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 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 unluteinised 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 hyperinsulinemic 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 unluteinised 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 errered 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-$(OH)_2$-D3 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-$(OH)_2$-D3 to decrease AMH expression, supports a role for 1,25-$(OH)_2$-D3 in follicle progression. Uniquely we have shown a direct effect of deficient levels of 1,25-$(OH)_2$-D3 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-$(OH)_2$-D3 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-$(OH)_2$-D3 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 D2, with the major source being the photolytic conversion of 7-dehydrocholesterol in the skin to cholecalciferol (vitamin D3) catalysed by UVB radiation (reviewed in Bouillon R *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 D3 *aka calcidiol* (25-(OH)-D3), an inert stable metabolite that circulates bound to VD-binding protein (VDBP). The stability of the bound circulating 25-(OH)-D3 means that it is used as an indicator of VD status in an individual (Holick, 2007)). 25-(OH)-D3 is then further metabolised to the biologically active form 1,25-$(OH)_2$-D3 (*aka calcitriol*), primarily in the kidneys, a reaction catalysed by the mitochondrial

123 enzyme 1α-hydroxylase (encoded for by *CYP27B1*) (Schuster, 2011); (Luk J, 2012). 1-α-
124 hydroxylase has also been found in extra-renal tissue indicating local conversion of 25-(OH)-
125 D3 to the active 1α,25-$(OH)_2$-D3.

126 The ability of active 1α,25-$(OH)_2$-D3 to exert its profound actions is mediated exclusively by
127 VDR, a member of the nuclear hormone receptor super-family. In addition to its non-
128 genomic actions outside of the nucleus, VDR acts as a ligand-inducible transcription factor
129 (Haussler MR, 2013), enabling it to regulate 3% of the human genome (Holick, 2007).
130 Normally 1α,25-$(OH)_2$-D3 enters the cell by diffusion where it binds to and activates VDR to
131 form a heterodimer complex with retinoid X receptor (RXR): this complex interacts with VD
132 response elements (VDREs) found in the promoter regions of both positively and negatively
133 controlled genes (Cheskis & Freedman, 1994). The localization of VDR to numerous cell
134 types highlights the importance of 1,25-$(OH)_2$-D3 as a regulatory hormone in many systems;
135 consequently, VD deficiency is recognised to be associated with numerous pathological
136 conditions which is usually reversed upon supplementation (Holick MF *et al*, 2011;2012).

137 Current guidelines define VD deficiency as 25-(OH)-D3 levels <20ng/ml (or <50nmol/L); with
138 levels of 21-29ng/ml (52.5-72.5nmol/L) characterized as VD insufficiency and >30ng/ml
139 (>75nmol/L) considered as sufficient (Holick MF *et al*, 2011; 2012) (Pilz S, 2019). Due to
140 avoidance of direct exposure to sunlight and/or nutrient-deficient diets, global deficiency of
141 VD is commonplace in both men and women, but this is exacerbated in northern climates
142 due to lack of sunshine.

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

153 PCOS is well-recognised as one of the most common endocrine disorders in women,
154 manifesting with increased androgen production, irregular menstrual cycles, and
155 dysregulated ovarian function, including an increase in antral follicle numbers associated
156 with increased AMH; but often a lack of ovulatory follicle selection in a sub-set of women
157 (Pellatt L *et al*, 2010). Additionally, there is a clear link between vitamin D, glucose clearance
158 and insulin secretion, such that insulin sensitivity is reduced in vitamin D-deficient women
159 with PCOS, sometimes independently of obesity (Łagowska *et al*, 2018). The compensatory
160 hyper-insulinaemia is known to further amplify the increased thecal androgen secretion in
161 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.

199 *Luteinised granulosa cells (GLC)* were obtained from follicular aspirates obtained during
200 oocyte retrieval from women undergoing *in-vitro* fertilisation (IVF) treatment (see
201 introduction) at various assisted conception units including those at King's College Hospital
202 and The Lister Hospital. Local ethics committee approval for each unit was sought and
203 informed consent was obtained from all women.

204 *KGN granulosa cell (KGN-GC) line,* that are established to correspond to immature granulosa
205 cells from smaller antral follicles, were used to provide mechanistic insight (Nishi *et al*.,
206 2001). The distribution of VDR and AMH protein in various ovarian tissue compartments was
207 assessed using *ovaries from mice (strain 129Sv)* aged between 4 and 5 months. These were
208 euthanised as part of separate ethically approved projects at SGUL and ovaries donated to
209 this study.

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

216 Briefly, slides were dewaxed and rehydrated, and the antigen epitopes exposed by heating
217 the slides in a 0.01M citrate buffer (pH 6, National Diagnostics) bath until boiling point for
218 20 minutes. Peroxidases were blocked by 3% hydrogen peroxide (VWR) in methanol
219 (National Diagnostics) for 10 minutes. Sections were then blocked with relevant anti-serum
220 to reduce non-specific binding for 1h at room temperature. Slides were then incubated with
221 the relevant primary antibody overnight at 4C, followed by 1-hour incubation at room
222 temperature with 0.1% monoclonal biotin-labelled secondary antibody (Vector) (Table 2).
223 Visualisation was carried out with Avidin Biotin Complex (ABC) peroxidase solution followed
224 by 3,3'-diaminobenzidine (DAB) (Vector Laboratories). A haematoxylin counterstain was also
225 used for 10 seconds to label nuclei blue. After a brief dehydration through an ethanol series,
226 slides were mounted using Histomount (National Diagnostics). Negative controls omitted
227 primary antibody. Images were captured using the Hamamatsu NanoZoomer 2.0-Rs Slide
228 Scanner for analysis with the NDP.view 2 software (courtesy of the Image Resource Facility,
229 SGUL).

230 **mRNA and protein expression of VDR in ovarian tissue/cellular compartments**
231 The mRNA expression of VDR in human ovarian cortex, stroma and theca tissue
232 compartments was assessed using archived frozen tissue collected as described above. All
233 tissue was stored and used under HTA regulations. Tissues were defrosted on ice and
234 homogenised in lysis buffer (RLT lysis buffer, Qiagen, Netherlands) using the FastPrep®
235 TissueLyser 24 and the FastPrep® Lysing Matrix A 2mL tubes containing a garnet matrix and
236 a ceramic sphere (MP Biomedicals). After homogenisation RNA was extracted, reverse
237 transcribed and real-time quantitative PCR (qPCR) performed for VDR relative to L19 (the

238 reference gene), as previously described (Rice S *et al*, 2006). In addition, the expression of
239 VDR in the KGN-GC was established using standard PCR as well as qPCR (see Table 3 for
240 details of primers and cycling conditions). For protein extraction the tissue was
241 homogenised in RIPA buffer with a cocktail of phosphatase inhibitors (Sigma-Aldrich, Merck,
242 Gillingham, Dorset), using a sonicator three times for 30s each time. Samples were
243 maintained on ice throughout to prevent overheating and denaturation of proteins. The
244 lysed samples were micro-centrifuged at 4C for 10min at maximum speed. Pelleted debris
245 was discarded, and the protein lysate stored at -80C prior to Bradford assays (for
246 measurement of protein concentration) and western blotting.

