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ORIGINAL ARTICLE

Uterine fluid cytokine/chemokine levels of women undergoing ART with and without oral Vitamin D supplementation

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STUDY QUESTION: Is oral Vitamin D supplementation able to modify the intrauterine milieu in terms of cytokine/chemokine pattern?

SUMMARY ANSWER: No significant differences were detected in cytokine and chemokine levels in endometrial secretions between patients undergoing ART with or without Vitamin D supplementation.

WHAT IS KNOWN ALREADY: Cytokines and chemokines secreted into the intrauterine environment are fundamental for the molecular crosstalk between the endometrium and the preimplantation embryo. Whether Vitamin D can regulate these mediators in the endometrial environment is still unclear.

STUDY DESIGN, SIZE, DURATION: This study was an analysis of a secondary outcome from the Supplementation of Vitamin D and Reproductive Outcomes—SUNDRO—clinical trial, a multicenter randomized double-blinded trial designed to explore the effects of Vitamin D replacement in women with Vitamin D levels below 30 ng/ml undergoing autologous ART cycles. Uterine fluid samples were collected from both patients supplemented with Vitamin D (n = 17) and from the placebo group (n = 32).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Based on cutoff points for Vitamin D insufficiency (20–29.9 ng/ml) or deficiency (<20 ng/ml), 67% of patients in the study were insufficient, and 33% deficient, in Vitamin D, although they were considered together for the analysis. Women received a single dose of 600 000 IU 25-hydroxyvitamin D or placebo from 2 to 12 weeks before oocyte retrieval. Inclusion criteria were female age 18–39 years, with a BMI between 18 and 25 kg/m^2. Serum 25-hydroxyvitamin D was assessed at the time of hCG administration. Uterine fluid samples were collected during the secretory phase of the menstrual cycle preceding oocyte retrieval. The quantitative determination of 27 cytokines in endometrial secretion samples was performed by using a multiplex immunoassay.

MAIN RESULTS AND THE ROLE OF CHANCE: Uterine fluid samples were collected after a median (range) of 21 (12–41) days after the oral Vitamin D supplementation. Both the supplemented and placebo groups had Vitamin D serum levels below 30 ng/ml at baseline/time of randomization ((median 23.4 ng/ml (interquartile range 19.5–28.4) and 23.4 ng/ml (17.8–25.9), respectively). At the time of hCG administration, serum Vitamin D in supplemented subjects was significantly raised compared to the placebo group ((median 52.9 ng/ml (interquartile range 40.7–64.1) and 24.6 ng/ml (19.3–29.2), respectively, $P < 0.001$). Our data revealed no significant differences in uterine fluid cytokine/chemokine composition of Vitamin D-supplemented women compared with the placebo group. This finding remained when the concentrations of all mediators studied were normalized to total protein. In a further analysis, no significant differences were found in

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the content of cytokines/chemokines in uterine fluid from women who conceived (n = 19) compared with the nonpregnant group (n = 30).

LIMITATIONS, REASONS FOR CAUTION: Using a randomized study design (a single dose of 600 000 IU 25-hydroxyvitamin D versus placebo), we found no significant differences between groups. However, we cannot exclude that any benefit of Vitamin D supplementation may be specific for some subgroups of patients, such as those with an imbalance of T-helper 1 and T-helper 2 cell populations. The uterine secretions were collected during the menstrual cycle that preceded oocyte retrieval; therefore, it is possible the uterine fluid collection and analysis in the same cycle of the embryo transfer might have resulted in different conclusions. Moreover, the small sample size could limit the power of the study.

WIDER IMPLICATIONS OF THE FINDINGS: Our analysis of the uterine secretome profiling failed to show any significant difference in endometrial cytokine/chemokine patterns between women with oral Vitamin D supplementation and the placebo group. Vitamin D may act on the uterine environment through a different mechanism.

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Key words: chemokines / cytokines / endometrium / immunomodulation / interleukin / IVF / randomized controlled trial / Vitamin D

WHAT DOES THIS MEAN FOR PATIENTS?

Vitamin D, taken through the diet or produced in the skin, represents a very important factor for human health. It has also been suggested that Vitamin D is important for the success of fertility treatments, and this may be especially important for women who have low blood levels of Vitamin D. Recently, our group set up a study to find out whether taking Vitamin D by mouth could increase the pregnancy rate in infertile patients undergoing fertility treatment. In particular, we have looked at whether taking oral Vitamin D could change the levels of substances found in the fluid in the uterus that are important during early pregnancy (these are proteins, called cytokines and chemokines). The results showed no significant difference between women who received Vitamin D and those who did not. However, further studies are needed to clarify whether Vitamin D may be of benefit in specific subgroups of patients such as those with recurrent abortion.

Introduction

In humans, Vitamin D, taken through the diet or produced in the skin, undergoes two reactions, one at the level of the liver and one at the level of the kidney, resulting in the active molecule 1,25-dihydroxyvitamin D_3. This compound represents the key molecule of a major endocrine system, with pleiotropic functions acting through two different molecular signaling pathways: the genomic action which takes a few hours before effects can be observed and mediated by RNA and proteins synthesis, and the nongenomic action with short-term effects (Pawlowska *et al.*, 2016).

