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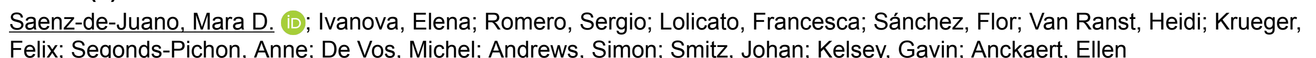
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ORIGINAL ARTICLE *Embryology*

DNA methylation and mRNA expression of imprinted genes in blastocysts derived from an improved *in vitro* maturation method for oocytes from small antral follicles in polycystic ovary syndrome patients

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STUDY QUESTION: Does imprinted DNA methylation or imprinted gene expression differ between human blastocysts from conventional ovarian stimulation (COS) and an optimized two-step IVM method (CAPA-IVM) in age-matched polycystic ovary syndrome (PCOS) patients?

SUMMARY ANSWER: No significant differences in imprinted DNA methylation and gene expression were detected between COS and CAPA-IVM blastocysts.

WHAT IS KNOWN ALREADY: Animal models have revealed alterations in DNA methylation maintenance at imprinted germline differentially methylated regions (gDMRs) after use of ARTs. This effect increases as more ART interventions are applied to oocytes or embryos. IVM is a minimal-stimulation ART with reduced hormone-related side effects and risks for patients. CAPA-IVM is an improved IVM system that includes a pre-maturation step (CAPA), followed by an IVM step, both in the presence of physiological compounds that promote oocyte developmental capacity.

STUDY DESIGN, SIZE, DURATION: For DNA methylation analysis 20 CAPA-IVM blastocysts were compared to 12 COS blastocysts. For RNA-Seq analysis a separate set of 15 CAPA-IVM blastocysts were compared to 5 COS blastocysts.

PARTICIPANTS/MATERIALS, SETTING, METHODS: COS embryos originated from 12 patients with PCOS (according to Rotterdam criteria) who underwent conventional ovarian stimulation. For CAPA-IVM 23 women were treated for 3–5 days with highly purified hMG (HP-hMG) and no hCG trigger was given before oocyte retrieval. Oocytes were first cultured in pre-maturation medium (CAPA for 24 h containing C-type natriuretic peptide), followed by an IVM step (30 h) in medium containing FSH and Amphiregulin. After ICSI, Day 5 or 6 embryos in both groups were vitrified and used for post-bisulphite adaptor tagging (PBAT) DNA methylation analysis or RNA-seq gene expression analysis of individual embryos. Data from specific genes and gDMRs were extracted from the PABT and RNA-seq datasets.

MAIN RESULTS AND THE ROLE OF CHANCE: CAPA-IVM blastocysts showed similar rates of methylation and gene expression at gDMRs compared to COS embryos. In addition, expression of major epigenetic regulators was similar between the groups.

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LIMITATIONS, REASONS FOR CAUTION: The embryos from the COS group were generated in a range of culture media. The CAPA-IVM embryos were all generated using the same sperm donor. The DNA methylation level of gDMRs in purely *in vivo*-derived human blastocysts is not known.

WIDER IMPLICATIONS OF THE FINDINGS: A follow-up of children born after CAPA-IVM is important as it is for other new ARTs, which are generally introduced into clinical practice without prior epigenetic safety studies on human blastocysts. CAPA-IVM opens new perspectives for patient-friendly ART in PCOS

STUDY FUNDING/COMPETING INTEREST(S): IVM research at the Vrije Universiteit Brussel has been supported by grants from the Institute for the Promotion of Innovation by Science and Technology in Flanders (Agentschap voor Innovatie door Wetenschap en Technologie-IWT, project 110680), the Fund for Research Flanders (Fonds voor Wetenschappelijk Onderzoek-Vlaanderen-FWO-AL 679 project, project G.0343.13), the Belgian Foundation Against Cancer (HOPE project, Dossier C69Ref Nr 2016-119) and the Vrije Universiteit Brussel (IOF Project 4R-ART Nr 2042). Work in G.K.'s laboratory is supported by the UK Biotechnology and Biological Sciences Research Council and Medical Research Council. The authors have no conflicts of interest.

Key words: IVM / oocyte / embryo / DNA methylation / genomic imprinting

Introduction

IVM can be offered as a 'patient friendly' treatment in polycystic ovary syndrome (PCOS) with minimal FSH stimulation and no hCG triggering of the follicles, eliminating the risk of ovarian hyperstimulation syndrome (OHSS). This treatment comes with reduced cost and monitoring needs (reviewed in Herta *et al.,* 2018). IVM of oocytes retrieved from extracorporeal ovarian tissue ('*ex vivo*' IVM) may be useful as a fertility preservation strategy in girls and women prior to receiving gonadotoxic cancer treatment (De Vos *et al.,* 2014; Segers *et al.,* 2015). In its genuine form, IVM involves the meiotic transition from Prophase I to Metaphase II, *in vitro*, of oocytes retrieved from small and mid-antral follicles in unstimulated or minimally stimulated cycles (De Vos *et al.,* 2016). Oocytes retrieved from small antral follicles without an hCG trigger still need to acquire full meiotic and developmental competence (Sánchez *et al.,* 2015), which are essential for successful fertilization and pre- and post-implantation embryo development; therefore, implantation and pregnancy rates are lower than in conventional ART. To improve the outcome of IVM, a major challenge is to synchronize oocyte nuclear and cytoplasmic maturation (Coticchio *et al.,* 2015). This can be achieved in a first culture step by preventing resumption of meiosis during a prematuration culture (PMC) of cumulus-oocyte complexes to promote cytoplasmic maturation prior to inducing meiotic maturation in a second culture step. Maintenance of a functional connection between the oocyte and cumulus during culture is crucial for oocyte competence acquisition (Gilchrist *et al.* 2008, Luciano *et al.* 2011, Lodde *et al.* 2013; Macaulay *et al.,* 2016). Based on these concepts, a new PMC approach based on the addition of C-type natriuretic peptide (CNP) was recently developed (Zhang *et al.,* 2010; Romero *et al.,* 2016; Sánchez *et al.,* 2017). CNP is a natural meiotic inhibitor produced and secreted by mural granulosa cells (Zhang *et al.,* 2010) that binds to the natriuretic peptide receptor 2 (NPR2) in the cumulus cells (Zhang *et al.,* 2010; Tsuji *et al.,* 2012) and increases cGMP levels in the cumulus-oocyte complexes (COCs). cGMP enters the oocyte via gap-junctional communication (Norris *et al.,* 2009) and prevents cAMP degradation to keep the oocyte under meiotic arrest (Zhang *et al.,* 2010; Kawamura *et al.,* 2011; Tsuji *et al.,* 2012). CNP maintains gap junction activity (Campen *et al.,* 2016), and translation of some maternal transcripts within the oocyte depends on the bi-directional communication between oocytes and their surrounding cumulus cells (Zamah *et al.,* 2010; Chen *et al.,* 2013). This PMC-IVM strategy has been tested for human oocytes from small (2–8 mm) antral follicles in PCOS patients in a prospective study involving sibling oocytes leading to a significant increase in oocyte maturation rates that resulted in a higher availability of cleaving (Day 3) embryos and good quality blastocysts (Days 5 and 6) (Sánchez *et al.,* 2017).