247 **GC, theca and GLC culture experiments**

248 The granulosa or theca cells were pooled from several follicles based on the experimental
249 protocol and cultured in McCoys 5A medium supplemented with penicillin/streptomycin, L-
250 glutamine and amphotericin B (all purchased from Invitrogen, UK). Only cells from those
251 follicles appearing healthy were used. Granulosa cells were plated in 96-well plates at $5x10^4$
252 and theca cells at $5x10^5$ cells per well. To increase the adhesion of theca cells to the plate,
253 plates were first coated in ECM gel (McCaig, 2002).
254 Cells were incubated in culture medium (plus experimental treatments) for 48 hours with a
255 range of 1,25-$(OH)_2$-D3 concentrations (0.2, 2 and 20nM serially diluted down from a stock
256 concentration of 2µM). Testosterone ($5x\ 10^{-7}$ M) was used as a substrate for conversion to
257 oestradiol in the granulosa cells and steroid levels measured in the medium by
258 radioimmunoassay (Hillier SG *et al*, 1980); (Gilling-Smith C *et al*, 1994); (Willis DS *et al*,
259 1996). To confirm that cells were healthy, 10ng/ml LH was used as a positive control in
260 luteinised GCs and theca, and 5ng/ml FSH in cells dissected from small follicles (LH and FSH
261 supplied by Endocrine Services, Biddeford-upon-Avon, UK). Isolation of GLC was performed
262 as previously described (Wright R *et al*, 2002). Cells were plated in 96-wells at $5x10^4$ cells per
263 well in 200µl of serum (5%) supplemented medium for 48 hours, after which the medium
264 was removed and replaced with experimental treatment as detailed above. GLCs were also
265 exposed to the vitamin D analogue ED1089 (kindly donated by Dr. Kay Colston, SGUL), to
266 examine comparative efficacy and demonstrate specificity.

267 **KGN-GC culture experiments**

268 To model the effect that VD deficiency may have on ovarian function, KGN-GC were grown
269 and passaged in 10% DMEM-F12 supplemented with L-glutamine and
270 penicillin/streptomycin (Invitrogen), at 37°C in 95% air/CO_2. Cells were plated in 12-well
271 plates (3×10^3 cells/well) and cultured in 1% DMEM-F12 (charcoal-stripped) overnight. Cells
272 were serum-starved for 2h and then treated with forskolin (25µM), to reproduce the effect
273 of LH on cAMP stimulation, ± 1,25-$(OH)_2$-D3 at 0.02, 0.2, 2 and 20nM and cultured for a
274 further 48h. Testosterone ($5x10^{-7}$M) was added to all cultures as the aromatase substrate
275 for conversion to oestrogen. Similarly, the effect of VD on AMH mRNA expression was
276 investigated in cells treated with forskolin and VD as described above, using only the upper
277 and lower doses of 1,25-$(OH)_2$-D3 from the range above at 0.02 and 20nM. At the end of the

278 relevant culture time, RNA was extracted, reverse transcribed and real-time quantitative
279 PCR (qPCR) performed for *CYP19A1* (encoding aromatase) as described previously (Rice S *et*
280 *al*, 2013).
281 To investigate the effect of VD on aromatase promoter II (PII) activity, cells were plated at a
282 density of $2x10^4$ cells/well in 96-well plates. After overnight incubation, they were
283 transfected with a *CYP19A1 PII-516* reporter construct expressing firefly Luciferase, along
284 with a control plasmid expressing Renilla luciferase from a constitutive promoter, and a
285 transcription enhancing element, PVAi, as previously described (Rice S *et al*, 2013). After 2h
286 serum-starvation, cells were treated as described above for 24 hours with quadruplicate
287 replicates/treatment, and luciferase reporter assays were carried out using the Dual-Glo
288 Luciferase Assay System (Promega).
289 To mimic a situation of chronic insulin exposure, cells were treated with insulin for 48h at
290 post-prandial (10ng/ml) and hyperinsulinemic (100ng/ml) levels in the presence and
291 absence of the lowest [0.02nM] and highest [20nM] 1,25-$(OH)_2$-D3 levels tested in the
292 experiments. The effect of 1,25-$(OH)_2$-D3 on aromatase and insulin receptor (InsR) mRNA
293 expression was measured by qPCR.
294
295 **Radioimmunoassay (RIA) for P, E2 and Androstenedione**
296 Conditioned media was collected from the cells at 48 hours, frozen at -20C and E2, P and
297 androstenedione measured using a modified 'in-house' RIA, with tritiated steroids and
298 charcoal separation (Hillier SG *et al*, 1980); (Gilling-Smith C *et al*, 1994); (Willis DS *et al*,
299 1996). The components of the assay were: tritiated steroid label (Amersham
300 Pharmaceuticals, Bucks, UK), steroid antiserum (sheep anti-human from Guildhay,
301 Guildford, UK) and standards and quality controls (QCs) (approximately 1, 6 and 35% of the
302 top standard), prepared from powdered steroid (Sigma Co Ltd, Poole, UK). Conditioned
303 medium was diluted to be within the midrange values for each assay and therefore on the
304 linear part of the standard curve. Each sample was tested in duplicate.
305
306 **Immunoprecipitation**
307 To investigate the effect of 1,25-$(OH)_2$-D3 on VDR:RXR dimer formation, KGN cells were
308 plated at a density of $5x10^5$ cells/well in a 6-well plate and treated as described above with
309 and without forskolin, testosterone and 1,25-$(OH)_2$-D3 at 0.02 and 20nM for 48h. At the end
310 of culture time, media was removed, cells washed with ice-cold PBS and lysed with ice-cold
311 RIPA buffer as previously described (Dilaver N *et al*, 2019). The protein concentration of the
312 lysate was measured by Bradford assay and prior to immunoprecipitation, equal amounts of
313 protein lysate from each treatment group were pre-cleared on the A/G-coupled Sepharose
314 beaded (Pierce) support for one hour at 4C to reduce non-specific ligand binding.
315 Immunoprecipitation of the cleared lysates was performed overnight at 4C with 5µg of anti-
316 VDR antibody or anti-RXRα monoclonal antibodies (Abcam – Table 2). The beaded
317 complexes were washed several times with NP-40 lysis buffer, after which protein
318 complexes were dissociated by boiling for 10 mins with 1X SDS-DTT reducing buffer