Recent data suggest a possible association between Vitamin D serum level and many pathophysiological processes of the female reproductive system including fertility (Irani and Merhi, 2014; Pilz *et al.*, 2018; Fichera *et al.*, 2020). Two recent meta-analyses showed that Vitamin D deficiency or insufficiency could be detrimental for the success of ART (Chu *et al.*, 2018; Zhao *et al.*, 2018). In addition, the first double-blind randomized controlled trial in infertile women showed a positive impact of maternal Vitamin D supplementation on clinical outcomes in ART cycles in terms of clinical pregnancy rate and quality of endometrium. No differences were, however, found in terms of number of retrieved oocytes and mature oocytes, fertilization rate and top quality embryo rate (Abedi *et al.*, 2019). Additional literature reported a beneficial clinical action of Vitamin D on endometrial tissue and during the implantation window (Asadi *et al.*, 2014; Polyzos *et al.*, 2014; Rudick *et al.*, 2014).

Molecular Vitamin D action on human endometrial cells remains under researched (Cermisoni *et al.*, 2018). During a successful implantation, synchronous modifications and a bidirectional crosstalk occur between a receptive endometrium and a competent embryo (Brosens et al., 2014; Giacomini *et al.*, 2019). The molecular crosstalk is mediated by proteins, cytokines and chemokines, secreted into the intrauterine environment, which are presumed to be critical for the establishment of an optimal setting for embryo implantation. Cytokine profile analysis with multiplex immunoassay/enzymatic assay showed a different production of proinflammatory and anti-inflammatory mediators by endometrial cells from women with recurrent implantation failure compared with fertile women (Rajaei *et al.*, 2011). This evidence supports a promising noninvasive approach to characterize the endometrial milieu and to profile the immunologic mediators that could be crucial for a successful implantation (Boomsma *et al.*, 2009; Lombardelli *et al.*, 2013).

In this context, studying the possible modulating action of Vitamin D on the maternal–fetal interface could be of the utmost importance (Dekel *et al.*, 2014; Mekinian *et al.*, 2016; D'Ippolito et al. 2018).

In 2017, our group started a multicenter randomized double-blinded clinical trial to test the effects of supplementation with 25-hydroxyvitamin D, a precursor for the synthesis of the active form 1,25-

dihydroxyvitamin D_3, in women with Vitamin D levels below 30 ng/ml undergoing ART cycles (the Supplementation of Vitamin D and Reproductive Outcomes—SUNDRO—trial; Paffoni *et al.*, 2019). The clinical results of the trial have been recently published (Somigliana *et al.*, 2021). Here, we present a report of a secondary outcome from the SUNDRO clinical trial, namely the effect of Vitamin D replacement on the molecular pattern of the uterine secretome. Analysis of the uterine secretome provides a noninvasive approach for studying the endometrial milieu as well as a snapshot of the environment that the preimplantation embryo will meet.

Materials and methods

Ethics approval and consent to participate

The study was conducted in accordance with the ethical principles of the Helsinki Declaration guidelines. It was approved by the Ethical Committees of the two participating centers (Comitato Etico Area B, Milan, protocol n° 602 05/04/2016 and Comitato Etico Istituto di Ricovero e Cura a Carattere Scientifico—Ospedale San Raffaele, protocol n° 189/2016). The study was approved by the Italian Medicines Agency (AIFA; Protocol AIFA/RSCP/P/65768) and registered (EUDRACT 2015-004233-27). Written informed consent was obtained from eligible patients.

Study design and subjects

The study has been designed according to the CONSORT methodology (Paffoni *et al.*, 2019). The randomized double-blinded placebo-controlled trial involved two Italian ART clinics (IRCCS Fondazione Ca' Granda, Ospedale Maggiore Policlinico, Milan and IRCCS San Raffaele Scientific Institute, Milan).

The selected population included infertile patients undergoing ART cycles with insufficient or deficient serum levels of Vitamin D (25-hydroxyvitamin D serum level $<$ 30 ng/ml), according to the most recent International Guidelines (Ross *et al.*, 2011). Inclusion criteria were female age 18–39 years, with a BMI between 18 and 25 kg/m^2 and undergoing autologous ART cycles with less than three previous cycles. Exclusion criteria included contraindications/side effects for consumption of Vitamin D, an anti-Müllerian hormone serum level $<$0.5 ng/ml and ART cycles with surgical retrieval of the spermatozoa or frozen gametes.

The first 50 eligible women that agreed to participate in both the randomized double-blinded clinical trial and the uterine fluid collection were enrolled. In one patient, the uterine fluid collection did not result in a sufficient sampling. Researchers were blinded to which treatment was being provided to enrolled women. At the time of randomization, from 2 to 12 weeks before oocyte retrieval, women received a single dose of 600 000 IU of 25-hydroxyvitamin D or placebo. Randomization was performed centrally by Fondazione IRCCS Ospedale Maggiore Policlinico. The computer-generated allocation sequence was hidden from the participants as well as from the physicians and biologists involved in the clinical management of the patients.

The two centers followed their own standard regarding ovarian stimulation, ART laboratory procedures and endometrial preparation, as described elsewhere (Faulisi *et al.*, 2017; Papaleo *et al.*, 2017; Viganò *et al.*, 2020). Serum 25-hydroxyvitamin D was assessed at the time of hCG administration. All Vitamin D levels measurements were performed with a commercially available kit (DiaSorin; Saluggia, Italy). Clinical pregnancy was defined after the ultrasound presence of at least one intrauterine gestational sac with viable fetus at about 6 weeks of pregnancy.

