Human Reproduction, Vol.36, No.1, pp. 130–144, 2021
Advance Access Publication on December 11, 2020 doi:10.1093/humrep/deaa262

human reproduction

ORIGINAL ARTICLE ***Reproductive biology***

Oral Vitamin D supplementation impacts gene expression in granulosa cells in women undergoing IVF

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Submitted on June 4, 2020; resubmitted on September 4, 2020; editorial decision on September 11, 2020

STUDY QUESTION: Does oral Vitamin D supplementation alter the hormonal milieu of follicular fluid (FF) and the transcriptomic profile of luteinised granulosa cells (GCs) in women with Vitamin D deficiency undergoing IVF?

SUMMARY ANSWER: A transcriptomic signature relevant to oral Vitamin D supplementation in luteinised GCs was demonstrated, although Vitamin D supplementation did not alter hormone levels in FF.

WHAT IS KNOWN ALREADY: Vitamin D deficiency is linked to lower live birth rates among women undergoing IVF. It is unclear whether Vitamin D elicits a targeted action in reproductive physiology or is a surrogate marker of overall well-being. Several *in-vitro* studies, but none *in vivo*, have examined the impact of Vitamin D on the periovulatory follicle, focusing on GCs as a proxy marker of oocyte competence.

STUDY DESIGN, SIZE, DURATION: We present a report of secondary outcomes from the SUNDRO clinical trial, which was launched in 2016 to determine whether Vitamin D supplementation can improve the IVF outcomes of women who are deficient in Vitamin D (<30 ng/ml). FF samples of 145 women who were randomised to receive Vitamin D or placebo from March 2017 to January 2019 were collected. All follicles that were aspirated in our study measured ≥11 mm on the day of hCG trigger. The first cohort of samples was collected from the dominant follicle of each participant and utilised for hormone profiling (n = 50 Vitamin D, n = 45 Placebo). For the second cohort, the follicle aspirates of each participant were pooled to create a single FF sample, which was used for the isolation of GCs for gene expression studies (n = 20 Vitamin D, n = 30 placebo). Six of the samples from the second cohort were used for RNA-sequencing analysis (n = 3 Vitamin D, n = 3 placebo).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Two academic infertility units were involved in the recruitment of the participants, who received a single dose of oral 25-hydroxyvitamin D (600 000 IU) or placebo, 2–12 weeks before oocyte retrieval. Women in both groups were deficient in Vitamin D, aged 18–39 years with a normal BMI (18–25 kg/m^2) and <3 previous IVF cycles. The FF was aspirated at the time of oocyte retrieval and stored. Liquid chromatography tandem mass spectrometry was used to measure FF abundance of 25-hydroxyvitamin D, aldosterone, androstenedione, cortisol, cortisone, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol, 21-deoxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, dihydrotestosterone, oestradiol (E2), 17-OH-hydroxyprogesterone, progesterone (P4) and testosterone. GCs were isolated from pooled FFs and the transcriptome was evaluated by RNA-sequencing and RT-PCR. Ingenuity pathway analysis (IPA) was used to assess the top canonical pathways and upstream regulators mediating the action of Vitamin D.

MAIN RESULTS AND THE ROLE OF CHANCE: At oocyte retrieval, FF concentration of 25-hydroxyvitamin D was 2.8-fold higher ($P < 0.001$) in the Vitamin D group (39.5 ng/ml; n = 50) compared to placebo (13.8 ng/ml; n = 45) but no other hormonal differences were detected. In the placebo group, but not the Vitamin D group, weak correlations of 25-hydroxyvitamin D concentration with P4 ($r = 0.31$, $P = 0.03$) and E2 ($r = 0.45$, $P = 0.002$) were observed. RNA-sequencing identified 44 differentially expressed genes in the

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GCs of patients who received Vitamin D (n = 3) compared to placebo (n = 3). RT-PCR demonstrated upregulation of VDR (vitamin D receptor), GSTA3 (glutathione S-transferase A3) and IL21R (interleukin 21 receptor), and downregulation of P T GS2 (prostaglandin-endoperoxide synthase 2), KLF4 (kruppel-like factor 4), T RP C4 (transient receptor potential cation channel subfamily C member 4), VEGF (vascular endothelial growth factor), RXRB (retinoid X receptor beta) and AGER (advanced glycosylation end-product specific receptor) genes in the Vitamin D (n = 17) versus placebo (n = 27) group. IPA suggested roles of Vitamin D in antioxidant defence.

LIMITATIONS, REASONS FOR CAUTION: Interpretation of the data is influenced by our intervention strategy (2–12 weeks prior to retrieval). As folliculogenesis may last 5–6 months, our protocol can only examine with confidence the impact of Vitamin D on the final stages of follicular growth. Furthermore, we examined the hormonal profile of the dominant follicle only, while the GC data reflect the transcriptome of all (pooled) follicles large enough to be used for IVF. Luteinised GCs from controlled ovarian stimulation were used in this study, which may be functionally distinct from the GCs of developing follicles. Moreover, the sample size for RNA-sequencing analysis was low (n = 3 per group), regardless of validation by RT-PCR that was performed on a larger cohort, introducing complexity to the IPA analysis, which required an input of data with *P*-adjusted <0.08 instead of <0.05 to be informative.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first *in-vivo* study to show that Vitamin D supplementation alters gene expression in luteinised GCs. In contrast to some *in-vitro* evidence, no effect of the intervention on expression of genes encoding steroidogenic enzymes was observed. Unlike other studies, our results suggest that supplementation with Vitamin D is unlikely to directly influence hormone availability in FF. Our findings instead reinforce the hypothesis that Vitamin D could be considered one of the gatekeepers in protecting against an exaggerated response to ovarian stimulation.

STUDY FUNDING/COMPETING INTEREST(S): The study has been funded by the Italian Ministry of Health (RF-2013-02358757) following peer review in the competitive 'Bando di Ricerca Finalizzata e Giovani Ricercatori 2013' for the clinical trial SUNDRO (EudraCT registration number 2015-004233-27). There are no competing interests.

TRIAL REGISTRATION NUMBER: EudraCT registration number 2015-004233-27.

Key words: granulosa cells / Vitamin D / clinical trial / follicular fluid / transcriptomics / RNA-sequencing / ovarian hyperstimulation syndrome / oxidative stress

Introduction

Vitamin D, or cholecalciferol, is synthesised in the skin in response to sunlight or is obtained from diet. Cholecalciferol travels to the liver where it converts into calcifediol (25-hydroxyvitamin D), which is subsequently converted to calcitriol (1,25-dihydroxyvitamin D) in the kidney. The association of Vitamin D with reproduction has been an intense area of research since the 1940s, when Obermer published his recommendation on Vitamin D supplementation during pregnancy in England (Obermer, 1947). Moreover, seasonal variations in conception rates have been long described, with summer and autumn being the most prolific seasons (Rojansky *et al.*, 1992). At the same time, Vitamin D deficiency has been linked to reduced fertility in ART cycles (Paffoni *et al.*, 2014, 2017; Vanni *et al.*, 2014; Pagliardini *et al.*, 2015) but not in natural cycles (Somigliana *et al.*, 2016).

The most recent meta-analysis available assessed the reproductive outcomes of 2700 subfertile women undergoing ART cycles and found favourable outcomes correlated with Vitamin D replete status (Chu *et al.*, 2018). In particular, among the 11 eligible studies, the clinical pregnancy and live birth rates were greater in calcifediol replete (>30 ng/ml) women when compared to women with a deficiency (<20 ng/ml) or insufficiency (20–30 ng/ml). It is noteworthy that the only comparison not favouring the replete group in their study was including cycles from oocyte donation. Hence, it is tempting to speculate that in homologous cycles, Vitamin D is acting upon the ovarian follicle and may improve oocyte quality rather than improving endometrial receptivity.

In support of this theory, women with higher concentration of Vitamin D in follicular fluid (FF) are more likely to conceive following ART (Ozkan *et al.*, 2010). However, negative (Anifandis *et al.*, 2010; Aleyasin *et al.*, 2011) and insignificant (Firouzabadi *et al.*, 2014) correlations in this context have also been reported. Regardless of this inconsistency, a direct relation between Vitamin D and ovarian follicle competence has been demonstrated in humans and animals. One study found higher numbers of mature antral follicles in women with higher serum Vitamin D (Arabian and Raoofi, 2018) while in women with polycystic ovary syndrome (PCOS), Vitamin D supplementation improved follicular growth and dominant follicle formation (Thys-Jacobs *et al.*, 1999; Fang *et al.*, 2017). In line with this, a multi-centre observational study suggested that Vitamin D deficiency in women >40 years of age correlated with a greater decrease in ovarian reserve (Merhi *et al.*, 2012).

A plausible mechanism to explain how Vitamin D impacts the follicle may involve the granulosa cells (GCs), which surround the developing oocyte and supply it with hormones and growth factors. To note, the Vitamin D receptor (VDR) has been identified in various tissues, including GCs (Thill *et al.*, 2009). In mice, VDR null females have ovarian insufficiencies that are characterised by impaired follicular development (Kinuta *et al.*, 2000). In goats, treatment of GCs with Vitamin D induced VDR expression and a number of genes involved in steroidogenesis (Yao *et al.*, 2017). VDR was predominantly localised in the GCs of the follicle and its expression increased with follicle diameter, being at its lowest in apoptotic atretic follicles (Yao *et al.*, 2020). A series of additional experiments including silencing or overexpression of VDR in luteinised GCs showed that VDR signalling is essential for regulation of GC proliferation and apoptosis. In the macaque monkey, Vitamin D supplementation improved preantral and antral follicle survival and growth, and anti-Müllerian hormone (AMH) and oestradiol (E2) production as well as oocyte maturation *in vitro* (Xu *et al.*, 2018). Regardless of these studies, the question remains of whether Vitamin

D is a surrogate marker for general well-being rather than a specific regulator of follicular function.

