Mechanism of action of Vitamin D (1,25-(OH)2-D3) on ovarian follicle growth: implications for women with Vitamin D deficiency and PCOS

Abstract

Study question: Do very low levels of 1,25-(OH)2-D3 [<20nmol/L or <10ng/ml] alter ovarian cell function?

Summary answer: An in vitro environment of severe vitamin D deficiency [<2nM] could contribute to impaired folliculogenesis/ovulation identified in women with PCOS, but levels above this [2-20nM] may have less impact; apart from in women who are also insulin-resistant.

What is known already: Vitamin D (VD) is known to regulate steroidogenic enzymes crucial for human granulosa and cumulus cell function (Merhi Z et al, 2014) and genes that play a critical role in folliculogenesis have a vitamin D response element on their promoters (Tiejun S et al, 1998); (Maestro B et al, 2003); (Krishnan AV et al, 2007 & 2010). Women with PCOS have lower serum 25-(OH)-D3 compared to fertile controls but studies investigating the effects of vitamin D deficiency on reproductive outcomes are conflicting.

Study design, size, duration: A variety of cells and tissues were used for the in vitro experiments of this study. Ovaries were used from mice (aged between 4-5 months) to initially establish expression and correlations of VDR and AMH protein. Human ovarian tissue was obtained from women undergoing trans-abdominal hysterectomy with bilateral oophorectomy for benign gynaecological conditions, and was dissected to provide cortex, stroma, theca and granulosa cells from various-sized follicles. Luteinised granulosa cells (GLC) were harvested from follicular aspirates taken during oocyte retrieval from women undergoing in-vitro fertilisation (IVF) treatment at various clinics. A granulosa cell line (KGN-GC), that is established to correspond to immature granulosa cells from smaller antral follicles, was used to provide mechanistic insight. All tissue was obtained with appropriate ethical permission.

Participants/materials, setting, methods: mRNA and protein expression of VDR/CYP27B1/AMH in various ovarian human and mice tissue compartments was assessed by qPCR, Westerns and IHC. GC, theca and GLC were cultured with a range of concentration of 1,25-(OH)2-D3 [0.02, 0.2, 2 and 20nM] with testosterone [5x10^{-7}M] as an aromatase substrate in the presence or absence of either FSH/LH depending on the cell type. Steroid levels (E2, Androstenedione, 17-OH-P and P) were measured in the spent medium using RIA. KGN cells were also cultured in a similar manner with VD concentrations in the presence or absence of forskolin [25µM]. Aromatase mRNA expression was assessed by qPCR and aromatase promoter II activity by transfection with CYP19A1 reporter construct. KGN cells were also cultured with insulin [10ng/ml and 100ng/ml] ± VD doses and the effect on mRNA expression of insulin receptor and AMH assessed by qPCR. KGN protein lysates were immunoprecipitated with anti-VDR antibodies to pull-down all existing forms of VDR (homodimers, heterodimers and solo). The immunoprecipitants were then immunoblotted with anti-RXR antibody to assess the heterodimer association between VDR-RXR in the
presence of different concentrations of VD ± forskolin (to activate cAMP-dependent signalling pathways).

**Main results and the role of chance:** VDR mRNA significantly increased in human theca from larger antral follicles (LAF) (7-12mm, n=11) compared to theca from smaller antral follicles (SAF) (5-6mm, n=4) (unpaired t-test *p=0.013). Additionally, VDR mRNA and protein was detected in both cortex and stroma from normal and PCO. *CYP27B1* (the enzyme that makes active 1,25-(OH)$_2$D3) mRNA expression was detected in stroma tissue, showing the human ovary can make local active 1,25-(OH)$_2$D3; however, the expression was extremely variable. Cell culture experiments demonstrated that 1,25-(OH)$_2$D3 was able to suppress androstenedione production in theca taken from LAF but not SAF (n=4-8; 2-way ANOVA, p=0.048 for treatment and p=0.0018 for follicle size). 1,25-(OH)$_2$D3 had no effect on either 17-OH-P or P production from theca cells. Likewise, 1,25-(OH)$_2$D3 had no effect on either basal or FSH-mediated E2 production from unlu teinised GC taken from AF. Surprisingly, 1,25-(OH)$_2$D3 significantly reduced both basal and LH-stimulated E2 production from luteinized GC (ANOVA p=0.0001; post-hoc t-test **p<0.005). Investigation of the mechanism behind this reduction in E2 production using the KGN-GC showed that extremely low doses of 1,25-(OH)$_2$D3 [0.02 and 0.2nM] significantly down-regulated forskolin-stimulated aromatase expression (n=6, One-way ANOVA **p=0.0018; post-hoc multiple t-test *p<0.05, **p<0.005). As higher doses [2 and 20nM] this attenuation was reversed. This pattern was also replicated using KGN cells transfected with a *CYP19A1-PII-516* reporter construct, showing that 1,25-(OH)$_2$D3 affected aromatase activity as well as expression (n=3; ANOVA****p<0.0001; post-hoc multiple t-tests for significance between treatments). Chronic exposure of KGN-GC to equivalent hyperinsulimemic doses [100ng/ml], significantly down-regulated insulin receptor mRNA expression, which was markedly potentiated by both lowest and highest doses of 1,25-(OH)$_2$D3 (n=5=8; One-way ANOVA*p=0.048; post-hoc t-tests *p<0.05, **p<0.005). Both doses of 1,25-(OH)$_2$D3 also reduced expression of insulin receptor in the presence of post-prandial levels of insulin; indicating the mechanism by which VD deficiency may reduce insulin sensitivity. Intriguingly, 1,25-(OH)$_2$D3 also further reduced the forskolin-induced downregulation of AMH mRNA expression (n=3; ANOVA *p=0.02; post-hoc t-test *p<0.05). Immunoprecipitation experiments showed a clear difference between levels of VDR associated with RXR in the presence of forskolin, compared with basal or VD ligands.

**Limitations, reasons for caution:** Ideally, we would have used primary unlu teinised GC and theca cells taken from various-sized follicles for the studies on the mechanism of action of 1,25-(OH)$_2$D3 and the VDR; but human ovaries are by necessity in short supply hence the use of the luteinized GLCs and KGN–GC line. By its nature human primary cells and tissues display considerable biological variability normally requiring large numbers for statistical analysis, which is not feasible due to the scarcity of such tissue. It is difficult to extrapolate from serum measurements of 25-(OH)-D3, the exact levels that the ovary would be exposed to in hypovitaminosis; hence the range chosen errred towards the lower end of VD deficiency.
**Wider implications of the findings:** Numerous observational and intervention studies have been conducted on VD deficiency and various reproductive outcomes, with contradictory results. Whilst other *in vitro* studies (Merhi *et al.*, 2014) have investigated effects of normal levels of vitamin D on steroidogenesis, by focusing on deficient levels our data has contributed significantly to understanding the mechanism of action of 1,25-(OH)$_2$-D$_3$ at a cellular level and its interaction with its receptor. We have shown for the first time an increase in VDR expression in theca of larger follicles, which along with its ability of 1,25-(OH)$_2$-D$_3$ to decrease AMH expression, supports a role for 1,25-(OH)$_2$-D$_3$ in follicle progression. Uniquely we have shown a direct effect of deficient levels of 1,25-(OH)$_2$-D$_3$ on inhibiting androstenedione production from human thecal cells; which together with a reduction in LH-mediated E2 production could contribute to anovulation seen in women with PCOS and vitamin D deficiency. Human thecal and unluteinised GC are incredibly hard to obtain and in short supply for research purposes, highlighting the uniqueness of our data set. For the first time, we have also discovered a mechanism accounting for the numerous studies showing a correlation between low levels of 1,25-(OH)$_2$-D$_3$ and reduced insulin sensitivity i.e. by reducing insulin receptor expression. This would have implications for treating women with PCOS, insulin resistance and vitamin D deficiency. The ability of the ovary to make local bioactive 1,25-(OH)$_2$-D$_3$ via expression of *CYP27B1*, could also explain the contradictory outcomes of clinical reproductive studies that rely on measurements of systemic VD levels. Encouragingly our data indicated that women (apart from those with PCOS and insulin resistance) must become severely deficient in VD before it impacts on their ovarian function.

