 (24.9)			228.7 (26.6)	232.8 (26.9)		
	Split ICSI[b]	164 (57.7)	349 (60.3)			526.1 (61.2)	516.1 (59.7)		
No. of embryos transferred per cycle: *n* (%)	Single	257 (90.5)	539 (93.1)	0.23	0.10	797.0 (92.7)	797.3 (92.3)	0.84	0.02
	Double	27 (9.5)	40 (6.9)			63.1 (7.3)	66.8 (7.7)		
Type of embryos transferred: *n* (%)	Cleavage	12 (4.2)	11 (1.9)	0.08	0.14	24.6 (2.9)	24.5 (2.8)	0.98	<0.01
	Blastocyst	272 (95.8)	568 (98.1)			835.6 (97.1)	839.5 (97.2)		

(*continued on next page*)

TABLE 2 (*Continued*)

Characteristic	Category	Original (unadjusted)				IPTW			
		PPOS	GnRH-antagonist	*P*-value[a]	SMD[a]	PPOS	GnRH-antagonist	*P*-value[a]	SMD[a]
		***n* = 284**	***n* = 579**			**weight = 860.2**	**weight = 864.1**		
No. of good-quality embryos transferred: *n* (%)	0	25 (8.8)	36 (6.2)	0.19	0.13	56.2 (6.5)	60.9 (7.0)	0.96	0.02
	1	252 (88.7)	535 (92.4)			789.6 (91.8)	788.8 (91.3)		
	2	7 (2.5)	8 (1.4)			14.5 (1.7)	14.3 (1.7)		
Endometrial preparation protocol: *n* (%)	NC	22 (7.7)	50 (8.6)	0.75	0.03	73.4 (8.5)	70.6 (8.2)	0.87	0.01
	HRC	262 (92.3)	529 (91.4)			786.8 (91.5)	793.5 (91.8)		
Endometrial thickness on day of FET (mm): median (IQR)		11.5 (10.2–13.6)	11.8 (10.4–13.4)	0.25	0.05	11.5 (10.2–13.7)	11.8 (10.4–13.4)	0.49	0.01
Progesterone concentration on day of FET (ng/ml): median (IQR)		9.5 (7.7–12.1)	10.6 (8.1–13.9)	**<0.001**	**0.29**	10.1 (7.9–12.6)	10.2 (7.9–13.2)	0.78	<0.01

[a] Bold indicates significance: *P*-value <0.05 and SMD >0.25. Weights applied without rounding; case numbers after weighting rounded to two decimal places for presentation purposes. *P*-values for continuous and categorical variables calculated using Wilcoxon rank sum and chi-squared tests, respectively. SMD were computed as difference in means divided by pooled SD. Statistical tests did not account for the estimation error associated with IPTW.

[b] Split ICSI = oocyte division into cIVF and ICSI, with simultaneous fertilization attempts using both methods.

cIVF, conventional IVF; HRC, hormone replacement cycle; NC, natural cycle; PPOS, progestin-primed ovarian stimulation; GnRH, gonadotrophin-releasing hormone; IPTW, inverse probability of treatment weighting; SMD, standardized mean difference; ICSI, intracytoplasmic sperm injection; FET, frozen embryo transfer.

PPOS has gained global recognition as an efficient and simple ovarian stimulation technique. *Kuang et al. (2015)* first demonstrated progestin as an effective suppressant of the LH surge during ovarian stimulation. Various reports have shown that PPOS is not inferior to GnRH-antagonist protocols, with several randomized controlled trials reporting their equivalence (*Beguería et al., 2019*; *Giles et al., 2021*). *Ata et al. (2021)* conducted a meta-analysis and reported no difference in the number of retrieved oocytes and mature oocytes between PPOS and GnRH-antagonist protocols. The present study found that the premature LH surge rate was lower in the PPOS group compared with the GnRH-antagonist group (3.1% versus 20.1%), which contrasts with previous findings (*Beguería et al., 2019*; *Giles et al., 2021*). This difference may be attributed to the alternate-day administration protocol of the GnRH-antagonist, which aligns with a previous study showing a similar premature LH surge rate of 18.1% in the GnRH-antagonist group (*Feng L et al., 2022*), designed to minimize GnRH-antagonist administration to avoid potential impairment of follicular development due to excessive suppression of FSH and LH. While the authors' GnRH-antagonist protocol is not commonly practised globally, it was effective in the study group of Japanese patients with relatively low BMI, resulting in a low early ovulation rate (0.5%). Therefore, despite the higher frequency of a premature LH surge in the GnRH-antagonist group, there was no significant difference in the number of oocytes, oocyte maturation or fertilization, consistent with previous reports (*Beguería et al., 2019*; *Giles et al., 2021*), because the premature LH surge was suppressed effectively by the timely administration of a GnRH-antagonist before it could induce irreversible ovulation. In terms of clinical implications, the present results suggest that PPOS may be particularly advantageous for patient groups prone to a premature LH surge. Additionally, PPOS may be beneficial for patients as it is generally more cost-effective and less burdensome due to its oral administration. Conversely, in patients who are at lower risk of experiencing a premature LH surge, the GnRH-antagonist protocol may be a more suitable alternative to PPOS. However, given that the premature LH surge rate in the GnRH-antagonist group was higher than typically observed, this interpretation should be approached with caution.