Only interventional studies could disentangle this uncertainty. Therefore, additional important information can be provided by a clinical trial. Indeed, in 2016 our group launched a randomised double-blinded clinical trial to test whether supplementation of 25-hydroxyvitamin D in deficient women undergoing IVF would improve outcomes (Paffoni *et al.*, 2019). While the trial is still ongoing, we addressed one of the secondary trial outcomes providing novel *in-vivo* findings on the impact of Vitamin D supplementation on FF hormonal content and GC transcriptomics in human.

Materials and methods

Study design

The study included a total of 145 patients forming a subgroup of the total 630 patients recruited in the SUNDRO clinical trial 'Vitamin D Supplementation on Assisted Reproduction Technology (ART) outcomes: a randomized clinical controlled trial and an investigation of the involved biological mechanisms' (Paffoni *et al.*, 2019). The clinical trial (EUDRACTNR—2015-004233-27) has been designed according to the CONSORT methodology. It is a multi-centre randomised superiority double-blinded placebo-controlled clinical trial with parallel groups and allocation 1:1. Two Italian academic infertility units are involved: IRCCS Fondazione Ca' Granda, Ospedale Maggiore, Policlinico, Milan, Italy; and IRCCS Ospedale San Raffaele, Milan, Italy. These subgroup analyses received ethical approval and were reported in the informed consent forms signed by the patients.

Briefly, the inclusion criteria included women undergoing ART, aged: 18–39 years, BMI: 18–25 kg/m^2 (normal), previous IVF cycles <3, circulating 25-hydroxyvitamin D < 30 ng/ml (low). Exclusion criteria were: AMH <0.5 µg/l (low ovarian reserve), IVF frozen cycle, testicular sperm extraction or contraindications/side effects for consumption of Vitamin D. The patients were randomly assigned to two groups following confirmation of the low 25-hydroxyvitamin D concentration in serum. At the time of randomisation, the Vitamin D group and the Placebo group were administered a single dose of oral calcifediol (25-hydroxyvitamin D; 600 000 IU) or Placebo (diluted in 2 ml olive oil), respectively. The intervention took place 2–12 weeks before oocyte retrieval. Patients were treated according to standard IVF protocols, as described elsewhere (Bartolacci *et al.*, 2018). Briefly, a GnRH agonist or antagonist was used for pituitary down-regulation and ovarian stimulation was achieved with either recombinant FSH (rFSH) alone or rFSH combined with recombinant LH or highly purified hMG alone (HP-hMG). Antral follicle (2–10 mm) count (AFC) was used to determine the strategy for stimulation and dose specifics were chosen on a case-by-case basis according to the response to gonadotrophins in previous attempts and patient's characteristics. Ovulation trigger was performed with HP-hCG when leading follicles had reached a diameter of ≥17–18 mm. On day of ovulation trigger, the AFC was not recorded but only follicles ≥11 mm were recorded. All follicles that were aspirated in our study measured ≥11 mm on the day of trigger (considering that 1 mm growth/day is expected until retrieval 2 days after trigger) and no antral follicles were aspirated. Oocytes were retrieved 36 hours after hCG administration. The serum calcifediol was assessed twice: before randomisation (baseline to verify deficiency) and at ovulation trigger. The FF calcifediol was assessed at oocyte retrieval.

FF collection

During the ultrasound-guided follicle aspiration, FF was retrieved following two different strategies. For assessment of hormones in FF, the dominant follicle was sampled (≥17–18 mm), and an aliquot was stored at −80°C. Following thawing, calcifediol and other hormones were measured with liquid chromatography tandem mass spectrometry (LC-MS/MS). A total of 95 patients formed this first cohort (Table I). For the isolation of GCs, all the follicles (≥11 mm measured at trigger) of a patient were sampled, and the FF was pooled. Following centrifugation of FF, the supernatant was removed, and the pellet was processed. A total of 50 patients formed the second cohort (Table II).

Vitamin D and hormone measurements

Vitamin D concentration was assessed by measuring 25-hydroxyvitamin D in serum and FF. In serum, Vitamin D was assayed at Fondazione IRCCS Ca' Grand Ospedale Maggiore Policlinico (Milan, Italy) using standard clinical procedure based on a chemiluminescent immunoassay. In FF, Vitamin D was assessed using the IVD-MS kit MassChrom® 25-hydroxyvitamin D3/D2 (#72072, Chromsystems, Gräfelfing, Germany) followed by LC-MS/MS analysis according to manufacturers' protocol. Briefly, an aliquot of 100 µl of FF, calibrator or quality control (QC) solution were combined with 200 µl of internal standard solution containing a Vitamin D deuterated analogue and 25 µl of precipitation reagent. The mixture was vortexed, incubated

Table I Characteristics of patients assessed for follicular fluid analysis.

Characteristics	Vitamin D n = 50	Placebo n = 45	*P*-value
Age (years)	36 [33–38]	36 [33–37]	0.96
BMI (Kg/m^2)	21.3 [19.5–22.9]	22.3 [19.9–23.4]	0.16
Smokers	7 (14%)	9 (20%)	0.58
Duration of infertility (years)	3 [2–4]	2 [2–3,5]	0.29
Previous pregnancy	12 (24%)	15 (33%)	0.37
Previous delivery	5 (10%)	7 (16%)	0.45
AMH (ng/ml)	2.7 [1.6–4.3]	3.0 [1.6–4.9]	0.47
FSH (mIU/ml)	6.5 [5.5–8.2]	7,0 [5.6–8.5]	0.31
Antral follicle count	13 [9–17]	12 [8–17]	0.71
Previous IVF cycle	22 (44%)	9 (20%)	0.02
Indication for IVF			0.63
Idiopathic	18 (36%)	11 (25%)	
Male factor	17 (34%)	18 (40%)	
Endometriosis	7 (14%)	8 (18%)	
Tube factor	4 (8%)	2 (4%)	
Multiple	4 (8%)	6 (13%)	

AMH, anti-Müllerian hormone.
Data are median (interquartile range (IQR)) or numeric value (%).

Table II Characteristics of patients assessed for transcriptomic analysis of granulosa cells.

Characteristics	Vitamin D n = 20	Placebo n = 30	*P*-value
Age (years)	34.6 ± 1.1	34.4 ± 0.7	0.89
Duration of infertility (months)	45.6 ± 3.5	51.5 ± 5.5	0.44
Race			0.33
Caucasian	18 (90%)	29 (97%)	
Other	2 (10%)	1 (3%)	
BMI (Kg/m^2)	21.38 ± 0.4	21.12 ± 0.8	0.66
E2 serum (ng/ml)	2932 ± 2476	2266 ± 1208	0.62
P4 serum (ng/ml)	1.189 ± 1.4	0.8485 ± 0.6	0.47
Oocytes recovered	10.471 ± 1.1	10.519 ± 1.0	0.97
Oocytes utilised	9.235 ± 1.0	7.778 ± 0.8	0.27
Oocytes fertilised	0.6876 ± 0.05	0.7415 ± 0.04	0.48

E2, oestradiol; P4, progesterone.

for 10 min at 4°C and centrifuged for 5 min at 15 000*xg* in a microcentrifuge (Abbott, Roma, Italy). The supernatant was then transferred to an autosampler glass vial, and a 10 µl aliquot was injected onto a liquid chromatography system (Agilent, Milan, Italy); the system was equipped with a trap column (operating at room temperature) for sample purification and an analytical column (operating at 25°C) for peak separation. Mobile phases (provided with the kit) were used for elution; the total run time was 5 min. The chromatographic system was interfaced with a Sciex 5500 QTRAP mass spectrometer (Sciex, Milan, Italy) equipped with an atmospheric pressure ionization source, operating in positive mode. For calibration, a blank calibrator matrix and three plasma calibrators were used. To assess within- and between-run precision and accuracy, two QC solutions, provided with the kit, were used. The FF of the first cohort was also analysed with LC-MS/MS to evaluate any differences in the hormonal content between Vitamin D- and Placebo-supplemented patients. To analyse aldosterone, androstenedione, cortisol, cortisone, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol, 21-deoxycortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S), dihydrotestosterone, E2, 17-OH-hydroxyprogesterone, progesterone (P4) and testosterone concentration by LC-MS/MS, an IVD-MS steroids kit (MassChrom, Steroids in Serum/Plasma, Chromsystems, Gräfelfing, Germany) was used. Samples were prepared according to the manufacturer's instructions. Briefly, 500 µl of each FF sample, calibrators, or QC were placed in solid phase extraction sample plate, previously equilibrated, with 50 µl of a deuterated internal standard mix solution and 450 µl extraction buffer. This mixed sample was then vortexed and centrifuged for 1 min at 400*xg*. The supernatant of the sample was evaporated under nitrogen to dryness, reconstituted with 100 µl of reconstitution buffer and two 40 µl aliquots were injected into the high-performance liquid chromatography system equipped with an analytical column (operating at 32°C) for peak separation. Mobile phases were used for steroids elution. As previously mentioned, a blank calibrator matrix and six multilevel serum calibrators provided with the kit were used for calibration and three certified QCs of serum were used to assess within- and between-run precision and accuracy. Analysis of duplicates was performed on 10% of the samples. Relative standard deviation was up to 10% for hormones and up to 4% for Vitamin D. Accuracy, evaluated for low, intermediate and high QC for hormones, and low and high QC for vitamin D, was in the range of 91–110% for hormones and 93–108% for Vitamin D; this refers to plasma QC samples as no FF QC is commercially available.