**Introduction**

Vitamin D (VD), is a fat soluble prohormone with a plethora of activities ranging from its classical role in calcium homeostasis and skeletal integrity (Dusso, 2005) to anti-inflammatory and immunosuppressive actions (Aranow, 2011). The discovery of vitamin D receptors (VDR) on male and female gonadal cells prompted investigations into the role of vitamin D in reproduction and fertility (reviewed in Lerchbaum & Obermayer-Pietsch, 2012; Lorenzen, 2017).

VD is derived minimally from the diet in the form of vitamin D$_2$, with the major source being the photolytic conversion of 7-dehydrocholesterol in the skin to cholecalciferol (vitamin D$_3$) catalysed by UVB radiation (reviewed in Bouillon *et al.*, 2008). Both forms of VD are biologically inert and require activation through sequential hydroxylation in the liver and kidneys. The initial hydroxylation in the liver by 25-hydroxylase enzyme (encoded by *CYP2R1*) results in 25-hydroxyvitamin D$_3$ *aka* calcidiol (25-(OH)-D$_3$), an inert stable metabolite that circulates bound to VD-binding protein (VDBP). The stability of the bound circulating 25-(OH)-D$_3$ means that it is used as an indicator of VD status in an individual (Holick, 2007). 25-(OH)-D$_3$ is then further metabolised to the biologically active form 1,25-(OH)$_2$-D$_3$ (*aka* calcitriol), primarily in the kidneys, a reaction catalysed by the mitochondrial
enzyme 1α-hydroxylase (encoded for by CYP27B1) (Schuster, 2011); (Luk J, 2012). 1-α-hydroxylase has also been found in extra-renal tissue indicating local conversion of 25-(OH)-D3 to the active 1α,25-(OH)2-D3.

The ability of active 1α,25-(OH)2-D3 to exert its profound actions is mediated exclusively by VDR, a member of the nuclear hormone receptor super-family. In addition to its non-genomic actions outside of the nucleus, VDR acts as a ligand-inducible transcription factor (Haussler MR, 2013), enabling it to regulate 3% of the human genome (Holick, 2007). Normally 1α,25-(OH)2-D3 enters the cell by diffusion where it binds to and activates VDR to form a heterodimer complex with retinoid X receptor (RXR): this complex interacts with VD response elements (VDREs) found in the promoter regions of both positively and negatively controlled genes (Chesnksis & Freedman, 1994). The localization of VDR to numerous cell types highlights the importance of 1,25-(OH)2-D3 as a regulatory hormone in many systems; consequently, VD deficiency is recognized to be associated with numerous pathological conditions which is usually reversed upon supplementation (Holick MF et al, 2011;2012).

Current guidelines define VD deficiency as 25-(OH)-D3 levels <20ng/ml (or <50nmol/L); with levels of 21-29ng/ml (52.5-72.5nmol/L) characterized as VD insufficiency and >30ng/ml (>75nmol/L) considered as sufficient (Holick MF et al, 2011; 2012) (Pilz S, 2019). Due to avoidance of direct exposure to sunlight and/or nutrient-deficient diets, global deficiency of VD is commonplace in both men and women, but this is exacerbated in northern climates due to lack of sunshine.

The first indication that VD affected female fertility was shown by VD-deficient rats with reduced fertility rates and litter sizes (reviewed in Lorenzen et al, 2017). VD has been shown to interact and regulate steroidogenic enzymes that are crucial for human granulosa and cumulus cell function (Merhi Z et al, 2014). Interestingly, genes such as insulin receptor (InsR), anti-Müllerian hormone (AMH) and CYP19A1 (encoding aromatase) that play a critical role in folliculogenesis have VDRE on their promoters (Tiejun S et al, 1998); (Maestro B et al, 2003); (Krishnan AV et al, 2007 & 2010), strongly supporting a role for VD in female fertility. Furthermore, there is a growing body of evidence that VD has a role to play in fertility outcomes in assisted reproductive technology and in women with polycystic ovary syndrome (PCOS), but the results are conflicting (reviewed Lorenzen M. B., 2017).

PCOS is well-recognised as one of the most common endocrine disorders in women, manifesting with increased androgen production, irregular menstrual cycles, and dysregulated ovarian function, including an increase in antral follicle numbers associated with increased AMH; but often a lack of ovulatory follicle selection in a sub-set of women (Pellatt L et al, 2010). Additionally, there is a clear link between vitamin D, glucose clearance and insulin secretion, such that insulin sensitivity is reduced in vitamin D-deficient women with PCOS, sometimes independently of obesity (Łagowska et al, 2018). The compensatory hyper-insulinaemia is known to further amplify the increased thecal androgen secretion in these ovaries (Nestler J.E., 1998) and vitamin D replacement has been shown to reduce
serum androgens in some cases (Menichini D., 2020). Interestingly this reduction in hyperandrogenemia can occur in the absence of changes in insulin sensitivity, suggesting a possible direct mechanism of action of VD (reviewed in Menichini D., 2020).

Women with PCOS have lower serum 25-(OH)-D3 levels compared to fertile controls (Krul-Poel YHM et al, 2018) (Eftekhar M et al, 2020) and yet studies investigating the effects of VD deficiency/VD levels on reproductive outcomes produce extremely variable results (reviewed in Lorenzen M. et al, 2017). Whilst this could be due to factors such as differing study design, low participant numbers, different ethnicities etc, there is also a lack of understanding of the mechanistic actions of VD in ovarian physiology, and in the effect and outcomes of varying levels of VD ligand interaction with its receptor.

The aim of our study was to investigate the function of ovarian cells in a low vitamin D environment (i.e., ≤20nM), thereby providing mechanistic insight to account for the variable reproductive outcomes observed clinically in women with deficient serum levels of VD.

Materials and Methods

All reagents were obtained from Sigma, Poole, UK, unless stated otherwise, and all plasticware was purchased from Fisher Scientific, UK.

Tissue samples for in vitro experiments

A variety of cells and tissues were used for the in vitro experiments of this study as detailed below.

Human Ovarian Tissues: Informed written consent was obtained from women undergoing trans-abdominal hysterectomy with bilateral oophorectomy for benign gynaecological conditions at St George’s Hospital, London and St Luke’s Hospital, Malta. Ethical approval was granted by the relevant committee in each institution and informed consent obtained. Clinical details were obtained including age, gynaecological history, menstrual frequency, and day of cycle. The ovaries removed from each patient were seen by a pathologist before a portion of each was taken to the laboratory for dissection. Morphology and ovulatory status were assigned as previously published and were based on ovarian size, follicle sizes and numbers, the presence of a dominant follicle or corpus luteum and the amount and density of stroma as determined by dissection, in conjunction with patient history (Mason HD et al, 1994; Gilling Smith C et al, 1994). The timing of surgery was random. Patient details, number of follicles obtained, and tissue used in each experiment are shown in Table 1. Follicles were isolated from the surrounding stroma, the diameter measured, and granulosa cells (GC) collected as previously described (Mason H. D. et al, 1990; 1996). Finally, the theca cell layer was carefully peeled off and digested in an enzyme cocktail for 30mins at 37C with gentle agitation (Mason H. D. et al, 1990; 1996). GC and theca cells were cultures as outlined in subsequent sections, other ovarian tissue samples were immersed in RNA-later or flash-frozen and archived at -80C for further analysis.
Luteinised granulosa cells (GLC) were obtained from follicular aspirates obtained during oocyte retrieval from women undergoing in-vitro fertilisation (IVF) treatment (see introduction) at various assisted conception units including those at King’s College Hospital and The Lister Hospital. Local ethics committee approval for each unit was sought and informed consent was obtained from all women.

