OXFORD

human reproduction

Human Reproduction, 2024, **39(2)**, 403–412
https://doi.org/10.1093/humrep/dead256
Advance Access Publication Date: December 18, 2023
Original Article

Reproductive endocrinology

Oral dydrogesterone versus micronized vaginal progesterone for luteal phase support: a double-blind crossover study investigating pharmacokinetics and impact on the endometrium

S. Loreti [1], K. Thiele[2], M. De Brucker[1], C. Olsen [3,4,5], J. Centelles-Lodeiro[6], C. Bourgain[7], W. Waelput[8], H. Tournaye[1], G. Griesinger [9], J. Raes[6], S. Vieira-Silva[10,11], P. Arck[2], C. Blockeel [1], and S. Mackens [1,7,*]

[1]Brussels IVF, Centre for Reproductive Medicine, Universitair Ziekenhuis Brussel, Jette, Belgium
[2]Division of Experimental Feto-Maternal Medicine, Department of Obstetrics and Fetal Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[3]Brussels Interuniversity Genomic High-Throughput Core Facility (BrightCore), Vrije Universiteit Brussel, Brussels, Belgium
[4]Vrije Universiteit Brussel (VUB), Universitair Ziekenhuis Brussel (UZ Brussel), Clinical Sciences, Research Group Reproduction and Genetics, Centre for Medical Genetics, Brussels, Belgium
[5]Interuniversity Institute of Bioinformatics in Brussels (IB)2, VUB-ULB, Brussels, Belgium
[6]Vlaams Instituut voor Biotechnologie (VIB), Katholieke Universiteit Leuven, Leuven, Belgium
[7]Reproductive Immunology and Implantation Research Group (REIM), Vrije Universiteit Brussel, Brussels, Belgium
[8]Department of Pathology, Universitair Ziekenhuis Brussel, Brussels, Belgium
[9]Department of Gynecological Endocrinology and Reproductive Medicine, University Hospital of Schleswig-Holstein, Campus Luebeck, Germany
[10]Institute of Medical Microbiology and Hygiene and Research Center for Immunotherapy (FZI), University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany
[11]Institute of Molecular Biology (IMB), Mainz, Germany

*Correspondence address. Brussels IVF, Centre for Reproductive Medicine, Universitair Ziekenhuis Brussel, Laarbeeklaan 101, 1090 Jette, Belgium. E-mail: shari.mackens@uzbrussel.be https://orcid.org/0000-0001-7012-0436

ABSTRACT

STUDY QUESTION: How do plasma progesterone (P) and dydrogesterone (D) concentrations together with endometrial histology, transcriptomic signatures, and immune cell composition differ when oral dydrogesterone (O-DYD) or micronized vaginal progesterone (MVP) is used for luteal phase support (LPS)?

SUMMARY ANSWER: Although after O-DYD intake, even at steady-state, plasma D and 20αdihydrodydrogesterone (DHD) concentrations spiked in comparison to P concentrations, a similar endometrial signature was observed by histological and transcriptomic analysis of the endometrium.

WHAT IS KNOWN ALREADY: O-DYD for LPS has been proven to be noninferior compared to MVP in two phase III randomized controlled trials. Additionally, a combined individual participant data and aggregate data meta-analysis indicated that a higher pregnancy rate and live birth rate may be obtained in women receiving O-DYD versus MVP for LPS in fresh IVF/ICSI cycles. Little data are available on the pharmacokinetic (PK) profiles of O-DYD versus MVP and their potential molecular differences at the level of the reproductive organs, particularly at the endometrial level.

STUDY DESIGN, SIZE, DURATION: Thirty oocyte donors were planned to undergo two ovarian stimulation (OS) cycles with dual triggering (1.000 IU hCG + 0.2 mg triptorelin), each followed by 1 week of LPS: O-DYD or MVP, in a randomized, cross-over, double-blind, double-dummy fashion. On both the first and eighth days of LPS, serial blood samples upon first dosing were harvested for plasma D, DHD, and P concentration analyses. On Day 8 of LPS, an endometrial biopsy was collected for histologic examination, transcriptomics, and immune cell analysis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: All oocyte donors were <35 years old, had regular menstrual cycles, no intrauterine contraceptive device, anti-Müllerian hormone within normal range and a BMI ≤29 kg/m^2. OS was performed on a GnRH antagonist protocol followed by dual triggering (1.000 IU hCG + 0.2 mg triptorelin) as soon as ≥3 follicles of 20 mm were present. Following oocyte retrieval, subjects initiated LPS consisting of MVP 200 mg or O-DYD 10 mg, both three times daily. D, DHD, and P plasma levels were measured using liquid chromatography–tandem mass spectrometry. Histological assessment was carried out using the Noyes criteria. Endometrial RNA-sequencing was performed for individual biopsies and differential gene expression was analyzed. Endometrial single-cell suspensions were created followed by flow cytometry for immune cell typing.

MAIN RESULTS AND THE ROLE OF CHANCE: A total of 21 women completed the entire study protocol. Subjects and stimulation characteristics were found to be similar between groups. Following the first dose of O-DYD, the average observed maximal plasma concentrations (C_{max}) for D and DHD were 2.9 and 77 ng/ml, respectively. The C_{max} for D and DHD was reached after 1.5 and 1.6 h

Received: August 18, 2023. **Revised:** November 6, 2023. **Editorial decision:** November 17, 2023.

(=T_{max}), respectively. On the eighth day of LPS, the first administration of that day gave rise to a C_{max} of 3.6 and 88 ng/ml for D and DHD, respectively. For both, the observed T_{max} was 1.5 h. Following the first dose of MVP, the C_{max} for P was 16 ng/ml with a T_{max} of 4.2 h. On the eighth day of LPS, the first administration of that day showed a C_{max} for P of 21 ng/ml with a T_{max} of 7.3 h. All 42 biopsies showed endometrium in the secretory phase. The mean cycle day was 23.9 (±1.2) in the O-DYD group versus 24.0 (±1.3) in the MVP group. RNA-sequencing did not reveal significantly differentially expressed genes between samples of both study groups. The average Euclidean distance between samples following O-DYD was significantly lower than following MVP (respectively 12.1 versus 18.8, Mann–Whitney $P = 6.98^{e-14}$). Immune cell profiling showed a decrease of CD3 T-cell, γδ T-cell, and B-cell frequencies after MVP treatment compared to O-DYD, while the frequency of natural killer (NK) cells was significantly increased.

LIMITATIONS, REASONS FOR CAUTION: The main reason for caution is the small sample size, given the basic research nature of the project. The plasma concentrations are best estimates as this was not a formal PK study. Whole tissue bulk RNA-sequencing has been performed not correcting for bias caused by different tissue compositions across biopsies.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first study comparing O-DYD/MVP, head-to-head, in a randomized design on a molecular level in IVF/ICSI. Plasma serum concentrations suggest that administration frequency is important, in addition to dose, specifically for O-DYD showing a rapid clearance. The molecular endometrial data are overall comparable and thus support the previously reported noninferior reproductive outcomes for O-DYD as compared to MVP. Further research is needed to explore the smaller intersample distance following O-DYD and the subtle changes detected in endometrial immune cells.