Luteinised GC isolation

Human luteinised GCs from Vitamin D- and Placebo-supplemented patients were isolated from FF according to the 'flask method' reported elsewhere (Ferrero *et al.*, 2012). This method provides good cell viability and a high percentage (average 65%) of recovered luteinised GCs and is considered one of the methods to give the highest percentage of recovered GCs. Briefly, cells present in FF were isolated by initial centrifugation (800*xg*, 10 min, room temperature) followed by gradient separation at density of 1.077 g/ml (Lymphoprep^{TM}, STEMCELL Technologies, Vancouver, Canada). Following the gradient centrifugation (500*xg*, 20 min, room temperature), the middle layer containing mononuclear cells was collected and supplemented with 3 ml of RPMI 1640 medium containing 10% foetal bovine serum. The mononuclear cell suspension was incubated in a Petri dish at 37°C for 15 min to allow macrophages to adhere to the plastic. The remaining suspension containing GCs and lymphocytes was transferred to a new culture dish containing 3 ml of RPMI 1640 medium with 10% foetal bovine serum for 24 h at 37°C. The spent media containing lymphocytes, which do not attach to the plastic, was removed and the GC monolayer was washed in PBS before being lysed using the RLT buffer (QIAGEN, Valencia, CA 91355, USA). The lysate was used to isolate total RNA.

RNA extraction

RNA was extracted from all GC samples using the RNeasy Mini Kit (#74134, QIAGEN) according to the manufacturer's instructions and assessed for quality and quantity using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA) and 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Only samples with A260/A280 and A260/A230 >1.8 and RIN number >8 were considered suitable for use.

RNA-sequencing

Six representative samples from the second cohort were used for RNA-sequencing analysis to identify transcriptomic differences in GCs between Vitamin D- (n = 3) and Placebo- (n = 3) supplemented patients. These patients were selected to be similar for certain characteristics: age (Vitamin D: 33.7 ± 1.05, Placebo: 35.3 ± 2.72, $P = 0.7$), baseline E2 serum (ng/ml) (Vitamin D: 1664 ± 224, Placebo: 3541 ± 1195, $P = 0.1$), baseline P4 serum (ng/ml) (Vitamin D: 0.62 ± 0.0, Placebo: 0.83 ± 0.2, $P = 0.2$), oocytes recovered (Vitamin D: 8 ± 1.00, Placebo: 10.33 ± 2.88, $P = 0.19$), oocytes utilised (Vitamin D: 6.66 ± 0.57, Placebo: 8.33 ± 3.05, $P = 0.65$) and oocytes fertilised (Vitamin D: 4.33 ± 1.52, Placebo: 8 ± 2.64, $P = 0.2$).

The total input RNA was 500 ng and the TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, USA) was used for

library preparation. The libraries were sequenced on an Illumina HiSeq2500 Rapid Run System, generating pair-end 50 base-pair reads (140 M reads on average per sample). Transcript abundance was estimated with Kallisto (Bray et al., 2016) and differentially expressed genes (DEGs) were identified using the DeSeq2 R package (Love et al., 2014) and an adjusted *P*-value <0.05. Raw data are available in Supplementary Tables SI and SII.

RT-PCR

The DEGs emerging from the RNA-sequencing analysis and other selected genes were subjected to RT-PCR analysis using the SYBR green system (QuantiTect SYBR® Green PCR Kits QIAGEN), QIAGEN prevalidated ready-to-use primer sets and the QIAGEN real-time PCR cycler Rotor-Gene Q. Briefly, a total of 550 ng of RNA were used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific, #4368813) according to the protocol instructions. Technical duplicates were included, and data were analysed using of the comparative Ct method and normalised to the housekeeping gene β-Actin (*ACTB*). The primers used were the QuantiTect Primer Assays 249900: Hs_PTGS2_1_SG QT00040586, Hs_KLF4_1_SG QT00061033, Hs_IL21R_1_SG QT00045822, Hs_ACTB_1_SG QT00095431, Hs_GSTA3_1_SG QT00017353, Hs_TRPC4_1_SG QT00046081, Hs_VEGFA_6_SG QT01682072, Hs_VDR_1_SG QT01010170, Hs_RXRB_1_SGQT00061117 and Hs_AGER_1_SG QT00000119.

Pathway analyses

Ingenuity pathway analysis (IPA) is a web-based bioinformatics application available from QIAGEN that builds on manually curated content of the Ingenuity Knowledge database to predict significant biological mechanisms and pathways (Krämer et al., 2014). The input for IPA was a file containing a column with the list of all genes resulting from the RNA-sequencing analysis comparing the Vitamin D and Placebo groups, a column for the fold change and a third column for the *P*-value. Initially, a false discovery rate (FDR)-adjusted *P*-value cut-off of 0.05 was set to identify molecules whose expression was significantly differentially regulated between the Vitamin D and Placebo group. The output included a limited number of genes and, consequently, was not informative. A secondary analysis with an FDR-adjusted *P*-value cut-off of 0.08 produced numerous molecules, which helped to identify the 'canonical pathways' regulated by the intervention. Molecules from the data set that met the FDR-adjusted *P*-values cut-off of $P < 0.08$ were associated with a canonical pathway in Ingenuity's Knowledge Base and considered for the analysis. The significance of the association between the input dataset and the canonical pathway shown as –log(*P*-value) on the Stacked Bar Chart was determined based on two parameters: the ratio of the number of molecules included in the input dataset that map to a canonical pathway divided by the total number of molecules belonging to the canonical pathway; and the *P*-value that deduced the probability that the observed association between the molecules and the pathway is attributed to chance alone, calculated with Fischer's exact test. An IPA 'upstream regulators' analysis was additionally performed to identify the transcriptional regulators involved in our dataset.

Statistical analysis

All data were initially examined for normality using the Kolmogorov–Smirnov test. The normally distributed data were analysed with the Student's *t*-test and the not normal data were analysed with the Mann–Whitney test. The frequency of patient's characteristics was analysed with the chi-square test. Data are presented as mean ± SD, SEM or interquartile range, and statistical significance is considered a result with a difference of $P < 0.05$. Statistical analyses were conducted with SPSS (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, USA) software version 26.0 and R software version 3.6.

Results

Oral Vitamin D supplementation increased the concentration of Vitamin D in serum and FF of women undergoing IVF

The serum and FF samples of women randomised to receive either 25-hydroxyvitamin D (Vitamin D; 600 000 IU) or Placebo in a single oral administration between 2 and 12 weeks prior to oocyte retrieval for IVF treatment were analysed. The two groups (Vitamin D n = 50 patients, Placebo n = 45 patients) did not differ with regard to various characteristics (Table I). Serum Vitamin D was assessed twice: on the day of randomisation (baseline) and on the day of ovulation trigger. The two groups had a similar Vitamin D concentration (<30 ng/ml) at the time of randomisation (Vitamin D 18.0 (13.8–22.9) ng/ml, Placebo 17.5 (10.4–22.7) ng/ml; Fig. 1A). However, at the time of ovulation trigger (Fig. 1B), women who received supplementation of Vitamin D had significantly higher ($P < 0.001$) serum Vitamin D (51.6 (40.0–68.2) ng/ml) compared to women who received Placebo (15.4 (11.1–20.6) ng/ml). In FF, assessed at oocyte retrieval, the concentration of Vitamin D was higher ($P < 0.001$; Fig. 2A) in women who received Vitamin D (39.5 (33.2–59.2) ng/ml) supplementation when compared to women receiving Placebo (13.8 (9.5–20.7) ng/ml). For both groups, Vitamin D levels were lower in FF compared to serum ($P < 0.001$). Regression analysis revealed a strong positive correlation between serum and FF Vitamin D concentrations in the full cohort (total n = 95 patients; Fig. 2B).