There is concern that ART, and oocyte culture in particular, might interfere with the process of genomic imprinting. As a fundamental part of this epigenetic mechanism, DNA methylation is established at imprinted germline differentially methylated regions (gDMRs) during oocyte growth (Obata and Kono, 2002; Lucifero *et al.,* 2004; Hiura *et al.,* 2006). Furthermore, maternal-effect products are transcribed and stored in the oocyte, which are necessary to maintain DNA methylation specifically at gDMRs after fertilization (reviewed by Kelsey and Feil, 2013). Correct establishment and maintenance (during pre-implantation development) of genomic imprinting are necessary for normal foetal and placental development. Studies in various animal models have revealed a link between ARTs, such as ovarian stimulation and pre-implantation embryo culture, and altered genomic imprinting (reviewed by Canovas *et al.,* 2017a). Furthermore, ART has been associated with an increased risk of imprinting disorders such as Beckwith–Wiedemann and Angelman syndromes in children (Cox *et al.,* 2002, DeBaun *et al.,* 2003; Maher *et al.,* 2003; Halliday *et al.,* 2004; Bowdin *et al.,* 2007; Cortessis *et al.,* 2018).

In human, we have previously shown that application of a standard IVM strategy (De Vos *et al.,* 2016) does not interfere with imprinting establishment at four selected genes in oocytes (Kuhtz *et al.,* 2014). Moreover, while mouse studies have revealed that neither *in vitro* follicle culture (from the early pre-antral stage) or superovulation perturb imprinting establishment in oocytes, the possibility remains that both ARTs lead to some loss of imprinted DNA methylation during pre-implantation development (Denomme *et al.,* 2011; Anckaert *et al.,* 2009; Saenz-de-Juano *et al.,* 2016).

The current study aims to assess the potential epigenetic impact of the novel two-step IVM culture protocol, including a pre-maturation ("Capacitation") step, followed by an IVM step (abbreviated 'CAPA-IVM'). We analyzed DNA methylation and RNA expression of imprinted genes in single human blastocysts derived from CAPA-IVM oocytes and from conventional controlled ovarian stimulation (COS) in age-matched PCOS patients. DNA methylation analysis was

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Table I Characteristics of blastocysts and patients selected for DNA methylation and gene expression analysis.

Analysis/ conditions	Number of blastocysts	Blastocyst grading (n (%))[a]				Vitrification day		Number of PCOS patients	Age (y; mean (SD))
		AA	AB	BA	BB	Day 5	Day 6		
DNA methylation									
CAPA-IVM	20	4 (20)	6 (30)	6 (30)	4 (20)	10	10	11	27.4 (3.9)
COS	12	3 (25)	6 (50)	3 (25)	0	4	8	9	30.7 (2.3)
Gene expression									
CAPA-IVM	15	4 (26.7)	2 (13.3)	3 (20)	6 (40)	8	7	12	26.9 (3.1)
COS	5	1 (20)	1 (20)	2 (40)	1 (20)	2	3	5	30.0 (1.8)

[a]Gardner et al. (1998)

performed using a recently developed low-input genome-wide DNA profiling method (post-bisulphite adaptor tagging, PBAT) and gene expression analysis using RNA-seq.

Materials and Methods

Study approval

The study was approved by the local ethics committee of the University Hospital UZ Brussel of the Vrije Universiteit Brussel (project 2008/068) and by the Federal Commission for Medical and Scientific Research on embryos *in vitro* (Adv043/2012). The consent form included details pertaining to the donation of immature COCs to test a novel IVM method and to assess efficacy and safety parameters (including embryo development) in comparison to the current standard of practice. Average patient age of each experimental group is shown in Table I.

Control COS blastocysts

A comparison of methodologies to obtain COS embryos and CAPA-IVM embryos is illustrated in Figure 1. In brief, vitrified blastocysts were obtained from age-matched PCOS patients who consented to donate stored supernumerary blastocysts for research. Mature oocytes obtained after COS were inseminated using ICSI and cultured to Day 5 or 6 before vitrification. Inclusion criteria for sperm quality parameters were concentration $> 15 \times 10^6$/ml, A+B motility >32% and normal morphology ≥4%. As for the CAPA-IVM group, only blastocysts graded AA, AB, BA and BB according to Gardner scoring method (Gardner *et al.,* 1998) were included for analysis.

IVM of oocytes

IVM of oocytes was performed as described in Sánchez *et al.* (2017). Briefly, PCOS patients were recruited for this study if 30 or more antral follicles were visible on the last pelvic ultrasound scan before oocyte retrieval. Highly purified hMG (HP-hMG, Menopur, Ferring, Saint-Prex, Switzerland) was started on cycle Day 5 of the menstrual period after discontinuation of one strip of the combined oral contraceptive pill. Subcutaneous injections of HP-hMG were administered for 3 to 5 consecutive days, at a maximum daily dose of 225IU, 225IU, 225IU, 150 IU and 150IU HP-hMG, respectively. If all follicles had a diameter of <6 mm on the third stimulation day, HP-hMG stimulation was given for 1 or 2 further days, but caution was taken for the diameter of the leading follicle not to exceed 10 mm.