TABLE 3 OUTCOMES OF PROGESTIN-PRIMED AND GONADOTROPHIN-RELEASING HORMONE ANTAGONIST OVARIAN STIMULATION PROTOCOLS IN THE CONTEXT OF FIRST INVERSE PROBABILITY OF TREATMENT WEIGHTING ANALYSIS

Outcomes	Category		Original (unadjusted)						IPTW					
			PPOS	GnRH-antagonist	Estimated[a]		95% CI	*P*-value[b]	PPOS	GnRH-antagonist	Estimated[a]		95% CI	*P*-value[b]
			***n* = 299**	***n* = 608**					**weight = 909.9**	**weight = 904.4**				
Primary outcome	Premature LH surge rate: *n* (%)		14 (4.7)	116 (19.1)	OR	0.21	(0.11–0.36)	<**0.001**	28.1 (3.1)	181.4 (20.1)	OR	0.13	(0.07–0.23)	<**0.001**
Other outcomes	Duration of ovarian stimulation (days): median (IQR)		9.0 (8.0–10.0)	9.0 (8.00–10.0)	Diff	0.17	(0.00–0.33)	0.05	9.0 (8.0–10 .0)	9.00 (8.00–9.00)	Diff	0.13	(0.07–0.23)	**0.01**
	Total gonadotrophin dose (IU): median (IQR)		2400 (1613–2700)	2400 (1800–2700)	Diff	-10.5	(-98.2 to 77.2)	0.81	2400 (1725–2700)	2400 (1725–2700)	Diff	51.2	(-47.5 to 149.9)	0.31
	Oral medications administered for ovarian stimulation *n* (%)	None	101 (33.8)	174 (28.6)	OR	1.28	(0.94–1.71)	0.11	304.7 (33.5)	262.0 (29.0)	OR	1.23	(0.90–1.69)	0.19
		Clomiphene citrate	105 (35.1)	243 (40.0)	OR	0.81	(0.61–1.08)	0.16	336.5 (37.0)	346.9 (38.4)	OR	0.94	(0.70–1.28)	0.71
		Letrozole	93 (31.1)	191 (31.4)	OR	0.99	(0.73–1.33)	0.92	268.7 (29.5)	295.5 (32.7)	OR	0.86	(0.63–1.19)	0.37
	Endocrine profiles on last visit before oocyte retrieval	LH (mIU/ml)	2.8 (1.5–4.5)	2.6 (1.4–4.5)	Diff	-0.31	(-0.74 to 0.13)	0.17	2.5 (1.3–4.2)	2.6 (1.5–4.6)	Diff	-0.62	(-1.02 to -0.23)	**0.002**
		Peak oestradiol (pg/ml)	2799.1 (2116.9–3958.7)	2578.6 (1936.4–3357.1)	Diff	310	(123–498)	<**0.001**	2740.0 (2084.3–3924.4)	2603.7 (1964.7–3373.7)	Diff	216	(-4.59 to 437)	0.06
	Elevated LH concentration[d]: median (IQR)		0.00 (-2.00 to 1.50)	1.00 (-0.83 to 3.73)	Diff	-2.74	(-3.43 to -2.05)	<**0.001**	0.10 (-1.60 to 1.60)	1.00 (-1.00 to 3.89)	Diff	-2.55	(-3.11 to -2.00)	<**0.001**
	Fertilization method: *n* (%)	cIVF	33 (11.0)	92 (15.1)	OR	0.70	(0.45–1.05)	0.09	119.3 (13.1)	121.8 (13.5)	OR	0.97	(0.62–1.51)	0.89
		ICSI	93 (31.1)	156 (25.7)	OR	1.31	(0.96–1.77)	0.08	276.0 (30.3)	236.3 (26.1)	OR	1.23	(0.89–1.70)	0.21
		Split ICSI[c]	173 (57.9)	360 (59.2)	OR	0.95	(0.72–1.25)	0.70	514.6 (56.6)	546.4 (60.4)	OR	0.85	(0.63–1.15)	0.30
	Incidence of moderate-to-severe OHSS: *n* (%)		1 (0.3)	3 (0.5)	OR	0.68	(0.03–5.31)	0.74	3.0 (0.3)	6.6 (0.7)	OR	0.45	(0.05–4.41)	0.49
	Ovulation findings before oocyte retrieval[e]: *n* (%)		0 (0.0)	3 (0.5)	OR	0.00	(NA, 7.75E+164)	1.00	0.0 (0)	4.4 (0.5)	OR	0.00	(5.65E−09 to 5.55E−08)	<**0.001**
	No. of oocytes retrieved: median (IQR)		15.0 (10.0–21.0)	14.0 (10.0–22.0)	Diff	-0.22	(-1.51 to 1.06)	0.73	15.0 (10.0–21.0)	15.0 (10.0–22.0)	Diff	-0.77	(-2.10 to 0.57)	0.26
	Oocyte maturation rate[f]: %, median (IQR)		80.0 (66.7–89.2)	77.8 (66.7–87.5)	RR	1.02	(0.99–1.05)	0.27	78.8 (66.7–88.9)	77.8 (66.7–87.5)	RR	1.02	(0.98–1.05)	0.33
	Fertilization rate[g]: %, median (IQR)		75.0 (59.6–87.5)	72.2 (60.0–83.3)	RR	1.03	(0.99–1.07)	0.12	73.3 (59.7–87.4)	72.0 (60.0–83.3)	RR	1.02	(0.98–1.06)	0.42
	No. of cryopreserved cleavage embryos: median (IQR)		1.0 (1.0–1.0)	1.0 (1.0–1.0)	Diff	0.09	(-0.02 to 0.20)	0.09	1.0 (1.0–1.0)	1.0 (1.0–1.0)	Diff	0.10	(-0.02 to 0.21)	0.11
	No. of cryopreserved blastocysts: median (IQR)		5.0 (2.0–8.0)	5.0 (2.0–8.0)	Diff	-0.10	(-0.68 to 0.48)	0.74	4.0 (2.0–7.0)	5.0 (3.0–8.0)	Diff	-0.40	(-1.02 to 0.23)	0.22