The hormonal milieu of FF was not affected by Vitamin D supplementation

The difference in FF hormonal abundance between the two groups (Vitamin D versus Placebo) was studied with LC-MS/MS. No differences were observed between the two groups for the hormones aldosterone, androstenedione, cortisol, cortisone, corticosterone, 11-desoxicorticosterone, 11-deoxicortisol, 21-deoxicortisol, DHEA, DHEA-S, E2, 17-OH-hydroxiprogesterone, P4 and testosterone (Table III). No difference in the concentration of the hormones in FF was found even when the two groups were stratified based on the concentration of circulating Vitamin D measured at randomisation (<20: deficiency and 20–30: insufficiency; Supplementary Table SIII). In the Vitamin D group, the concentration of Vitamin D in FF did not correlate with the concentration of any studied hormone, as revealed by regression analysis using Vitamin D as independent and the hormone as dependent variables (Supplementary Table IV). However, in

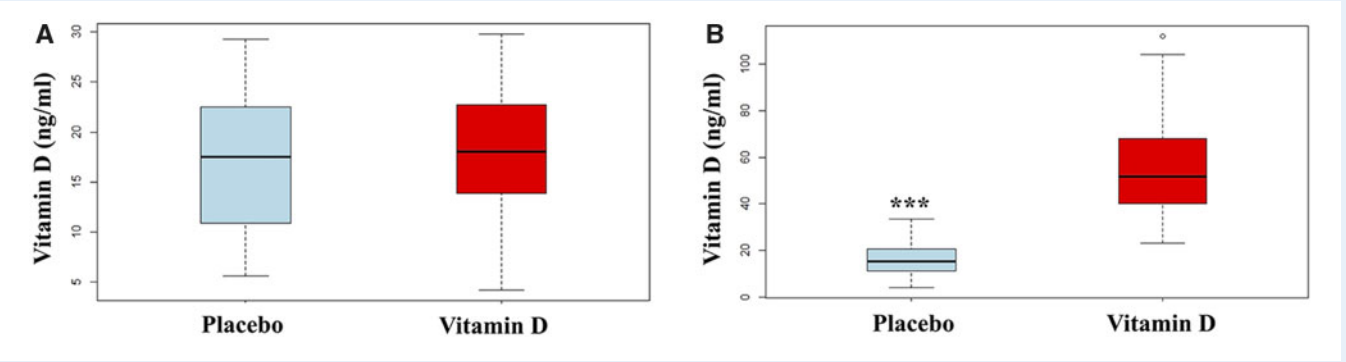

Figure 1. Concentration of circulating Vitamin D at randomisation and at ovulation trigger in women with Vitamin D deficiency undergoing IVF. Vitamin D was measured on the day of randomisation (**A**) and on the day of ovulation trigger (**B**) in the serum of patients receiving Vitamin D (n = 50) or Placebo (n = 45). Data analysed with Mann–Whitney test and presented with Whisker plots (interquartile range (IQR)), ***$P < 0.001$.

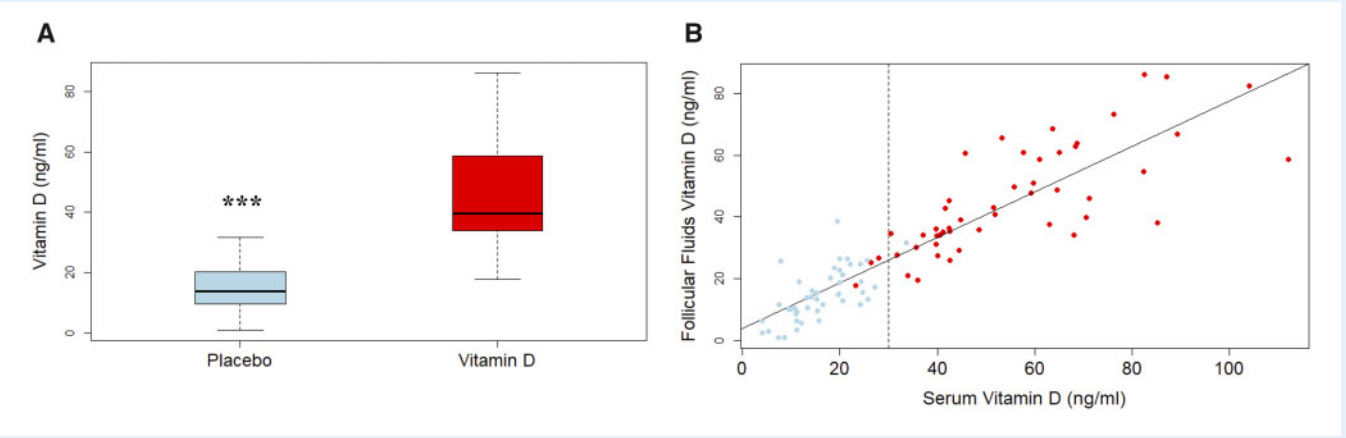

Figure 2. Concentration of Vitamin D in follicular fluid. (**A**) The concentration of Vitamin D was assessed in follicular fluid (FF) on the day of oocyte retrieval. Data analysed with Mann–Whitney test and presented with Whisker plots (IQR), Vitamin D (n = 50) versus Placebo (n = 45) ***$P < 0.001$. (**B**) Regression analysis showing the correlation of Vitamin D concentration between FF (assessed at oocyte retrieval) and serum (assessed at ovulation trigger); $P < 0.001$, $\beta = 0.74$, $R^2 = 0.80$, n = 95 FF samples from patients belonging to both groups (blue dots represent Placebo, red dots represent Vitamin D).

the Placebo group, a weak negative correlation was observed between the concentration of Vitamin D and E2 ($\beta = -12.9$, $P = 0.002$, $r = 0.45$; Fig. 3A). In addition, P4 had a positive weak correlation with Vitamin D ($\beta = 6.8$, $P = 0.03$, $r = 0.31$; Fig. 3B). Calculated *Z*-values for E2 and P4 were 2.15 and 0.55, respectively. A *Z*-value ≥ 2 represents significant difference between the two correlation coefficients (*r*) in Placebo and Vitamin D groups.

GCs isolated from FF of patients supplemented with Vitamin D exert an intervention-relevant transcriptomic response

We next assessed whether the observed increase in the follicular Vitamin D of patients who received supplementation with Vitamin D had affected gene expression in GCs. An RNA-sequencing analysis followed by PCR validation was performed on a subgroup of samples with homogeneous characteristics selected for gene expression studies (Table II). The RNA-sequencing revealed a distinct transcriptomic profile in GCs of patients receiving Vitamin D (Fig. 4). Principal component analysis (PCA) performed on normalised data showed a different distribution for gene expression profiles of samples from the Vitamin D (n = 3) group compared to Placebo (n = 3) group (Fig. 4A). An interpatient variation of gene expression within each group was observed, as expected (Supplementary Fig. S1). The transcriptomic profile associated with Vitamin D supplementation was characterised by up-regulation of 20 transcripts and down-regulation of 22 transcripts (Fig. 4B). The list of DEGs (Table IV) was further explored with pathway analysis strategies. An ARCHS4 tissue expression atlas allocated the DEG list to a 'Granulosa Cell Type' (Supplementary Table SV),

Table III Hormonal abundance in follicular fluid of patients after receiving Vitamin D or Placebo.

Hormone	Vitamin D n = 50	Placebo n = 45	*P*-value
Aldosterone (μg/l)	0.05 [0.03–0.07]	0.05 [0.03–0.07]	0.87
Cortisol (μg/l)	66.0 [56.6–72.8]	64.6 [56.1–84.7]	0.92
Cortisone (μg/l)	16.6 [14.2–21.0]	18.2 [13.6–20.5]	0.36
11-DeoxyCortisol (μg/l)	1.7 [1.1–2.4]	1.7 [0.9–2.7]	0.92
21-DeoxyCortisol (μg/l)	0.16 [0.09–0.28]	0.13 [0.06–0.25]	0.75
Corticosterone (μg/l)	2.8 [2.3–4.0]	3.0 [2.1–4.1]	0.77
11-DeoxyCorticosterone (μg/l)	33.3 [20.5–48.7]	35.5 [19.1–44.0]	0.92
DHEA-S (μg/l)	1 502 [1 015–2 091]	1 579 [1 207–2 051]	0.92
DHEA (μg/l)	7.9 [3.8–14.8]	6.8 [2.9–11.6]	0.75
Androstenedione (ng/l)	1.1 [0.4–2.8]	1.3 [0.5–3.4]	0.92
Testosterone (μg/l)	0.19 [0.05–0.89]	0.13 [0.02–0.65]	0.75
17-OH-Progesterone (μg/l)	318.1 [199.2–443.6]	377.3 [246.3–499.2]	0.61
E2 (μg/l)	384.7 [187.8–633.3]	369.3 [160.6–596.2]	0.75
P4 (μg/l)	623.4 [541.1–808.4]	576.8 [499.8–821.3]	0.12

Data analysed with Mann–Whitney test and reported as median [IQR].
DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate.