Endometrial secretion aspiration

Uterine fluid samples were collected during the secretory phase of the menstrual cycle that preceded the oocyte retrieval. Dating was estimated according to the previous cycles and to the presence of a corpus luteum cyst at ultrasound. After insertion of a sterilized speculum, vaginal secretions were cleaned by cotton buds. The uterine flushing was performed by using a disposable catheter for sonohysterography with a balloon opening the cavity when it is inserted in the uterus to minimize vaginal contamination (Wallace® Trial Transfer Catheter, CooperSurgical Fertility & Genomic Solutions, Denmark). To obtain representative sampling of uterine secretions, 1.5 ml of physiologic solution was injected and gently suctioned. Samples were immediately centrifuged at 600x*g* for 15 min in order to separate cell debris, mucus and minimal blood contamination from the liquid fraction. The liquid fractions were stored at −80°C. After thawing, total protein concentration was measured by Bradford assay (Quick Start™ Bradford, Bio-Rad Laboratories, Hercules, CA, USA) for normalization purposes.

Determination of cytokine concentrations in endometrial secretions using bead-based multiplex immunoassays

After thawing, the quantitative determination of IL-1beta, IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, and IL-17, fibroblast growth factor (FGF), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), eotaxin, interferon gamma (IFNγ), interferon-inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1-α), macrophage inflammatory protein 1 beta (MIP-1-β), platelet-derived growth factor-BB (PDGF-BB), regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor alpha (TNF)-α and vascular endothelial growth factor (VEGF) secretion samples was performed by using a bead-based multiplex immunoassay (Bio-Rad Laboratories, Hercules, CA, USA) and the Bioplex 200 system (Bio-Rad Laboratories, Hercules, CA, USA), as previously described (Lombardelli *et al.*, 2013). In brief, in a 96-well filter plate (Bio-Rad Laboratories), 50 µl of each serum sample was added to 50 µl of antibody-conjugated beads directed against the cytokines listed above (Bio-Rad Laboratories). After a 30-min incubation, the plate was washed and 25 µl of biotinylated anti-cytokine antibody solution was added to each well before another 30-min incubation. The plate was then washed and 50 µl of streptavidin-conjugated phycoerythrin was added to each well. After a final wash, each well was resuspended with 125 µl of assay buffer (Bio-Rad Laboratories) and analyzed by Bioplex 200. Standard curves were constructed using various concentrations of the cytokine standards and followed the same protocol as for the endometrial secretion samples. The concentration of the 27 cytokines (pg/ml) in each endometrial secretion sample was calculated using Bioplex 200 software.

Table I Basal and clinical characteristics of infertile women in the Vitamin D supplementation and placebo groups.

Characteristics	Vitamin D n = 17	Placebo n = 32	P
Age (years)	33.5 ± 3.6	35.2 ± 3.0	0.09
BMI (kg/m^2)	21.5 ± 1.9	21.0 ± 1.9	0.44
Smokers, n (%)	1 (6%)	2 (6%)	1.00
Duration of infertility (years)*	2 [2–3.5]	2 [1–4]	0.72
Previous pregnancy, n (%)	3 (18%)	6 (19%)	1.00
Previous delivery, n (%)	2 (12%)	2 (6%)	0.60
Serum AMH (ng/ml)*	2.7 [1.2–7.6]	2.4 [1.3–5.0]	0.51
Serum FSH (mIU/ml)*	6.4 [4.8–8.0]	7.4 [5.8–9.2]	0.15
Antral follicle count*	9 [6–16]	14 [10–20]	0.09
Previous IVF cycle, n (%)	3 (18%)	5 (16%)	1.00
Serum Vitamin D at baseline*,** (ng/ml)	23.4 [19.5–28.4]	23.4 [17.8–25.9]	0.57
Indication for IVF [all, n (%)]			0.11
Idiopathic	4 (23%)	12 (38%)	
Male factor	6 (35%)	10 (31%)	
Endometriosis	3 (18%)	3 (9%)	
Tube factor	3 (18%)	0 (0%)	
Genetic	1 (6%)	5 (16%)	
Male and female factor	0 (0%)	2 (6%)	
Serum Vitamin D at oocyte retrieval (ng/ml)*	52.9 [40.7–64.1]	24.6 [19.3–29.2]	<0.001
Oocyte retrieved*	9 [5–12]	8 [5–11]	0.98
Metaphase II oocytes*	8 [4–10]	7 [4–9]	0.91
Zygotes*	5 [3–7]	5 [3–8]	0.88
Cleavage embryos*	5 [2–7]	5 [3–7]	0.80
Top quality embryos*	0 [0–1]	1 [0–2]	0.21
Blastocysts*	2 [2–4]	3 [1–4]	0.91
Top quality blastocysts*	0 [0–1]	0 [0–0]	0.20
Fresh embryo transfer,* n (%)	9 (53%)	15 (47%)	0.77
Reason for no fresh embryo transfer			0.16
High risk of OHSS, n (%)	4 (50%)	13 (77%)	
No oocyte retrieved, n (%)	1 (12%)	1 (6%)	
No viable cleavage embryos, n (%)	3 (38%)	1 (6%)	
Other, n (%)	0 (0%)	2 (12%)	
Frozen embryo transfer, n (%)	7 (41%)	13 (41%)	1.00
Cumulative pregnancy rate, n (%)	7 (41%)	12 (38%)	1.00
Cumulative live birth rate, n (%)	5 (29%)	11 (34%)	1.00

*Data are presented as mean ± SD, median [interquartile range] or n (%). Data were compared using Student's *t*-test, Mann–Whitney test or Chi-square test.
**Baseline was time of randomization.
OHSS, ovarian hyperstimulation syndrome; AMH, anti-Müllerian hormone.