[a] OR, RR and Diff were calculated for PPOS versus GnRH-antagonist groups.

[b] Bold type indicates statistical significance: *P*-value <0.05.

[c] Split ICSI = division of oocytes into cIVF and ICSI, with simultaneous fertilization attempts using both methods.

[d] Elevated LH concentration = serum LH concentration increase from basal to peak concentration.

[e] Ovulation findings before oocyte retrieval = serum progesterone ≥5.0 ng/ml or ultrasonographical evidence of ovulation.

[f] Oocyte maturation rate = number of metaphase II oocytes/total number of oocytes retrieved.

[g] Fertilization rate = ratio of two-pronuclear embryos to number of retrieved oocytes in IVF or mature oocytes with ICSI.

Weights applied without rounding; case numbers after weighting rounded to two decimal places for presentation purposes. *P*-values were calculated based on the sandwich variance in the generalized linear regression procedure with IPTW. cIVF, conventional IVF; OHSS, ovarian hyperstimulation syndrome; PPOS, progestin-primed ovarian stimulation; GnRH, gonadotrophin-releasing hormone; IPTW, inverse probability of treatment weighting; ICSI, intracytoplasmic sperm injection; RR, risk ratio; Diff, risk difference.

TABLE 4 PREGNANCY OUTCOMES WITH PROGESTIN-PRIMED AND GONADOTROPHIN-RELEASING HORMONE ANTAGONIST OVARIAN STIMULATION AND FIRST FROZEN EMBRYO TRANSFER PROTOCOLS IN THE CONTEXT OF THE SECOND INVERSE PROBABILITY OF TREATMENT WEIGHTING ANALYSIS

Outcome	Category	Original (unadjusted)					IPTW				
		PPOS	GnRH-antagonist	Estimate	95% CI	*P*-value[a]	PPOS	GnRH-antagonist	Estimate	95% CI	*P*-value[a]
		***n* = 284**	***n* = 579**	**OR**			**weight = 860.2**	**weight = 864.1**	**OR**		
Primary outcome	Live birth rate: *n* (%)	90 (31.7)	244 (42.1)	0.64	(0.47–0.86)	**0.003**	270.6 (31.5)	365.2 (42.3)	0.63	(0.46–0.86)	**0.004**
Other outcomes	Implantation rate: *n* (%)	135/311 (43.4)	322/620 (51.9)	-	-	**0.02**	-	-	-	-	-
	Biochemical pregnancy rate: *n* (%)	154 (54.2)	374 (64.6)	0.65	(0.49–0.87)	**0.003**	467.9 (54.4)	555.2 (64.3)	0.66	(0.49–0.90)	**0.009**
	Clinical pregnancy rate: *n* (%)	133 (46.8)	318 (54.9)	0.72	(0.54–0.96)	**0.03**	411.1 (47.8)	474.2 (54.9)	0.75	(0.56–1.02)	0.07
	Early miscarriage rate: *n* (%)	28 (9.9)	57 (9.8)	1.00	(0.61–1.60)	0.99	91.7 (10.7)	85.2 (9.9)	1.09	(0.66–1.80)	0.74
	Multiple pregnancy rate: *n* (%)	2 (0.7)	5 (0.9)	0.81	(0.12–3.80)	0.81	4.0 (0.5)	8.4 (1.0)	0.47	(0.09–2.53)	0.38
	Ongoing pregnancy rate: *n* (%)	94 (33.1)	247 (42.7)	0.67	(0.49–0.89)	**0.007**	281.7 (32.8)	369.9 (42.8)	0.65	(0.48–0.89)	**0.007**

[a] Bold type indicates significance: *P*-value <0.05.

Weights applied without rounding; case numbers after weighting rounded to two decimal places for presentation purposes. *P*-values were calculated based on the sandwich variance in the generalized linear regression procedure with IPTW. Live birth, delivery of viable infant after 24 weeks of gestation; implantation rate, number of gestational sacs on transvaginal ultrasonography/embryo number transferred; biochemical pregnancy, serum human chorionic gonadotrophin ≥5 mIU/ml; clinical pregnancy, presence of one or more gestational sacs confirmed by ultrasound; early miscarriage, before 12 weeks of gestation; ongoing pregnancy, fetal heartbeat after 12 weeks of gestation; PPOS, progestin-primed ovarian stimulation; GnRH, gonadotrophin-releasing hormone; IPTW, inverse probability of treatment weighting.

Regarding pregnancy outcomes, multiple studies have demonstrated that PPOS is comparable to GnRH-antagonist protocols; randomized controlled trials have indicated similar effectiveness between the two approaches (*Chen et al., 2024*; *Giles et al., 2021*; *Guo et al., 2020*), along with comparable euploidy rates (*La Marca et al., 2020*; *Vidal et al., 2024*; *Yang L et al., 2022*). Furthermore, a meta-analysis by *Ata et al. (2021)* revealed no significant differences in live birth rate, clinical pregnancy rate and miscarriage rate per embryo transfer cycle between PPOS and GnRH-antagonist protocols. Consequently, PPOS has been endorsed as an effective treatment for patients who will not undergo fresh embryo transfer (*Ata et al., 2021*; *Yamada et al., 2022*). However, several studies have raised concerns about its effectiveness (*Beguería et al., 2019*; *Chen et al., 2022*; *Zhang et al., 2021a*; *Zhou et al., 2023a*, b). Specifically, the number and ratio of good-quality blastocysts are reduced significantly with PPOS compared with GnRH-antagonist protocols (*Zhou et al., 2023a*, b). Moreover, in preimplantation genetic testing (PGT) cycles, PPOS was negatively associated with the cumulative live birth rate (CLBR) and the number and ratio of good-quality euploid blastocysts compared with GnRH-antagonist protocols (*Zhou et al., 2023b*). These findings are consistent with the present results, which also showed a lower rate of good-quality embryos and a lower pregnancy rate in the PPOS group. Although differences in patient populations and protocols should be considered, these results suggest the potential non-genomic adverse effects of progestin on oocytes. The deterioration of early embryo quality is attributed to oocyte factors, particularly the cytoplasm (*Levy et al., 2004*). The present results, specifically the lower pregnancy rate in the PPOS group with similar rates of good-quality embryos transferred across groups, suggest that progestin may impair oocyte cytoplasmic functions.