KGN granulosa cell (KGN-GC) line, that are established to correspond to immature granulosa cells from smaller antral follicles, were used to provide mechanistic insight (Nishi et al., 2001). The distribution of VDR and AMH protein in various ovarian tissue compartments was assessed using ovaries from mice (strain 129Sv) aged between 4 and 5 months. These were euthanised as part of separate ethically approved projects at SGUL and ovaries donated to this study.

Immunohistochemistry of AMH and VDR in mouse ovarian sections.

Ovaries were fixed in paraformaldehyde (4%) at 4C, dehydrated in a gradient ethanol series, cleared in methyl salicylate, embedded in wax and 5μm serial sections cut. Every 3rd section was stained with haematoxylin and eosin (H&E). Adjacent sections were used for immunohistochemistry with anti-VDR (1:100, rat) and anti-AMH (1:50, mouse) monoclonal antibodies (Abcam, Cambridge, UK) as per manufacturers’ protocol with modifications.

Briefly, slides were dewaxed and rehydrated, and the antigen epitopes exposed by heating the slides in a 0.01M citrate buffer (pH 6, National Diagnostics) bath until boiling point for 20 minutes. Peroxidases were blocked by 3% hydrogen peroxide (VWR) in methanol (National Diagnostics) for 10 minutes. Sections were then blocked with relevant anti-serum to reduce non-specific binding for 1h at room temperature. Slides were then incubated with the relevant primary antibody overnight at 4C, followed by 1-hour incubation at room temperature with 0.1% monoclonal biotin-labelled secondary antibody (Vector) (Table 2). Visualisation was carried out with Avidin Biotin Complex (ABC) peroxidase solution followed by 3,3’-diaminobenzidine (DAB) (Vector Laboratories). A haematoxylin counterstain was also used for 10 seconds to label nuclei blue. After a brief dehydration through an ethanol series, slides were mounted using Histomount (National Diagnostics). Negative controls omitted primary antibody. Images were captured using the Hamamatsu NanoZoomer 2.0-Rs Slide Scanner for analysis with the NDP.view 2 software (courtesy of the Image Resource Facility, SGUL).

mRNA and protein expression of VDR in ovarian tissue/cellular compartments

The mRNA expression of VDR in human ovarian cortex, stroma and theca tissue compartments was assessed using archived frozen tissue collected as described above. All tissue was stored and used under HTA regulations. Tissues were defrosted on ice and homogenised in lysis buffer (RLT lysis buffer, Qiagen, Netherlands) using the FastPrep® TissueLyser 24 and the FastPrep® Lysing Matrix A 2mL tubes containing a garnet matrix and a ceramic sphere (MP Biomedicals). After homogenisation RNA was extracted, reverse transcribed and real-time quantitative PCR (qPCR) performed for VDR relative to L19 (the
reference gene), as previously described (Rice S et al, 2006). In addition, the expression of VDR in the KGN-GC was established using standard PCR as well as qPCR (see Table 3 for details of primers and cycling conditions). For protein extraction the tissue was homogenised in RIPA buffer with a cocktail of phosphatase inhibitors (Sigma-Aldrich, Merck, Gillingham, Dorset), using a sonicator three times for 30s each time. Samples were maintained on ice throughout to prevent overheating and denaturation of proteins. The lysed samples were micro-centrifuged at 4C for 10min at maximum speed. Pelleted debris was discarded, and the protein lysate stored at -80C prior to Bradford assays (for measurement of protein concentration) and western blotting.

**GC, theca and GLC culture experiments**

The granulosa or theca cells were pooled from several follicles based on the experimental protocol and cultured in McCoys 5A medium supplemented with penicillin/streptomycin, L-glutamine and amphotericin B (all purchased from Invitrogen, UK). Only cells from those follicles appearing healthy were used. Granulosa cells were plated in 96-well plates at 5x10⁴ and theca cells at 5x10⁵ cells per well. To increase the adhesion of theca cells to the plate, plates were first coated in ECM gel (McCag, 2002).

Cells were incubated in culture medium (plus experimental treatments) for 48 hours with a range of 1,25-(OH)₂-D₃ concentrations (0.2, 2 and 20nM serially diluted down from a stock concentration of 2µM). Testosterone (5x 10⁻⁷ M) was used as a substrate for conversion to oestradiol in the granulosa cells and steroid levels measured in the medium by radioimmunoassay (Hillier SG et al, 1980); (Gilling-Smith C et al, 1994); (Willis DS et al, 1996). To confirm that cells were healthy, 10ng/ml LH was used as a positive control in luteinised GCs and theca, and 5ng/ml FSH in cells dissected from small follicles (LH and FSH supplied by Endocrine Services, Biddeford-upon-Avon, UK). Isolation of GLC was performed as previously described (Wright R et al, 2002). Cells were plated in 96-wells at 5x10⁴ cells per well in 200µl of serum (5%) supplemented medium for 48 hours, after which the medium was removed and replaced with experimental treatment as detailed above. GLCs were also exposed to the vitamin D analogue ED1089 (kindly donated by Dr. Kay Colston, SGUL), to examine comparative efficacy and demonstrate specificity.

**KGN-GC culture experiments**

To model the effect that VD deficiency may have on ovarian function, KGN-GC were grown and passaged in 10% DMEM-F12 supplemented with L-glutamine and penicillin/streptomycin (Invitrogen), at 37ºC in 95% air/CO₂. Cells were plated in 12-well plates (3x10³ cells/well) and cultured in 1% DMEM-F12 (charcoal-stripped) overnight. Cells were serum-starved for 2h and then treated with forskolin (25µM), to reproduce the effect of LH on cAMP stimulation, ± 1,25-(OH)₂-D₃ at 0.02, 0.2, 2 and 20nM and cultured for a further 48h. Testosterone (5x10⁻⁷M) was added to all cultures as the aromatase substrate for conversion to oestrogen. Similarly, the effect of VD on AMH mRNA expression was investigated in cells treated with forskolin and VD as described above, using only the upper and lower doses of 1,25-(OH)₂-D₃ from the range above at 0.02 and 20nM. At the end of the
relevant culture time, RNA was extracted, reverse transcribed and real-time quantitative PCR (qPCR) performed for CYP19A1 (encoding aromatase) as described previously (Rice S et al., 2013).

To investigate the effect of VD on aromatase promoter II (PII) activity, cells were plated at a density of 2x10^4 cells/well in 96-well plates. After overnight incubation, they were transfected with a CYP19A1 PII-516 reporter construct expressing firefly luciferase, along with a control plasmid expressing Renilla luciferase from a constitutive promoter, and a transcription enhancing element, PVAi, as previously described (Rice S et al., 2013). After 2h serum-starvation, cells were treated as described above for 24 hours with quadruplicate replicates/treatment, and luciferase reporter assays were carried out using the Dual-Glo Luciferase Assay System (Promega).

To mimic a situation of chronic insulin exposure, cells were treated with insulin for 48h at post-prandial (10ng/ml) and hyperinsulinemic (100ng/ml) levels in the presence and absence of the lowest [0.02nM] and highest [20nM] 1,25-(OH)_2-D3 levels tested in the experiments. The effect of 1,25-(OH)_2-D3 on aromatase and insulin receptor (InsR) mRNA expression was measured by qPCR.

**Radioimmunoassay (RIA) for P, E2 and Androstenedione**

Conditioned media was collected from the cells at 48 hours, frozen at -20°C and E2, P and androstenedione measured using a modified ‘in-house’ RIA, with tritiated steroids and charcoal separation (Hillier SG et al., 1980); (Gilling-Smith C et al., 1994); (Willis DS et al., 1996). The components of the assay were: tritiated steroid label (Amersham Pharmaceuticals, Bucks, UK), steroid antiserum (sheep anti-human from Guildhay, Guildford, UK) and standards and quality controls (QCs) (approximately 1, 6 and 35% of the top standard), prepared from powdered steroid (Sigma Co Ltd, Poole, UK). Conditioned medium was diluted to be within the midrange values for each assay and therefore on the linear part of the standard curve. Each sample was tested in duplicate.

