Oral Vitamin D Supplementation does not Impact Cytokine Levels in Uterine Fluid of Women Undergoing Assisted Reproduction Technology (ART)

Greta Chiara Cermisoni
IRCCS Ospedale San Raffaele
Marco Reschini
IRCCS Foundation Maggiore Policlinico Hospital: Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico
Marie-Pierre Piccinni
Università degli Studi di Firenze
Letizia Lombardelli
Universita degli Studi di Firenze
Federica Logiodice
Universita degli Studi di Firenze
Veronica Sarais
IRCCS Ospedale San Raffaele
Elisa Giacomini
IRCCS: IRCCS Ospedale San Raffaele
Simona Signorelli
IRCCS: IRCCS Ospedale San Raffaele
Edgardo Somigliana
IRCCS Foundation Maggiore Policlinico Hospital: Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico
Paola Viganò (vigano.paola@hsr.it)
Ospedale San Raffaele https://orcid.org/0000-0003-3674-5912

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Abstract

Background: Cytokines and chemokines, secreted in the intrauterine environment, are fundamental for the molecular crosstalk between endometrium and preimplantation embryo. Whether Vitamin D can have a beneficial effect on endometrial district and immune cells is still unclear. To fill this gap, the aim of this study was to explore if Vitamin D supplementation can act on intrauterine milieu as immuno-modulator.

Methods: We present a report of a secondary outcome from the SUNDRO clinical trial, a multi-center randomized double-blinded trial aimed to explore the effects of Vitamin D replacement (a single dose of 600,000 IU of 25-hydroxy Vitamin D or placebo) in insufficient women undergoing autologous ART cycles. Inclusion criteria were female age between 18-39 years, with body mass index between 18 and 25 Kg/m^2. Uterine fluid samples were collected during the secretory phase of the menstrual cycle that proceeded the oocyte retrieval. The quantitative determination of twenty-seven cytokines in endometrial secretion samples was performed by using a multiplex immunoassay.

Results: Forty-nine uterine fluid samples (UF) were collected during the secretory phase of the menstrual cycle prior to oocyte retrieval. Our data revealed no differences in UF composition of Vitamin D supplemented women (*n*=17) compared with the placebo group (*n*=32), also when protein normalization was performed. In addition, no significant differences were found in mediators relative content of UFs from women who conceived (*n*=19) compared with the non-pregnant group (*n*=30).

Conclusions: The explored *in vivo* vitamin D replacement regimen is unlikely to directly influence the cytokine and chemokine endometrial milieu.

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Background

In human 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 non-genomic action with short-term effects [1].

Recent data suggest a possible association between Vitamin D serum level and many pathophysiological processes of the female reproductive system including fertility [2–4]. Two recent meta-analyses showed that Vitamin D deficiency or insufficiency could be detrimental for the success of Assisted Reproductive Technology (ART) treatments [5, 6]. In addition, the first double-blind randomized controlled trial on 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 [7]. Additional literature reported a beneficial clinical action of Vitamin D on the endometrial tissue and during the implantation window [8–10].

Molecular Vitamin D action on human endometrial cells remains under-researched [11]. During a successful implantation, synchronous modifications and a bidirectional crosstalk occurs between a receptive endometrium and a competent embryo [12, 13]. The molecular crosstalk is mediated by proteins, cytokines and chemokines, secreted in 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 pro-inflammatory and anti-inflammatory mediators by endometrial cells from women with recurrent implantation failure compared with fertile women [14]. These evidence support a promising noninvasive approach to characterize the endometrial milieu and to profile the immunologic mediators that could be crucial for a successful implantation [15, 16].

In this context, studying the possible modulating action of Vitamin D on the maternal-fetal interface could be of utmost importance [17–19].