The lower detection limits of the multiplex immunoassay kit were as follows: IL-1β (0.39 pg/ml), IL-1RA (42.01 pg/ml), IL-2 (0.33 pg/ml), IL-4 (0.1 pg/ml), IL-5 (0.86 pg/ml), IL-6 (0.22 pg/ml), IL-7 (29.25 pg/ml), IL-8 (1.09 pg/ml), IL-9 (2.28 pg/ml), IL-10 (0.98 pg/ml), IL-12 (0.23 pg/ml), IL-13 (0.09 pg/ml), IL-15 (8.96 pg/ml), IL-17 (1.7 pg/ml), Eotaxin (0.13 pg/ml), FGF (2.62 pg/ml), G-CSF (2.33 pg/ml), GM-CSF (0.43 pg/ml), IFNγ (0.2 pg/ml), IP-10 (5.64 pg/ml), MCP-1 (0.45 pg/ml), MIP-1α (0.05 pg/ml), MIP-1β (0.46 pg/ml), PDGF-BB (4.56 pg/ml), (4.56 pg/ml), RANTES (3.76 pg/ml), TNF-α (3.28 pg/ml) and VEGF (10.38 pg/ml). Concentrations of uterine mediators were normalized to total protein concentration of each sample, as measured by Bradford assay.

Statistical analysis

All data were initially examined for normality using the Kolmogorov–Smirnov test: the normally distributed data were analyzed with the Student's *t*-test, while the non-normally distributed data were analyzed

Table II Detection and concentrations of soluble mediators in human endometrial secretion aspirations collected 12–41 days after the oral Vitamin D supplementation.

Mediators	Vitamin D n = 17	Placebo n = 32	P
IL-2	3 (18%)	9 (28%)	0.50
IL-4	8 (47%)	16 (50%)	1.00
IL-6	7 (41%)	17 (53%)	0.55
IL-7	1 (6%)	0 (0%)	0.35
IL-9	1 (6%)	6 (19%)	0.40
IL-10	0 (0%)	1 (3%)	1.00
IL-15	4 (24%)	5 (16%)	0.70
IL-17	0 (0%)	1 (3%)	1.00
EOTAXIN	8 (47%)	13 (41%)	0.77
FGF	5 (29%)	14 (44%)	0.37
GM-CSF	3 (18%)	8 (25%)	0.73
PDGF-BB	3 (18%)	8 (25%)	0.73
RANTES	8 (47%)	13 (41%)	0.77
TNF-α	6 (35%)	15 (47%)	0.55
IL-1β (pg/ml)	2.91 [0.39–29.00]	4.77 [0.39–33.94]	0.68
IL-1RA (pg/ml)	2187.88 [654.81–6486.14]	3259.74 [1029.50–5347.27]	0.83
IL-8 (pg/ml)	630.24 [113.53–1539.21]	784.91 [139.33–4342.58]	0.31
G-CSF (pg/ml)	49.14 [2.33–784.46]	77.93 [16.14–843.48]	0.58
IFNγ (pg/ml)	0.58 [0.20–5.81]	3.91 [0.20–22.06]	0.31
IP-10 (pg/ml)	226.01 [5.64–1066.92]	117.50 [5.64–653.62]	0.98
MCP-1 (pg/ml)	29.52 [7.45–245.59]	63.49 [19.56–237.18]	0.53
MIP-1-α (pg/ml)	0.17 [0.05–4.91]	1.38 [0.05–7.80]	0.39
MIP-1-β (pg/ml)	0.46 [0.46–26.90]	0.68 [0.46–49.62]	0.86
VEGF (pg/ml)	133.67 [18.07–466.56]	127.92 [10.38–500.02]	0.65

Upper panel: Data are presented as number of samples (%) in which the mediator was detected. The concentrations of 14 soluble mediators were below the reliable detection limit in <50% of the samples.
Lower panel: When a mediator was detected in more than 50% of the samples (n = 10), the specific concentration in endometrial secretions is reported as median [interquartile range] level of mediators.
Three mediators (IL-5, IL-12 and IL-13) were not detectable in any of the samples. Data were compared using Chi-square test or Mann–Whitney test.
IL-1RA, IL-1 receptor antagonist; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFNγ, interferon gamma; IP-10, interferon-inducible protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1-α, macrophage inflammatory protein 1-alpha; MIP-1-β, macrophage inflammatory protein 1-beta; PDGF-BB, platelet-derived growth factor-BB; RANTES, Regulated on Activation, Normal T Cell Expressed and Secreted; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

with the Mann–Whitney test. The frequency of patients' characteristics was analyzed with the Chi-square test. Data are presented as number (%), mean ± SD and median interquartile range (IQR). Soluble mediators in endometrial secretion aspirations in which more than 50% of the samples were detectable were analyzed as continuous variables (median (IQR)) and the difference between the two groups was analyzed by Mann–Whitney test. For the remaining molecules, a dichotomous analysis (presence or absence in the sample) was carried out and they were tested by Chi-square test. The significance level was defined at $\alpha = 0.002$ after correcting for multiple testing of 24 mediators with the Bonferroni method. For the calculation of the sample size, we could not find any previous publication on the effect of vitamin D on the cytokine/chemokine profile in the uterine fluid to be used as assumptions. However, from two previous studies of the effect of Vitamin D on the secretion of specific cytokines (IL-8 and IL-6) by the endometrial cells *in vitro* (Tavakoli *et al.*, 2011; Rajaei *et al.*, 2012), we expected that our sample size could detect a 1-fold increase in levels of IL-8 and 1-fold decrease in levels of IL-6 with a type I error of 0.002 and II error of 0.20, respectively.