STUDY FUNDING/COMPETING INTEREST(S): Not related to this work, C.Bl. has received honoraria for lectures, presentations, manuscript writing, educational events, or scientific advice from Abbott, Ferring, Organon, Cooper Surgical, Gedeon-Richter, IBSA, and Merck. H.T. has received honoraria for lectures, presentations, manuscript writing, educational events, or scientific advice from Abbott, Ferring, Cooper Surgical, Gedeon-Richter, Cook, and Goodlife. S.M. has received honoraria for lectures, presentations, educational events, or scientific advice from Abbott, Cooper Surgical, Gedeon-Richter, IBSA, and Merck and Oxolife. G.G. has received honoraria for lectures, presentations, educational events, or scientific advice from Merck, MSD, Organon, Ferring, Theramex, Gedeon-Richter, Abbott, Biosilu, ReprodWissen, Obseva, PregLem, Guerbet, Cooper, Igyxos, and OxoLife. S.V.-S. is listed as inventor on two patents (WO2019115755A1 and WO2022073973A1), which are not related to this work.

TRIAL REGISTRATION NUMBER: EUDRACT 2018-000105-23

Keywords: luteal phase support / progesterone / dydrogesterone / receptivity / IVF / ICSI

Introduction

In IVF/ICSI treatment, following ovarian stimulation (OS) the luteal phase needs additional support with exogeneous progesterone (P) (van der Linden *et al.*, 2015). In daily clinical practice, great divergence exists among reproductive health physicians concerning the preferred and prescribed route of P supplementation. Especially in Europe, micronized vaginal progesterone (MVP) is most frequently applied, although oral dydrogesterone (O-DYD) is a very patient-friendly and cost-efficient alternative (Vaisbuch *et al.*, 2014; Griesinger *et al.*, 2018b; Di Guardo *et al.*, 2020). Thanks to its specific stereoisomer structure, O-DYD has higher bioavailability and specificity for progesterone receptors than MVP, inducing the required endometrial transformation at lower doses (Rižner *et al.*, 2011; Stanczyk *et al.*, 2013). Two large phase III randomized controlled trials, each involving more than 1000 patients, have demonstrated that O-DYD is noninferior to MVP in supporting pregnancy rates in fresh IVF/ICSI cycles at equivalent maternal and fetal safety (Tournaye *et al.*, 2017; Griesinger *et al.*, 2018a). More recently, an individual participant data and aggregate data meta-analysis indicated that a higher pregnancy rate and live birth rate may be obtained in women receiving O-DYD versus MVP for luteal phase support (LPS) in fresh IVF/ICSI cycles (Griesinger *et al.*, 2020).

In contrast to these extensive clinical datasets, very little information is available on the differences in pharmacokinetic (PK) profiles between the two medication strategies in reproductive-aged women undergoing ART. Furthermore, while P modulates immune responses and plays a key role in mediating immune tolerance toward the embryo in early pregnancy (Mincheva-Nilsson, 2003; Griesinger *et al.*, 2019), no studies have investigated the potential differences between LPS strategies on a more molecular level that could have an impact on endometrial functionality and local immune modulation.

The study presented compares for the first time O-DYD and MVP, head-to-head, with a specific focus on plasma P, dydrogesterone (D) and 20αdihydrodydrogesterone (DHD) concentrations, endometrial histology, and transcriptome signatures, as well as endometrial immune cell composition when these medications are used as the LPS strategy after OS for fresh embryo transfer.

Materials and methods

Ethical approval and quality assurance

The study (EUDRACT 2018-000105-23) was approved by the Ethical Committee of the UZ Brussel and the Federal Agency for Medicines and Health Products (FAGG) and performed in accordance with the endorsed guidelines. Brussels IVF is fully accredited by the Association for the Accreditation of Human Research Protection Programme (AAHRPP); all study data were collected by authorized staff and stored in a restricted directory on the hospital's network system.

Study design

This was a randomized, cross-over, double-blind, double-dummy study including 30 oocyte donors between 1 June 2019 and 30 June 2020. The study subjects each underwent two OS cycles. Between the two OS cycles an interval period of a minimum of 2 to a maximum of 12 months was respected. After each OS cycle, a different active LPS strategy was administered so that every patient received O-DYD once and MVP once and could serve as her own control (Fig. 1).

Sample size calculation

The sample size calculation was performed following the guidelines for sample size calculation for RNA-sequencing (RNA-seq) experiments by Hart *et al.* (2013), using SPSS SamplePower® version 3.0 (IBM, USA). To detect with 90% power, at a false-positive detection rate of 0.01 and assuming a variation coefficient of 0.74, all genes with a ≥2-fold change in gene expression, a minimum of 36 endometrial biopsies are needed (respectively, 18 after O-DYD versus 18 after MVP, i.e. 18 sample pairs in the given cross-over design). To account for dropouts, 30 subjects were included, each of them delivering two endometrial biopsies.

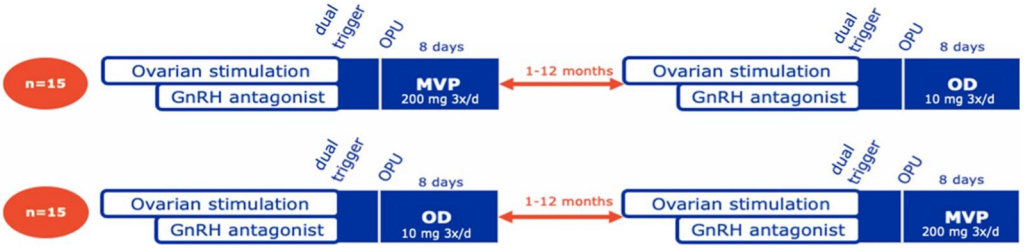

Figure 1. Study design: a randomized, double-blind, double-dummy cross-over study of oral dydrogesterone versus micronized vaginal progesterone for luteal phase support. The study included 30 oocyte donors who underwent two ovarian stimulation cycles, each followed by 1 week of oral dydrogesterone or micronized vaginal progesterone for luteal phase support. OPU: oocyte pick-up; MVP: micronized vaginal progesterone; OD: oral dydrogesterone.

Randomization, allocation, and blinding

A computer-generated block randomization list was used to allocate the double-blind, double-dummy study medication packages (provided by Abbott Healthcare Products B.V.) to the participants. Only one dedicated study nurse from the study site was not blinded to the link between the block randomization list, a six-digit randomization number and the content of the study medication packages, in order to allocate and provide the subject with the correct medication package. Neither the subject, the investigators, Abbott Healthcare Products B.V., nor any other involved laboratory was aware of the treatment allocation until after all planned analyses were finalized. Throughout the entire course of the study, there was no need for emergency unblinding (owing to adverse events) of the randomization numbers.

Study medication

Production, packaging, and labeling was controlled by the Clinical Supply Management (Drug Product Development) of Abbott Healthcare Products BV, Weesp, The Netherlands. All packaging and labeling as well as the production of study drug were following Good Manufacturing Practices (GMP) specifications, as mentioned in the Manufacturing of Investigation and Medicinal Products Volume 4 Annex 13 and in accordance with other applicable laws or local regulations. An Abbott Qualified Person formally released all the clinical supplies prior to shipment to the investigational site. A Certificate of Compliance was issued stating the expiry date of the medication packages, which was respected throughout the entire course of the study. All medication packages were stored in a secure, monitored, limited-access area in accordance with labeled storage conditions prior to distribution to the study participants. For both treatment medications, O-DYD and MVP, a placebo version was available and administered in both study cycles. All the tablets and capsules were identical in appearance, shape, smell and taste, and packaged in the proper proportion to assure desired dosages and maintenance of the blinding. Any intake of further products during the treatment phase was not allowed.