Granulosa cells are crucial for oocyte development and maturation (*Buccione et al., 1990*; *Gilchrist et al., 2008*; *Jiang et al., 2010*; *Liu et al., 2017*), and a close association exists between the mitochondrial status of granulosa cells and embryo quality (*Cecchino and Garcia-Velasco, 2019*). Mitochondria are directly involved in the cell's energetic metabolism (*Vakifahmetoglu-Norberg et al., 2017*), supplying ATP to cells through the OXPHOS pathway. Various factors,

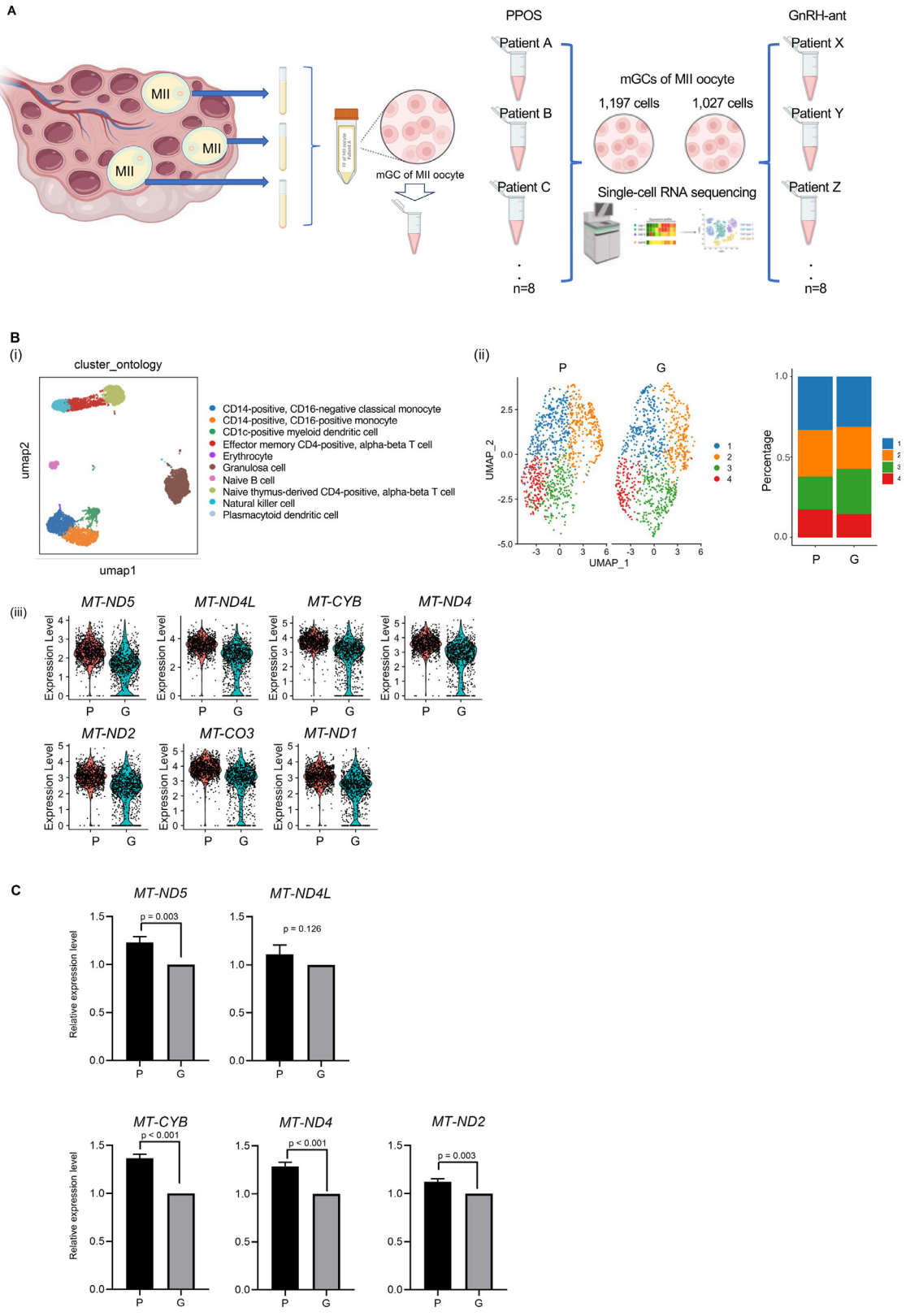

FIGURE 2 Integrated analysis of single-cell RNA-sequencing (scRNA-seq), uniform manifold approximation and projection (UMAP), and quantitative real-time polymerase chain reaction (RT-qPCR) validation based on metaphase MII (MII) oocyte mural granulosa cells (mGC). (A) Schematic overview of scRNA-seq protocol: MII oocyte mGC collection, preparation and sequencing. Created with BioRender (BioRender.com, Canada). BioRender. Han, M. (2025) https://BioRender.com/0ekz2ku. (B) scRNA-seq analysis of mGC. (i) UMAP projection of captured cells, with granulosa cells highlighted in the red dotted circle. (ii) Distribution of granulosa cells in the progestin-primed ovarian stimulation (PPOS)_MII (P) and gonadotrophin-releasing hormone (GnRH)-antagonist_MII (G) groups. (iii) Differential gene expression analysis of mtDNA genes between the PPOS_MII (P) and GnRH-antagonist_MII (G)

including oxidative stress, can lead to mitochondrial dysfunction, significantly affecting energy synthesis in oocytes and early embryos (*Babayev and Seli, 2015*; *Lan et al., 2020*). The quantification of mtDNA content in cumulus granulosa cells could be a non-invasive method to evaluate oocyte quality and associated metabolic processes (*Desquiret-Dumas et al., 2017*; *Lan et al., 2020*; *Ogino et al., 2016*). In the present study, the cell proportion analysis revealed comparable cluster distributions between the PPOS and GnRH-antagonist groups (Supplemental Figure 1). Even when focusing on granulosa cells, the clustering analysis showed similar distributions between the PPOS_MII and GnRH-antagonist_MII