**Immunoprecipitation**

To investigate the effect of 1,25-(OH)_2-D3 on VDR:RXR dimer formation, KGN cells were plated at a density of 5x10^5 cells/well in a 6-well plate and treated as described above with and without forskolin, testosterone and 1,25-(OH)_2-D3 at 0.02 and 20nM for 48h. At the end of culture time, media was removed, cells washed with ice-cold PBS and lysed with ice-cold RIPA buffer as previously described (Dilaver N et al., 2019). The protein concentration of the lysate was measured by Bradford assay and prior to immunoprecipitation, equal amounts of protein lysate from each treatment group were pre-cleared on the A/G-coupled Sepharose beaded (Pierce) support for one hour at 4°C to reduce non-specific ligand binding. Immunoprecipitation of the cleared lysates was performed overnight at 4°C with 5μg of anti-VDR antibody or anti-RXRα monoclonal antibodies (Abcam – Table 2). The beaded complexes were washed several times with NP-40 lysis buffer, after which protein complexes were dissociated by boiling for 10 mins with 1X SDS-DTT reducing buffer.
Western Blotting of VDR and RXR

Protein concentrations were measured using the Bradford assay (BioRad, Hertfordshire) and equal amounts of protein obtained from lysing ovarian tissues or from immunoprecipitation experiments, were resolved with Western blotting as previously described (Dilaver N et al, 2019). PVDF(FL) (Immobilon, Millipore Merk, Massachusetts) membranes with transferred proteins were incubated with rat anti-human VDR or rabbit anti-human RXR (both used at 1:1000) and/or mouse anti-human β-actin (1:2000). Fluorescently conjugated relevant secondary antibodies (1:5000) (see Table 2 for details of antibodies) were used for visualisation using the Odyssey Imaging System (Li-Cor Biosciences) (Dilaver N et al, 2019).

Experimental Quantification and Statistical Analysis

All data are represented as the mean ± SEM of triplicate or more observations (detail in legends) from a minimum of 3 or more independent experiments, unless otherwise stated. qPCR data were analysed using the ΔΔCt method as described in detail previously (Rice S et al, 2006), with normalisation to L19 and subsequent normalisation to the Ct value of the control (untreated). To use the ΔΔCt method, the amplification efficiency for each GOI and reference gene must be in the recommended range of 90-100%. This was rigorously applied to our study by the inclusion of a standard curve for every qPCR assay conducted. Data from Western blots represent the mean densitometry measurements taken from all individual experiments using Image Studio software (Licor™) and normalised to β-actin (loading control) and where relevant, to the untreated (control) samples. Results for steroid RIA were calculated using Assay Zap V2.69 software (Biosoft, Cambridge, UK). Intra- and inter-assay coefficients of variation were below 5% and 6% respectively. Statistical significance was determined by ANOVA followed by post hoc multiple comparison tests; unpaired Student’s or paired t test when 2 groups were compared (depending on the design of the experiment) or a one-sample t test when comparing with normalised control values, using GraphPad Prism™. Significance was set at P ≤ 0.05.

Results

Expression of VDR in ovarian tissue and cellular compartments

IHC was used to detect the extent of VDR protein expression in sequential mouse ovary sections in which an anti-VDR antibody revealed strong and extensive staining throughout all mouse ovarian tissue compartments including the cellular components of the follicles (figure 1a and 1b). Closer examination revealed more intense staining in thecal cells of LAF compared to SAF (inset figures 1b). The general uniformity and ubiquity of VDR expression was in marked contrast to staining with anti-AMH antibody in the sequential section, which showed a distinctive pattern of expression i.e., greater staining in GC of SAF compared to...
LAF, with no staining in theca or ovarian compartments (figure 1c). This also demonstrated the rigour of the IHC protocol.

This finding in the mouse ovary was extended by measuring VDR mRNA levels in theca cells dissected from human follicles of varying sizes to ascertain whether expression levels changed with follicle size and to determine if there was a difference between tissue taken from normal and polycystic ovaries. VDR mRNA levels were significantly higher in theca from larger antral follicles (7-12mm, n=11) and those approaching preovulatory sizes, compared to those from smaller antral follicles (5-6mm, n=4) (unpaired t-test, *p=0.013; figure 2a). A further analysis of data comparing theca from normal and PCO, revealed a trend for PCO follicles to express more VD mRNA but this did not reach statistical significance as numbers were small (figure 2b). In addition, VDR mRNA and protein expression were also detected in human cortex and stroma (fig 2c and 2d respectively). There appeared to be increased levels in cortex compared to stroma, but levels were quite variable. VDR protein expression was detected in both cortex and stroma from normal and polycystic ovaries (both ovulatory and anovulatory) (fig 2e), however there were insufficient samples numbers to conduct statistical analysis. We also saw higher levels of VDR protein in human theca from a 17mm follicle compared to a whole 6mm follicle (fig 2e), supporting the trend seen with human mRNA expression (fig 2a) and in the mouse ovary (fig 1b).

Expression of CYP27B1 in human ovarian tissue

CYP27B1 mRNA was expressed in ovarian stroma, but there was a wide variation in CYP27B1 mRNA expression in individual samples of stroma from normal or polycystic human ovaries (figure 2f). There was however a trend for higher levels in stroma from normal when compared to polycystic ovaries, though this did not reach statistical significance.

Effect of 1,25(OH)2-D3 on steroid production in ovarian cells.

VD and theca steroidogenesis: Overall, in theca from large follicles (15-22mm), all doses of 1,25-(OH)2-D3 consistently suppressed Androstenedione production (mean 22% suppression) but had no effect on androstenedione production from small follicles (n=4-8, Two-way ANOVA, p=0.048 for treatment and p=0.002 for follicle size) (fig 3a). 1,25-(OH)2-D3 had no effect on either 17-OH-P or P production (supplemental figures S1 and S2).

VD and GC steroidogenesis: There was no effect of 1,25-(OH)2-D3 at any dose on E2 production of GCs taken from follicles <10mm diameter (n=4; figure 3b); however, in follicles >10mm there appeared to be a trend to increasing E2 with 1,25-(OH)2-D3 though this was not significant (figure 3b). In addition, 1,25-(OH)2-D3 did not alter the normal responsiveness to FSH in SAF (n=4, ANOVA p<0.0001) (figure 3c).
**VD and E2 production in GLC:** The effect of VD on E2 production from luteinised granulosa cell culture was then investigated. As expected, exposure of the cells to LH produced a substantial stimulation in the production of E2, which surprisingly was considerably attenuated by 1,25-(OH)\(_2\)-D3 at both 2nM and 20nM doses (figure 3d) (ANOVA p<0.0001; post-hoc t-test **p<0.005). This contrasts with the unluteinised GC from small follicles where 1,25-(OH)\(_2\)-D3 had no effect on FSH-stimulated E2 production (figure 3c).

Interestingly culturing GLC with the VD analogue EB1089 also appeared to suppress E2 production in a dose-related manner (figure 3e), though the data did not reach statistical significance (ANOVA n/s). Exposure of GLCs to all low doses of VD had no effect on progesterone production (supplemental figure S3).

**Effect of VD on aromatase expression in KGN-GC:** To determine the mechanism by which 1,25-(OH)\(_2\)-D3 was altering E2 production in primary cells, KGN cells were cultured with forskolin to increase cAMP and hence aromatase activity (equivalent to stimulation by either gonadotrophin). Very low 1,25-(OH)\(_2\)-D3 levels [0.02 & 0.2nM] significantly down-regulated Fsk-stimulated aromatase mRNA expression (n=6, One-way ANOVA**p=0.0018; post-hoc t-tests *p<0.05, **p<0.005), but with doses approaching sufficiency [2 and 20nM] this attenuation was reversed. An identical pattern was seen in PII transfected KGN cells demonstrating that 1,25-(OH)\(_2\)-D3 affected aromatase activity as well as expression (figures 4a & 4b) (n=3; ANOVA****p<0.0001; post-hoc t-tests *p=0.02, **p=0.0095).