319 (protocol adapted from Luderer HF *et al*, 2011). The collected supernatant was stored at -
320 80C for subsequent Western blotting analysis.
321
322 **Western Blotting of VDR and RXR**
323 Protein concentrations were measured using the Bradford assay (BioRad, Hertfordshire) and
324 equal amounts of protein obtained from lysing ovarian tissues or from immunoprecipitation
325 experiments, were resolved with Western blotting as previously described (Dilaver N *et al*,
326 2019). PVDF(fl) (Immobilon, Millipore Merk, Massachusetts) membranes with transferred
327 proteins were incubated with rat anti-human VDR or rabbit anti-human RXR (both used at
328 1:1000) and/or mouse anti-human β-actin (1:2000). Fluorescently conjugated relevant
329 secondary antibodies (1:5000) (see Table 2 for details of antibodies) were used for
330 visualisation using the Odyssey Imaging System (Li-Cor Biosciences) (Dilaver N *et al*, 2019).
331
332 **Experimental Quantification and Statistical Analysis**
333 All data are represented as the mean ± SEM of triplicate or more observations (detail in
334 legends) from a minimum of 3 or more independent experiments, unless otherwise stated.
335 qPCR data were analysed using the ΔΔCt method as described in detail previously (Rice S *et*
336 *al*, 2006), with normalisation to L19 and subsequent normalisation to the Ct value of the
337 control (untreated). To use the ΔΔCt method, the amplification efficiency for each GOI and
338 reference gene must be in the recommended range of 90-100%. This was rigorously applied
339 to our study by the inclusion of a standard curve for every qPCR assay conducted. Data from
340 Western blots represent the mean densitometry measurements taken from all individual
341 experiments using Image Studio software (Licor™) and normalised to ß-actin (loading
342 control) and where relevant, to the untreated (control) samples. Results for steroid RIA
343 were calculated using Assay Zap V2.69 software (Biosoft, Cambridge, UK). Intra- and inter-
344 assay coefficients of variation were below 5% and 6% respectively.
345 Statistical significance was determined by ANOVA followed by post hoc multiple comparison
346 tests; unpaired Student's or paired t test when 2 groups were compared (depending on the
347 design of the experiment) or a one-sample t test when comparing with normalised control
348 values, using GraphPad Prism™. Significance was set at $P \leq 0.05$.
349
350 ## Results
351 **Expression of VDR in ovarian tissue and cellular compartments**
352 IHC was used to detect the extent of VDR protein expression in sequential mouse ovary
353 sections in which an anti-VDR antibody revealed strong and extensive staining throughout
354 all mouse ovarian tissue compartments including the cellular components of the follicles
355 (figure 1a and 1b). Closer examination revealed more intense staining in thecal cells of LAF
356 compared to SAF (inset figures 1b). The general uniformity and ubiquity of VDR expression
357 was in marked contrast to staining with anti-AMH antibody in the sequential section, which
358 showed a distinctive pattern of expression i.e., greater staining in GC of SAF compared to

359 LAF, with no staining in theca or ovarian compartments (figure 1c). This also demonstrated
360 the rigour of the IHC protocol.
361
362 This finding in the mouse ovary was extended by measuring VDR mRNA levels in theca cells
363 dissected from human follicles of varying sizes to ascertain whether expression levels
364 changed with follicle size and to determine if there was a difference between tissue taken
365 from normal and polycystic ovaries. VDR mRNA levels were significantly higher in theca from
366 larger antral follicles (7-12mm, n=11) and those approaching preovulatory sizes, compared
367 to those from smaller antral follicles (5-6mm, n=4) (unpaired t-test, *p=0.013; figure 2a). A
368 further analysis of data comparing theca from normal and PCO, revealed a trend for PCO
369 follicles to expresses more VD mRNA but this did not reach statistical significance as
370 numbers were small (figure 2b). In addition, VDR mRNA and protein expression were also
371 detected in human cortex and stroma (fig 2c and 2d respectively). There appeared to be
372 increased levels in cortex compared to stroma, but levels were quite variable. VDR protein
373 expression was detected in both cortex and stroma from normal and polycystic ovaries
374 (both ovulatory and anovulatory) (fig 2e), however there were insufficient samples numbers
375 to conduct statistical analysis. We also saw higher levels of VDR protein in human theca
376 from a 17mm follicle compared to a whole 6mm follicle (fig 2e), supporting the trend seen
377 with human mRNA expression (fig 2a) and in the mouse ovary (fig 1b).
378
379
380 **Expression of *CYP27B1* in human ovarian tissue**
381 *CYP27B1* mRNA was expressed in ovarian stroma, but there was a wide variation in *CYP27B1*
382 mRNA expression in individual samples of stroma from normal or polycystic human ovaries
383 (figure 2f). There was however a trend for higher levels in stroma from normal when
384 compared to polycystic ovaries, though this did not reach statistical significance.
385
386 **Effect of 1,25 (OH)2-D3 on steroid production in ovarian cells.**
387 ***VD and theca steroidogenesis:*** Overall, in theca from large follicles (15-22mm), all doses of
388 1,25-$(OH)_2$-D3 consistently suppressed Androstenedione production (mean 22%
389 suppression) but had no effect on androstenedione production from small follicles (n=4-8,
390 Two-way ANOVA, p=0.048 for treatment and p=0.002 for follicle size) (fig 3a). 1,25-$(OH)_2$-D3
391 had no effect on either 17-OH-P or P production (supplemental figures S1 and S2).
392
393 ***VD and GC steroidogenesis***: There was no effect of 1,25-$(OH)_2$-D3 at any dose on E2
394 production of GCs taken from follicles <10mm diameter (n=4; figure 3b); however, in
395 follicles >10mm there appeared to be a trend to increasing E2 with 1,25-$(OH)_2$-D3 though
396 this was not significant (figure 3b). In addition, 1,25-$(OH)_2$-D3 did not alter the normal
397 responsiveness to FSH in SAF (n=4, ANOVA p<0.0001) (figure 3c).
398