In 2017, our group started a multi-center randomized double-blinded clinical trial to test the effects of the supplementation with 25-hydroxyvitamin D, a precursor for the synthesis of the active form 1,25-dihydroxyvitamin D_3, in deficient women undergoing ART cycles (SUNDRO trial) [20]. We herein present a report of a secondary outcome from the SUNDRO clinical trial, the effect of Vitamin D replacement on the molecular pattern of uterine secretome. The analysis of the uterine secretome benefits of the non-invasive approach for studying the endometrial milieu and provides a shapshot of the environment that preimplantation embryo will meet.

Methods

Study Design And Subjects

The study has been designed according to the CONSORT methodology [20]. The randomized superiority 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 study has been conducted in accordance to the ethical principles of the Helsinki Declaration guidelines. The study 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).

Selected population were infertile patients undergoing ART cycles with insufficient serum levels of Vitamin D (25-hydroxyvitamin D serum level < 30 ng/ml) according to the most recent International Guidelines [21]. Inclusion criteria were female age between 18–39 years, with body mass index (BMI)

between 18 and 25 Kg/m^2 undergoing autologous ART cycles with less than three previous cycles. Exclusion criteria included contraindications/side effects for consumption of Vitamin D, anti-mullerian hormone serum level < 0.5 ng/ml and ART cycles with surgical retrieval of the spermatozoa or frozen gametes.

Written informed consents were obtained from eligible patients. The first 50 eligible women that agree to participate to both the randomized double-blinded clinical and the uterine fluid collection were enrolled. In one patient, the uterine fluid collection did not result in an adequate sampling. Researchers were unaware of 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 allocation sequence were computer-generated and 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 [22–24]. Serum 25-hydroxyvitamin D was assessed at the time of hCG administration. All Vitamin D levels measurements was performed with a commercially available kit (DiaSorin). Clinical pregnancy was defined after the ultrasound presence of at least one intrauterine gestational sac with viable fetus.

Endometrial Secretion Aspiration

Uterine fluid samples were collected during the secretory phase of the menstrual cycle that proceeded 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 get representative sampling of uterine secretion, 1.5 mL of physiologic solution was injected and gently suctioned. Samples were immediately centrifuged at 1200xr.p.m. for 15 min in order to separate cell debris, mucus and minimal blood contamination from 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) for normalization purposes.

Determination of endometrial Secretion cytokine concentrations with bead-based multiplex immunoassays

After thawing, the quantitative determination of IL-1beta, IL-1Ralpha, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, IFN-alpha, TNF-alpha, G-CSF, GM-CSF, VEGF, PDGF, FGF, IP-10, MCP-1, RANTES, eotaxin, MIP-1-alpha, and MIP-1-beta in endometrial secretion samples was performed by using a bead-based multiplex immunoassay (Biorad Laboratories, Hercules, CA, USA) and the Bioplex 200 system

(Biorad Laboratories, Hercules, CA, USA), as we have previously described elsewhere [24]. In brief, in a 96-well filter plate (Bio-Rad) 50µl of each serum sample were added to 50 µl of antibody-conjugated beads directed against the cytokines listed above (Bio-Rad). After a 30-min incubation, the plate was washed and 25 µl of biotinylated anti-cytokine antibody solution were added to each well before another 30-min incubation. The plate was then washed and 50 µl of streptavidin-conjugated phycoerithrin were added to each well. After a final wash, each well was resuspended with 125 µl of assay buffer (Bio-Rad) and analyzed by Bioplex 200 (Biorad Laboratories, Hercules, CA). Standard curves were derived from various concentrations of the cytokine standards and followed the same protocol as the endometrial secretion samples. The concentration of the 27 cytokines (pg/ml) in each endometrial secretion sample was calculated thanks to the Bioplex 200 software.