Results

Uterine cytokine profile and Vitamin D supplementation

Forty-nine patients underwent endometrial secretion aspiration and were included in this analysis: n = 17 samples were collected from Vitamin D-supplemented patients and n = 32 samples from the

Table III Detection and relative concentrations of soluble mediators in human endometrial secretion aspirations after normalization to total protein.

Mediators	Vitamin D n = 15	Placebo n = 28	P
IL-2	3 (20%)	8 (29%)	0.72
IL-7	1 (7%)	0 (0%)	0.35
IL-9	1 (7%)	6 (21%)	0.39
IL-10	0 (0%)	1 (4%)	1.00
IL-15	4 (27%)	3 (11%)	0.22
IL-17	0 (0%)	1 (4%)	1.00
EOTAXIN	7 (47%)	13 (46%)	1.00
FGF	5 (33%)	14 (50%)	0.35
GM-CSF	3 (20%)	8 (29%)	0.72
PDGF-BB	2 (13%)	8 (29%)	0.45
RANTES	7 (47%)	13 (46%)	1.00
TNF-α	5 (33%)	15 (54%)	0.34
IL-1β (pg/mg)	4.77 [1.24–49.74]	8.39 [2.02–51.56]	0.59
IL-1RA (pg/mg)	3960.3 [2080.49–27 372.55]	3925.71 [997.24–8159.30]	0.59
IL-4 (pg/mg)	0.42 [0.16–1.98]	0.72 [0.19–4.15]	0.48
IL-6 (pg/mg)	1.52 [0.50–37.93]	4.10 [0.58–99.36]	0.52
IL-8 (pg/mg)	1061.22 [319.62–2629.86]	2119.28 [593.29–8867.66]	0.20
GCSF (pg/mg)	152.3 [16.13–401.72]	258.85 [27.96–927.42]	0.58
IFNγ (pg/mg)	1.38 [0.33–7.48]	6.34 [0.54–33.20]	0.30
IP-10 (pg/mg)	262.14 [9.25–2478.03]	265.77 [43.19–1154.81]	0.92
MCP-1 (pg/mg)	77.59 [18.16–343.37]	143.13 [52.53–509.11]	0.30
MIP-1-α (pg/mg)	0.35 [0.08–6.48]	3.39 [0.38–13.16]	0.34
MIP-1-β (pg/mg)	2.20 [0.75–55.60]	2.84 [1.21–80.74]	0.58
VEGF (pg/mg)	493.68 [71.28–3702.16]	41.23 [16.67–448.42]	0.06

Data are presented as number of samples (%) with detectable mediator (*upper panel*) or median [interquartile range] (*lower panel*). In the lower panel, units are reported as pg/ml normalized on mg/ml total protein (pg/mg).
Three mediators (IL-5, IL-12 and IL-13) were not detectable in any of the samples. In six samples, normalization was not possible due to the limited protein concentration for the analysis. Data were compared using Chi-square test or Mann–Whitney test.
FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFNγ, interferon gamma; IP-10, interferon-inducible protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1-α, macrophage inflammatory protein 1-alpha; MIP-1-β, macrophage inflammatory protein 1-beta; PDGF-BB, platelet-derived growth factor-BB; RANTES, Regulated on Activation, Normal T Cell Expressed and Secreted; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

placebo group. There were no significant differences between the groups regarding basal and clinical characteristics (Table I). As expected, both groups had serum levels of Vitamin D below 30 ng/ml at the time of randomization (median 23.4 ng/ml (IQR 19.5–28.4) and 23.4 ng/ml (17.8–25.9), respectively). Based on the cutoff points advocated for Vitamin D insufficiency (20–29.9 ng/ml) or deficiency (<20 ng/ml) (Somigliana et al., 2021), 67% of the patients were insufficient and 33% deficient although they were considered together for the analysis. At the time of hCG administration, the serum level of Vitamin D-supplemented subjects was significantly raised compared with the placebo group (median 52.9 ng/ml (interquartile range 40.7–64.1) and 24.6 ng/ml (19.3–29.2), respectively, $P < 0.001$).

Uterine fluid samples were collected after a median (range) of 21 (12–41) days after the oral supplementation. Levels of 27 cytokines were investigated in uterine fluids of both Vitamin D supplemented and placebo groups. None of the samples were excluded because of inadequate sample conditions. Our data showed some differences in the detection frequency of cytokines and chemokines. The comparison of the uterine fluid mediators between supplemented patients and placebo group is represented in Table II. Three mediators (IL-5, IL-12 and IL-13) were not detectable in any of the samples. The concentrations of 14 soluble mediators were below the reliable detection limit in 50% of the samples: data were presented as the number of samples in which these soluble mediators were detected (Table II, upper panel). When a mediator was detected in more than 50% of the samples (n = 10), the specific concentration of the soluble mediator was reported (Table II, lower panel). No significant differences were found in uterine fluid composition.

The total protein content in the samples varied from 0.002 to 1.760 mg/ml. Given the heterogeneity of total protein concentration among samples, concentrations of all studied mediators were also normalized to total protein concentration. Comparison of the relative

Table IV Characteristics of pregnant and nonpregnant women in the study population.