COCs were retrieved 42 h after the last HP-hMG injection and collected in human tubal fluid supplemented with 50 µM 3-isobutyl-1-methylxanthine (IBMX) (Sigma, Schnelldorf, Germany) and heparin at 20 IU/ml. After collection, COCs were washed and transferred to a four-well dish, containing CAPA medium (IVM System, Medicult, Origio) supplemented with 1 mIU/ml recombinant FSH (Puregon MSD, Australia), 5 ng/ml insulin (Roche, Mannheim, Germany), 10 nM estradiol (E2) (Sigma; Schnelldorf, Germany), 10 mg/ml human serum albumin (Vitrolife, Göteborg, Sweden) and 25 nM CNP (Tocris Bioscience; Bristol, UK). COCs were cultured in 500 µl of CAPA medium, in groups of 10 COCs per well under oil for 24 h at 37°C, 6% CO_2 in air.

Following 24 h of incubation in CAPA media, COCs were thoroughly washed and transferred into Medicult IVM medium containing 5 ng/ml Insulin, 10 nM E2, 100 ng/ml human recombinant amphiregulin (rhAREG; R&D systems, Minneapolis, USA) and 100 mIU/ml recombinant FSH, and incubated for 30 h under the same incubation conditions as detailed above.

ICSI, embryo culture and blastocyst vitrification

Thirty hours after IVM culture, oocytes were mechanically and enzymatically denuded and mature oocytes were inseminated using ICSI with donated sperm. Fertilization was confirmed 16–18 h post-insemination by the presence of two pronuclei. Fertilized oocytes and embryos were cultured in individual droplets of 25 µl Cook medium with oil overlay (Ovoil, Vitrolife) until Day 5 or 6 after ICSI. Blastocysts were vitrified according to the method described by Van Landuyt *et al.* (2011). Only blastocysts graded AA, AB, BA and BB according to Gardner scoring method (Gardner *et al.,* 1998) were included for analysis.

DNA methylation analysis of blastocysts

Because of the limited amount of starting material, DNA isolation, bisulphite conversion and sequencing library preparation were performed using the PBAT protocol (Miura *et al.,* 2012) including the modifications described before (Canovas *et al.,* 2017b). Prior to processing, blastocysts were thawed, washed in PBS to remove

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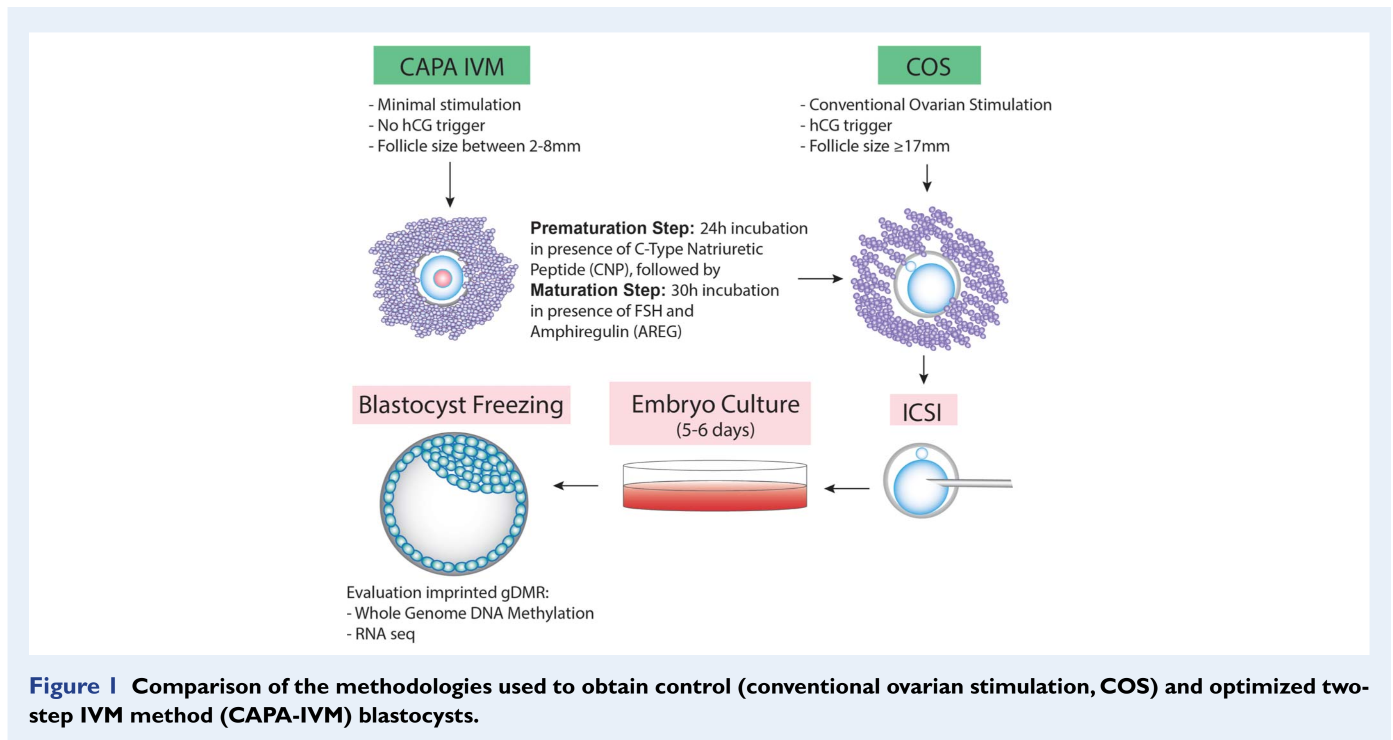

Figure 1 Comparison of the methodologies used to obtain control (conventional ovarian stimulation, COS) and optimized two-step IVM method (CAPA-IVM) blastocysts.

the cryoprotectant medium and directly transferred into 10 µl RLT buffer (Qiagen). DNA was isolated using Agencourt AMPure XP beads (Beckman Coulter, A63881) in excess (ratio 2:1) and eluted in 10 µl of elution buffer. After bisulphite conversion and PBAT according to Canovas *et al.* (2017b), libraries were generated using 15 rounds of amplification. Library quantity and quality were assessed using Bioanalyzer 2100 (High-Sensitivity DNA chips, Applied Biosystems) and KAPA Library Quantification Kit for Illumina (KAPA Biosystems). Each library was tagged with an individual identification sequence and sequenced on HiSeq2500.