groups (**FIGURE 2B-II**). Given these findings, the decision was made to focus on expression levels. Interestingly, among the top 20 DEG, 12 mtDNA genes, encoding 13 subunits of the respiratory chain complexes, exhibited notably higher expression in the PPOS group. Therefore, the mitochondrial OXPHOS pathway in mGC, which line the follicle wall and can influence the microenvironment of the follicle and subsequently affect the quality of oocytes, is predominantly and selectively affected by PPOS. mtDNA accumulation is correlated with an increase in mRNA encoding essential replication factors (*Lan et al., 2020*; *Mahrous et al., 2012*). Moreover, mitochondrial gene copy numbers in mGC increase with ageing, possibly as a compensatory response (*Liu et al., 2017*). Accordingly, the increase in mtDNA gene expression further suggests enhanced mitochondrial functions as a compensatory response to meet the heightened energy demands due to the concurrent deterioration in oocyte quality during oocyte growth in the PPOS group (*de Los Santos et al., 2018*). The genetic findings suggest that PPOS may be less suitable for obese or older patients, who are likely to have experienced oxidative stress and mitochondrial dysfunction, resulting in diminished mitochondrial compensatory capacity. Moreover, the functional impairment of mGC associated with increased mtDNA gene expression, as seen with PPOS, may be alleviated by the concurrent use of antioxidants, which could improve pregnancy outcomes. The mtDNA copy number in cumulus granulosa cells undergoes a transition, decreasing from the germinal vesicle phase to the metaphase I phase and remaining steady from the metaphase I to MII stages. This implies that mtDNA expression in cumulus granulosa cells is inversely correlated with oocyte cytoplasm maturation. Although similar observations have not been reported for mGC, it might be inferred that cytoplasmic maturity could be less advanced in MII oocytes with more mtDNA in mGC in this study. This could be one of the factors contributing to the lower pregnancy outcomes in the PPOS group.