Participants

Oocyte donors were between 18 and 35 years old with regular menstrual cycles and without an intra-uterine contraceptive device. Oral contraceptive pill (OCP) intake prior to study cycles was allowed and recorded. The anti-Müllerian hormone (AMH) value of participants was within the normal range (90th and 10th percentile for healthy women aged 25–29 years according to the applied Elecsys® AMH kit by Roche) and BMI was between 18 and 29 kg/m^2. In addition, the subjects had serum levels of prolactin, testosterone and thyroid-stimulating hormone within the normal limits for the clinical laboratory, tested <6 months prior to enrollment in the study, and they did not have an endometrial pathology as determined by transvaginal ultrasonographic scanning. All participants were nonsmokers and participated in the trial after written informed consent. Specified exclusion criteria were: acute urogenital disease during the course of the study, known allergic reactions to progesterone/dydrogesterone products (active substance or to any of the excipients), intake of any experimental drug or any participation in any other clinical trial within 30 days prior to study start, mental disability, current or recent substance abuse, including alcohol, or any other lack of fitness in the investigator's opinion to preclude subjects to participate in or to complete the study.

ART protocols and study medication intake

OS was performed in a GnRH antagonist protocol with a fixed dose of recombinant FSH (rFSH) or highly purified HMG (hp-HMG). The same medication dose and product was used throughout both cycles of a given participant to minimize inter-cycle variation. A dual trigger (1.000 U hCG + 0.2 mg triptorelin) was administered as soon as ≥3 follicles of 20 mm were present. Patients with serum P levels >1.5 ng/ml at ovulation triggering were excluded to avoid any impact of elevated serum late follicular phase P levels on subsequent analyses. Oocyte retrieval was performed according to the center's standard of practice. Following the oocyte retrieval, more specifically after exactly 1 h, subjects initiated the LPS consisting of MVP 200 mg (Utrogestan®) or O-DYD 10 mg (Duphaston®), both three times daily (at 11 a.m., 4 p.m., and 10 p.m.) and this in a double-blind, double-dummy, placebo-controlled manner, as described above. The last dose of LPS was administered by the subject (on site) on the eighth day, after which all study medication intake ended, meaning that on the eighth day only the morning dose was given to follow the plasma concentration profiles after medication withdrawal.

Sampling

Blood samples for measuring plasma P, D, and DHD concentrations were performed according to the following scheme:

- on Day 1 of treatment: 1 h before morning dosing (at the moment of the oocyte retrieval), 0.5 h after dosing, 1 h after dosing, 1.5 h after dosing, 2 h after dosing, 2.5 h after dosing, 3 h after dosing, 4 h after dosing, and 5 h after dosing immediately before next dose (in total, nine sampling time-points for Day 1);
- on Day 8 of treatment: 1 h before (last) morning dosing, 0.5 h after dosing, 1 h after dosing, 1.5 h after dosing, 2 h after dosing, 2.5 h after dosing, 3 h after dosing, 4 h after dosing, and 5 h after dosing (in total, nine sampling time-points for Day 8);

- after the treatment: 24 h after last dosing, 48 h after last dosing, and 72 h after last dosing (in total, three sampling time-points).

Owing to its more invasive nature and the potential impact of a first biopsy on a second one, an endometrial biopsy (Pipelle de Cornier®, Laboratoire CCD, France) for subsequent histologic, transcriptional, and immune cell analysis was taken only once per cycle on the eighth day of treatment with O-DYD or MVP, i.e. during the window of implantation, when a blastocyst is expected to start the implantation process. An overview of the entire sampling set-up is available in Fig. 2.

Sample processing and analysis

Plasma concentrations

All blood samples were harvested into 6 ml EDTA tubes and handled in an ice bath. Subsequently, plasma was separated (centrifuged within an hour for a duration of 10 min at 1900*g* at 4°C) and stored at −70°C in two aliquots. Sample shipment was by courier on dry ice in separate batches to the Abbott assigned research organization for the measurements. As both D and DHD are progestogenic, D, DHD, and P plasma levels were established using a validated liquid chromatography–tandem mass spectrometry assay.

Endometrial assessment

The endometrial biopsy was split immediately after harvesting. The first part was fixed in 10% neutral-buffered formalin for conventional histology using hematoxylin/eosin staining. Biopsies were assessed by two pathologists specialized in histological endometrial dating and unaware of the active LPS compound used by the participant, nor of the association between biopsies originating from the same participant in different study cycles. Dating was performed according to the Noyes criteria (Noyes *et al.*, 1975) and scored as in-phase when the dating corresponded to expected cycle day 23 ± 2 (Bourgain *et al.*, 1990).

The second part of the endometrial biopsy was submerged in RNAlater® (Qiagen, Germany) and kept at 4° for 24–72 h after which it was transferred to liquid nitrogen for long-term storage until finalization of the study. RNA extraction was performed using the RNeasy Plus Mini Kit® (Qiagen, Germany) for subsequent RNA-seq by BrightCore, the Brussels Interuniversity Genomics High Throughput Core Facility (Vrije Universiteit Brussel). RNA quality was determined with Agilent 2100 Bioanalyzer. Endometrial RNA-seq (Illumina Novaseq 6000) was performed for individual biopsies, and raw reads were processed using STAR and htseq-count and the differential gene expression was evaluated with EdgeR. The results were corrected for multiple hypothesis testing, the differentially expressed genes were compared, and a principal component analysis (PCA) plot was drawn. The average Euclidean distance between samples of the O-DYD group and the MVP group was also calculated and compared using the Mann–Whitney test.

A third part of the endometrial biopsy was used to create a single-cell suspension that was cryopreserved in liquid nitrogen until finalization of the study. More specifically, the tissue was transported to the laboratory in DMEM/F12 medium. Immediately upon arrival, the tissue was washed and minced using a scalpel followed by enzymatic digestion by collagenase Ia (134 U/ml, Sigma-Aldrich, Belgium) and deoxyribonuclease I (156 U/ml, Roche, Belgium) for 1 h at 37°C, 5% CO_2. After enzyme inactivation, centrifugation was performed for 5 min at 200*g*. The resuspended cell pellet was then carefully layered on top of 6 ml Biocoll (Sigma-Aldrich, Belgium) and centrifuged for 25 min at 450*g*. In the boundary layer, the milky film of endometrial mononuclear cells (EnMCs) was harvested and resuspended in phosphate buffered saline (PBS), followed by two additional centrifugation steps (10 min at 350*g* and 5 min at 250*g*). Finally, EnMCs were resuspended in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide (Sigma-Aldrich, Belgium), frozen using a Mr Frosty® (ThermoFisher Scientific, Belgium), and stored in liquid nitrogen. Cryopreserved EnMC suspensions were shipped on dry ice to the University Medical Center Hamburg-Eppendorf, Department of Obstetrics and Fetal Medicine, Germany for immunological assessment. Frozen EnMCs were thawed in a 37°C warm water bath until cells thawed. Cells were washed using pre-warmed (37°C) RPMI1640 medium supplemented with 5% of heat-inactivated FBS and centrifuged for 5 min with 450×*g* at room temperature. Subsequently, cells were incubated in normal rat serum (1:100, eBioscience) and anti-mouse CD16/32 Mouse Fragment crystallizable (Fc) Block (1:200, TruStain fcX, Biolegend) to block nonspecific FcγRII/III binding, followed by extracellular immune cell staining for 30 min at room temperature. Antibodies used are specified in Supplementary Table S1. For dead cell identification, a dead/live staining was performed using eFluor 506 viability dye (eBioscience). After a final washing and centrifugation