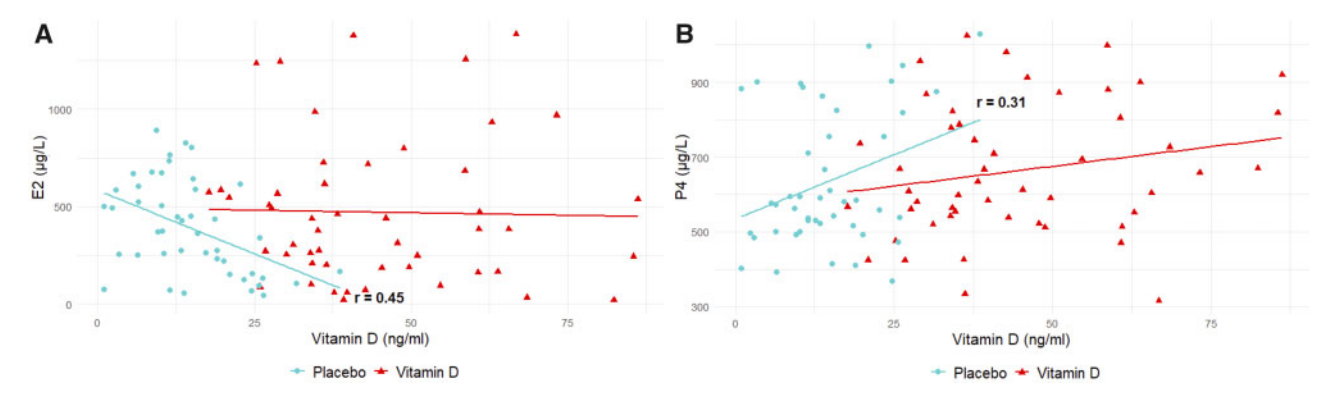

Figure 3. Correlation of Vitamin D with progesterone and oestradiol concentrations in FF. Vitamin D level was used as independent variable with progesterone (P4) and oestradiol (E2) as dependent variables. Coefficient β, R^2 and *P*-values were calculated using linear regression. Different trends in correlation are evident for the Placebo (n = 45) and Vitamin D (n = 50) groups. The Vitamin D concentration weakly correlates with E2 (**A:** β = −12.952, $P = 0.002$, $R^2 = 0.205$) and P4 (**B:** β = 6.889, $P = 0.03$, $R^2 = 0.101$) in the Placebo group but not in the Vitamin D group.

verifying the experiment, and an IPA analysis performed on a list of the complete dataset (*P*-adjusted cut-off set at 0.08) was utilised to identify the top canonical pathways affected by Vitamin D supplementation in GCs (Fig. 5). The analysis revealed an overlap of our data with 17 IPA canonical pathways, including the activation of Liver X Receptor (LXR), Retinoid X Receptor (RXR) and VDR, as well as numerous pathways relevant to pro-oxidant/antioxidant balance. Specifically, out of the 77 molecules registered in the IPA repository for VDR-RXR activation, 30 molecules in our full dataset (39%) appeared up-regulated and 32 (41.6%) down-regulated while 15 (19.5%) of molecules in the pathway did not overlap (Table V). To deduce what factors could be driving the DEGs, we performed an IPA upstream analysis to predict the top transcriptional regulators (Table VI). Amongst the top upstream regulators were TNF (tumor necrosis factor), MAPK11 (mitogen-activated protein kinase 11), MAPK3 (mitogen-activated protein kinase 3), JUN (Jun Proto-Oncogene), P LCE1 (1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase epsilon-1) and EDN1 (Endothelin 1), which are involved in the signalling of Advanced Glycation End (AGE)-Receptor of AGE (RAGE). The AGE-RAGE signalling pathway plays major roles in cell stress and toxicity and is considered pro-inflammatory.

Finally, based on the above findings, we sought to examine the differential expression of genes using RT-PCR, both to validate the genes that were identified with the RNA-sequencing analysis and to explore other genes that could be of importance in the context of IPA findings.

The RT-PCR analysis, performed on samples from the second cohort (Table II), revealed that Vitamin D down-regulated *PTGS2*

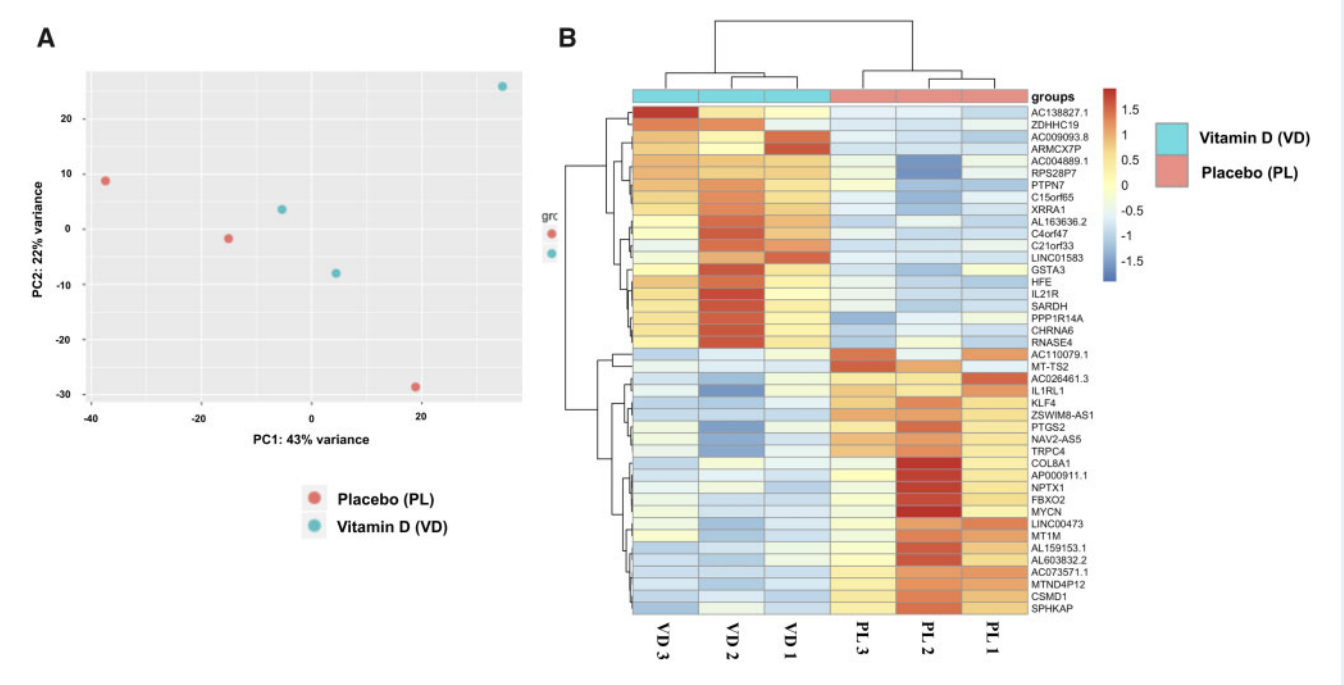

Figure 4. RNA-sequencing analysis of granulosa cells from patients. Cells were isolated from FF recovered from patients receiving Vitamin D (VD, n = 3) or Placebo (PL, n = 3) and analysed. (**A**) Principal component analysis (PCA) distribution of the samples. (**B**) Heatmap of the differential expressed genes. At a false discovery rate <0.05, 20 genes were up-regulated and 22 genes were down-regulated.

(prostaglandin-endoperoxide synthase 2, $P = 0.014$, fold change: 2.23 ± 0.08), *KLF4* (kruppel-like factor 4, $P = 0.03$, fold change: 2.13 ± 0.052) and *TRPC4* (transient receptor potential cation channel subfamily C member 4, $P = 0.04$, fold change: 3.54 ± 0.084), as shown in Fig. 6. On the other hand, an up-regulation of *IL21R* (interleukin 21 receptor, $P = 0.008$, fold change: 2.08 ± 0.41) and *GSTA3* (glutathione S-transferase A3, $P = 0.02$, fold change: 2.16 ± 0.54) was observed in GCs of the Vitamin D group compared to Placebo group, in line with the RNA-sequencing data. Additionally, we found *VDR* to be up-regulated ($P = 0.02$, fold change: 1.9 ± 0.30) and its signalling contributor *RXRB* ($P = 0.04$, fold change: 1.35 ± 0.06) to be down-regulated in the group of patients receiving Vitamin D supplementation. Interestingly, vascular endothelial growth factor (VEGF) was additionally down-regulated by Vitamin D in GCs ($P = 0.04$, fold change: 1.35 ± 0.06. Finally, the gene *AGER* (Advanced Glycosylation End-Product Specific Receptor) encoding the RAGE, receptor of AGE products, was also down-regulated with Vitamin D treatment ($P = 0.018$, fold change: 1.47 ± 0.62).

Overall, our findings show that oral Vitamin D supplementation directly affects the ovarian follicle, as reflected by the increase of Vitamin D concentration in FF and modified gene expression in GCs.

Discussion

It is essential that we address any beneficial role of Vitamin D supplementation in the context of natural and assisted reproduction, as this remains a topic of considerable clinical controversy. A recent meta-analysis concluded that a high circulating Vitamin D concentration in women undergoing IVF was associated with a higher live birth rate (Chu *et al.*, 2018). In the absence of evidence to support a causative relation between Vitamin D and IVF outcomes, our group launched a randomised double-blinded clinical trial to deduce whether supplementation with 600 000 IU 25-hydroxyvitamin D would improve pregnancy rates in deficient (<30 ng/ml) IVF patients (Paffoni *et al.*, 2019). In anticipation of the results, we performed a subgroup mid-term analysis, to determine whether any prospective effects of Vitamin D supplementation would manifest as changes in the follicular microenvironment, focusing on FF and GCs. The trial arms included a Vitamin D group and a Placebo group. We highlight that the Placebo participants were Vitamin D deficient, which is important to bear in mind when interpreting the findings. No previous studies have explored the total GC transcriptome in women with Vitamin D deficiency let alone examined how that changes in response to *in-vivo* Vitamin D administration. The hormonal constitution of FF has only been compared between women with replete and deficient Vitamin D levels with or without PCOS (Masjedi *et al.*, 2019). Our cohort included women with no underlying pathology.

We found that Vitamin D supplementation increased 25-hydroxyvitamin D concentration in both serum and FF considerably above the deficiency threshold. A strong positive correlation ($r = 0.9$) between 25-hydroxyvitamin D in serum and FF was evident, in line with previous reports on endogenous levels (Potashnik *et al.*, 1992; Firouzabadi *et al.*, 2014). Despite 25-hydroxyvitamin D reaching the ovarian follicle, the supplementation itself did not change the hormonal constitution of FF. The abundance of all investigated hormones, including P4 and

Table IV List of differentially expressed genes.