**Effect of VD on other factors implicated in regulation of follicle growth.**

**Insulin:** There is compelling evidence that systemic VD levels are correlated with insulin sensitivity (Łagowska et al, 2018); however, there is little supportive evidence at a cellular level. Chronic exposure of granulosa cells to high (100ng/ml) insulin significantly downregulated expression of total InsR mRNA (p=0.001), and this reduction was surprisingly potentiated by both doses of 1,25-(OH)\(_2\)-D3: 20nM (<50% of basal) and 0.02nM (<25% of basal) (n=5-8; One-way ANOVA *p=0.048, one-sample t-tests to the control ***p<0.0005, **p<0.005, *p<0.05) (figure 5a). This profound attenuation of insulin receptor expression by 1,25-(OH)\(_2\)-D3 in the presence of chronic insulin exposure did not have a concomitant reduction on aromatase expression in the same cells. In fact, the presence of insulin reversed the attenuation of basal aromatase levels brought about by low dose VD (figure 5b).

**AMH:** Forskolin down-regulated AMH expression compared to basal (<50%), which was further potentiated in the presence of 1,25-(OH)\(_2\)-D3, with a more potent attenuation by 20nM 1,25-(OH)\(_2\)-D3 compared to 0.02nM (figure 5c) (n=3, ANOVA *p=0.02; multiple comparison t-test *p<0.05).

**Investigating the differential effects of varying doses of VD ligand.**

Data presented so far indicate that those doses of 1,25-(OH)\(_2\)-D3 equivalent to extremely deficient levels have differential effects compared to those approaching sufficiency. To
determine a possible mechanism, the relationship between levels of VD ligand and the
proportion of either VDR or RXR was investigated. KGN-GC were treated with extremely low
or sufficient doses of 1,25-(OH)$_2$-D3 in the presence of Fsk, immunoprecipitated with anti-
VDR antibodies, to pull down all forms of VDR: homodimers, heterodimers and solo. The
immuno-precipitated samples were then immunoblotted for RXR to assess only the
heterodimer association between RXR and VDR (figure 6a). There was a clear difference in
levels of VDR associated with RXR in the presence of Fsk compared to basal or either 20nM
of 0.02nM 1,25-(OH)$_2$-D3, but no measurable difference between the two doses of 1,25-
(OH)$_2$-D3. (figures 6a, n=4).

**Discussion**

We have discovered widespread distribution of VDR in the human ovary, pointing to a likely
critical role for VD in female reproduction. We have demonstrated for the first time, a clear
relationship between increasing VDR expression and follicle size, in that both VDR mRNA
and protein levels were significantly up-regulated in human theca taken from large AF (7-
12mm) compared to smaller AF (5-6mm), highlighting the importance of VDR in antral
follicle progression.

Our findings in human theca were supported by the results of histological analysis of mouse
ovarian follicles showing an increase in protein levels of VDR in theca as follicles progress
from early antral through to pre-ovulatory stages. That activation of these receptors is
playing a role in follicle growth was demonstrated by the fact that VD3 supplementation
promoted the survival and growth of *in vitro* cultured preantral follicles to the antral stage
in the primate ovary (Xu J, 2018). Our finding of protein expression of VDR in human cortex
and stroma was also matched by extensive and uniform expression of VDR protein in
granulosa cells, cortex, and stroma of mouse ovaries, further highlighting the importance of
VD throughout the ovary. This is also supported by the fact that female VDR knockout mice
have impaired folliculogenesis with no progression beyond primary and secondary stages
and low oestradiol levels (Yoshizawa T, 1997).

The detection of *CYP27B1* mRNA expression in human ovarian stroma was intriguing as it
indicated that the human ovary is capable of being an extra-renal site of active 1,25-(OH)$_2$-
D3 synthesis, as supported by studies in other species (Xu J *et al*, 2018). This local
contribution of bioactive 1,25-(OH)$_2$-D3 is difficult to estimate and account for, especially
with respect to observational clinical studies that measure systemic levels of VD. Active
1,25-(OH)$_2$-D3 negatively regulates expression of *CYP27B1*, hence regulating its own
circulating concentrations (Bouillon *et al*, 2008).

This increasing expression of VDR in the growing follicle at the LAF stage corresponds to
increasing steroidogenesis and the acquisition of LH receptors. It had been documented that
incubation of human cumulus GC with VD at normal levels affects steroid output (Merhi *et
al*, 2014), but we wished to investigate the extent of and mechanisms by which incubating
cells in a 1,25-(OH)$_2$-D3-deficient environment affected follicle growth and steroidogenesis. To that end we incubated cells with 20, 2, 0.2 or 0.02 nmol/L (since serum levels <50nmol/L is defined as severe deficiency) (Holick MF et al, 2011). A recent study of women of childbearing age in rural northern China found the prevalence of severe VD deficiency was 16% in the 1151 women studied, with a median serum level of only 5.63ng/ml (equivalent to 14nmol/L) (Lin S et al, 2021); indicating that severe hypovitaminosis of this magnitude occurs in the population. Interestingly, 1,25-(OH)$_2$-D3 is established to act at nanomolar, or even picomolar, concentrations, as a direct regulator of specific target genes in VDR-expressing cell and tissues. It also binds to VDR with an affinity of 0.1nM (reviewed in Carlsberg, 2022).

In GC, gonadotrophins stimulate oestrogen synthesis via cAMP-mediated signalling, and it is well-established that a cAMP-response element (CRE) sequence has been identified within promoter II (PII); the predominant aromatase promoter used in the ovary (Michael MD, 1997). Changes in the 1,25-(OH)$_2$-D3 environment had no effect on E2 production from GC of either small or large AFs, in the presence or absence of FSH and FSH responsiveness was retained. Conversely in luteinised granulosa cells (GLCs), 1,25-(OH)$_2$-D3 did prevent the LH-mediated stimulation of E2 production. It is without doubt that 1,25-(OH)$_2$-D3 can decrease E2 production as the same effect was observed with the VD analogue in GLCs. This would imply that attenuation by VD only occurred when GC acquired LHR or else is dependent on levels of cAMP generated, since LH stimulates more cAMP activity than FSH (Aharoni D et al, 1995). This is supported by the observation that extremely low doses of 1,25-(OH)$_2$-D3 down-regulated forskolin-stimulated aromatase expression and activity in KGN cells (which do not have LHR), an effect that was lost once dosing levels were increased.

This contrasted with theca, where a low 1,25-(OH)$_2$-D3 significantly attenuated androstenedione production from theca of LAF (15-22mm) but not of SAF (<10mm). Surprisingly, 1,25-(OH)$_2$-D3 had no effect on progesterone or 17-OH-P production from theca of all sized follicles indicating that this inhibitory action presumably occurs in the CYP17 pathway, but only in large follicles when they have upregulated VDR expression. Like observations in theca, there was no effect of 1,25-(OH)$_2$-D3 on progesterone production from GLCs.

The bi-phasic dose-response effects of 1,25-(OH)$_2$-D3 on aromatase expression, was a direct influence on the transcriptional activity of PII, as seen in the PII transfection assay results. Analysis of PII has revealed the presence of two VDRE - proximal and distal, with an overlap between the proximal VDRE and CRE (Krishnan AV et al, 2010). A key factor could be the mechanism of ligand-binding of VD to its receptor. VDR functions as both a monomer, homodimer, and a heterodimer with RXR, and the alteration of the ratio of these complexes within a cell is dependent on the amount of VD ligand present (Chesksis B et al, 1994). Depending on the proportion of each, these complexes will bind to VDRE on genes and
attract either co-repressors/activators to enhance or suppress gene expression and activity. Increased levels of VD decreased the amount of DNA-bound VDR homodimer complexes and promote the formation of VDR-RXR heterodimers (Carlsberg C, 2022; Cheskis B et al, 1994; Haussler MR et al, 2013).