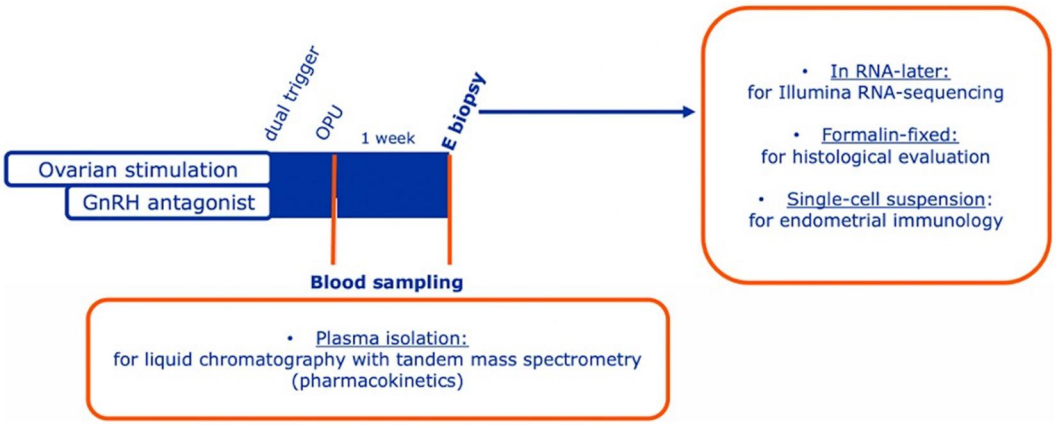

Figure 2. Overview of sampling set-up. On both the first and eighth day of luteal phase support, serial blood samples upon first dosing were harvested for plasma D, DHD, and P concentration analyses. On Day 8 of luteal phase support, an endometrial biopsy was collected for histologic examination, transcriptomics, and immune cell analysis. OPU: oocyte pick-up; E biopsy: endometrial biopsy; D: dydrogesterone; DHD: 20αdihydrodydrogesterone; P: progesterone.

step, the cells are resuspended in PBS. Flow cytometric data were acquired using a BD FACSymphony™ A3 Cell Analyzer (BD Biosciences) and analyzed using FlowJo (Tree Star, Ashland, OR, USA).

Results

Following the randomization of 30 participants, a total of 21 women completed the entire study protocol. The other nine subjects were excluded from the analysis owing to elevated serum P in the late follicular phase in either or both OS cycles (n = 5), drop-out after one cycle on patient request (n = 3) or because of a spontaneous pregnancy that occurred in the period between the two OS cycles (n = 1). Six of the 21 included participants used OCP prior to the study cycles. In that case, OS started 5 days after OCP discontinuation, for both study cycles.

Subjects and stimulation characteristics were compared and found to be similar between groups, supporting the effectiveness of the randomization process. The mean age (±SD) of the participants was 27.4 years (±3.8), BMI was 24.0 (±3.2) kg/m^2, and AMH 2.6 (±1.3) µg/l. rFSH was administered in both cycles in 10/21 participants, while the other 11/21 received hp-HMG. The mean daily gonadotrophin dose was 254.8 IU (±53.4), the mean total gonadotrophin dose 2750.7 IU (±53.4) and the mean length of OS 10.7 days (±1.8). The mean number of oocytes retrieved was 19.7 ± 10. No adverse events were reported during the intake of the study medication.

Plasma concentrations

Following intake of the first (single) dose of O-DYD the average observed maximal plasma concentrations (C_{max}) for D and DHD were 2.9 and 77 ng/ml, respectively. The C_{max} for D and DHD was reached after 1.5 and 1.6 h (=T_{max}), respectively. On the eighth day of LPS, the first administration of that day (but thus following multiple doses during previous days) gave rise to a C_{max} of 3.6 and 88 ng/ml for D and DHD, respectively. For both D and DHD, the observed T_{max} was 1.5 h. Following intake of the first dose of MVP, the C_{max} for P was 16 ng/ml with a T_{max} of 4.2 h. On the eighth day of LPS, the first administration of that day showed a C_{max} for P of 21 ng/ml with a T_{max} of 7.3 h. Arithmetic mean plasma D, DHD, and P concentrations are presented in Fig. 3.

Endometrial assessment

For the endometrial histology and RNA-seq, 21 pairs of samples (n = 42 biopsies) were available from participants having a biopsy after O-DYD as well as after MVP. All 42 biopsies showed an endometrium in the secretory phase. Histological dating according to Noyes was not possible for three biopsies as there was too little tissue to adequately determine the cycle day (one biopsy in the O-DYD group versus two in the MVP group). The mean cycle day was 23.9 (±1.2) in the O-DYD group versus 24.0 (±1.3) in the MVP group. Using the assignment of Days 20–24 as 'in-phase', five biopsies (5/20) were out of phase in the O-DYD group and five (5/19) following MVP.

At the molecular level, the RNA integrity number was >7 for all samples, indicating a high quality of RNA prior to analysis. The read quality was evaluated using FASTQC. Using EdgeR to run a differential expression analysis and after correction for multiple hypothesis testing, no differentially expressed genes could be withheld between study groups and the PCA plot showed one mixed O-DYD/MVP cluster (PCA1 25% variance, PCA2 16% variance, Fig. 4A). The Euclidean distance between samples of the O-DYD group was significantly lower than for the MVP group (respectively 12.1 versus 18.8, comparison of the two-group wise Euclidean distributions, $P = 6.98^{e-14}$ using the Mann–Whitney test, Fig. 4B).

From an initial 21 paired endometrial samples, the immune cell profiling could be completed for only 17 samples owing to insufficient cell numbers. In a pairwise comparison, we identified the frequency of CD3+T cells and γδT cells to be significantly decreased after MVP treatment compared to O-DYD treatment (Fig. 5A and B). The same trend was observed for B-cell frequency, but this did not reach significance ($P = 0.0684$) (Fig. 5C). In contrast, the frequency of NK cells was significantly increased after MVP treatment compared to O-DYD treatment (Fig. 5D). The frequency of conventional dendritic cells did not differ between the two treatment options (Fig. 5E). Albeit a minor immune cell population in the endometrium, monocyte frequency was significantly reduced after MVP treatment compared to O-DYD treatment (Fig. 5F). Visualization of the endometrial immune cell profiles by dimensionality reduction (UMAP plots) after O-DYD and MVP treatment are shown in Fig. 5G.

Discussion

The current study investigated the PK, endometrial molecular profile, and immune cell profiles in oocyte donors who, for study purposes, received O-DYD once and MVP once within two subsequent OS cycles in a randomized fashion. In summary, the PK profile of O-DYD showed a rapid absorption and clearance, even at steady-state, but endometrial assessment via histology and whole tissue bulk RNA-seq revealed no difference in the overall ability of O-DYD/MVP to induce a putative receptive state. Nevertheless, it is noteworthy that the intersample distance of RNA-profiles was significantly smaller following O-DYD when compared to MVP and that endometrial immune cell profiles also differed significantly between groups.

This is a unique study comparing O-DYD and MVP on a molecular level, head-to-head, in a randomized, cross-over, double-blind double-dummy controlled manner, mimicking LPS strategies for fresh embryo transfer after OS. The major strengths of the study are its prospective design, in which participants served as their own controls, and the collaboration between expert teams in the field to process, analyze, and interpret molecular data originating from a multitude of biological samples. The limitations are more specific, dependent on the performed harvestings and analyses, and are discussed for each section separately below.