Reads were mapped using Bismark software v.0.16 (http://www.bioinformatics.babraham.ac.uk/projects/bismark/) to the human reference genome GRCh38. DNA methylation analysis was done using SeqMonk software v.1.40.0 (http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). The full analysis of the genome-wide datasets will be presented elsewhere. For the present study, methylation calls for the CpG sites within 21 gDMRs (Okae *et al.*, 2014) were extracted from the mapped data. Note that all extracted methylation calls were from uniquely mapped, deduplicated reads, therefore excluding PCR duplicates. For analysis of methylation consistency within gDMRs, methylation calls for CpGs within individual sequence reads were extracted from BAM files. The comparison of gDMR methylation between COS and CAPA-IVM blastocysts was performed using Student *t*-tests. The *P* values were adjusted for multiple comparisons with a Benjamini–Hochberg correction. Statistical significance was established with P value < 0.05.

RNA-seq analysis of blastocysts

Individual blastocysts were thawed and washed in PBS to remove the cryoprotectant medium. RNA extraction was performed using the ARCTURUS® PicoPure® RNA Isolation kit (KIT0204, Life Technologies), according to the manufacturer's instructions, after which the Ovation RNA-Seq System V2 (NuGEN, Cat. 7102-08) kit was used to generate the RNA-seq libraries. Final amplification of libraries was performed with NEB Next DNA Library Prep Master Mix for Illumina (NEB, Cat. E6040S) according to the manufacturer's guidelines. iPCRTag reverse primer with individual indices was used to generate RNA-seq libraries. One hundred bp single end reads were sequenced on Illumina HiSeq 1000. For RNA-seq libraries, raw sequence reads were trimmed using Trim Galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to remove adapter contamination and reads with poor quality defined by low PHRED score. Mapping was performed using Hisat software (http://www.ccb.jhu.edu/software/hisat/) to human genome GRCH38, and data were visualized using Seqmonk (https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/).

Reads over transcripts for imprinted genes and selected epigenetic regulator genes were merged across exons correcting for feature length and quantitated using the RNA-Seq quantitation pipeline. After quantification, reads were entered into DESeq2 for differential expression analysis using a *P* value cut-off of 0.05 and not applying independent filtering.

Results

Similar maintenance of DNA methylation at gDMR in COS and CAPA-IVM embryos

To investigate the epigenetic safety of CAPA-IVM, genome-wide DNA methylation of 32 single human blastocysts was profiled individually using the PBAT protocol. Twenty blastocysts obtained after CAPA-IVM were compared to 12 blastocysts generated after COS (control). Patient characteristics and blastocyst grading scores are presented in Table 1. There were no significant differences in age between control and CAPA-IVM patients.

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In both cases, oocytes were fertilized using ICSI and cultured *in vitro* for 5–6 days until the blastocyst stage. From the 12 control blastocysts used for DNA methylation analysis, 9 had been cultured in Sage medium, 2 in Medicult medium and 1 in Vitrolife medium. On the other hand, all CAPA-IVM blastocysts were cultured in Cook medium. To avoid parental effects, all CAPA-IVM oocytes had been inseminated by sperm from the same donor for ICSI.

The sequence yield per embryo varied between 6.65×10^6 and 23.3×10^6 unique reads. The percentage of CpGs covered with 1, 3 or 5 reads in each sample is shown in Supplementary Figure S1. The global CpG methylation level per embryo varied from 15% to 36% for control embryos and from 23% to 44% for CAPA-IVM embryos. In addition to CpG methylation rate methylation values for CHG and CHH sites (non-CpG methylation) were evaluated. Non-CpG methylation was very low (between 0.4% and 0.8%), indicating a bisulphite conversion efficiency of $\geq$99.2%. A full analysis of the genome-wide datasets will be presented elsewhere.

Despite the extensive DNA methylation reprogramming events in preimplantation embryos, gDMRs are expected to retain methylation levels derived from the oocyte or the sperm. We analysed methylation levels of 21 individual imprinted genes previously described by Okae *et al.* (2014). Twenty of these gDMRs correspond to CpG islands that are fully methylated in oocytes and are expected to maintain DNA methylation only on the maternal allele in blastocysts. The remaining gDMR was the *H19* Upstream Region, which has a paternal methylation imprint. Initially, in order to increase sequencing coverage and the robustness of the comparison between the two groups, we extracted a methylation score for the combined gDMRs in each blastocyst. All blastocysts but one had more than 900 calls (being the total number of calls at CpG sites), the average being 1495.3. For the COS blastocysts, the gDMR methylation per blastocyst ranged between 28% and 41% and for the CAPA-IVM between 30% and 44% (Fig. 2A, Supplementary Table SI). ANOVA comparison showed that there was no significant difference in methylation between COS and CAPA-IVM blastocysts (P value = 0.08). We also evaluated the methylation level of each gDMR in the combined COS and combined CAPA-IVM blastocysts (Table II) and compared our values to a deeply sequenced reference dataset (Okae *et al.* 2014). In this analysis, the range in gDMR methylation levels in the CAPA-IVM blastocysts was similar to the reference dataset, while the COS group exhibited greater variation (Fig. 2B).