Gene name	Description	log2FoldChange	lfcSE	Stat	*P*-value	*P*-adj
MTND4P12	Mitochondrially encoded NADH: ubiquinone oxidoreductase core subunit 4 pseudogene 12	−4.00877	0.479035	−8.36842	5.84E−17	1.46E−12
AC073571.1		−3.63657	0.498794	−7.29072	3.08E−13	3.85E−09
CSMD1	CUB and Sushi multiple domains	−2.78125	0.503768	−5.5209	3.37E−08	0.000281
AL163636.2		2.724487	0.509324	5.349219	8.83E−08	0.000551
CHRNA6	Cholinergic receptor nicotinic alpha 6 subunit	2.41979	0.463428	5.221505	1.77E−07	0.000886
PTPN7	Protein tyrosine phosphatase, non-receptor type 7	1.867619	0.400504	4.663169	3.11E−06	0.012952
TRPC4	Transient receptor potential cation channel subfamily C member 4	−1.97243	0.425912	−4.63106	3.64E−06	0.01297
ARMCX7P	Armadillo repeat containing, X-linked 7, pseudogene	2.28023	0.511599	4.457064	8.31E−06	0.018852
AC138827.1		2.282522	0.512085	4.457313	8.3E−06	0.018852
LINC01583	Long intergenic non-protein coding RNA 1583	2.154138	0.479692	4.490671	7.1E−06	0.018852
AC110079.1		−2.19066	0.490046	−4.47032	7.81E−06	0.018852
NAV2-AS5	NAV2 antisense RNA 5	−2.01883	0.45962	−4.39238	1.12E−05	0.022078
IL21R	Interleukin 21 receptor	1.923137	0.438387	4.386849	1.15E−05	0.022078
C4orf47	Chromosome 4 open reading frame 47	1.921149	0.445922	4.308259	1.65E−05	0.028358
XRRA1	X-ray radiation resistance associated 1	1.47311	0.342547	4.300466	1.7E−05	0.028358
C21orf33	Chromosome 21 open reading frame 33	1.879672	0.442204	4.250686	2.13E−05	0.033242
RPS28P7	Ribosomal protein S28 pseudogene 7	1.662736	0.392672	4.234415	2.29E−05	0.03364
ZSWIM8-AS1	ZSWIM8 antisense RNA 1	−2.15956	0.51194	−4.21838	2.46E−05	0.034116
AL603832.2		−2.05275	0.488596	−4.20132	2.65E−05	0.034857
AC026461.3		−1.92908	0.465764	−4.14174	3.45E−05	0.035842
C15orf65	Chromosome 15 open reading frame 65	1.678325	0.40449	4.149239	3.34E−05	0.035842
HFE	Homeostatic iron regulator	1.574188	0.37755	4.169486	3.05E−05	0.035842
GSTA3	Glutathione S-transferase alpha 3	1.92195	0.460516	4.17347	3E−05	0.035842
KLF4	Kruppel-like factor 4	−1.39557	0.336401	−4.14854	3.35E−05	0.035842
PPP1R14A	Protein phosphatase 1 regulatory inhibitor subunit 14A	1.785163	0.434836	4.105371	4.04E−05	0.040297
AC009093.8		1.995731	0.491841	4.057679	4.96E−05	0.040845
FBXO2	F-box protein 2	−2.003	0.490265	−4.08554	4.4E−05	0.040845
MYCN	MYCN proto-oncogene, bHLH transcription factor	−2.06983	0.509505	−4.06242	4.86E−05	0.040845
LINC00473	Long intergenic non-protein coding RNA 473	−1.78821	0.439045	−4.07296	4.64E−05	0.040845
COL8A1	Collagen type VIII alpha 1 chain	−1.9875	0.492253	−4.03757	5.4E−05	0.040845
AC004889.1		1.520299	0.376338	4.039723	5.35E−05	0.040845
SPHKAP	SPHK1 interactor, AKAP domain containing	−1.50724	0.372473	−4.04659	5.2E−05	0.040845
PTGS2	Prostaglandin-endoperoxide synthase 2	−1.76705	0.437074	−4.04291	5.28E−05	0.040845
MT1M	Metallothionein 1M	−1.86004	0.46315	−4.01606	5.92E−05	0.043439
MT-TS2	Mitochondrially encoded tRNA serine 2 (AGU/C)	−1.99995	0.499372	−4.00493	6.2E−05	0.044235
ZDHHC19	Zinc finger DHHC-type containing 19	1.76034	0.441592	3.986349	6.71E−05	0.045699
SARDH	Sarcosine dehydrogenase	1.590494	0.399215	3.984049	6.78E−05	0.045699
NPTX1	Neuronal pentraxin 1	−2.0177	0.508592	−3.96722	7.27E−05	0.047756
AP000911.1		−1.91379	0.486379	−3.93476	8.33E−05	0.049714
AL159153.1		−1.84324	0.468583	−3.93365	8.37E−05	0.049714
RNASE4	Ribonuclease A family member 4	1.684382	0.427986	3.9356	8.3E−05	0.049714
IL1RL1	Interleukin 1 receptor-like 1	−1.59333	0.403941	−3.94447	8E−05	0.049714

False discovery rate <0.05.

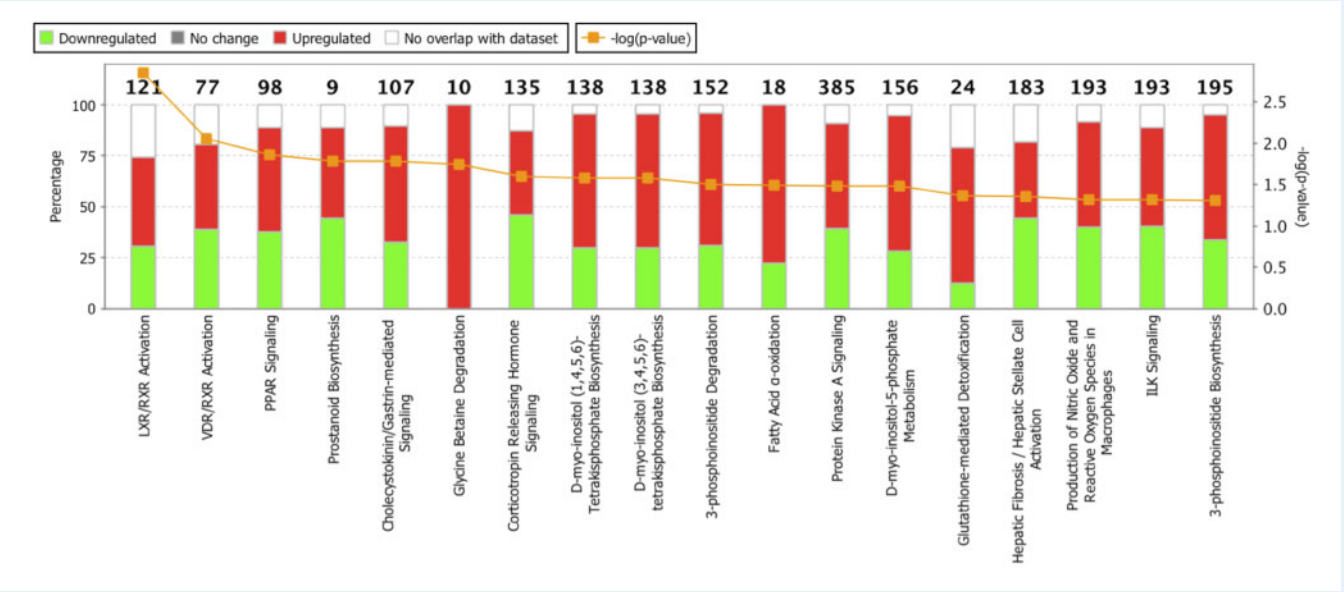

Figure 5. Analysis of RNA-sequencing data and differentially expressed genes in granulosa cells from patients. Ingenuity pathway analysis of the complete RNA-sequencing dataset presented as a Stacked Bar Chart showing the overlap of the analysis with the top canonical pathways affected by Vitamin D supplementation in granulosa cells. The total number of genes belonging to each pathway is displayed above each bar. The overlap of these genes with our dataset is indicated: the % of up-regulated (red) and % down-regulated (green) molecules. The right *x*-axis displays the −log of *P*-value calculated by Fisher's exact test right-tailed.

Table V Ingenuity pathway analysis top canonical pathways.