Plasma concentrations

Individualization of LPS for fresh embryo transfer has recently gained more interest (Benmachiche *et al.*, 2021) and insight into the PK profiles of progestogens is essential to correctly interpret the potential impact of circulating hormone levels on reproductive outcomes. This is the first study comparing plasma D, DHD, and P concentrations following O-DYD/MVP intake for LPS in IVF/ICSI. The plasma concentration profiles differed strongly between the two LPS administration strategies, with a rapid absorption and clearance of O-DYD. This observation has ramifications for studies in which D/DHD levels are measured and associated with outcomes. These results also suggest administration frequency to be as important as dose, especially for O-DYD. However, peripheral concentrations do not necessarily reflect the steroidogenic effect on endometrial progesterone receptors (Labarta *et al.*, 2021). Extrapolation to clinical practice is therefore difficult and this is the reason why this study protocol also focused on molecular analyses of endometrial tissue harvested within the same participants to further investigate PK and the progestogenic impact on endometrial receptivity during the

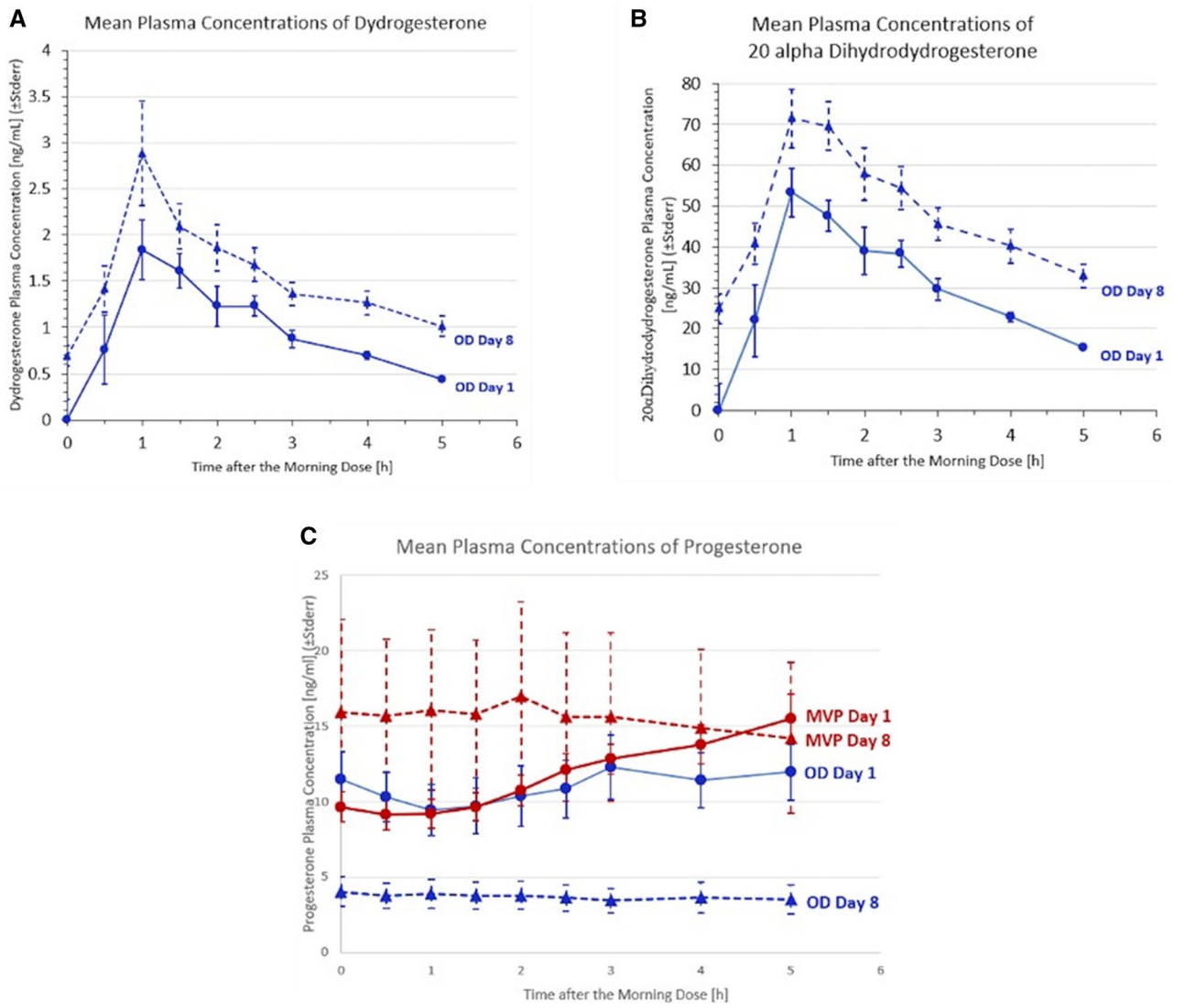

Figure 3. Plasma concentrations of dydrogesterone, 20αdihydrodydrogesterone, and progesterone from the first day of oral dydrogesterone or micronized vaginal progesterone intake until the eighth day of luteal phase support. (**A**) Plasma concentrations of dydrogesterone. (**B**) Plasma concentrations of 20αdihydrodydrogesterone. (**C**) Plasma concentrations of progesterone. All data are arithmetic mean values. MVP: micronized vaginal progesterone; OD: oral dydrogesterone.

embryo implantation period. Although of major interest to the field, the plasma concentration profiles, as investigated here, harbor several limitations. A first one is that these results are best estimates as sampling was reduced compared to a formal PK study to keep it feasible to be executed in an ambulatory, clinical setting with oocyte donors participating on a voluntary basis. Also, given this setting, we did not control nor correct for dietary intake on the days of sampling, potentially leading to less homogeneous results, but on the other hand reflecting real-life circumstances. It should be re-stated that all oocyte donors participating in the trial had a BMI ranging between 18 and 29 kg/m^2, thus excluding underweight as well as obese women. Furthermore, a recent study did not find any relevant association between BMI (or body weight) and D and DHD levels (Neumann *et al.*, 2022), making BMI an unlikely source of interindividual variation of D and DHD PK parameters. Another element prone for discussion within the study concept is the administration of a dual trigger that consisted of 1000 IU hCG + 0.2 mg triptorelin; this choice was the consensus of the research team aiming to mimic one of the most common methods in daily clinical practice, for triggering after OS when planning a fresh embryo transfer (i.e. with hCG). However, to avoid severe ovarian hyperstimulation syndrome in the participating oocyte donors, we decided to not administer more than 1000 IU of hCG; the applied trigger is a limitation of the study. It is important to acknowledge that in both study arms endogeneous serum P originating from the corpora lutea post-oocyte retrieval was circulating. This is visible in the serum P curves of the O-DYD-cycles, with a similar initial profile compared to MVP cycles on Day 1 of LPS, but with a severe deficiency reflecting the endogenous P luteal phase defect toward Day 8. More studies are needed to additionally investigate serum levels in relation to different methods of LPS in ART settings other than the fresh embryo transfer, especially in (artificially prepared) frozen embryo transfer cycles without endogenous P production to further optimize LPS individualization strategies (Neumann *et al.*, 2022).