Unliganded VDR-RXR heterodimers are initially bound to a VDRE and recruit co-repressor complexes, which prevents basal transcription through the activity of histone deacetylase. Once sufficient ligand has bound, the repressors are substituted by co-activator complexes, allowing gene transcription to commence (Carlsberg C, 2022; Dwivedi PP et al, 1998; Perissi V et al, 2010). This was to some extent corroborated by the immunoprecipitation studies which indicted a greater ratio of VDR:RXR at basal levels, with alterations in association in the presence of forskolin and compared to decreasing quantities of 1,25-(OH)_{2}-D3 ligand. It is not possible from our experiments to determine the proportion of heterodimers in the cytoplasmic or nuclear compartments as we used whole cell lysates. To answer this, we would need to carry out further experiments (beyond scope of this study) to explore VDR sub-cellular trafficking between nuclear and cytoplasmic compartments and components of the repressor/activator complexes bound to chromatin. Interestingly, the ability of forskolin to alter the association of the intracellular dimers would indicate the presence of a ligand-independent cAMP activated pathway outside the nucleus (Luk J et al, 2012); (Haussler MR et al, 2013) which could account for the different outcomes observed when using either FSH or LH.

Interestingly, theca from PCO follicles expressed higher levels of VDR mRNA than those from normal ovaries, though this was not statistically significant. This apparent up-regulation in VDR expression could be in response to VD deficiency, which is commonly prevalent in women with PCOS, as seen in population cohort studies (Krul-Poel YHM et al, 2018); (Wang L et al, 2020). There is also evidence for autoregulation of VDR by 1,25-(OH)_{2}-D3 via VDRE on the VDR gene itself (Zella LA et al, 2007).

As stated earlier, women with PCOS commonly present with increased serum levels of AMH (reflecting the pool of stalled SAF) and hyperinsulinemia, both of which are also linked to VD status (Luk J, 2012; Lorenzen M. B., 2017). To investigate this link, we replicated hyperinsulinemia and insulin resistance in vitro by chronically exposing KGN cells to insulin at either post-prandial (10ng/ml) or hyperinsulinemic (100ng/ml) doses. The HI dose downregulated expression of insulin receptor mRNA by more than 50% reproducing insulin resistance, whereas there was no significant effect noted at post-prandial treatment levels. To our surprise, culture of cells in an extremely low and deficient VD environment caused an even further attenuation of insulin receptor expression in the presence of both doses of insulin. This effect only occurred in the presence of insulin, as 1,25-(OH)_{2}-D3 alone had no effect on insulin receptor expression. Systemic VD deficiency is clearly linked to a reduction in insulin sensitivity (independent of BMI) as others have shown (Muscogiuri G et al, 2017), and this insulin receptor reduction may be a contributory mechanism. Interestingly, in the same cells a combination of insulin and 1,25-(OH)_{2}-D3 had no effect on aromatase, unlike in
the forskolin experiments, again indicating that this was a cAMP-driven process, possibly linked to overlap between VDRE and CRE on PII as described previously.

It is well established that follicle progression in the normal human ovary requires down-regulation of AMH expression to permit FSH-driven activity (Pellatt L et al, 2010). The IHC analysis of mouse ovaries also revealed high AMH expression in SAF which was substantially reduced in pre-ovulatory follicles. This is thought to normally occurs via LH down-regulating AMH expression (Pierre A et al, 2013). Using forskolin instead of LH to achieve down-regulation, we demonstrated that this reduction in AMH was potentiated by 1,25-(OH)$_2$-D$_3$ [0.02nM] with a further reduction occurring with higher doses of 1,25-(OH)$_2$-D$_3$ [20nM]. Interestingly treatment with 1,25-(OH)$_2$-D$_3$ alone had no significant effect on AMH mRNA levels, indicating again that this only occurs when the cAMP pathway is activated.

In hen GC from 3-5mm and 6-8mm follicles, vitamin D3 was shown to substantially decrease AMH mRNA expression, with a more robust effect seen in the larger AF (Wojtusik J, 2012).

In addition, Mehri et al showed that in SAF (<14mm) from women with insufficient/deficient follicular fluid levels of 25-OH-D3, there was significantly higher AMHR-II mRNA level compared to those with normal VD levels (Merhi Z et al, 2014). In the same study, 25-OH-D3 was shown to decrease AMH-mediated pSMAD 1/5/8 nuclear localisation in cumulus GC, indicative of reduced AMH signalling. Hence at a local ovarian level, our data and other studies, clearly showed that VD is involved in AMH regulation and expression, which could impact on follicle progression particularly in conditions such as PCOS. At a systemic level however, evidence for correlations between serum vitamin D and serum AMH levels are contradictory and dependent on a woman’s ovulatory status (Moridi I et al, 2020).

To summarise, we have shown that Vitamin D clearly has a role to play in follicle growth, as shown by the increased expression of VDR in follicles of increasing size. In addition, we have shown that Vitamin D may promote follicle progression by downregulating the expression of AMH, thereby reducing AMH’s well-documented inhibitory effect on follicle growth (Pellatt L et al, 2010). Vitamin D is known to affect steroidogenesis and we have demonstrated that levels of 1,25-(OH)$_2$-D$_3$ equivalent to hypovitaminosis, inhibited thecal production of androstenedione. In addition, extremely low levels of vitamin D had an attenuating effect on cAMP-driven aromatase expression, which translated to decreased E2 production. Encouragingly this reduction in E2 is reversed as levels of 1,25-(OH)$_2$-D$_3$ increased; apart from in the presence of LH in luteinised GC, which could have consequences for regular ovulation in women with severe Vitamin D deficiency. For the first time we have demonstrated that deficient levels of 1,25-(OH)$_2$-D$_3$ also down-regulated insulin receptor expression, potentially reducing insulin sensitivity. This could have serious implications for women with PCOS and insulin resistance; indicating that insulin resistant women should try and maintain sufficient levels of systemic vitamin D for regular ovarian function. The ability of the ovary to make local bioactive 1,25-(OH)$_2$-D$_3$, was demonstrated by the expression of CYP27B1. This together with the upregulation of VDR expression in all ovarian cellular compartments, could potentially counteract the effect of systemic VD deficiency and
protect the local ovarian environment. To conclude a severely deficient VD environment (<2nM or <1ng/ml) could contribute to the impaired folliculogenesis/ovulation identified in women with PCOS, but levels associated with mild deficiency may have less impact, apart from on those who are also insulin resistant.
References


Eftekhari, M., Mirhashemi, E. S., Molaei, B., & Pourmasumi, S. "Is there any association between vitamin D levels and polycystic ovary syndrome (PCOS) phenotypes?" Archives of endocrinology and metabolism 64, no. 1 (2020): 11–16.


**Statement regarding use of archived tissue:** Research and the Human Tissue Act 2004 - Consent IS REQUIRED to use and store tissues for research; UNLESS: The relevant material is classed as an existing holding i.e., held prior to 1st September 2006 and/or the relevant material is imported.

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Finally, and most importantly, we would like to acknowledge and thank all the patients who donated tissue and cells which allowed us to conduct this study.
Figure Legends:

**Fig 1a:** H&E of a sectioned mouse ovary displaying a large preovulatory follicle (black arrowhead) with adjacent small antral follicle (SAF) (white arrowhead) (cross-section sizes displayed).

**Fig 1b:** IHC of VDR expression in the corresponding adjacent section of the same mouse ovary. There is extensive expression of VDR protein throughout cortex, stroma and cellular compartments i.e. both GC and theca of both SAF and LAF. Though the intensity of staining in theca appears to be greater in theca of LAF compared to SAF (inset pictures enlarged).