Canonical pathway	Molecules in pathway	Down-regulated	Up-regulated	No overlap
LXR/RXR activation	121	37/121 (30.6%)	53/121 (43.8%)	31/121 (25.6%)
VDR/RXR activation	77	30/77 (39.0%)	32/77 (41.6%)	15/77 (19.5%)
PPAR signalling	98	37/98 (37.8%)	50/98 (51.0%)	11/98 (11.2%)
Prostanoid biosynthesis	9	49 (44.4%)	4/9 (44.4%)	1/9 (11.1%)
Cholecystokinin/gastrin-mediated signalling	107	35/107 (32.7%)	61/107 (54.0%)	11/107 (10.3%)
Corticotropin releasing hormone signalling	135	62/135 (45.9%)	56/135 (41.5%)	17/135 (12.6%)
Production of nitric oxide and reactive oxygen species in machrophages	193	77/193 (39.9%)	100/193 (51.8%)	16/193 (8.3%)
Fatty acid oxidation	18	4/18 (22.2%)	14/18 (77.8%)	0/18 (0.0%)
Glutathione-mediated detoxification	24	3/24 (12.5%)	16/24 (66.7%)	5/14 (20.8%)
ILK signalling	193	78/193 (40.4%)	94/193 (48.7%)	21/193 (10.9%)
3-phosphoinositide biosynthesis	195	66/195 (33.8%)	120/195 (62.5%)	9/195 (4.6%)
3-phosphoinositide degradation	152	47/152 (31.0%)	93/152 (61.2%)	12/152 (7.9%)
Glycine betaine degradation	10	0/10 (0.0%)	10/10 (100%)	0/10 (0.0%)
D-myo-inositol (3,4,5,6)-tetrakisphosphate biosynthesis	138	43/138 (31.2%)	82/138 (59.4%)	13/138 (9.4%)
Protein A kinase signalling	385	151/385 (39.2%)	190/385 (49.4%)	44/385 (11.4%)
D-myo-inositol-5-phospht metabolism	156	47/156 (30.2%)	97/156 (62.1%)	12/156 (7.7%)
Hepatic fibrosis/hepatic stellate cell activation	183	86 (46.9%)	59 (32.6%)	38 (20.5%)

Table VI Upstream regulators analysis on ingenuity pathway analysis.

Upstream regulator	Full name	Function	*P*-value
HGF	Hepatocyte growth factor	Growth factor	0.00143
SIGLEC7	Sialic acid-binding Ig-like lectin 7	Transmembrane receptor	0.00179
PRC1	Protein Regulator of cytokinesis 1	Other	0.00179
LRRN1	Leucine Rich Repeat Neuronal 1	Other	0.00179
CREBBP	CREB-binding protein	Transcription regulator	0.00221
MYCNOS	MYCN Opposite Strand	Other	0.00262
MAPK11	Mitogen-activated protein kinase 11	Kinase	0.00357
ITGA3	Integrin Subunit Alpha 3	Other	0.00536
P2RY2	P2Y purinoceptor 2	G-protein-coupled receptor	0.00536
FFAR4	Free fatty acid receptor 4	G-protein-coupled receptor	0.00536
PLCE1	1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase epsilon-1	Enzyme	0.00714
CDF9	Growth differentiating factor 9	Growth factor	0.00714
ADM2	Adrenomedullin 2	Other	0.00891
TLR4	Toll-like receptor 4	Transmembrane receptor	0.00923
TNF	Tumour necrosis factor	Cytokine	0.00972
ZFP36	Zinc finger protein 36 homolog	Transcription regulator	0.0107
EDN1	Endothelin 1	Cytokine	0.0125
LPAR1	Lysophosphatidic acid receptor 1+E4E16: E40	G-protein-coupled receptor	0.0142
MAPK3	Mitogen-Activated Protein Kinase 3	Kinase	0.0142
NTRK1	Neurotrophic Receptor Tyrosine Kinase 1	Kinase	0.0142
KAT2B	K(lysine) acetyltransferase 2B	Transcription regulator	0.016
TGFA	Transforming Growth Factor Alpha	Growth Factor	0.0248
MXI1	MAX-interacting protein 1	Transcription regulator	0.0248
MIF	Macrophage migration inhibitory factor	Cytokine	0.0248
FGFR1	Fibroblast growth factor receptor 1	Kinase	0.025
JUN	Jun proto-oncogene	Transcription regulator	0.0253
ADORA2A	Adenosine A2a Receptor	G-protein-coupled receptor	0.0265
NPC2	NPC Intracellular Cholesterol Transporter 2	Transporter	0.0314
NFKBIA	NF-kappa-B inhibitor alpha	Transcription regulator	0.0335
PCDH11Y	Protocadherin 11 Y-linked	Other	0.0369
IL33	Interleukin 33	Cytokine	0.0386
GATA2	GATA-binding factor 2	Transcription regulator	0.0421
DUSP1	Dual specificity protein phosphatase 1	Phosphatase	0.0438
ASAH1	N-Acylsphingosine Amidohydrolase 1	Enzyme	0.0455
HMGA1	High Mobility Group AT-Hook 1	Transcription regulator	0.0472

E2, was similar in the two study groups. Conversely, Masjedi *et al.* (2019), who performed an observational study in women with and without PCOS, reported hormonal differences in the pre-ovulatory FF associated with the Vitamin D status. Using ELISAs, Masjedi *et al.* (2019) demonstrated that the PCOS group had lower 25-hydroxyvitamin D (23 ng/ml versus 41 ng/ml, respectively), E2 and P4, but higher testosterone, compared to the control group. These diverse findings may be attributed to several factors. Firstly, Masjedi *et al.* based the grouping of deficient and replete patients on endogenous 25-hydroxyvitamin D levels, while in our study the introduction of exogenous 25-hydroxyvitamin D may have established different hormonal dynamics. Secondly, our Placebo participants had an extremely low median concentration of 25-hydroxyvitamin D compared to the Vitamin D group (14 ng/ml versus 40 ng/ml, respectively), which could have been comparable to the PCOS group of Masjedi *et al.* (2019). However, this is not the case as their observations with regard to E2, P4 and testosterone may be more relevant to the PCOS phenotype itself rather than the concentration of Vitamin D. Lastly, we utilised LC-MS/MS methodology, which is more sensitive when compared to ELISA assays, generating lower values with less background (Kushnir *et al.*, 2009). An interesting finding in our study was the negative correlation of E2 with 25-hydroxyvitamin D in FF of Placebo patients, a correlation matrix that was significantly different to the one observed in the Vitamin D group ($z = 2.15$). Although Vitamin D *in vitro* has been

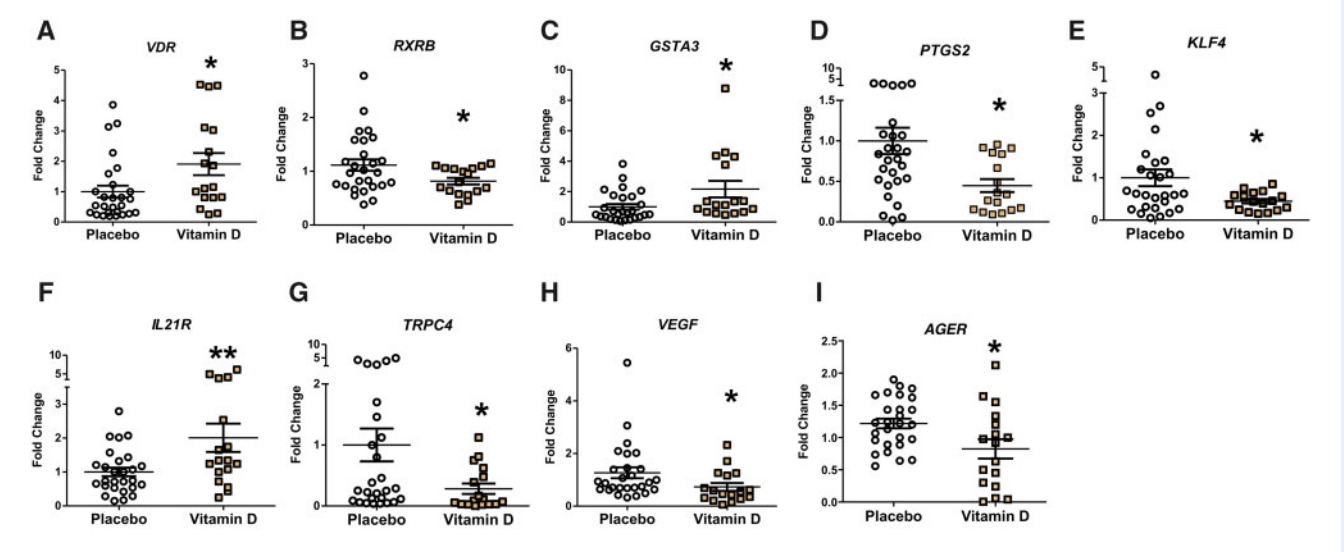

Figure 6. Differential expression assessed by RT-PCR in granulosa cells from patients. (**A**) *VDR* (vitamin D receptor) $^{*}P = 0.02$ fc: 1.9 ± 0.30. (**B**) *RXRB* (retinoid X receptor beta) $^{*}P = 0.04$ fc: 1.35 ± 0.06. (**C**) *GSTA3* (glutathione S-transferase A3) $^{*}P = 0.02$ fc: 2.16 ± 0.54. (**D**) *PTGS2* (prostaglandin-endoperoxide synthase 2) $^{*}P = 0.014$ fc: 2.23 ± 0.08. (**E**) *KLF4* (kruppel-like factor 4) $^{*}P = 0.03$ fc: 2.13 ± 0.052. (**F**) *IL21R* (interleukin 21 receptor) $^{**}P = 0.008$ fc: 2.08 ± 0.41. (**G**) *TRPC4* (transient receptor potential cation channel subfamily C member 4) $^{*}P = 0.04$ fc: 3.54 ± 0.084. (**H**) *VEGF* (vascular endothelial growth factor) $^{*}P = 0.013$ fc: 1.73 ± 0.14. (**I**) *AGER* (advanced glycosylation end-product specific receptor) $^{*}P = 0.018$ fc: 1.47 ± 0.62. Data analysed with Mann–Whitney test and errors bars denote ±SEM. Vitamin D: n = 17, Placebo: n = 27.

shown to both induce and inhibit E2 synthesis in GCs in animals (Bakhshalizadeh *et al.*, 2017; Hong *et al.*, 2017) and humans (Merhi *et al.*, 2014), it is not clear how the introduction of exogenous Vitamin D may have impacted E2 availability in our study.