As a further analysis, we sought to test individual gDMRs in individual blastocysts. Because PBAT is an unbiased whole-genome profiling method, it is expected that individual gDMRs will be sampled at different coverage per blastocyst; in addition, the variable sequencing depths obtained for the libraries will contribute to variation in the number of calls per gDMR. Thus, some gDMRs were represented by as few as 3 or 4 CpG calls at the level of individual blastocysts (e.g. *NAP1L5* in UZ7; Supplementary Table SII), but for many gDMRs the number of calls could provide a robust quantification in individual blastocysts, with longer CG-rich gDMRs like *TRAPPC9* yielding up to 799 calls in a single embryo (UZ10; Supplementary Table SII). Therefore, we plotted observed gDMR methylation levels with 95% CIs, shaded for actual number of observations (Fig. 3). As expected, longer gDMRs with greater numbers of observations (e.g. *TRAPPC9*) showed the greatest confidence in methylation estimates. It was also evident that, despite variation in individual gDMR confidence levels, no single blastocyst had a consistent skew in individual gDMR methylation values towards hypomethylation or hypermethylation. Importantly, in comparing the two groups, none of the methylation levels of the gDMRs differed significantly in CAPA-IVM blastocysts from those of COS blastocysts (all adjusted P values > 0.25; Supplementary Table SIII).

Table II **Average DNA Methylation (%) at imprinted gDMR for COS (*n* = 12) and CAPA-IVM blastocysts (*n* = 20).**

Imprinted germline differentially methylated region	COS	CAPA-IVM
DIRAS3 (DMR3)	18.352	36.89
DIRAS3(DMR2)	20.47	43.03
NAP1L5	38.55	42.72
FAM50B	37.20	49.32
PLAGL1	44.64	48.39
PEG10/SGCE	28.811	38.37
MEST	39.29	34.77
HTR5A	28.35	37.06
TRAPPC9	36.31	37.81
INPP5F	42.24	34.89
H19 upstream	38.68	31.28
KCNQ1OT1	41.87	36.48
SNRPN	46.91	53.25
ZNF331	36.05	36.49
PEG3	48.14	40.76
PSIMCT-1	33.57	37.36
NNAT	36.07	34.38
L3MBTL1	38.37	49.57
GNAS	36.82	35.88
GNAS complex locus	32.05	38.71
PPIEL	41.17	33.32

Finally, as a measure of methylation consistency at gDMRs, we evaluated CpG methylation calls on individual sequence reads from the PBAT libraries for reads with a minimum of 3 CpG positions. In this analysis, ~50% reads in each blastocyst are fully unmethylated, with the remainder being fully or partially methylated. However, there was no difference in the proportions of unmethylated, partially or fully methylated reads between the two groups (Fig. 2C). These results demonstrate that CAPA-IVM blastocysts maintain fidelity of imprinted methylation to similar levels as COS blastocysts.

CAPA-IVM is not associated with changes in blastocyst imprinted gene expression

DNA methylation regulates allelic expression but changes in expression of the active allele could occur in the absence of DNA methylation differences. For that reason, we evaluated whether CAPA-IVM influenced the gene expression of imprinted genes, even in the absence of significant DNA methylation differences between CAPA-IVM and COS blastocysts. RNA-seq libraries were generated from 20 individual blastocysts unrelated to those profiled by PBAT: 5

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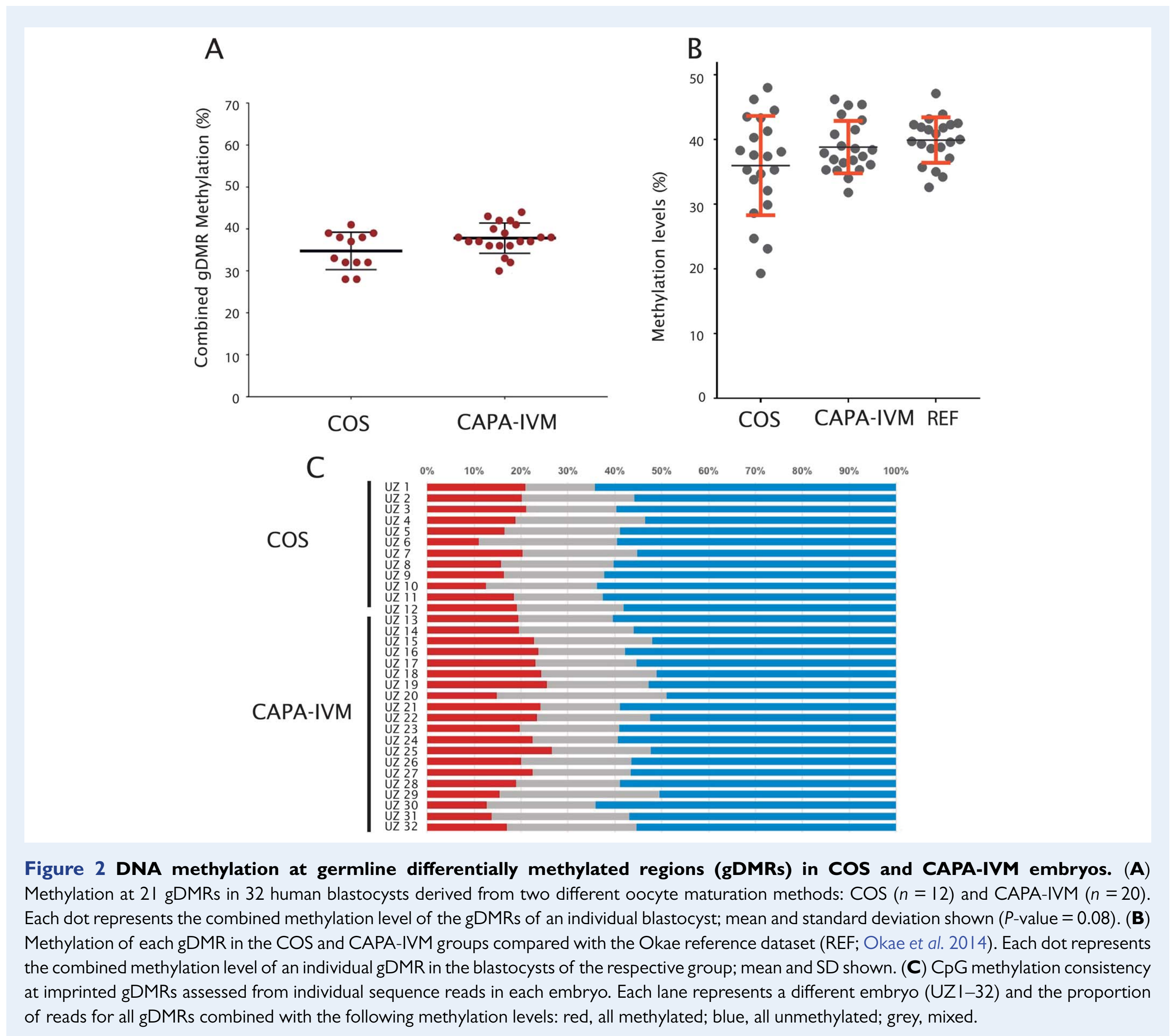