Endometrial assessment

Little data are available on endometrial histology upon administration of O-DYD to support the luteal phase. A small (n = 6)

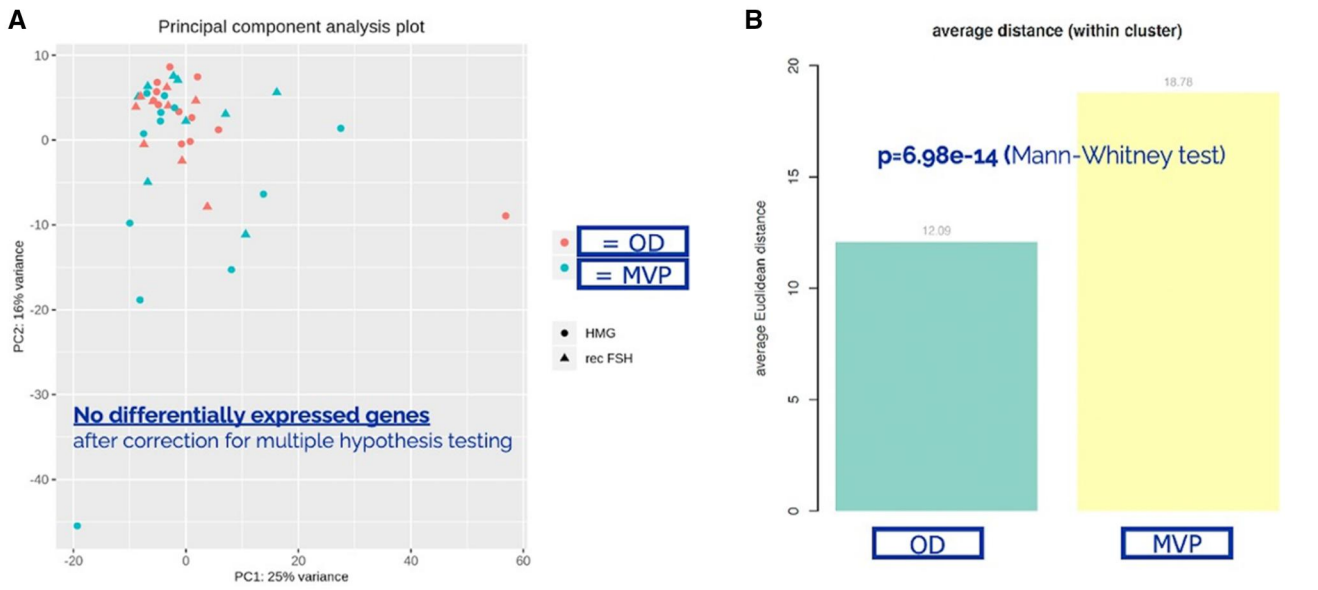

Figure 4. Endometrial RNA-sequencing data as a function of the luteal phase support medication groups. (**A**) Principal component analysis following RNA-sequencing of endometrial biopsies harvested after 1 week of luteal phase support. (**B**) Average Euclidean distances between the samples of the study groups based on RNA-sequencing data. MVP: micronized vaginal progesterone; OD: oral dydrogesterone; PC: principal component; recFSH: recombinant FSH.

prospective pilot study with cross-over design previously raised caution for O-DYD potentially being less effective than MVP in creating an 'in-phase' secretory endometrium in artificial cycles for patients with premature ovarian insufficiency (Fatemi *et al.*, 2007). As already mentioned above, the set-up of the current study cannot be compared as LPS was administered post-OS and post-dual trigger. Nevertheless, it is reassuring that histological assessment of the study endometrial biopsies confirmed a secretory phenotype for all specimens. Interesting to point out is the confirmation of an overall histological advancement of the endometrium following OS, with O-DYD as well as with MVP, as published consistently before by our research group (but previously always following MVP: Bourgain and Devroey, 2003; Kolibianakis *et al.*, 2005; Van Vaerenbergh *et al.*, 2009). Indeed, while endometrial biopsies were all harvested on cycle Day 22, the mean cycle day after dating according to Noyes was 24. The ratio of biopsies determined to be 'out-of-phase' (i.e. <Day 20 or >Day 24, according to Bourgain *et al.* (1990)) was similar in the O-DYD and the MVP group.

Supportive of the extensive clinical, reproductive outcome datasets derived from the Lotus I and II program reporting noninferiority O-DYD when compared to MVP for fresh embryo transfer LPS (Tournaye *et al.*, 2017; Griesinger *et al.*, 2018a), are the similar high-throughput RNA-seq data for both study arms as investigated here. After correction for multiple hypothesis testing, not a single gene was withheld as being differentially expressed when comparing O-DYD versus MVP. This is suggestive of the fact that both LPS strategies have the ability to induce a putative receptive phenotype corresponding to the window of implantation. When interpreting in detail the PCA-plot visualizing each individual sample in function of its transcriptomic signature, the ones following O-DYD seem to cluster more together. Indeed, when calculating the Euclidean distance for each study group, the intersample distance of RNA-profiles was significantly smaller following O-DYD. A potential explanation for this finding could be that interindividual variations are lower following the oral administration route when compared to the vaginal one, especially as BMI could interfere with the absorption of MVP (Maignien *et al.*, 2022), but additional research is needed to further explore this finding. It also needs to be mentioned in this section that the technique of bulk/whole tissue endometrial RNA-seq data has several limitations in reflecting the true biological functionality of the tissue, an important one being the potential risk of bias of different tissue compartment compositions between biopsies (Mackens *et al.*, 2020). Future studies could attempt to circumvent this issue with innovative techniques that are currently under development, such as single-cell RNA-seq (Koel *et al.*, 2022).

The immune paradox surrounding pregnancy supporting growth and development of a semi-allogenic fetus is still not fully solved. However, the local immune environment is critical for successful implantation of the conceptus (Robertson and Moldenhauer, 2014). Despite a reduced antigenicity and attenuated expression of major histocompatibility complex (MHC) genes of trophoblast cells, it is evident that the maternal immune system responds to the inherited paternal antigens. Key features involve the inhibition of T- and B-cell proliferation, reduced NK cell cytotoxicity, and the arrest of dendritic cells in a tolerogenic state mediated by ovarian sex hormones, e.g. progesterone (Arck and Hecher, 2013; Abu-Raya *et al.*, 2020). A detriment of the present analysis is a missing baseline, which would have been preferable to properly interpret the immune response to O-DYD and MVP, but was omitted to avoid the additional burden of a third biopsy prior to treatment. Based on the immune data from nonpregnant women reported in the literature (Feyaerts *et al.*, 2021), it is evident that both medications induce a reduction of T cells, which is more prominent with MVP treatment. However, the T-cell compartment is diverse and while the proliferation of alloreactive T effector cells should be diminished, the frequency of CD4+ regulatory T cells is essential to establish a tolerant immune environment for the fetus. Hence, a more detailed investigation of the T-cell compartment would be informative. The most abundant lymphocyte population in the endometrium is uterine NK cells. While conventional NK cells are characterized by their natural cytotoxicity, uterine NK cells support placental vascular remodeling and produce growth-promoting factors,