In PPOS, the effects of different progestins and their administration methods on treatment outcomes are still uncertain. While progestins such as medroxyprogesterone (MPA), dydrogesterone (DYG) and natural micronized progesterone are widely studied, the optimal type and dosage are still unclear. Some studies report no significant differences in reproductive outcomes based on the type of progestin (*Ata et al., 2021*; *Huang et al., 2019*), while others indicate that variations in pharmacokinetics and pharmacodynamics may influence ovarian response and embryo quality (*Guo et al., 2020*; *Yu et al., 2018*). For instance, one study found that while oocyte retrieval numbers and pregnancy rates were similar between different progestins, DYG showed a tendency for higher LH concentrations compared with MPA (*Yu et al., 2018*). The present study, which utilized CMA — a progestin commonly used in Japan but less so globally — adds valuable insight into the variability associated with different progestins. Similarly, the method of progestin administration, whether fixed or flexible, is also subject to differing opinions. Some research indicates comparable outcomes between these methods (*Ata and Kalafat 2024*; *Kalafat et al., 2022*), while others have suggested that the timing of follicular development and ovulation suppression might be better managed with one approach depending on the patient's hormonal profile (*Ata and Kalafat, 2024*; *Chen et al., 2023*; *Doğan et al., 2023*). These inconsistencies underscore the need for further research to refine PPOS and tailor these protocols to individual patient needs. This study employed a 'modified' protocol based on the fixed protocol, where the dosage of progestin is increased according to serum LH concentration. This method starts with a low dose of progestin at the beginning of ovarian stimulation, and then adjustment of the dose based on serum LH concentration. This strategy allows use of the minimum necessary amount of progestin, potentially minimizing its adverse effects on follicular development. Unlike the flexible protocol, the authors' method begins progestin administration at the start of stimulation, reducing the risk of delayed suppression of the LH surge. The low rate of premature LH surge and absence of early ovulation in this study suggests the effectiveness of this protocol. Nevertheless, further research is needed to determine whether these findings with the 'modified' protocol are applicable to other protocols.

The role of progesterone in oocytes remains ambiguous. Although progesterone receptors A and B are expressed in granulosa cells, they are absent in the oocyte, indicating an indirect influence of progesterone mediated by granulosa cells (*Revelli et al., 1996*). Some studies suggest that elevated progesterone concentrations, when ovulation is triggered, are associated with fewer high-quality early embryos and lower pregnancy and birth rates (A*li et al., 2023*; *Bu et al., 2014*; *Huang et al., 2015*, *2016*; *Li et al., 2023*; *Pal et al., 2004*; *Racca et al., 2021*; *Vanni et al., 2017*; *Villanacci et al., 2023*). Whereas these reports suggest that progesterone could compromise embryo quality (*Santos-Ribeiro et al., 2014*), contrasting reports also exist, precluding a definitive conclusion (*Baldini et al., 2018*; *Pardiñas et al., 2021*; *Racca et al., 2020*). This clinical outcome analysis suggests that progestin could have an adverse effect on the quality of retrieved oocytes, which is correlated with early embryo development.

This study also had some limitations. First, it was conducted at a single centre, focusing exclusively on Japanese patients with normal ovarian reserves using CMA. This may limit the generalizability of the findings to broader populations undergoing IVF. For instance, while PPOS has shown equivalence in high responders