**Fig 1c:** IHC of AMH expression in the corresponding adjacent section of mouse ovary. AMH protein is expressed strongly in GC of SAF (white arrowhead) with minimal expression in GC of LAF (black arrowhead). There is no expression in theca cells (black arrows) or in cortex/stroma.

**Fig 2a:** mRNA expression of VDR in human theca taken from both normal and polycystic ovaries is significantly lower in small (5-6mm ▲) compared to large antral follicles (7-12mm ▲). *(Unpaired t-test, two-tailed *p*=0.0128; 5-6mm (n=4); 7-12mm (n=11)).

**Fig 2b:** mRNA expression of VDR in human theca from 5-12mm sized follicles dissected from PCO (white bar ▲; n=10) appears to be greater than that from normal ovaries (dark bar ■; n=5), but without statistical significance. *Data expressed as mean±SEM*

**Fig 2c:** VDR mRNA expression in human cortex (grey bar ▲, n=6) and stroma (white bar ■, n=5). There was no significant difference in expression.

**Fig 2d:** Densitometry analysis of VDR protein expression in human ovarian cortex (n=5; grey bar ■) and stroma (n=4; white bar ▲), also showed no significant difference in expression. *Densitometry values on y-axis multiplied by 1000 for scaling purposes.*

**Fig 2e:** *(Top panel):* Representative image of western blot using anti-VDR antibody (1:1000) and β-actin (1:2000), visualised with relevant secondary antibody (1:5000) conjugated with infra-red dye at 780nm (green band) and 680nm (red band) respectively. *Lanes 1=normal stroma, 2=anovPCO stroma, 3=normal theca (17mm follicle), 4=ovPCO cortex, 5&8=anovPCO cortex, 6&7=normal cortex, L=marker.* *(Bottom panel):* Densitometry analysis of VDR protein from a variety of human ovarian cell types - cortex from normal ovaries (n=2), ovulatory PCO (n=1) and anovulatory PCO (n=2) (grey bars); stroma from normal ovaries (n=2), ovulatory PCO (n=1) and anovulatory PCO (n=1) (white bars); theca from 17mm ovaries.
follicle from a normal ovary \((n=1)\), 6mm follicle from anovulatory PCO \((n=1)\) (black bars).

**Densitometry values on y-axis multiplied by 1000 for scaling purposes**

**Fig 2f:** (Left-hand panel): Representative image of standard PCR gel illustrating the expression of CYP27B1 mRNA in various ovarian tissue compartments (Lane 1=stroma, 2=cortex, 3=stroma, 4=corpus luteïm, 5=KGN cells, 6=HEK cells, 7=negative control). (Right-hand panel): CYP27B1 mRNA expression in human ovarian stroma samples from women with normal ovaries \([\text{□}]\) \((n=5)\) and polycystic ovaries \([\text{■}]\) \((n=3)\) was subsequently quantified by qPCR and showed no statistically significant difference between the two ovarian morphologies.

**Fig 3a:** Effect of \(1,25(OH)_2D_3\) on androstenedione production from theca taken from small (<10mm, white bars; \(n=4\) subjects, total follicles=15) and large follicles (15-22mm, grey bars, \(n=5\) subjects, total follicles=5) from normal ovaries. \(1,25(OH)_2D_3\) suppressed androstenedione production significantly but only in theca from large follicles. Results expressed as a percentage change from control, where the control was taken as 100%. (Two-way ANOVA \(p=0.05\), source of variation follicle size \(*p=0.002\), treatment\(^*p=0.048\); Tukey’s post-hoc multiple comparison \(*p<0.05\) )

**Fig 3b:** \(1,25(OH)_2D_3\) had no effect on \(E_2\) production from granulosa cells taken from small antral follicles (<10mm, white bars; \(n=3\) subjects, total follicles=11) and large antral follicles (>10mm, grey bars; \(n=6\) subjects, total follicles=8) from normal ovaries. Results showed no effect of \(1,25(OH)_2D_3\) \((vd\ 0.2-20nM)\) on \(E_2\) production. The results are expressed as mean percentage change from control where the control is taken as 100%.

**Fig 3c:** \(1,25(OH)_2D_3\) at 2 (spotted bar) & 20nM (hashed bar) had no effect on \(E_2\) production from granulosa cells in the absence (white bars)/presence (dark grey bars) of FSH (5ng/ml), which as expected significantly simulated \(E_2\) production. The results are expressed as mean percentage change from control where the control is taken as 100%. \((n=4\) subjects; total follicles=49. ANOVA ****\(p<0.0001\); **p<0.005 Sidak’s multiple comparison test)

**Fig 3d:** Effect of \(1,25(OH)_2D_3\) (at 2 & 20nM) on \(E_2\) production from granulosa-luteïn cells in the presence of LH (10ng/ml). LH significantly stimulated \(E_2\) production, which was significantly attenuated in the presence of VD \((n=4; \text{ANOVA **p}<0.005\); Post-hock Sidak’s multiple comparison test. Alphabetical annotations are used to denote differences in statistical significance).

**Fig 3e:** A similar dose-dependent reduction in \(E_2\) production was seen in granulosa-luteïn cells cultured in the presence of the VD analogue EB1089, though this did not reach statistical significance \((n=3)\).
Fig 4a: Aromatase mRNA expression levels were measured in KGNs cultured with forskolin (Fsk) to stimulate aromatase and different doses of VD (0.02-20nM). Testosterone (5x10^{-7}M) was used as an aromatase substrate. VD at the lowest two doses significantly down-regulated Fsk-stimulated aromatase expression, but as the doses increased this attenuation was lost. (One-way ANOVA ***p=0.0002; Tukey’s multiple comparisons test con vs fsk **p<0.005; con vs 2 **p<0.005; con vs 20 *p<0.05; fsk vs VD0.02 *p<0.05; fsk vs VD0.2*p<0.05 (n=6). Alphabetical annotations are used to denote differences in statistical significance).

Fig 4b: KGN cells were transfected with PII-specific-Luc reporter construct and treated as for the qPCR experiments. The luciferase assay showed that the effect of VD doses on aromatase expression was direct action on aromatase transcription, with very low doses down-regulating Fsk-stimulated PII activity. As VD concentrations increased this attenuation was lost. (One-way ANOVA****p<0.0001; Tukey’s multiple comparison- C/C+T vs Fsk ***; Con vs 2***; Con vs 20***; Fsk vs 0.02**; Fsk vs 0.2*; 0.02 vs 2* (n =3). Alphabetical annotations are used to denote differences in statistical significance).

Fig 5a: Expression of insulin receptor (InsR) mRNA expression ± insulin and VD at various doses. Chronic exposure to high insulin (100ng/ml) significantly reduced InsR expression (white bars), which was further attenuated in the presence of very low VD (light grey bars). This combined suppression of InsR was seen even with VD at sufficient levels compared to basal (dark grey bars). One column t-test p=0.0014 Ins100; p=0.001 VD20+Ins10; p=0.0003 VD0.02+Ins100; p=0.0522 VD20+Ins10; p=0.0318 VD20+Ins100. One-way ANOVA*p=0.048; (n=5-8). Values expressed as mean±SEM

Fig 5b: Expression of aromatase mRNA expression ± insulin and VD at various doses. Insulin had no effect on aromatase expression but reversed the attenuation of basal aromatase expression brought about by low dose VD exposure (light grey bars). One way ANOVA *p=0.0307; Unpaired t-test: VD0.02 vs VD0.02+Ins100 *p=0.0118 (2 tail); VD0.02 vs VD20 +p=0.0324 (1-tail) (n=5-8) Values expressed as mean±SEM

Fig 5c: mRNA expression of AMH in the presence of VD (at 0.02 and 20nM) and forskolin (Fsk). Fsk reduced AMH expression below basal and this was further attenuated in the presence of 1,25(OH)_{2}D_{3} with the strongest reduction seen in the presence of 20nM VD. (ANOVA*p=0.02; Tukey’s multiple comparison *p<0.05))

Fig 6: Representative image of western blot of protein from KGN cells cultured ± Fsk and VD at 0.02 and 20nM; immuno-precipitated with anti-VDR antibody (1:1000) and western blotted with anti-RXR antibody (1:1000) to detect RXR:VDR heterodimers. Equal quantities of protein were loaded onto each lane to allow for comparisons. There is a demonstrable reduction in the association of RXR:VDR from the basal state compared to in the presence of forskolin or low VD ligand. Lanes: 1=control, 2=Fsk, 3=Fsk+VD20.4=Fsk+VD0.02 Red band=anti-RXRα, Green band=anti-VDR (IP-VDR)
Supplemental Figure Legends:

Fig S1: 1,25(OH)$_2$D$_3$ had no effect on progesterone production from theca taken from large follicles (15-22mm, grey bars, $n=5$ subjects, total follicles=5) from normal ovaries. (ANOVA n/s).