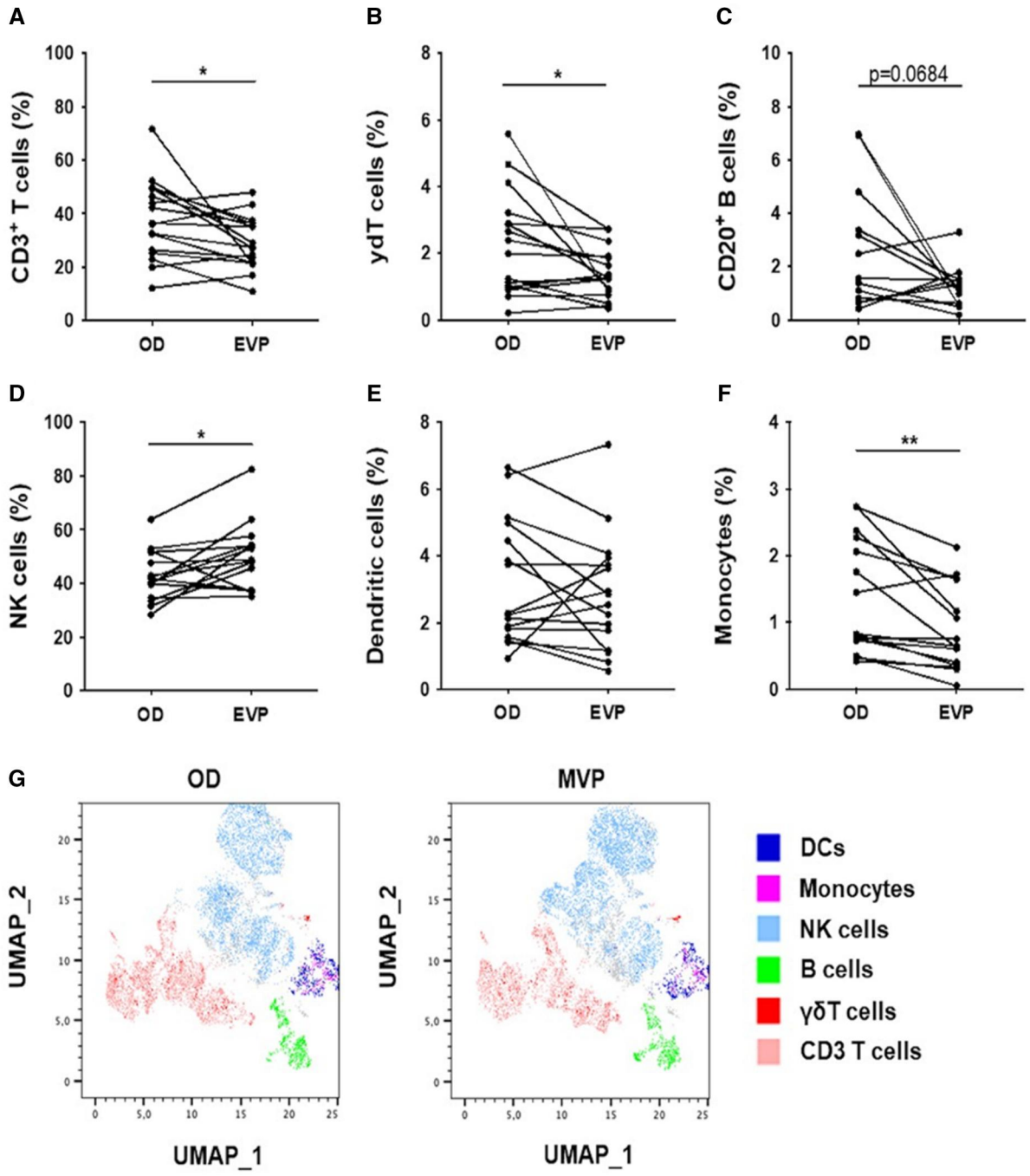

Figure 5. Endometrial immune cell analysis as a function of the luteal phase support medication groups. When comparing MVP treatment to O-DYD treatment, there was a significant decrease in the frequency of CD3+T cells and γδT cells (**A** and **B**, paired t test, *$P < 0.05$). Regarding B-cell frequency, the same trend was seen, although the result was not statistically significant ($P = 0.0684$) (**C**). On the other hand, NK cell frequency was noticeably higher following MVP treatment as opposed to O-DYD treatment (**D**, paired t test, *$P < 0.05$). There was no difference in the frequency of conventional dendritic cells between the two treatment options (**E**). Monocyte frequency was considerably lower following MVP treatment compared to O-DYD treatment (**F**, paired t test, **$P < 0.01$). (**G**) The endometrial immune cell profiles using dimensionality reduction (UMAP plots) following O-DYD and MVP treatment. MVP: micronized vaginal progesterone; OD/O-DYD: oral dydrogesterone; DC: dendritic cells; NK: natural killer cells.

facilitating embryo development (Sojka *et al.*, 2019). Hence, in early pregnancy, NK cells show an increased abundance, which we also observed in response to both medications. However, again the effect of MVP appears to be stronger than O-DYD. Crucial, especially for CD4+ regulatory T-cell activation and proliferation, are dendritic cells (Robertson and Moldenhauer, 2014). The functionality of dendritic cells is strongly dependent on the hormonal microenvironment (Thiele *et al.*, 2019; Diao *et al.*, 2021). Hence, although the overall frequency of dendritic cells is similar in response to both treatments, an in-depth characterization might reveal distinct tolerogenic phenotypes that may also account for a shift within the T-cell compartment that has yet to be determined.

In conclusion, this study showed that although plasma D, DHD, and P concentrations differ, both LPS strategies lead to overall comparable molecular findings at the level of the end-organ. Endometrial histology and RNA-seq data were aligned, but further research is needed to explore the smaller intersample

distance following O-DYD compared to MVP, and the subtle changes detected in endometrial immune cells.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

Authors' roles

S.M., C.Bl., G.G., and H.T. are responsible for the concept and study design. M.D.B. performed all oocyte retrievals and endometrial biopsies. S.M. and S.L. performed the data collection. C.Bo. and W.W. are responsible for the histological analyses. S.M. and C.O. are responsible for the RNA-seq analysis. S.M. isolated and cryopreserved endometrial immune cells. K.T. and P.A. profiled endometrial immune cells. S.M. and S.L. drafted the article. All authors contributed to the interpretation, discussion, and editing of the article. All authors approved the last version.

Funding

This investigator-initiated study was supported by a grant from Abbott Products Operations AG.

Conflict of interest

Not related to this work, C.Bl. has received honoraria for lectures, presentations, manuscript writing, educational events, or scientific advice from Abbott, Ferring, Organon, Cooper Surgical, Gedeon-Richter, IBSA, and Merck. H.T. has received honoraria for lectures, presentations, manuscript writing, educational events, or scientific advice from Abbott, Ferring, Cooper Surgical, Gedeon-Richter, Cook, and Goodlife. S.M. has received honoraria for lectures, presentations, educational events, or scientific advice from Abbott, Cooper Surgical, Gedeon-Richter, IBSA, and Merck and Oxolife. G.G. has received honoraria for lectures, presentations, educational events, or scientific advice from Merck, MSD, Organon, Ferring, Theramex, Gedeon-Richter, Abbott, Biosilu, ReprodWissen, Obseva, PregLem, Guerbet, Cooper, Igyxos, and OxoLife. S.V.-S. is listed as inventor on two patents (WO2019115755A1 and WO2022073973A1), which are not related to this work.

References

Abu-Raya B, Michalski C, Sadarangani M, Lavoie PM. Maternal immunological adaptation during normal pregnancy. *Front Immunol* 2020;**11**:575197.

Arck PC, Hecher K. Fetomaternal immune cross-talk and its consequences for maternal and offspring's health. *Nat Med* 2013; **19**:548–556.

Benmachiche A, Benbouhedja S, Zoghmar A, Al Humaidan PSH. The impact of preovulatory versus midluteal serum progesterone level on live birth rates during fresh embryo transfer. *PLoS One* 2021;**16**:e0246440.

Bourgain C, Devroey P. The endometrium in stimulated cycles for IVF. *Hum Reprod Update* 2003;**9**:515–522.