The lower detection limits of the multiplex immunoassay kit were as follows: Interleukin (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), Fibroblast Growth Factor (FGF) (2.62 pg/ml), Granulocyte-colony stimulating factor (G-CSF) (2.33 pg/ml), Granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.43 pg/ml), Interferon (IFN)-γ (0.2 pg/ml), IFN-γ-inducible 10 kDa protein (IP-10) (5.64 pg/ml), Monocyte chemo-attractant protein-1 (MCP-1) (0.45 pg/ml), Macrophage Inflammatory Protein(MIP)-1α/CCL3 (0.05 pg/ml), MIP-1β/CCL4 (0.46 pg/ml), Platelet-Derived Growth Factor-BB (PDGF-BB) (4.56 pg/ml), (4.56 pg/ml), Chemokine ligand 5 also known as regulated on activation, normal T cell expressed and secreted (RANTES) (3.76 pg/ml), Tumor Necrosis Factor-α (TNF-α) (3.28 pg/ml), Vascular Endothelial Growth Factor (VEGF) (10.38 pg/ml). Relative concentration of uterine mediators was performed based on total protein concentration of each sample measured by Bradford assay (Quick Start™ Bradford, Bio-Rad).

Statistical Analysis

All data were initially examined for normality using the Kolmogorov Smirnov test: the normal distributed data was analyzed with the Student's *t* test, while the not normal data was analyzed with the Mann-Whitney test. The frequency of patients' characteristics was analyzed with the chi-square test. Data are presented as Number (%), Mean ± SD, Median [IQR] as described in each legend. Significance was set at $p < 0.05$. Soluble mediators in endometrial secretion aspiration 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 tested 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.

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 placebo group. There were no significant differences between the studied groups regarding basal and clinical characteristics (Table 1). As expected, both groups had deficient serum levels of Vitamin D at the time of randomization ((median 23.4 (range 19.5-28.4) and 23.4 (17.8-25.9), respectively); at the time of hCG administration, serum level of Vitamin D supplemented subjects significantly raised compared with the placebo group ((median 52.9 ng/ml (range 40.7 - 64.1) and 24.6 (19.3 - 29.2), respectively, p<0.001).

Levels of 27 cytokines were investigated in uterine fluids of both Vitamin D supplemented and placebo groups. No sample was excluded because of inadequate sampling. 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 2. Three mediators (IL-5, IL-12 and IL-13) were not detectable in any of the samples. The concentration 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 was detected (Table 2, *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 2, *lower panel*). No 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 by total protein concentration. The comparison of the relative quantification of uterine fluid mediators between supplemented patients and placebo group after protein normalization is represented in Table 3. The results remained similarly not significantly different between samples.

Uterine Cytokine Profile and Clinical Outcome

In order to investigate the association between uterine environment and establishment of a clinical pregnancy, an analysis of cytokine profiles in women who conceived was performed. The analysis revealed a significantly different number of retrieved oocytes and MII oocytes in women who conceived compared to non-pregnant women (p=0.004 and p=0.007, respectively) (Table 4).

The comparison of the mediators in uterine fluid between pregnant and not-pregnant women are represented in Table 5. No significant differences in total protein content in samples from pregnant and not pregnant women were found, except for PDGF-BB which was found in a greater number of non-pregnant samples (p=0.03). Soluble mediator concentrations were expressed as relative quantification to total protein content in Table 6. The concentration of PDGF-BB was not different in women who conceived compared with the not-pregnant group (p=0.06).

Discussion

A recent systematic review reported a negative correlation between Vitamin D deficiency/insufficiency and reproductive outcomes achieved in women undergoing ART cycles [26]. In the absence of evidence

supporting a causative relationship between Vitamin D and ART outcomes, our group launched a randomized double-blinded clinical trial to investigate whether supplementation with 600.000 UI 25-hydroxvitamin D could improve pregnancy rates in deficient and insufficient infertile patients. In anticipation of the trial results, 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. This study showed, with a randomized *in vivo* study design, that a single high dose of 25-hydroxyvitamin D does not markedly impact on the cytokine/chemokine profile in the endometrial environment.