Characteristics	Cumulative clinical pregnancy n = 19	Not pregnant n = 30	P
Age (years)	35.1 ± 2.5	34.2 ± 3.7	0.87
BMI (kg/m^2)	21.0 ± 1.8	21.3 ± 1.9	0.63
Smokers	1 (5%)	2 (7%)	1.00
Duration of infertility (years)	2 [2–3]	2 [2–4]	0.71
Previous pregnancy	1 (5%)	8 (27%)	0.13
Previous delivery	0 (0%)	4 (13%)	0.15
AMH (ng/ml)	3.0 [1.9–5.9]	2.0 [1.2–3.8]	0.11
FSH (mIU/ml)	6.4 [5.0–8.3]	7.9 [6.2–8.9]	0.13
Antral Follicle Count	13 [9–19]	13 [7–18]	0.50
Previous IVF cycle	2 (11%)	6 (20%)	0.46
Vitamin D at baseline (ng/ml)	23.6 [19.9–25.2]	23.3 [17.9–26.5]	0.92
Indication for IVF			0.49
Idiopathic	8 (42%)	8 (27%)	
Male factor	6 (32%)	10 (33%)	
Endometriosis	1 (5%)	5 (17%)	
Tubal factor	2 (11%)	1 (3%)	
Genetic	1 (5%)	5 (17%)	
Male and female factor	1 (5%)	1 (3%)	
Vitamin D at oocyte retrieval (ng/ml)	29.1 [23.6–48.2]	28.2 [20.3–52.3]	0.63
Oocyte retrieved	11 [8–17]	7 [4–10]	0.004
Metaphase II Oocytes	8 [6–12]	5 [3–8]	0.007

Data are presented as number (%) or median [interquartile range]. Concentrations expressed as picogram of mediator per milliliter. Data were compared using Student's *t*-test, Mann–Whitney test or Chi-square test.
AMH, anti-Müllerian hormone.

quantification of uterine fluid mediators between supplemented patients and placebo group after protein normalization is presented in Table III. The results did not change after normalization to total protein, in that no significant differences between groups were detected.

The correlation between uterine cytokine levels and serum levels of Vitamin D for mediators detected in more than 50% of the samples is shown in Supplementary Fig. S1.

Uterine cytokine profile and clinical outcome

As a further analysis conducted to investigate the association between uterine environment and establishment of a clinical pregnancy, cytokine/chemokine profiles in women who conceived were evaluated. The analysis revealed a significantly higher number of retrieved oocytes and metaphase II oocytes in women who conceived compared to nonpregnant women ($P = 0.004$ and $P = 0.007$, respectively; Table IV).

The comparison of mediators in uterine fluid between pregnant and nonpregnant women is represented in Table V. No significant differences in number of samples from pregnant and nonpregnant women with detectable mediator were found, except for PDGF-BB, which was detected in a greater number of nonpregnant samples ($P = 0.03$). Mediator concentrations in uterine fluid from pregnant and nonpregnant women were also calculated after normalizing to total protein content (Table VI). The detection of PDGF-BB was not different in women who conceived compared with the nonpregnant group ($P = 0.06$) and also considering the Bonferroni correction.

Discussion

A recent systematic review reported a negative correlation between Vitamin D deficiency/insufficiency and reproductive outcomes in women undergoing ART cycles (Cozzolino *et al.*, 2020). In the absence of evidence supporting a causative relation between Vitamin D and ART outcomes, our group launched a randomized double-blinded clinical trial to investigate whether supplementation with 600 000 IU 25-hydroxyvitamin D could improve pregnancy rates in infertile patients with a deficiency or insufficiency of Vitamin D. Clinical results of the trial have been recently published, demonstrating no beneficial effect of Vitamin D supplementation on ART outcomes (Somigliana *et al.*, 2021). On the other hand, we cannot exclude some local effects in the various tissues that are not clinically detectable under our supplementation conditions. In the present study, we performed a subgroup analysis to determine whether Vitamin D supplementation could modulate locally the endometrial microenvironment, focusing on the characterization of a cytokine panel of 27 molecules. In line with the findings shown in the randomized trial (Somigliana *et al.*, 2021), we did not detect significant differences in the endometrial cytokine/

Table V Detection and concentrations of soluble mediators in human endometrial secretion aspirations in pregnant and nonpregnant women.

Mediators	Cumulative clinical pregnancy n = 19	Not pregnant n = 30	P
IL-2	5 (26%)	7 (23%)	1.00
IL-4	8 (42%)	16 (53%)	0.56
IL-6	9 (47%)	15 (50%)	1.00
IL-7	1 (5%)	0 (0%)	0.39
IL-9	2 (11%)	5 (17%)	0.69
IL-10	0 (0%)	1 (3%)	1.00
IL-15	3 (16%)	6 (20%)	1.00
IL-17	0 (0%)	1 (3%)	1.00
EOTAXIN	7 (37%)	14 (47%)	0.56
FGF	7 (37%)	12 (40%)	1.00
GM-CSF	3 (16%)	8 (27%)	0.49
PDGF-BB	1 (5%)	10 (33%)	0.03
RANTES	8 (42%)	13 (43%)	1.00
TNF-α	7 (37%)	14 (47%)	0.56
IL-1β (pg/ml)	2.91 [0.39–19.89]	4.76 [0.39–38.38]	0.59
IL-1RA (pg/ml)	2187.88 [593.15–4256.54]	3667.17 [1061.80–7931.62]	0.15
IL-8 (pg/ml)	717.08 [141.98–3177.16]	780.43 [114.23–4559.32]	0.97
G-CSF (pg/ml)	109.98 [2.33–446.98]	65.39 [12.31–976.83]	0.76
IFNγ (pg/ml)	1.18 [0.20–12.43]	0.64 [0.20–10.24]	0.57
IP-10 (pg/ml)	75.29 [5.64–372.08]	258.77 [5.64–787.14]	0.31
MCP-1 (pg/ml)	35.02 [7.45–155.19]	71.87 [22.67–240.13]	0.29
MIP-1-α (pg/ml)	0.05 [0.05–7.12]	1.42 [0.05–6.66]	0.26
MIP-1-β (pg/ml)	0.46 [0.46–20.78]	1.82 [0.46–49.34]	0.33
VEGF (pg/ml)	43.44 [10.38–668.64]	131.21 [10.38–426.96]	0.86