Fig S2: 1,25(OH)$_2$D$_3$ had no effect on 17-hydroxy-progesterone (17-OH-P) production from theca taken from large follicles (15-22mm, grey bars, $n=5$ subjects, total follicles=5) from normal ovaries. (ANOVA n/s).

Fig S3: 1,25(OH)$_2$D$_3$ had no effect on E$_2$ production from granulosa-lutein cells ($n=4$; ANOVA n/s).
Fig 1c
Fig 2f

CYP27B1 →

[Image of gel electrophoresis with lanes L to 7 and a box plot showing CYP27B1 fold expression in Normal and PCO conditions]
Fig 3a

![Graph showing % change in A1dione for different follicle diameters and treatments.]

Fig 3b

![Graph showing % change in E2 for different follicle diameters and treatments.]

Legend:
- GC
- Theca
- <10mm follicles
- >10mm follicles
- 0.2nM
- 2nM
- 20nM
Fig 5c

AMH:L19 mRNA expression

Fsk  
VD 0.02nM  
VD 20nM

-  +  -  +  -  +  
-  -  +  +  -  -  
-  -  -  -  +  +

Fig 6
Table 1: Patient details, ovarian morphology and follicles from which GC/theca harvested

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Morphology</th>
<th>Day of cycle/cycle length</th>
<th>No. Follicles used (size range mm)</th>
<th>Experiment used for</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43</td>
<td>Normal</td>
<td>7/28</td>
<td>2 (13+15 pooled)</td>
<td>GC steroids</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>Normal</td>
<td>7/28</td>
<td>3 (5+7 pooled + 10)</td>
<td>VD +/- FSH</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>Normal</td>
<td>22/30</td>
<td>2 (7+17)</td>
<td>GC/theca steroids</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>Normal</td>
<td>14/28</td>
<td>1 (10)</td>
<td>GC steroids</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>Normal</td>
<td>2/28-30</td>
<td>2 (8+9)</td>
<td>GC steroids</td>
</tr>
<tr>
<td>6</td>
<td>51</td>
<td>Normal</td>
<td>20/28</td>
<td>3 (6+9+19)</td>
<td>GC/theca steroids</td>
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<tr>
<td>7</td>
<td>40</td>
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<tr>
<td>8</td>
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<td>Normal</td>
<td>21/28-30</td>
<td>2 (11+12 pooled)</td>
<td>GC/theca steroids</td>
</tr>
<tr>
<td>9</td>
<td>32</td>
<td>ov PCO</td>
<td>10/27-30</td>
<td>17 (3-9 pooled)</td>
<td>VD +/- FSH</td>
</tr>
<tr>
<td>10</td>
<td>38</td>
<td>ov PCO</td>
<td>11/28</td>
<td>12 (4-14 pooled)</td>
<td>VD +/- FSH</td>
</tr>
<tr>
<td>11</td>
<td>33</td>
<td>anov PCO</td>
<td>0</td>
<td>18 (3-9 pooled)</td>
<td>VD +/- FSH</td>
</tr>
<tr>
<td>12</td>
<td>42</td>
<td>Normal</td>
<td>10/28</td>
<td>8 (&lt;10 pooled + 15)</td>
<td>Theca steroids</td>
</tr>
<tr>
<td>13</td>
<td>51</td>
<td>Normal</td>
<td>15/28</td>
<td>1 (39)</td>
<td>Theca steroids</td>
</tr>
<tr>
<td>14</td>
<td>48</td>
<td>Normal</td>
<td>2/28-30</td>
<td>3 (8,9+12 pooled)</td>
<td>Theca steroids</td>
</tr>
<tr>
<td>15</td>
<td>35</td>
<td>Normal</td>
<td>14/28</td>
<td>1 (22)</td>
<td>Theca steroids</td>
</tr>
</tbody>
</table>
Table 2: List of antibodies used and conditions.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conc</th>
<th>Species raised</th>
<th>Source</th>
<th>Secondary used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-VDR</td>
<td>1:1000</td>
<td>53kDa</td>
<td>Rat monoclonal</td>
<td>Abcam ab8756 Biotinylated monoclonal anti-rat (Vector) 0.1% [HIC] Goat anti-rat (Licor) 1:5000 conjugated 780nm IR dye [WB]</td>
</tr>
<tr>
<td>Anti-AMH</td>
<td>1:50</td>
<td>Mouse monoclonal</td>
<td>Abcam ab84952</td>
<td>Biotinylated monoclonal anti-mouse (Vector) 0.1%</td>
</tr>
<tr>
<td>Anti-RXR</td>
<td>1:1000</td>
<td>51kDa</td>
<td>Rabbit monoclonal</td>
<td>Abcam ab125001 Goat anti-rabbit (Licor) 1:5000 conjugated 680nm IR dye</td>
</tr>
<tr>
<td>Anti-βactin</td>
<td>1:2000</td>
<td>45kDa</td>
<td>Mouse monoclonal</td>
<td>Abcam ab125001 Goat anti-mouse (Licor) 1:5000 conjugated 680nm IR dye</td>
</tr>
</tbody>
</table>

Table 3: List of Primer used and conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence 5′-3′</th>
<th>Reverse sequence 5′-3′</th>
<th>Annealing temp °C</th>
<th>Primer conc [nM]</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP19A1 (Aromatase)</td>
<td>F – GACTCTAAATTGCCCTCTCTG</td>
<td>R – CAGAGATCCAGACTGCATG</td>
<td>60</td>
<td>100</td>
<td>NM_000103.4</td>
</tr>
<tr>
<td>VDR</td>
<td>F-GACTTTGACCGGAA</td>
<td>R-CATCATGGCGATGCTCCACA</td>
<td>55</td>
<td>300</td>
<td>NM_001017535.2</td>
</tr>
<tr>
<td>AMH</td>
<td>F- GCATGTTGACACATAGGC</td>
<td>R-GAGTGCCCTTCTCAAAGAC</td>
<td>60</td>
<td>100</td>
<td>NM_000479.5</td>
</tr>
<tr>
<td>L19</td>
<td>F – GCCGAAGGGTGACCCAAAT</td>
<td>R – GCAGCGGCGCGCAA</td>
<td>60</td>
<td>100</td>
<td>NM_000981.4</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>F-CACCTGCCAAGCTGCTGTA</td>
<td>R-GCTTTCTGCGCCGAACTTTC</td>
<td>60</td>
<td>300</td>
<td>NM_000785.4</td>
</tr>
<tr>
<td>Insulin Receptor</td>
<td>F-GTGTCAGGCGTGTGACTTAC</td>
<td>R-GTCATCAACGGGCAGTTTG</td>
<td>60</td>
<td>300</td>
<td>NM_000208.4</td>
</tr>
</tbody>
</table>
Supplemental Figures

S1. Progesterone production from theca

S2. 17-OH-P production from theca

S3. Progesterone production from GLC