The VDR expression in endometrial cells and in some immune cells could suggest a role for vitamin D as regulator of endometrial physiology [27] but the exact molecular mechanisms involved are still to be defined [11]. Some *in vitro* assays have demonstrated an immunomodulatory effect of 1,25-dihydroxyvitamin D_3 on endometrial cells derived from women with repeated implantation failure or unexplained recurrent spontaneous abortion compared to untreated samples [28, 29]. Similarly, a Japanese group detected decreased levels of IFN-γ, 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 ones [30]. A recent paper reported similar local immunomodulatory action in deficient women with repeated implantation failure following Vitamin D supplementation (at a dose of 0.5 μg per day for 2 months). NK cell cytotoxicity, Th cell and CD68 + macrophage populations were significantly reduced in endometrial samples collected during the mid-luteal phase of supplemented patients suggesting a local beneficial effect of Vitamin D treatment in a subgroup of infertile women [31]. However, these data are not supported by the analysis of the uterine secretome profiling *in vivo*. Our results failed to show any difference in endometrial cytokine/chemokine pattern between *in vivo* supplemented women compared with the placebo group.

It is plausible that a number of limitations may have influenced the results obtained. Firstly, Vitamin D might not be ensured adequately in these districts. Although we failed to observe any effect in the endometrial district, we observed significantly higher levels of Vitamin D in follicular fluid associated with a differently expression of 44 genes in granulosa cells of supplemented patients compared with the placebo group [32]. Secondly, we cannot exclude that Vitamin D acts on the uterine environment without impact as cytokine and chemokine pattern modulator. Thirdly, the small sample size could limit the power of the study. Finally, this study compared unequal sample size data sets. However, this issue is due to the researchers' unawareness of the treatment that patients received (as subgroups of a randomized double-blinded clinical trial). In this context, it should be considered a strength rather than a limit of the study.

Another major finding of the present study was the comparison of levels of immune mediators detected 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, the endometrial immune transcriptional profiling has been proposed as an innovative tool to test the local endometrial functioning [33] based on the idea that alterations in cytokine pattern at the feto-maternal interface could impact on the establishment and maintenance of pregnancy [34, 35]. Boomsa and colleagues demonstrated that the multiplex

immunoassays of aspirated uterine secretions offer a non-invasive method to characterize the endometrial *in vivo* milieu, providing the simultaneously detection of different mediators in a small volume [15]. Our data have revealed no significant differences in mediators relative content in uterine fluid samples from women who conceived compared with the non-pregnant group, thus not allowing to identify an immunological profile of a receptive endometrium. It is worth mentioning that uterine secretions were collected during 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 literature suggests that data and considerations can be generally applied between subsequent cycles, due to a low variability over cycles [36]. Finally, given the small sample size, caution should be applied in interpreting the data.

Conclusion

In conclusion, this study has explored the immunological impact of an *in vivo* preconception Vitamin D supplementation on the uterine milieu in infertile women. We documented no local modulation of endometrial secretome profile following a single high dose of oral Vitamin D supplementation in insufficient women undergoing ART cycles. Nonetheless, more studies are needed to investigate whether other doses and durations (modalities) of supplementation may result in different outcomes.

Abbreviations

ART: Assisted Reproduction Technology; IVF: in vitro fertilization; SUNDRO: Supplementation of Vitamin D and reproductive outcome; UF: uterine fluid samples.

Declarations

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Authors’ contributions

Study design: GCC and MR; Data collection: GCC, VS, EG and SS; Experimental Assay: MPP, LL and FL; Data analysis and interpretations: GCC, EG, MR and PV and ES; Drafting the manuscript: GCC and PV. All the above authors revised and approved the manuscript and take responsibility for the integrity of the data.

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Availability of data and materials

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

Ethics approval and consent to participate

The study will be conducted in accordance to the ethical principles of the Helsinki Declaration guidelines. The study 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). Patients that are enrolled into the study are covered by indemnity for

negligent/ non-negligent harm through a specific insurance to cover for harm associated with the protocol. Written informed consents were obtained from eligible patients.

Consent for publication

All participating women will sign an institutional authorization regarding the use of their clinical data for scientific publications.

Competing interests

The authors declare that they have no conflict of interest.