399 ***VD and E2 production in GLC:*** The effect of VD on E2 production from luteinised granulosa
400 cell culture was then investigated. As expected, exposure of the cells to LH produced a
401 substantial stimulation in the production of E2, which surprisingly was considerably
402 attenuated by 1,25-$(OH)_2$-D3 at both 2nM and 20nM doses (figure 3d) (ANOVA $p<0.0001$;
403 post-hoc t-test **$p<0.005$). This contrasts with the unluteinised GC from small follicles
404 where 1,25-$(OH)_2$-D3 had no effect on FSH-stimulated E2 production (figure 3c).
405 Interestingly culturing GLC with the VD analogue EB1089 also appeared to suppress E2
406 production in a dose-related manner (figure 3e), though the data did not reach statistical
407 significance (ANOVA n/s). Exposure of GLCs to all low doses of VD had no effect on
408 progesterone production (supplemental figure S3).
409
410 **Effect of VD on aromatase expression in KGN-GC**: To determine the mechanism by which
411 1,25-$(OH)_2$-D3 was altering E2 production in primary cells, KGN cells were cultured with
412 forskolin to increase cAMP and hence aromatase activity (equivalent to stimulation by
413 either gonadotrophin). Very low 1,25-$(OH)_2$-D3 levels [0.02 & 0.2nM] significantly down-
414 regulated Fsk-stimulated aromatase mRNA expression (n=6, One-way ANOVA**$p=0.0018$;
415 post-hoc t-tests *$p<0.05$, **$p<0.005$), but with doses approaching sufficiency [2 and 20nM]
416 this attenuation was reversed. An identical pattern was seen in PII transfected KGN cells
417 demonstrating that 1,25-$(OH)_2$-D3 affected aromatase activity as well as expression (figures
418 4a & 4b) (n=3; ANOVA****$p<0.0001$; post-hoc t-tests *$p=0.02$, **$p=0.0095$).
419
420 **Effect of VD on other factors implicated in regulation of follicle growth.**
421 *Insulin:* There is compelling evidence that systemic VD levels are correlated with insulin
422 sensitivity (Łagowska *et al*, 2018); however, there is little supportive evidence at a cellular
423 level. Chronic exposure of granulosa cells to high (100ng/ml) insulin significantly
424 downregulated expression of total InsR mRNA ($p=0.001$), and this reduction was surprisingly
425 potentiated by both doses of 1,25-$(OH)_2$-D3: 20nM (<50% of basal) and 0.02nM (<25% of
426 basal) (n=5-8; One-way ANOVA *$p=0.048$, one-sample t-tests to the control ***$p<0.0005$,
427 **$p<0.005$, *$p<0.05$) (figure 5a). This profound attenuation of insulin receptor expression by
428 1,25-$(OH)_2$-D3 in the presence of chronic insulin exposure did not have a concomitant
429 reduction on aromatase expression in the same cells. In fact, the presence of insulin
430 reversed the attenuation of basal aromatase levels brought about by low dose VD (figure
431 5b).
432 *AMH:* Forskolin down-regulated AMH expression compared to basal (<50%), which was
433 further potentiated in the presence of 1,25-$(OH)_2$-D3, with a more potent attenuation by
434 20nM 1,25-$(OH)_2$-D3 compared to 0.02nM (figure 5c) (n=3, ANOVA *$p=0.02$; multiple
435 comparison t-test *$p<0.05$).
436
437 **Investigating the differential effects of varying doses of VD ligand.**
438 Data presented so far indicate that those doses of 1,25-$(OH)_2$-D3 equivalent to extremely
439 deficient levels have differential effects compared to those approaching sufficiency. To

440 determine a possible mechanism, the relationship between levels of VD ligand and the
441 proportion of either VDR or RXR was investigated. KGN-GC were treated with extremely low
442 or sufficient doses of 1,25-$(OH)_2$-D3 in the presence of Fsk, immunoprecipitated with anti-
443 VDR antibodies, to pull down all forms of VDR:homodimers, heterodimers and solo. The
444 immuno-precipitated samples were then immunoblotted for RXR to assess only the
445 heterodimer association between RXR and VDR (figure 6a). There was a clear difference in
446 levels of VDR associated with RXR in the presence of Fsk compared to basal or either 20nM
447 of 0.02nM 1,25-$(OH)_2$-D3, but no measurable difference between the two doses of 1,25-
448 $(OH)_2$-D3. (figures 6a, n=4).
449
450 **Discussion**

451 We have discovered widespread distribution of VDR in the human ovary, pointing to a likely
452 critical role for VD in female reproduction. We have demonstrated for the first time, a clear
453 relationship between increasing VDR expression and follicle size, in that both VDR mRNA
454 and protein levels were significantly up-regulated in human theca taken from large AF (7-
455 12mm) compared to smaller AF (5-6mm), highlighting the importance of VDR in antral
456 follicle progression.

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

468 The detection of *CYP27B1* mRNA expression in human ovarian stroma was intriguing as it
469 indicated that the human ovary is capable of being an extra-renal site of active 1,25-$(OH)_2$-
470 D3 synthesis, as supported by studies in other species (Xu J *et al*, 2018). This local
471 contribution of bioactive 1,25-$(OH)_2$-D3 is difficult to estimate and account for, especially
472 with respect to observational clinical studies that measure systemic levels of VD. Active
473 1,25-$(OH)_2$-D3 negatively regulates expression of CYP27B1, hence regulating its own
474 circulating concentrations (Bouillon R *et al*, 2008).

475 This increasing expression of VDR in the growing follicle at the LAF stage corresponds to
476 increasing steroidogenesis and the acquisition of LH receptors. It had been documented that
477 incubation of human cumulus GC with VD at normal levels affects steroid output (Merhi Z *et*
478 *al*, 2014), but we wished to investigate the extent of and mechanisms by which incubating

479 cells in a 1,25-$(OH)_2$-D3-deficient environment affected follicle growth and steroidogenesis.
480 To that end we incubated cells with 20, 2, 0.2 or 0.02nmol/L (since serum levels <50nmol/L
481 is defined as severe deficiency) (Holick MF *et al*, 2011). A recent study of women of
482 childbearing age in rural northern China found the prevalence of severe VD deficiency was
483 16% in the 1151 women studied, with a median serum level of only of 5.63ng/ml (equivalent
484 to 14nmol/L) (Lin S *et al*, 2021); indicating that severe hypovitaminosis of this magnitude
485 occurs in the population. Interestingly, 1,25-$(OH)_2$-D3 is established to act at nanomolar, or
486 even picomolar, concentrations, as a direct regulator of specific target genes in VDR-
487 expressing cell and tissues. It also binds to VDR with an affinity of 0.1nM (reviewed in
488 Carlsberg, 2022).

489 In GC, gonadotrophins stimulate oestrogen synthesis via cAMP-mediated signalling, and it is
490 well-established that a cAMP-response element (CRE) sequence has been identified within
491 promoter II (PII); the predominant aromatase promoter used in the ovary (Michael MD,
492 1997). Changes in the 1,25-$(OH)_2$-D3 environment had no effect on E2 production from GC
493 of either small or large AFs, in the presence or absence of FSH and FSH responsiveness was
494 retained. Conversely in luteinised granulosa cells (GLCs), 1,25-$(OH)_2$-D3 did prevent the LH-
495 mediated stimulation of E2 production. It is without doubt that 1,25-$(OH)_2$-D3 can decrease
496 E2 production as the same effect was observed with the VD analogue in GLCs.

497 This would imply that attenuation by VD only occurred when GC acquired LHR or else is
498 dependent on levels of cAMP generated, since LH stimulates more cAMP activity than FSH
499 (Aharoni D *et al*, 1995). This is supported by the observation that extremely low doses of
500 1,25-$(OH)_2$-D3 down-regulated forskolin-stimulated aromatase expression and activity in
501 KGN cells (which do not have LHR), an effect that was lost once dosing levels were
502 increased.