groups. Violin plots highlight differentially expressed mtDNA genes. (C) RT-qPCR validation of the relative gene expression in mGC of MII oocytes in the PPOS_MII and GnRH-antagonist_MII groups. The GnRH-antagonist_MII group was set as the baseline (expression level = 1), and relative expression levels in the PPOS_MII group were calculated accordingly. Each measurement is based on three biological replicates. Values presented as mean ± SEM (error bars). Statistical analyses were performed using a two-tailed unpaired Student's *t*-test.

in a prospective study (*Chen et al., 2024*), the GnRH-antagonist protocol may be more appropriate for patients with low to normal ovarian reserves. Additionally, the variability in protocols across different centres could influence the applicability of these results to other settings. Hence, the applicability of these results to other populations and different types of progestin remains uncertain and warrants further study. Second, the retrospective nature of this study inherently carries the risk of selection bias and the presence of confounding variables that could skew the results. To mitigate these biases, the IPTW method was used, adjusting for 12 factors in the first IPTW analysis and 14 factors in the second IPTW analysis, based on previous reports that could impact reproductive outcomes. The number of adjustment factors in this study is comparable to or slightly higher than those used in previous studies (*Chen et al., 2022*; *Wang et al., 2024*). While it is impossible to eliminate the influence of confounding variables completely, the authors believe that this approach strengthens the robustness of the findings. Furthermore, the E-value was used, which represents the minimum strength of association that an unmeasured confounder would need to have with both the treatment and the outcome to fully explain the observed treatment–outcome association. The E-value analysis in this study suggests that the likelihood of unmeasured confounding factors overturning the results is low. Nonetheless, it is recognized that these inherent biases may reduce the strength and reliability of the results, and the authors will aim to address these limitations in future prospective studies. Third, the genetic analysis in this study was based on a relatively small sample size of 16 patients, which could limit the robustness and generalizability of the findings. While a larger sample size would improve these aspects, it is important to note that, in the context of scRNA-seq studies, the sample size in this study is higher than that in other similar studies (*Choi et al., 2023*; *Wagner et al., 2020*). These preliminary findings provide a foundation for further investigation. Fourth, PGT for aneuploidy was not included in this study due to the strict regulations in Japan during the study period, which may have affected the assessment of embryo quality. Finally, this assessment was based solely on pregnancy rates from the first embryo transfer, rather than CLBR in the cohort. While CLBR provides a more comprehensive picture of the long-term efficacy of the protocols, defining CLBR can be challenging – such as determining the observation period or the number of transfers to include – which complicates the execution of such studies. Consequently, many studies, including the present study, have focused on the outcomes from the first FET cycle (*Caetano et al., 2022*; *Chen et al., 2024*; *Dinç et al., 2024*; *Yang AM et al., 2022*). Given that the highest quality embryos were transferred consistently, and similar numbers of cryopreserved cleavage embryos and blastocysts were used in both groups, it is believed that the outcomes from the first FET cycle can partially reflect CLBR. However, further confirmation of these findings will require reassessment of the CLBR or conducting an analysis on a per-person basis once all embryos in this cohort have been utilized. Additionally, the scRNA-seq analysis of mGC from MII oocytes provided a complementary perspective to the comparison of clinical outcomes, enriching the conclusions with a molecular dimension that extends beyond traditional clinical metrics.