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Tables

Table 1. Basal and clinical characteristics of women in Vitamin D Supplementation and Placebo groups. Data is presented as Mean ± Standard Deviation, Median (Interquartile Range-IQR) or Number (%).

Characteristics	Vitamin D	Placebo	p
	n=17	*n*=32	
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	1 (6%)	2 (6%)	1.00
Duration of infertility (years)	2 [2 - 3.5]	2 [1 - 4]	0.72
Previous pregnancy	3 (17%)	6 (19%)	1.00
Previous delivery	2 (12%)	2 (6%)	0.60
AMH (ng/ml)	2.7 [1.2 - 7.6]	2.4 [1.3 - 5.0]	0.51
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	3 (18%)	5 (16%)	1.00
Vitamin D at baseline (ng/ml)	23.4 [19.5 - 28.4]	23.4 [17.8 - 25.9]	0.57
Indication for IVF			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%)	
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
MII Oocytes	8 [4 - 10]	7 [4 - 9]	0.91
Cumulative Ongoing Pregnancy rate	7 (41%)	12 (38%)	1.00
Cumulative Live birth rate	5 (29%)	11 (34%)	1.00

Table 2: Concentrations of soluble mediators in endometrial secretion aspirations in Vitamin D supplemented Women and Placebo groups. Data is presented as Number (%) or Median [Interquartile

Range-IQR].

Mediators	Vitamin D	Placebo	p
	n=17	*n*=32	
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 [0.00 - 29.00]	4.77 [0.20 - 33.94]	0.54
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 [0 - 784.46]	77.93 [16.14 - 843.48]	0.58
IFNγ (pg/ml)	0.58 [0.00 - 5.81]	3.91 [0.00 - 22.06]	0.28
IP-10 (pg/ml)	226.01 [0.00 - 1067.00]	117.50 [0.00 - 653.25]	0.98
MCP-1 (pg/ml)	30.00 [7.00 - 245.5]	63.50 [19.75 - 237.50]	0.52
MIP-1-α (pg/ml)	0.00 [0.00 - 5.00]	1.00 [0.00 - 7.75]	0.31
MIP-1-β (pg/ml)	0.00 [0.00 - 27.00]	0.50 [0.00 - 49.75]	0.87
VEGF (pg/ml)	134.00 [13.00 - 466.34]	128.00 [0.00 - 499.75]	0.65

Table 3: Relative Concentrations of soluble mediators in endometrial secretion aspirations in Vitamin D supplemented women and Placebo groups after protein normalization. Data is presented as Number (%) or Median [Interquartile Range-IQR].

Mediators	Vitamin D	Placebo	p
	n=15	*n*=28	
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
TNFα	5 (33%)	15 (54%)	0.34
IL-1β (pg/mg)	4.77 [0.00 - 42.46]	8.06 [0.43 - 47.90]	0.40
IL-1RA (pg/mg)	3960.3 [2080.49 - 27372.55]	3925.71 [997.24 - 8159.30]	0.59
IL-4 (pg/mg)	0.00 [0.00 - 1.98]	0.52 [0.00 - 3.15]	0.34
IL-6 (pg/mg)	0.00 [0.00 - 29.01]	2.06 [0.00 - 91.21]	0.34
IL-8 (pg/mg)	1061.22 [319.62 - 2629.86]	2119.28 [593.29 - 8867.66]	0.20
GCSF (pg/mg)	152.3 [0.00 - 359.50]	186.63 [25.57 - 750.00]	0.59
IFNγ (pg/mg)	0.97 [0.00 - 7.48]	5.69 [0.00 - 22.23]	0.22
IP-10 (pg/mg)	205.98 [0.00 - 2478.03]	212.44 [6.36 - 907.33]	0.81
MCP1 (pg/mg)	49.62 [12.22 - 343.37]	143.13 [52.53 - 509.11]	0.18
MIP-1-α (pg/mg)	0.03 [0.00 - 6.48]	2.32 [0.00 - 11.59]	0.39
MIP-1-β (pg/mg)	0.00 [0.00 - 54.47]	2.3 [0.00 - 66.15]	0.54
VEGF (pg/mg)	493.68 [71.28 - 3702.16]	40.81 [0.00 - 448.42]	0.07

Table 4: Baseline characteristics of pregnant and non-pregnant women. Data is presented as Number (%) or Median [Interquartile Range-IQR]. Concentrations expressed as picogram of mediator per milliliter. P-values calculated with the Mann-Whitney test.