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

510 The bi-phasic dose-response effects of 1,25-$(OH)_2$-D3 on aromatase expression, was a direct
511 influence on the transcriptional activity of PII, as seen in the PII transfection assay results.
512 Analysis of PII has revealed the presence of two VDRE - proximal and distal, with an overlap
513 between the proximal VDRE and CRE (Krishnan AV *et al*, 2010). A key factor could be the
514 mechanism of ligand-binding of VD to its receptor. VDR functions as both a monomer,
515 homodimer, and a heterodimer with RXR, and the alteration of the ratio of these complexes
516 within a cell is dependent on the amount of VD ligand present (Cheskis B *et al*, 1994).
517 Depending on the proportion of each, these complexes will bind to VDRE on genes and

518 attract either co-repressors/activators to enhance or suppress gene expression and activity.
519 Increased levels of VD decreased the amount of DNA-bound VDR homodimer complexes
520 and promote the formation of VDR-RXR heterodimers (Carlsberg C, 2022; Cheskis B *et al*,
521 1994; Haussler MR *et al*, 2013).

522 Unliganded VDR-RXR heterodimers are initially bound to a VDRE and recruit co-repressor
523 complexes, which prevents basal transcription through the activity of histone deacetylase.
524 Once sufficient ligand has bound, the repressors are substituted by co-activator complexes,
525 allowing gene transcription to commence (Carlsberg C, 2022; Dwivedi PP *et al*, 1998; Perissi
526 V *et al*, 2010). This was to some extent corroborated by the immunoprecipitation studies
527 which indicted a greater ratio of VDR:RXR at basal levels, with alterations in association in
528 the presence of forskolin and compared to decreasing quantities of 1,25-$(OH)_2$-D3 ligand. It
529 is not possible from our experiments to determine the proportion of heterodimers in the
530 cytoplasmic or nuclear compartments as we used whole cell lysates. To answer this, we
531 would need to carry out further experiments (beyond scope of this study) to explore VDR
532 sub-cellular trafficking between nuclear and cytoplasmic compartments and components of
533 the repressor/activator complexes bound to chromatin. Interestingly, the ability of forskolin
534 to alter the association of the intracellular dimers would indicate the presence of a ligand-
535 independent cAMP activated pathway outside the nucleus (Luk J *et al*, 2012); (Haussler MR
536 *et al*, 2013) which could account for the different outcomes observed when using either FSH
537 or LH.

538 Interestingly, theca from PCO follicles expressed higher levels of VDR mRNA than those from
539 normal ovaries, though this was not statistically significant. This apparent up-regulation in
540 VDR expression could be in response to VD deficiency, which is commonly prevalent in
541 women with PCOS, as seen in population cohort studies (Krul-Poel YHM *et al*, 2018); (Wang
542 L *et al*, 2020). There is also evidence for autoregulation of VDR by 1,25-$(OH)_2$-D3 via VDRE on
543 the *VDR* gene itself (Zella LA *et al*, 2007).

544 As stated earlier, women with PCOS commonly present with increased serum levels of AMH
545 (reflecting the pool of stalled SAF) and hyperinsulinemia, both of which are also linked to VD
546 status (Luk J, 2012; Lorenzen M. B., 2017). To investigate this link, we replicated
547 hyperinsulinemia and insulin resistance *in vitro* by chronically exposing KGN cells to insulin
548 at either post-prandial (10ng/ml) or hyperinsulinemic (100ng/ml) doses. The HI dose
549 downregulated expression of insulin receptor mRNA by more than 50% reproducing insulin
550 resistance, whereas there was no significant effect noted at post-prandial treatment levels.
551 To our surprise, culture of cells in an extremely low and deficient VD environment caused an
552 even further attenuation of insulin receptor expression in the presence of both doses of
553 insulin. This effect only occurred in the presence of insulin, as 1,25-$(OH)_2$-D3 alone had no
554 effect on insulin receptor expression. Systemic VD deficiency is clearly linked to a reduction
555 in insulin sensitivity (independent of BMI) as others have shown (Muscogiuri G *et al*, 2017),
556 and this insulin receptor reduction may be a contributory mechanism. Interestingly, in the
557 same cells a combination of insulin and 1,25-$(OH)_2$-D3 had no effect on aromatase, unlike in

558 the forskolin experiments, again indicating that this was a cAMP-driven process, possibly
559 linked to overlap between VDRE and CRE on PII as described previously.

560 It is well established that follicle progression in the normal human ovary requires down-
561 regulation of AMH expression to permit FSH-driven activity (Pellatt L *et al*, 2010). The IHC
562 analysis of mouse ovaries also revealed high AMH expression in SAF which was substantially
563 reduced in pre-ovulatory follicles. This is thought to normally occurs via LH down-regulating
564 AMH expression (Pierre A *et al*, 2013). Using forskolin instead of LH to achieve down-
565 regulation, we demonstrated that this reduction in AMH was potentiated by 1,25-$(OH)_2$-D3
566 [0.02nM] with a further reduction occurring with higher doses of 1,25-$(OH)_2$-D3 [20nM].
567 Interestingly treatment with 1,25-$(OH)_2$-D3 alone had no significant effect on AMH mRNA
568 levels, indicating again that this only occurs when the cAMP pathway is activated.

569 In hen GC from 3-5mm and 6-8mm follicles, vitamin D3 was shown to substantially decrease
570 AMH mRNA expression, with a more robust effect seen in the larger AF (Wojtusik J, 2012).
571 In addition, Mehri *et al* showed that in SAF (<14mm) from women with insufficient/deficient
572 follicular fluid levels of 25-OH-D3, there was significantly higher AMHR-II mRNA level
573 compared to those with normal VD levels (Merhi Z *et al*, 2014). In the same study, 25-OH-D3
574 was shown to decrease AMH-mediated pSMAD 1/5/8 nuclear localisation in cumulus GC,
575 indicative of reduced AMH signalling. Hence at a local ovarian level, our data and other
576 studies, clearly showed that VD is involved in AMH regulation and expression, which could
577 impact on follicle progression particularly in conditions such as PCOS. At a systemic level
578 however, evidence for correlations between serum vitamin D and serum AMH levels are
579 contradictory and dependent on a woman's ovulatory status (Moridi I *et al*, 2020).

580 To summarise, we have shown that Vitamin D clearly has a role to play in follicle growth, as
581 shown by the increased expression of VDR in follicles of increasing size. In addition, we have
582 shown that Vitamin D may promote follicle progression by downregulating the expression of
583 AMH, thereby reducing AMH's well-documented inhibitory effect on follicle growth (Pellatt
584 L *et al*, 2010). Vitamin D is known to affect steroidogenesis and we have demonstrated that
585 levels of 1,25-$(OH)_2$-D3 equivalent to hypovitaminosis, inhibited thecal production of
586 androstenedione. In addition, extremely low levels of vitamin D had an attenuating effect on
587 cAMP-driven aromatase expression, which translated to decreased E2 production.
588 Encouragingly this reduction in E2 is reversed as levels of 1,25-$(OH)_2$-D3 increased; apart
589 from in the presence of LH in luteinised GC, which could have consequences for regular
590 ovulation in women with severe Vitamin D deficiency. For the first time we have
591 demonstrated that deficient levels of 1,25-$(OH)_2$-D3 also down-regulated insulin receptor
592 expression, potentially reducing insulin sensitivity. This could have serious implications for
593 women with PCOS and insulin resistance; indicating that insulin resistant women should try
594 and maintain sufficient levels of systemic vitamin D for regular ovarian function. The ability
595 of the ovary to make local bioactive 1,25-$(OH)_2$-D3, was demonstrated by the expression of
596 *CYP27B1*. This together with the upregulation of VDR expression in all ovarian cellular
597 compartments, could potentially counteract the effect of systemic VD deficiency and

598 protect the local ovarian environment. To conclude a severely deficient VD environment
599 (<2nM or <1ng/ml) could contribute to the impaired folliculogenesis/ovulation identified in
600 women with PCOS, but levels associated with mild deficiency may have less impact, apart
601 from on those who are also insulin resistant.