Concentrations expressed as picogram of mediator per milliliter. Data are presented as number of samples with detectable mediator (%) (*upper panel*) or median [interquartile range] (*lower panel*).
Three mediators (IL-5, IL-12 and IL-13) were not detectable in any of the samples. Data were compared using Chi-square test or Mann–Whitney test.
IL-1RA, IL-1 receptor antagonist; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFNγ, interferon gamma; IP-10, interferon-inducible protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1-α, macrophage inflammatory protein 1-alpha; MIP-1-β, macrophage inflammatory protein 1-beta; PDGF-BB, platelet-derived growth factor-BB; RANTES, Regulated on Activation, Normal T Cell Expressed and Secreted; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

chemokine profile between patients who did or did not receive a single high oral dose of 25-hydroxyvitamin D.

Vitamin D receptor expression in endometrial cells and in some immune cells could suggest a role for Vitamin D as a regulator of endometrial physiology (Viganò *et al.*, 2006) but the exact molecular mechanisms involved are still to be defined (Cermisoni *et al.*, 2018). Some *in vitro* assays have evaluated the effect of 1,25-dihydroxyvitamin D_3 on endometrial cells derived from both fertile women and patients with repeated implantation failure or unexplained recurrent spontaneous abortion (Tavakoli *et al.*, 2011; Rajaei *et al.*, 2012; Ghanavatinejad *et al.*, 2021). Interestingly, results derived from the various studies are very controversial. In fertile women, endometrial IL-8 secretion *in vitro* was increased upon Vitamin D treatment in the study by Rajaei *et al.*(2012) but it was similar or even decreased, considering only stromal cells, in the study by Tavakoli *et al.* (2011). This *in vitro* effect was not maintained when cells from women with repeated implantation failure or unexplained spontaneous abortion were treated. Similarly, Vitamin D treatment *in vitro* decreased endometrial IL-6 production in endometrial cells from fertile women in the studies by Rajaei *et al.* (2012) and Tavakoli *et al.* (2011) but not in the study by Ghanavatinejad *et al.* (2021). In patients with unexplained spontaneous abortion or with repeated implantation failure, the production of IL-6 by endometrial cells *in vitro* was reduced by Vitamin D treatment but the extent of the reduction was different in the two subgroups of patients. A Japanese group detected decreased levels of IFNγ, but not of IL-4, in conditioned media of decidualized human endometrial stromal cells from infertile patients following treatment with 1,25-dihydroxyvitamin D_3 for 4 days when compared with untreated cells (Ikemoto *et al.*, 2018). Moreover, they showed that Vitamin D supplementation would regulate T-helper (Th) cell populations of these patients through an inhibition of Th1 cell proliferation and promotion of Th2 cells. In particular, when focusing on patients who reached sufficient Vitamin D levels (≥30 ng/ml) after oral supplementation, a decrease in serum Th1 cell level and Th1/Th2 cell ratio was recognized (Ikemoto *et al.*,

Table VI Detection and relative concentrations of soluble mediators in human endometrial secretion aspirations in pregnant and nonpregnant women after normalization to total protein.

Mediators	Cumulative clinical pregnancy n = 16	Not pregnant n = 27	*P*
IL-2	4 (25%)	7 (26%)	1.00
IL-7	1 (6%)	0 (0%)	0.37
IL-9	2 (13%)	5 (19%)	0.70
IL-10	0 (0%)	1 (4%)	1.00
IL-15	2 (13%)	5 (19%)	0.70
IL-17	0 (0%)	1 (4%)	1.00
EOTAXIN	7 (44%)	13 (48%)	1.00
FGF	7 (44%)	12 (44%)	1.00
GM-CSF	3 (19%)	8 (30%)	0.49
PDGF-BB	1 (6%)	9 (33%)	0.06
RANTES	7 (44%)	13 (48%)	1.00
TNF-α	7 (44%)	13 (48%)	1.00
IL-1β (pg/mg)	8.39 [1.04–56.74]	8.04 [2.06–51.21]	0.56
IL-1RA (pg/mg)	4035.98 [944.16–8159.30]	3960.30 [2024.68–29 099.56]	0.48
IL-4 (pg/mg)	0.31 [0.18–2.09]	0.69 [0.18–4.14]	0.40
IL-6 (pg/mg)	4.10 [0.57–48.60]	1.57 [0.40–92.29]	0.94
IL-8 (pg/mg)	2163.09 [387.82–3377.93]	1223.69 [570.12–4687.83]	0.90
G-CSF (pg/mg)	192.16 [9.81–1009.95]	152.30 [28.62–795.94]	0.71
IFNγ (pg/mg)	2.64 [0.59–19.50]	4.66 [0.33–23.03]	0.85
IP-10 (pg/mg)	148.90 [10.38–2648.02]	480.84 [30.22–1763.56]	0.37
MCP-1 (pg/mg)	54.45 [18.53–425.69]	153.90 [53.68–503.48]	0.16
MIP-1-α (pg/mg)	0.81 [0.07–12.11]	3.58 [0.35–12.37]	0.26
MIP-1-β (pg/mg)	1.42 [0.82–64.07]	12.48 [1.19–70.27]	0.29
VEGF (pg/mg)	84.53 [12.56–1159.15]	209.85 [18.89–560.33]	0.60

Data are presented as number of samples with detectable mediator (%) (*upper panel*) or median [interquartile range] (*lower panel*). *P*-values were calculated with the Mann–Whitney test. In the lower panel, units are reported as pg/ml, normalized on mg/ml total protein (pg/mg).