Figure 2 DNA methylation at germline differentially methylated regions (gDMRs) in COS and CAPA-IVM embryos. (**A**) Methylation at 21 gDMRs in 32 human blastocysts derived from two different oocyte maturation methods: COS ($n = 12$) and CAPA-IVM ($n = 20$). Each dot represents the combined methylation level of the gDMRs of an individual blastocyst; mean and standard deviation shown (P-value = 0.08). (**B**) Methylation of each gDMR in the COS and CAPA-IVM groups compared with the Okae reference dataset (REF; Okae *et al.* 2014). Each dot represents the combined methylation level of an individual gDMR in the blastocysts of the respective group; mean and SD shown. (**C**) CpG methylation consistency at imprinted gDMRs assessed from individual sequence reads in each embryo. Each lane represents a different embryo (UZ1–32) and the proportion of reads for all gDMRs combined with the following methylation levels: red, all methylated; blue, all unmethylated; grey, mixed.

embryos for the COS group and 15 for the CAPA-IVM group. The COS embryos were cultured in Sage ($n = 2$), Medicult ($n = 2$) or Vitrolife ($n = 1$) while all CAPA-IVM blastocysts were cultured in Cook medium.

Library quantification results assessed by qPCR before the sequencing step and duplication plots showing that our libraries had low duplication levels are shown in Supplementary Table SIV and Supplementary Figure S2, respectively. Annotated human mRNA features were quantitated as $\log_2$RPM (reads per million reads of library) and globally normalized to the 75th percentile of the data using Seqmonk software. Values for genes controlled by gDMRs were obtained and compared between the groups.

Despite a wide range in transcript abundance for some of the genes, there were no significant differences in imprinted gene expression between CAPA-IVM and COS blastocysts (P value > 0.05; Fig. 4A). Importantly, we could observe that the transcripts for the genes *DIRAS3*, *NAP1L5*, *FAM50B*, *PLAGL1* and *HTR5A* were of low abundance at the blastocyst stage, while *GNAS*, *SNRPN* and *PPIEL* had the highest expression levels.

Moreover, we also observed that expression of genes encoding key factors involved in maintenance of methylation, demethylation and remethylation, such as DNA methyltransferases and auxiliary factors (*DNMT1*, *DNMT3A*, *DNMT3B*, *DNMT3L* and *UHRF1*), demethylase activities (*TET1*, *TET2* and *TET3*) and factors involved in DNA methylation maintenance specifically at gDMRs (*TRIM28*, *ZFP57* and *ZNF445*), also did not differ significantly between the CAPA-IVM and COS groups (Fig. 4B).

Discussion

We recently published a novel IVM strategy in which COCs are retrieved from small antral follicles (2–8 mm) without prior hCG trigger and subjected to PMC in the presence of CNP for the first step followed by IVM using FSH + AREG in a second step

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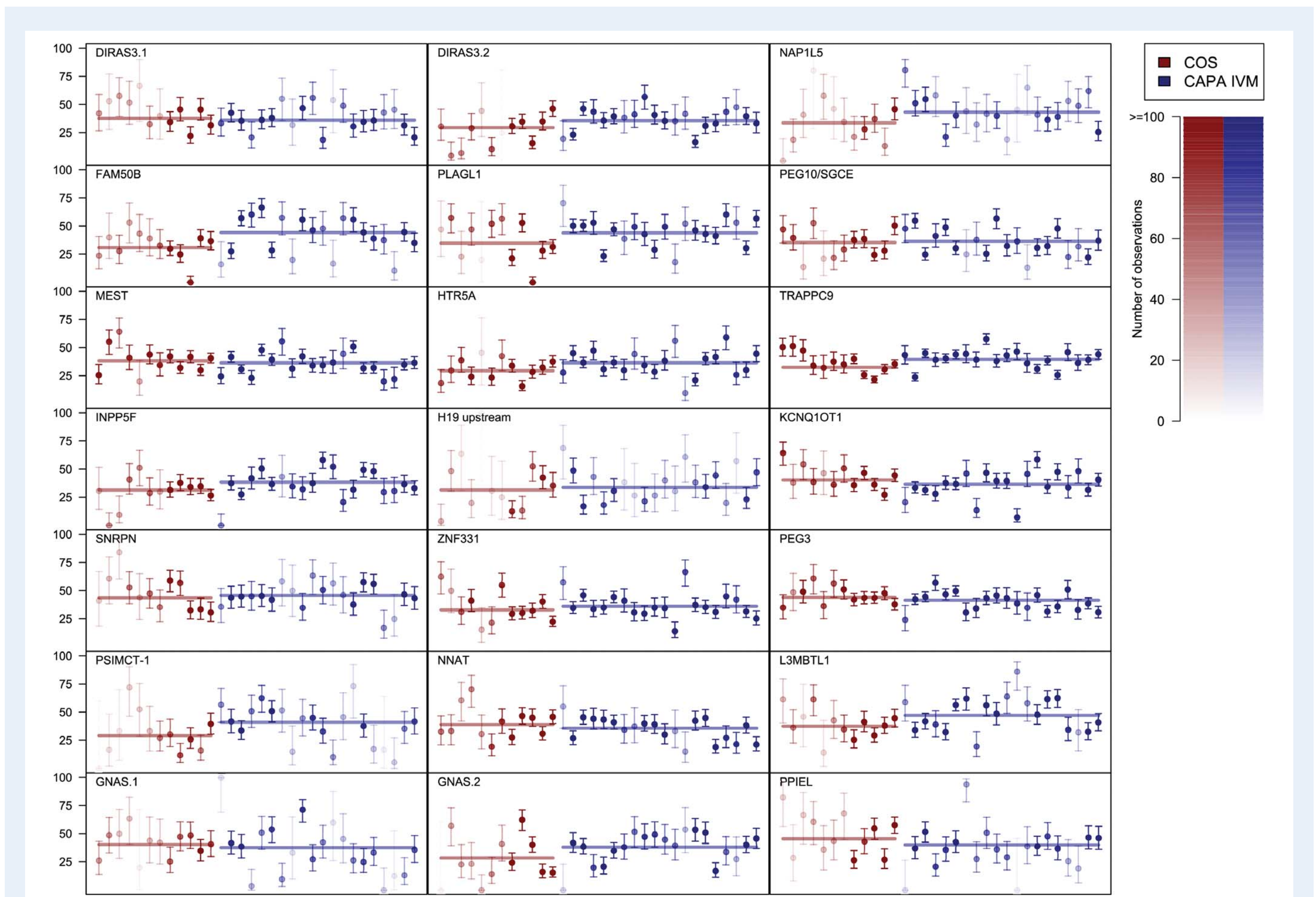