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738 transcriptional autoregulation by 1,25-dihydroxyvitamin D3." *J Steroid Biochem Mol Biol.* 103, no. 3-5
739 (2007): 435-9.

740

741

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

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

756

757 **Figure Legends:**

758

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

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

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

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

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

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

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

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

789 follicle from a normal ovary (*n=1*), 6mm follicle from anovulatory PCO (*n=1*) *(black bars).*
790 *Densitometry values on y-axis multiplied by 1000 for scaling purposes*

791 **Fig 2f: (Left-hand panel):** Representative image of standard PCR gel illustrating the
792 expression of CYP27B1 mRNA in various ovarian tissue compartments (*Lane 1=stroma,*
793 *2=cortex, 3-stroma, 4=corpus luteim, 5= KGN cells, 6= HEK cells, 7=negative control*). **(Right-**
794 **hand panel):** CYP27B1 mRNA expression in human ovarian stroma samples from women
795 with normal ovaries (□) (*n=5*) and polycystic ovaries (■) (*n=3*) was subsequently quantified
796 by qPCR and showed no statistically significant difference between the two ovarian
797 morphologies.

798 **Fig 3a:** Effect of $1,25(OH)_2D_3$ on androstenedione production from theca taken from small
799 (<10mm, white bars, *n=4 subjects, total follicles=15*) and large follicles (15-22mm, grey bars,
800 *n=5 subjects, total follicles=5*) from normal ovaries. $1,25(OH)_2D_3$ suppressed
801 androstenedione production significantly but only in theca from large follicles. Results
802 expressed as a percentage change from control, where the control was taken as 100%.
803 *(Two-way ANOVA p=0.05, source of variation follicle size **p=0.002, treatment*p=0.048;*
804 *Tukeys post-hoc multiple comparison *p<0.05)*

805 **Fig 3b**: $1,25(OH)_2D_3$ had no effect on E_2 production from granulosa cells taken from small
806 antral follicles (<10mm, white bars; *n=3 subjects, total follicles=11*) and large antral follciles
807 (>10mm, grey bars; *n=6 subjects, total follicles=8*) from normal ovaries. Results showed no
808 effect of $1,25(OH)_2D_3$ (vd 0.2-20nM) on E_2 production. The results are expressed as mean
809 percentage change from control where the control is taken as 100%.

810 **Fig 3c:** $1,25(OH)_2D_3$ at 2 (spotted bar) & 20nM (hashed bar) had no effect on E_2 production
811 from granulosa cells in the absence (white bars)/presence(dark grey bars) of FSH (5ng/ml),
812 which as expected significantly simulated E2 production. The results are expressed as mean
813 percentage change from control where the control is taken as 100%. *(n=4 subjects; total*
814 *follicles=49. ANOVA ****p<0.0001; **p<0.005 Sidak's multiple comparison test)*

815 **Fig 3d:** Effect of $1,25(OH)_2D_3$ (at 2 & 20nM) on E_2 production from granulosa-lutein cells in
816 the presence of LH (10ng/ml). LH significantly stimulated E2 production, which was
817 significantly attenuated in the presence of VD *(n=4; ANOVA **p<0.005; Post-hock Sidak's*
818 *multiple comparison test. Alphabetical annotations are used to denote differences in*
819 *statistical significance).*

820 **Fig 3e:** A similar dose-dependent reduction in E2 production was seen in granulosa-lutein
821 cells cultured in the presence of the VD analogue EB1089, though this did not reach
822 statistical significance (*n=3*).

823 **Fig 4a:** Aromatase mRNA expression levels were measured in KGNs cultured with forskolin
824 (Fsk) to stimulate aromatase and different doses of VD (0.02-20nM). Testosterone ($5x10^{-7}$M)
825 was used as an aromatase substrate. VD at the lowest two doses significantly down-
826 regulated Fsk-stimulated aromatase expression, but as the doses increased this attenuation
827 was lost. *(One-way ANOVA ***p=0.0002; Tukey's multiple comparisons test con vs fsk*
828 ***p<0.005; con vs 2 **p<0.005; con vs 20 *p<0.05; fsk vs VD0.02 *p<0.05; fsk vs*
829 *VD0.2*p<0.05 (n=6). Alphabetical annotations are used to denote differences in statistical*
830 *significance).*

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

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

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

850 **Fig 5c:** mRNA expression of AMH in the presence of VD (at 0.02 and 20nM) and forskolin
851 (Fsk). Fsk reduced AMH expression below basal and this was further attenuated in the
852 presence of $1,25(OH)_2D_3$ with the strongest reduction seen in the presence of 20nM VD.
853 *(ANOVA*p=0.02; Tukey's multiple comparison *p<0.05))*

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

861

862 **Supplemental Figure Legends:**

863 **Fig S1:** 1,25$(OH)_2D_3$ had no effect on progesterone production from theca taken from large
864 follicles (15-22mm, grey bars, *n=5 subjects, total follicles=5*) from normal ovaries. *(ANOVA*
865 *n/s).*

866 **Fig S2:** 1,25$(OH)_2D_3$ had no effect on 17-hydroxy-progesterone (17-OH-P) production from
867 theca taken from large follicles (15-22mm, grey bars, *n=5 subjects, total follicles=5*) from
868 normal ovaries. *(ANOVA n/s).*

869 **Fig S3:** 1,25$(OH)_2D_3$ had no effect on E_2 production from granulosa-lutein cells *(n=4; ANOVA*
870 *n/s).*