Bourgain C, Devroey P, Van Waesberghe L, Smitz J, Van Steirteghem AC. Effects of natural progesterone on the morphology of the endometrium in patients with primary ovarian failure. *Hum Reprod* 1990;**5**:537–543.

Di Guardo F, Midassi H, Racca A, Tournaye H, De Vos M, Blockeel C. Luteal phase support in IVF: comparison between evidence-based medicine and real-life practices. *Front Endocrinol (Lausanne)* 2020;**11**:500.

Diao L, Hierweger AM, Wieczorek A, Arck PC, Thiele K. Disruption of glucocorticoid action on $CD11c^{+}$ dendritic cells favors the generation of $CD4^{+}$ regulatory T cells and improves fetal development in mice. *Front Immunol* 2021;**12**:729742.

Fatemi HM, Bourgain C, Donoso P, Blockeel C, Papanikolaou EG, Popovic-Todorovic B, Devroey P. Effect of oral administration of dydrogestrone versus vaginal administration of natural micronized progesterone on the secretory transformation of endometrium and luteal endocrine profile in patients with premature ovarian failure: a proof of concept. *Hum Reprod* 2007; **22**:1260–1263.

Feyaerts D, Gillard J, van Cranenbroek B, Rigodanzo Marins L, Baghdady MMS, Comitini G, Lely AT, van Hamersvelt HW, van der Heijden OWH, Joosten I *et al.* Maternal, decidual, and neonatal lymphocyte composition is affected in pregnant kidney transplant recipients. *Front Immunol* 2021;**12**:735564.

Griesinger G, Blockeel C, Kahler E, Pexman-Fieth C, Olofsson JI, Driessen S, Tournaye H. Dydrogesterone as an oral alternative to vaginal progesterone for IVF luteal phase support: a systematic review and individual participant data meta-analysis. *PLoS One* 2020;**15**:e0241044.

Griesinger G, Blockeel C, Sukhikh GT, Patki A, Dhorepatil B, Yang DZ, Chen ZJ, Kahler E, Pexman-Fieth C, Tournaye H. Oral dydrogesterone versus intravaginal micronized progesterone gel for luteal phase support in IVF: a randomized clinical trial. *Hum Reprod* 2018a;**33**:2212–2221.

Griesinger G, Blockeel C, Tournaye H. Oral dydrogesterone for luteal phase support in fresh in vitro fertilization cycles: a new standard? *Fertil Steril* 2018b;**109**:756–762.

Griesinger G, Tournaye H, Macklon N, Petraglia F, Arck P, Blockeel C, van Amsterdam P, Pexman-Fieth C, Fauser BC. Dydrogesterone: pharmacological profile and mechanism of action as luteal phase support in assisted reproduction. *Reprod Biomed Online* 2019; **38**:249–259.

Hart SN, Therneau TM, Zhang Y, Poland GA, Kocher JP. Calculating sample size estimates for RNA sequencing data. *J Comput Biol* 2013;**20**:970–978.

Koel M, Krjutškov K, Saare M, Samuel K, Lubenets D, Katayama S, Einarsdottir E, Vargas E, Sola-Leyva A, Lalitkumar PG *et al.* Human endometrial cell-type-specific RNA sequencing provides new insights into the embryo-endometrium interplay. *Hum Reprod Open* 2022;**2022**:hoac043.

Kolibianakis EM, Bourgain C, Papanikolaou EG, Camus M, Tournaye H, Van Steirteghem AC, Devroey P. Prolongation of follicular phase by delaying hCG administration results in a higher incidence of endometrial advancement on the day of oocyte retrieval in GnRH antagonist cycles. *Hum Reprod* 2005; **20**:2453–2456.

Labarta E, Sebastian-Leon P, Devesa-Peiro A, Celada P, Vidal C, Giles J, Rodriguez-Varela C, Bosch E, Diaz-Gimeno P. Analysis of serum and endometrial progesterone in determining endometrial receptivity. *Hum Reprod* 2021;**36**:2861–2870.

Mackens S, Santos-Ribeiro S, Racca A, Daneels D, Koch A, Essahib W, Verpoest W, Bourgain C, Van Riet I, Tournaye H *et al.* The proliferative phase endometrium in IVF/ICSI: an in-cycle molecular

analysis predictive of the outcome following fresh embryo transfer. *Hum Reprod* 2020;**35**:130–144.

Maignien C, Bourdon M, Marcellin L, Guibourdenche J, Chargui A, Patrat C, Plu-Bureau G, Chapron C, Santulli P. Clinical factors associated with low serum progesterone levels on the day of frozen blastocyst transfer in hormonal replacement therapy cycles. *Hum Reprod* 2022;**37**:2570–2577.

Mincheva-Nilsson L. Pregnancy and gamma/delta T cells: taking on the hard questions. *Reprod Biol Endocrinol* 2003;**1**:120.

Neumann K, Masuch A, Vonthein R, Depenbusch M, Schultze-Mosgau A, Eggersmann TK, Griesinger G. Dydrogesterone and 20α-dihydrodydrogesterone plasma levels on day of embryo transfer and clinical outcome in an anovulatory programmed frozen-thawed embryo transfer cycle: a prospective cohort study. *Hum Reprod* 2022;**37**:1183–1193.

Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. *Am J Obstet Gynecol* 1975;**122**:262–263.

Rižner TL, Brožič P, Doucette C, Turek-Etienne T, Müller-Vieira U, Sonneveld E, van der Burg B, Böcker C, Husen B. Selectivity and potency of the retroprogesterone dydrogesterone in vitro. *Steroids* 2011;**76**:607–615.

Robertson SA, Moldenhauer LM. Immunological determinants of implantation success. *Int J Dev Biol* 2014;**58**:205–217.

Sojka DK, Yang L, Yokoyama WM. Uterine Natural Killer Cells. *Front Immunol* 2019;**10**:960.

Stanczyk FZ, Hapgood JP, Winer S, Mishell DR Jr. Progestogens used in postmenopausal hormone therapy: differences in their pharmacological properties, intracellular actions, and clinical effects. *Endocr Rev* 2013;**34**:171–208. Apr

Thiele K, Hierweger AM, Riquelme JIA, Solano ME, Lydon JP, Arck PC. Impaired progesterone-responsiveness of $CD11c^{+}$ dendritic cells affects the generation of $CD4^{+}$regulatory T cells and is associated with intrauterine growth restriction in mice. *Front Endocrinol (Lausanne)* 2019;**10**:96.

Tournaye H, Sukhikh GT, Kahler E, Griesinger G. A phase III randomized controlled trial comparing the efficacy, safety and tolerability of oral dydrogesterone versus micronized vaginal progesterone for luteal support in in vitro fertilization. *Hum Reprod* 2017;**32**:2152.

Vaisbuch E, de Ziegler D, Leong M, Weissman A, Shoham Z. Luteal-phase support in assisted reproduction treatment: real-life practices reported worldwide by an updated website-based survey. *Reprod Biomed Online* 2014;**28**:330–335.

van der Linden M, Buckingham K, Farquhar C, Kremer JA, Metwally M. Luteal phase support for assisted reproduction cycles. *Cochrane Database Syst Rev* 2015;**2015**:CD009154.

Van Vaerenbergh I, Van Lommel L, Ghislain V, In't Veld P, Schuit F, Fatemi HM, Devroey P, Bourgain C. In GnRH antagonist/rec-FSH stimulated cycles, advanced endometrial maturation on the day of oocyte retrieval correlates with altered gene expression. *Hum Reprod* 2009;**24**:1085–1091.