Figure 3 Methylation levels of the 21 individual gDMRs in individual blastocysts. Each point represents the observed methylation level of the indicated gDMR in an individual blastocyst, with 95% confidence limits shown shaded according to the number of observations (CpG calls; scale bar right). Blastocysts are ordered from left to right: UZ1–12 (COS group, red), UZ13–32 (CAPA-IVM, blue). The horizontal lines are the mean values for the COS and CAPA-IVM groups. There was no significant difference between the two groups for any of the gDMRs (statistical analysis given in Supplementary Table SIII).

(Sánchez *et al*. 2017). With this novel system, named CAPA-IVM, we significantly improved the meiotic and developmental competence of human oocytes, resulting in a higher proportion of Day 3 embryos and good quality blastocysts (Sánchez *et al.,* 2017). In the current study the possible impact on imprinting of this extended culture is addressed by evaluating DNA methylation at gDMRs in blastocysts derived from oocytes undergoing CAPA-IVM. Imprinted genes are important for normal fetal and placental development, and dysregulation of imprinted genes in humans causes a variety of imprinting syndromes, with associated effects on fetal growth and/or longer-term health outcomes (Cortessis *et al*. 2018). In oocytes, DNA methylation is established at gDMRs as the oocyte grows during the transition from primordial to antral follicle stages (Lucifero *et al.,* 2004; Hiura *et al.,* 2006; reviewed by Stewart *et al.,* 2016). Furthermore, maternal effect factors stored in the oocyte play a pivotal role in maintaining imprints during pre-implantation development (reviewed by Denomme and Mann, 2013 and Hanna *et al.,* 2018). Our results indicate that CAPA-IVM does not alter DNA methylation maintenance of gDMRs when compared to control blastocysts derived from COS in age-matched PCOS patients.

The global DNA methylation for all the embryos varied between 15% and 44%. This wide range of global DNA methylation was also observed by Li *et al*. (2017). Methylome analysis of 57 individual human blastocysts (obtained after COS and IVF) revealed that for high morphological grade embryos (AA) the global DNA methylation ranged from 27% to 32% while for low morphological grade embryos (CC) the methylation was more variable, ranging from 23% to 46%. For that reason, we evaluated the effect of the embryo grade (AA, AB, BA and BB according to Gardner scoring method; Gardner *et al.,* 1998) on gDMRs but did not observe any significant variation between different embryo grades (*P* value = 0.24; Supplementary Figure S3).

Both embryo culture medium and superovulation have been shown to affect imprinted DNA methylation in mouse blastocysts (Market-Velker *et al.,* 2010a*,* 2010b). As it is not possible to study imprinted methylation in naturally conceived human embryos, White *et al*. (2015) used DNA from human buccal cells and human embryonic stem cells for bisulphite conversion and sequencing cloned PCR products in an attempt to define 'normal' DNA methylation ranges of gDMRs. Specifically, for the *SNRPN* gene the inferred normal methylation range was 30–54%, for *KCNQ1OT1* 42–78% and for *H19* 40–72%, with

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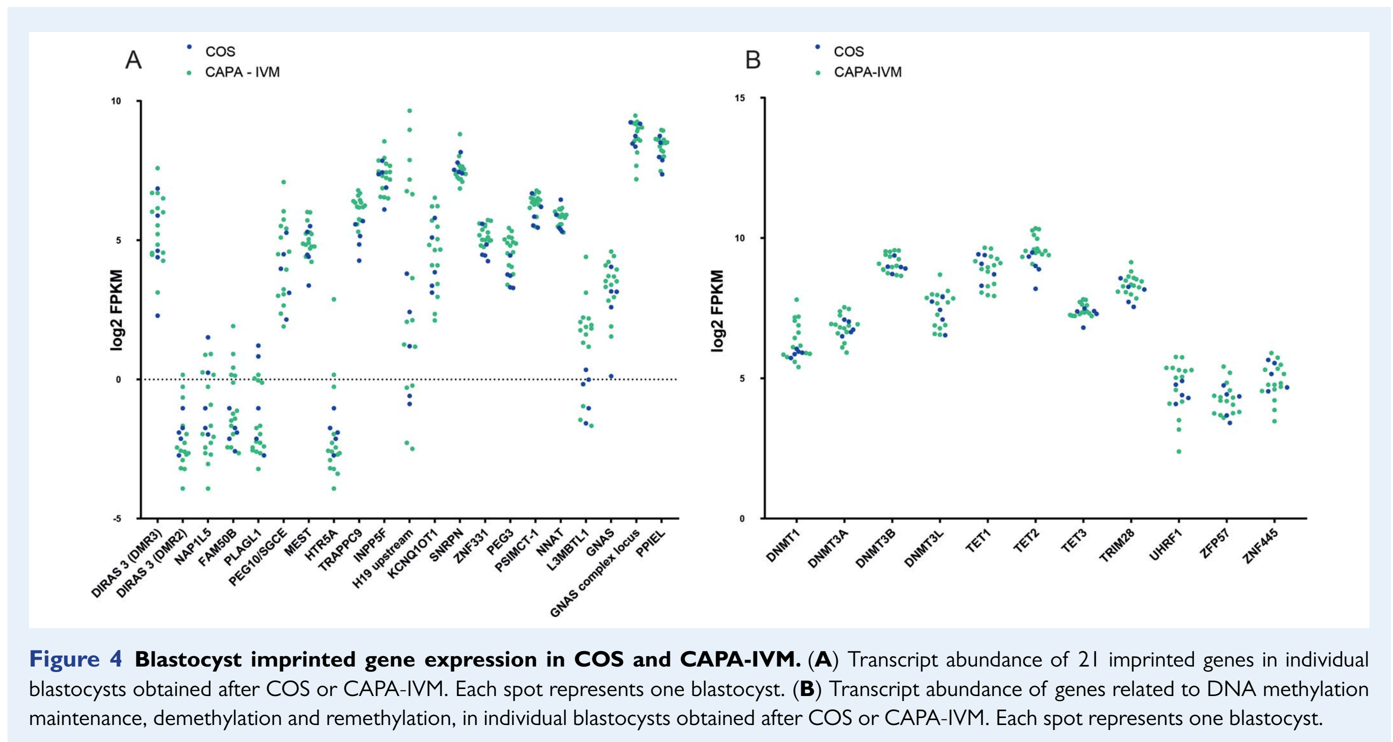