Characteristics	Cumulative Clinical Pregnacy	Not pregnant	p
	n=19	*n*=30	
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%)	
Tube 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
MII Oocytes	8 [6 - 12]	5 [3 - 8]	0.007

Table 5: Concentrations of soluble mediators in endometrial secretion aspirations in pregnant and non-pregnant women. Concentrations expressed as picogram of mediator per milliliter. Data is presented as Number (%) or Median [Interquartile Range-IQR].P-values calculated with the Mann-Whitney test.

Mediators	Cumulative Clinical Pregnancy	Not Pregnant	p
	n=19	*n*=30	
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
TNFα	7 (37%)	14 (47%)	0.56
IL-1B (pg/ml)	2.91 [0.12 - 19.89]	4.77 [0.07 - 38.38]	0.62
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 [0.00 - 446.98]	65.39 [12.31 - 976.83]	0.67
IFNγ (pg/ml)	1.18 [0.00 - 12.43]	0.64 [0.00 - 10.24]	0.63
IP-10 (pg/ml)	75.00 [0.00 - 372.00]	259.00 [0.00 - 787.25]	0.31
MCP-1 (pg/ml)	35.00 [7.00 - 155.00]	71.50 [22.75 - 240.50]	0.30
MIP-1-α (pg/ml)	0.00 [0.00 - 7.00]	1.00 [0.00 - 7.00]	0.27
MIP-1-β (pg/ml)	0.00 [0.00 - 21.00]	1.50 [0.00 - 49.25]	0.32
VEGF (pg/ml)	43.00 [0.00 - 669.00]	131.50 [0.00 - 427.00]	0.86

Table 6: Relative Concentrations of soluble mediators in endometrial secretion aspirations in pregnant and non-pregnant women after protein normalization. Data is presented as Number (%) or Median [Interquartile Range-IQR]. P-values calculated with the Mann-Whitney test.

Mediators	Cumulative Clinical Pregnancy	Not pregnant	p
	n=16	*n*=27	
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
TNFα	7 (44%)	13 (48%)	1.00
IL-1β (pg/mg)	8.39 [0.38 - 56.74]	6.90 [0.15 - 42.46]	0.84
IL-1RA (pg/mg)	4035.98 [944.16 - 8159.30]	3960.30 [2024.68 - 29099.56]	0.48
IL-4 (pg/mg)	0.00 [0.00 - 2.09]	0.42 [0.00 - 3.26]	0.56
IL-6 (pg/mg)	4.10 [0.00 - 48.60]	0.00 [0.00 - 71.42]	0.77
IL-8 (pg/mg)	2163.09 [387.82 - 3377.93]	1223.69 [570.12 - 4687.83]	0.90
G-CSF (pg/mg)	192.16 [4.60 - 1009.95]	119.03 [24.85 - 612.21]	0.96
IFNγ (pg/mg)	2.63 [0.00 - 19.50]	3.65 [0.00 - 15.22]	0.96
IP-10 (pg/mg)	148.90 [0.00 - 2648.02]	279.31 [0.00 - 1209.91]	0.76
MCP-1 (pg/mg)	54.45 [18.53 - 425.69]	153.90 [48.90 - 503.48]	0.26
MIP-1-α (pg/mg)	0.73 [0.00 - 12.11]	2.66 [0.00 - 9.25]	0.44
MIP-1-β (pg/mg)	0.00 [0.00 - 64.07]	2.45 [0.00 - 54.47]	0.48
VEGF (pg/mg)	84.53 [0.00 - 1159.15]	209.85 [0.00 - 560.33]	0.68