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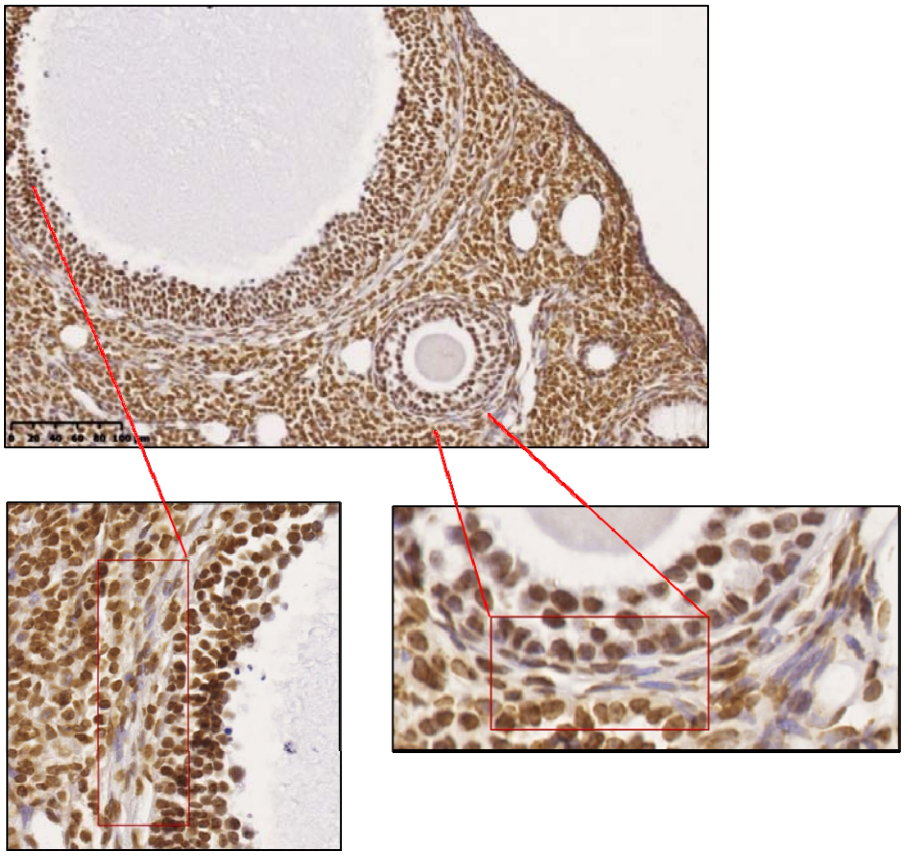
Fig 1a

Fig 1b



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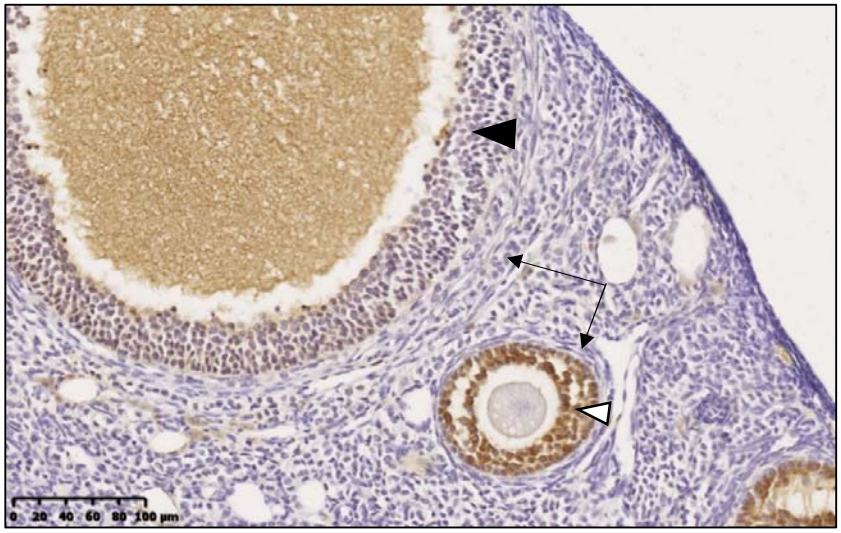
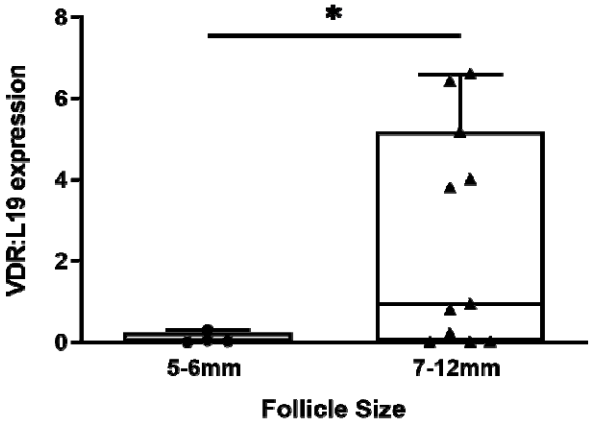


Fig 2a



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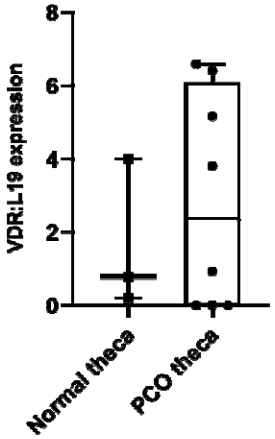

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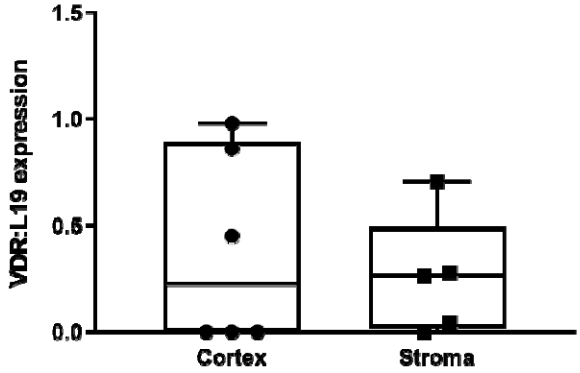

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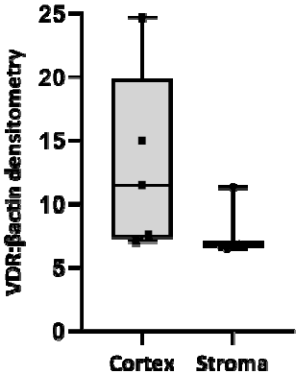


Fig 2e (top)

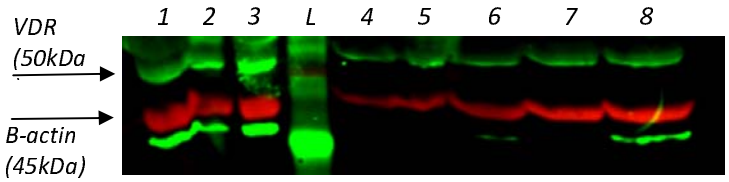
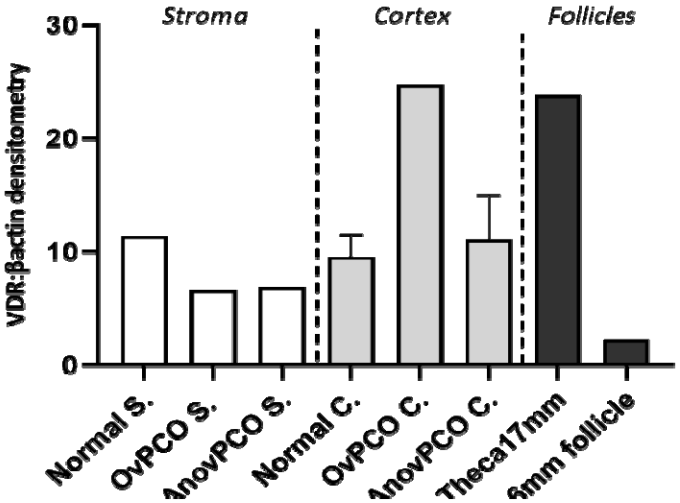