To closely inspect whether 25-hydroxyvitamin D supplementation imposed gene expression changes in GCs, we performed an RNA-sequencing analysis. To our surprise, none of the genes directly involved in steroidogenesis, and specifically E2 biosynthesis, changed in response to the exogenous signal even when validated with RT-PCR (Supplementary Table SVI). These results were at first glance surprising as they contradicted the literature, where evidence of *in-vitro* Vitamin D supplementation influencing the expression of numerous steroidogenic genes in GCs is provided for humans and animals (Merhi *et al.*, 2014; Hong *et al.*, 2017; Yao *et al.*, 2017; Bakhshalizadeh *et al.*, 2018; Xu *et al.*, 2018). We highlight the higher biological relevance of our *in-vivo* findings compared to the findings of others, more so in the presence of a credible difference in 25-hydroxyvitamin D levels between the deficient and replete groups.

Despite finding no direct effects on the gene expression of key enzymes in steroidogenesis, our findings inform on functions of Vitamin D in promoting anti-inflammatory and antioxidant events. These conclusions were largely derived using the validated and highly credible computational IPA analysis (Krämer *et al.*, 2014; Ma *et al.*, 2016), which considers a complete RNA-sequencing dataset, including the fold changes and *P*-values between the groups for all genes detected in GCs (Supplementary Table SII) and not only the list of DEGs (Table IV). IPA's top canonical pathways regulated by the intervention appeared to include signalling mediated by the interconnected three crucial ligand-activated nuclear receptors LXR, RXR and VDR. The VDR/RXR is not unexpected, as it is the main pathway that mediates Vitamin D signalling, and the *VDR* gene was up-regulated in our study in response to 25-hydroxyvitamin D supplementation. Indeed, 25-hydroxyvitamin D is an additional ligand for the VDR besides its active metabolite 1,25-dihydroxyvitamin D (Lou *et al.*, 2010). Upon binding to its ligand, VDR forms a heterodimer, most commonly with RXRA, to translocate to the nucleus and act as transcription factor for various anti-inflammatory genes within the female reproductive tract (Arck *et al.*, 2010). One of these is the cyclo-oxygenase-2 (COX-2) encoding gene *PTGS2*, which was down-regulated by Vitamin D supplementation in our study. VDR activation has been shown to inhibit *PTGS2* in both placenta (Wang *et al.*, 2018) and decidua (Zhou *et al.*, 2017) while ablation of VDR from myometrial cells promoted the overexpression of *PTGS2* (Mohamed *et al.*, 2018). Thill *et al.* (2009) showed that VDR and COX-2 proteins are co-expressed in the human GCs but did not investigate their expression dynamics. COX-2 is induced in the ovarian follicle in response to inflammatory stimuli, including cytokines and growth factors, and is necessary for physiological ovulation as evident by *PTGS2* null mice that exhibit impaired ovulatory function and infertility (Lim *et al.*, 1997). Our upstream regulators analysis with IPA (Table VI) suggests possible upstream mediators that could have up-regulated *VDR* in order to suppress *PTGS2* in response to exogenous Vitamin D supplementation. It is fair to wonder why Vitamin D supplementation would down-regulate *PTGS2* when its induction is essential for ovulation.

One plausible hypothesis is inspired by evidence where Vitamin D supplementation has been investigated in the prevention of ovarian hyperstimulation syndrome (OHSS) (Turan *et al.*, 2015b), an undesirable outcome of controlled ovarian stimulation (COS) in IVF patients. OHSS involves ovarian enlargement, a high level of E2 and increased vascular permeability associated with high expression of COX-2 and

VEGF (Kitsou et al., 2014). Our results show down-regulation of both *PTGS2* and *VEGF* in response to Vitamin D supplementation. It is possible that up-regulation of VDR signalling stimulated by 25-hydroxyvitamin D attenuated COX-2 and VEGF with the aim to protect the ovary from OHSS. This hypothesis is supported by the fact that COX-2 is increased in GCs of women who experienced OHSS (Cheng et al., 2016), while the inhibition of COX-2 in rodents has been shown to reduce the incidence of OHSS (Quintana et al., 2008; Elia et al., 2013). A preclinical randomised study showed that intervention with inhibitors of COX-2 and VEGF in a rat model of OHSS resulted in the decrease of corpus luteum numbers, suggesting a protective effect against the disease (Kitsou et al., 2014). Moreover, a randomised placebo-controlled trial assessing VEGF in the serum of patients with PCOS, at high risk of OHSS, who were deficient in Vitamin D found that 25-hydroxyvitamin D supplementation decreased VEGF and improved the clinical presentation of PCOS, suggesting possible benefit in prevention of OHSS (Irani et al., 2017).

More clues in agreement with our notion that Vitamin D supplementation may protect the follicle from the processes resulting in OHSS stem from the interpretation of the IPA top canonical pathways. The majority of these pathways have been associated with OHSS: for example, the LXR/RXR activation pathway (Mouzat et al., 2009) and pathways relevant to inositol metabolism (3-phosphoinositide Degradation, 3-phosphoinositide Biosynthesis D-myo-inositol-5-phospht Metabolism D-myo-inositol (3,4,5,6)-Tetrakisphosphate Biosynthesis, D-myo-inositol-5-phospht Metabolism and Glycine Betaine Degradation). Indeed, inhibitors of myo-inositol can decrease COX-2 and VEGF, and reduce the incidence of OHSS in rats (Turan et al., 2015a).

Besides OHSS, another complication of COS can be the disruption of oxidative-antioxidative balance, which is detrimental to normal follicular development (Tulić et al., 2017). A number of IPA top canonical pathways that were regulated by Vitamin D supplementation in our study are implicated in oxidative stress: glutathione-mediated detoxification, production of nitric oxide and reactive oxygen species and fatty acid oxidation. In agreement, Vitamin D supplementation in our study up-regulated the transcript encoding the antioxidative GSTA3 and down-regulated AGER, that encodes the pro-oxidant AGE receptor called RAGE. Accumulation of AGE in GCs was shown to impair follicular growth and insulin signalling, increasing susceptibility to OHSS (Lin et al., 2019). A recent *in-vitro* study showed that Vitamin D supplementation in human GC cultures reversed the AGE-induced damage that caused altered expression of genes critical for steroidogenesis (Merhi et al., 2018). Lastly, we show for the first time that another promoter of oxidative stress, namely KLF4 (Xu et al., 2017), is down-regulated in the intervention group. Collectively, our findings may suggest that Vitamin D supplementation could enhance the antioxidant defence in GCs. Indeed, a recent report demonstrated a direct correlation of Vitamin D concentration in FF with various indicators of oxidative status (Masjedi et al., 2019). We call attention to possible molecular players that warrant further mechanistic investigation in VDR signalling: KLF4, GSTA3, RAGE, TRPC4 and VEGF.

Our molecular observations reinforce the hypothesis that Vitamin D could be considered a gatekeeper against an exaggerated response to ovarian stimulation. Despite this being the first *in-vivo* study to investigate the molecular imprint of Vitamin D supplementation on the ovarian follicle, several limitations in the experimental design should not be ignored. We used GCs that were luteinised in response to COS; therefore, the findings may not be conserved in the absence of COS and the cells may be functionally distinct from the GCs of developing follicles. The interpretation of the data is also influenced by our intervention strategy (2–12 weeks prior to retrieval). As folliculogenesis has been claimed to last 5–6 months, our protocol can only examine with confidence the impact of Vitamin D on the final stages of follicular growth. In this context, we examined the hormonal profile of only the dominant follicle, while the GCs data reflect the transcriptome of all follicles large enough to be used for IVF. Finally, the sample size of RNA-sequencing analysis was low ($n = 3$ per group) introducing complexity to the IPA analysis, which required an input of data with *P*-adjusted <0.08 instead of <0.05 to be informative.

Conclusion

This is the first *in-vivo* evidence of the impact of Vitamin D supplementation on human GC transcriptomics and the follicular hormonal milieu. We did not find differences in the abundance of follicular hormones when comparing Vitamin D deficient to supplemented women. Transcriptomic studies revealed several areas for further investigation in order to elucidate whether Vitamin D supplementation may defend the ovarian follicle from the negative impacts of COS, including oxidative damage and the risk of OHSS.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

All the raw datasets generated during the current study are available from the corresponding author on reasonable request. All datasets analysed during this study are included in this published article as Supplementary information files.

Acknowledgements

The authors would like to acknowledge the assistance of visiting student Gianluca Puglisi for technical input in the experiments.

Authors' roles

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

Funding

The study has been funded by the Italian Ministry of Health (RF-2013-02358757) following peer review in the competitive 'Bando di Ricerca

Finalizzata e Giovani Ricercatori 2013' for the clinical trial SUNDRO with EudraCT registration number 2015-004233-27.

Conflict of interest

None of the authors have conflicts of interest to disclose in relation to the presented study.

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