CONCLUSIONS

These results suggest a hypothetical mechanism through which progestin indirectly affects oocyte development, leading to a compensatory increase in mitochondrial gene expression in mGC. In patients with normal ovarian reserves, PPOS suppressed the premature LH surge rate significantly, but was associated with a lower pregnancy rate and a lower proportion of good-quality cleavage embryos compared with the GnRH-antagonist protocol. The elevated expression of mtDNA genes in mGC may also indicate a decline in oocyte quality with PPOS. Considering these findings, caution should be exercised when employing PPOS for ART.

DATA AVAILABILITY

The sequencing data are accessible via the NBDC (National Bioscience Database Center) website under controlled access (https://humandbs.dbcls.jp/en/hum0490-v1). The NBDC number is hum0490, and the JGA (Japanese Genotype-phenotype Archive) accession number is JGAS000770. As this dataset is classified as controlled-access (Type I), a formal application procedure is required as outlined at https://humandbs.dbcls.jp/en/data-use.

AUTHOR CONTRIBUTIONS

Mika Handa: writing – original draft, methodology, conceptualization, investigation, funding acquisition, data curation, visualization. Tsuyoshi Takiuchi: writing – review and editing, writing – original draft, conceptualization, supervision, project administration. Sumika Kawaguchi: investigation, visualization, formal analysis. Sho Komukai and Tetsuhisa Kitamura: methodology, investigation, visualization, formal analysis. Jonathan Moody, Chung-Chau Hon, Kokoro Ozaki and Yasushi Okazaki: investigation resources. Yasuhiro Ohara, Masakazu Doshida, Takumi Takeuchi, Hidehiko Matsubayashi, Tomomoto Ishikawa, Fumie Saji and Tatsuya Muyake: resources. Masafumi Horie, Yoshinari Ando and Jay W. Shin: methodology, supervision. Tasdashi Kimura: supervision, project administration.

ACKNOWLEDGEMENTS

The authors wish to thank all individuals who helped to conduct this study, including Saki Ishino and Takashi Enjoji (Centre of Medical Innovation and Translational Research, Graduate School of Medicine, Osaka University) for their technical assistance; Lee Yuna, Chiho Kumeta, Kazumi Suehara and Shimpei Mizuta (Reproduction Clinic Osaka) for their expertise in embryology laboratory work and sample collection; and Tsugumi Kawashima, Nozomi Moritsugu, Tomoko Hirata, Shohei Noma, Michihira Tagami, Chitose Takahashi and Riichiroh Manabe (RIKEN Centre for Integrative Medical Sciences) for technical assistance in sequencing. In addition, the authors wish to thank Tomoe Nobusada and Takeya Kasukawa (RIKEN Centre for Integrative Medical Sciences) for their valuable contributions in supporting the deposition of datasets. Special appreciation goes to Editage (www.editage.jp) for English language editing. Artwork in the graphical abstract was created in BioRender. Han, M. (2025) https://BioRender.com/0ekz2ku.

FUNDING

This research was supported by a research grant to RIKEN Centre for Integrative Medical Sciences from the Ministry of Education, Culture, Sports, Science and Technology.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2025.104833.

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Received 15 June 2024; received in revised form 15 August 2024; accepted 14 January 2025.