Data were compared using Chi-square test or Mann–Whitney test. Three mediators (IL-5, IL-12 and IL-13) were not detectable in any samples. In six samples, normalization was not possible owing to the limited protein concentration for the analysis.

IL-1RA, IL-1 Receptor Antagonist; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFNγ, interferon gamma; IP-10, interferon-inducible protein 10; MCP1, monocyte chemoattractant protein 1; MIP-1-α, macrophage inflammatory protein 1-alpha; MIP-1-β, macrophage inflammatory protein 1-beta; PDGF-BB, platelet-derived growth factor-BB; RANTES, Regulated on Activation, Normal T Cell Expressed and Secreted; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

2018). Since balancing of Th cells is known to be critical to maternal immune tolerance, an optimal Vitamin D level may have a significant role in the regulation of immunologic embryo receptivity. A recent publication evaluated some functions of peripheral and endometrial immune cells in women with repeated implantation failure before and after a Vitamin D supplementation (at a dose of 0.5 µg per day for 2 months). Vitamin D insufficiency or deficiency caused a marked increase in peripheral natural killer cell cytotoxicity and in the percentage of endometrial CD68+ macrophages. The supplementation of vitamin D could suppress the natural killer cell cytotoxicity as well as reduce endometrial CD68+ macrophages (Chen *et al.*, 2020). Overall, although our results showed no significant differences in endometrial cytokine/chemokine patterns between Vitamin D-supplemented women undergoing ART compared with the placebo group, we cannot exclude that Vitamin D might be of particular benefit in specific groups of patients such as those with an unbalanced Th1/Th2 ratio.

Another major aspect of the present study was the further comparison of levels of immune mediators in uterine fluid from the secretory phase of the menstrual cycle prior to oocyte retrieval between women that achieved a clinical pregnancy or not. Recently, endometrial immune transcriptional profiling has been proposed as an innovative tool to test the local endometrial functioning (Lédée et al., 2020), based on the idea that alterations in cytokine pattern at the feto–maternal interface could impact on the establishment and maintenance of pregnancy (Franasiak and Scott, 2017; Taima *et al.*, 2020). Boomsma *et al.* (2009) demonstrated that multiplex immunoassay of aspirated uterine secretions offers a noninvasive method to characterize the endometrial *in vivo* milieu, providing the simultaneous detection of different mediators in a small volume. Our data have revealed no significant differences in mediator content in uterine fluid samples from women who conceived versus the nonpregnant group, thus not allowing us to identify an immunological profile of a receptive endometrium. It is worth

mentioning that uterine secretions were collected during the menstrual cycle which preceded the oocyte retrieval. Therefore, we cannot exclude that the uterine fluid collection and analysis in the same cycle of the embryo transfer might have resulted in different conclusions. However, robust evidence from the literature suggests that most data can generally be applied between subsequent cycles, owing to a low variability among cycles (Evans *et al.*, 2018). We have to recognize that this further analysis was unrelated to the main study question and the results should be, however, interpreted with caution, given the small sample size.

It is possible that a number of limitations may have influenced the results obtained. Firstly, we cannot be sure that oral Vitamin D supplementation might be adequately provided at endometrial level and the broad range of timing of the single dose of Vitamin D supplementation might have influenced this. Although we failed to observe any effects in the endometrial tissue, previously we observed that significantly higher levels of Vitamin D in follicular fluid were associated with different expression of 44 genes in the granulosa cells of supplemented patients compared with the placebo group (Makieva *et al.*, 2021). Secondly, we cannot exclude that Vitamin D acts on the uterine environment without an impact as cytokine and chemokine pattern modulator. Thirdly, the sample size could be a limitation. The reason for the unequal sample size between the groups has to be attributed to the researchers' blindness of the treatment received by the patients (as subgroups of a randomized double-blinded clinical trial). In this context, a blinded approach should be considered a strength rather than a limitation of the study. Conversely, a type II error might result from the small sample size. However, we calculated the sample size based on previous assumptions for two cytokines from two previous studies (Tavakoli *et al.*, 2011; Rajaei *et al.*, 2012). This allowed us to calculate the greatest difference that we can foresee from our data, but smaller differences cannot be excluded.

Conclusion

In conclusion, this *in vivo* study has explored the impact of preconception oral Vitamin D supplementation on the uterine milieu in infertile women, in particular on chemokine/cytokine levels in uterine fluid. We found no significant differences in the endometrial secretome profile between women undergoing ART cycles who did or did not receive a single high dose of oral Vitamin D. Nonetheless, more studies are needed to investigate whether other doses and durations (modalities) of supplementation may result in different outcomes.

Supplementary data

Supplementary data are available at *Human Reproduction Open* online.

Data availability

The datasets analyzed during the current study are available from the corresponding author.

Authors' roles

Study design: G.C.C. and M.R.; Data and sample collection: G.C.C., V.S., and S.S.; Experimental Assay: M.-P.P., L.L. and F.L.; Data analysis and interpretations: G.C.C., E.G., M.R., A.C. and P.V.; Drafting the manuscript: G.C.C. and P.V. All the above authors revised and approved the manuscript and take responsibility for the integrity of the data.

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Conflict of interest

The authors declare that they have no conflict of interest.

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