Figure 4 Blastocyst imprinted gene expression in COS and CAPA-IVM. (**A**) Transcript abundance of 21 imprinted genes in individual blastocysts obtained after COS or CAPA-IVM. Each spot represents one blastocyst. (**B**) Transcript abundance of genes related to DNA methylation maintenance, demethylation and remethylation, in individual blastocysts obtained after COS or CAPA-IVM. Each spot represents one blastocyst.

blastocysts exhibiting gDMR methylation outside these ranges deemed to have abnormal methylation. In our study, we observed slightly lower levels of methylation, ranging from 18% to 53%. These differences might arise from the different methodology used or from sampling effects from the relatively low sequencing coverage in some embryos. In a study from Okae *et al*. (2014), in which methylation was assessed by a similar method on pooled blastocysts but with a higher sequencing depth, methylation at gDMRs ranged from 32% to 46%. As we showed, the methylation level estimates of gDMRs in the CAPA-IVM blastocysts were very similar to this range, despite the lower sequencing coverage. Ultimately, however, without knowing the normal level of methylation of gDMRs in human blastocysts, it is difficult to evaluate whether variations observed could be within limits tolerated in normal embryonic development, or the extent to which variation at this stage is part of a normal dynamic of stabilizing imprinted methylation. More substantial departures from normality would be expected to lead to misregulation of imprinted gene expression with potential consequences, in particular, on placental development and function, and foetal growth.

Despite the fact that DNA methylation was not altered, we wanted to evaluate whether gene expression levels of the associated imprinted genes was affected in blastocysts derived from oocytes undergoing CAPA-IVM. For this reason, we performed RNA-seq on a separate set of twenty individual blastocysts. We did not observe any significant difference in imprinted gene expression levels between CAPA-IVM and control blastocysts. We also found no differences in expression of some of the key factors in DNA methylation reprogramming and maintenance, consistent with the results of the DNA methylation analysis and strengthening the safety evaluation of the CAPA-IVM system.

Our study has a number of possible limitations. First, as only limited numbers of human blastocysts are available for research, the number of blastocysts analysed may be too low to detect subtle increases in imprinting error rates. Therefore, follow-up of the health of children born after CAPA-IVM will be important as it is for other new ARTs that are generally introduced into clinical practice without prior epigenetic safety studies in human blastocysts. Second, the sequencing depth was not sufficient to assess methylation of all individual gDMRs with confidence in all individual embryos. We are therefore not able completely to exclude the presence of abnormalities in individual blastocysts, but the analysis is sufficiently robust to conclude that if any change in imprinted gene methylation between CAPA-IVM treatment and conventional ART exists it must be very slight.

Inherent to research on scarcely available human blastocysts, there may be a number of confounding variables in the current study. Notably, CAPA-IVM blastocysts were all fertilized by sperm from the same donor and cultured in the same medium; in contrast, the control blastocysts were fertilized by sperm from different fathers and cultured in several other media. Moreover, CAPA-IVM treatment requires no or only minimal ovarian stimulation, while the controls received COS. Embryo culture medium as well as superovulation have been shown to affect imprinted DNA methylation in mouse blastocysts (Saenz-de-Juano *et al.,* 2016). Therefore, the greater range in gDMR methylation values observed in the COS blastocysts could reflect the variety of culture media used. Finally, cumulus-oocyte complexes for CAPA-IVM are derived from small antral follicles, the majority having a diameter less than 6 mm.

In conclusion, we did not find any significant differences in imprinted gene DNA methylation or mRNA expression in blastocysts derived from oocytes cultured in the CAPA-IVM system compared to blastocysts derived from oocytes retrieved following COS in age-matched PCOS patients. However, follow-up of health of children conceived with the technique is mandatory to monitor the epigenetic safety of CAPA-IVM. CAPA-IVM is an optimized IVM method that promotes oocyte developmental capacity. Therefore, the results from the current study may not be extended to all IVM protocols.

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Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

E.A., G.K. and J.S. conceived and designed the study. F.L., H.V.R., F.S. and S.R. performed culture procedures and experiments. E.I., M.D.S., G.K. and E.A. analysed and interpreted the data, with assistance from A.S.-P., S.A. and F.K. M.D.V. recruited and managed the patients and supervised the clinical activities. E.A. supervised the hormonal monitoring and safety analysis. All authors discussed the results and implications of the study. M.D.S., E.I., G.K., J.S. and E.A. wrote the manuscript. All authors revised, edited and approved the manuscript.

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Conflict of Interest

There are no conflicts of interest to declare.

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