Fig 2e (bottom)



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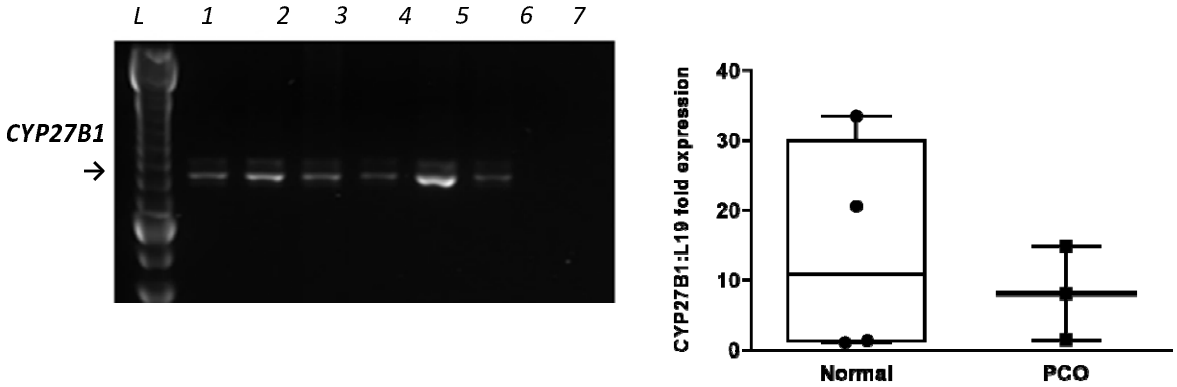
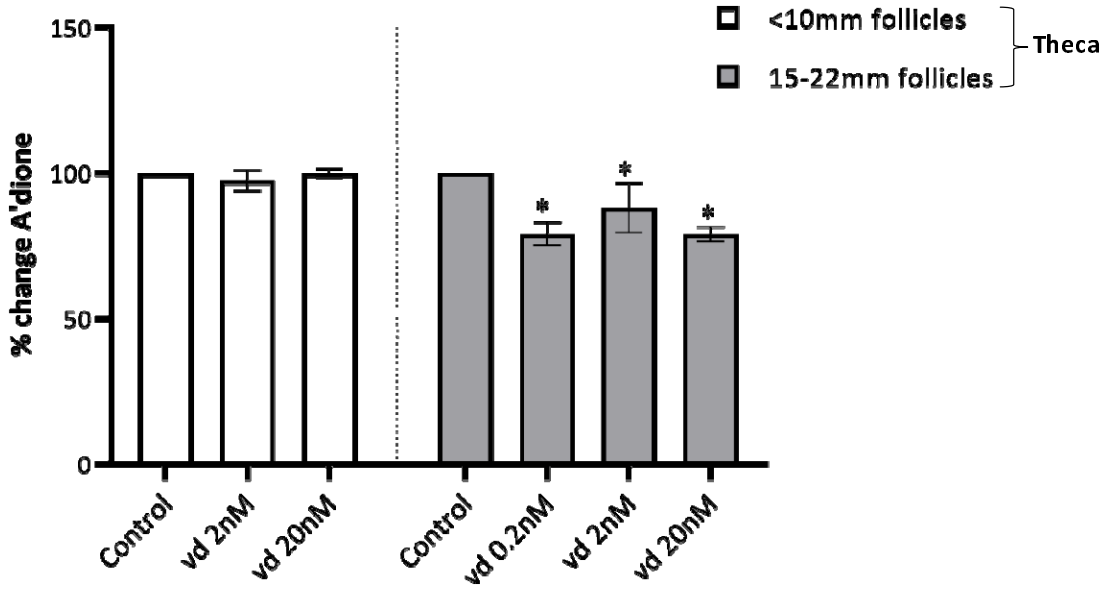

L
1
2
3
4
5
6
7
CYP27B1
CYP27B1:L19 fold expression
40
30
20
10
0
Normal
PCO

Fig 3a


<10mm follicles
15-22mm follicles
Theca
% change A'dione
150
100
50
0
*
*
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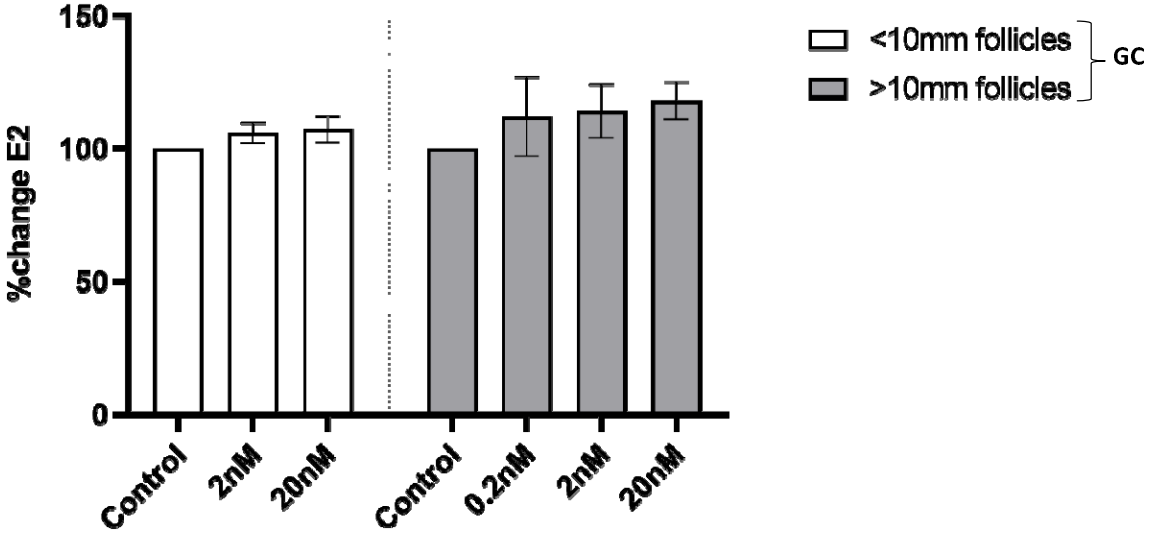
Control
vd 2nM
vd 20nM
Control
vd 0.2nM
vd 2nM
vd 20nM

Fig 3b


<10mm follicles
>10mm follicles
GC
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Control
2nM
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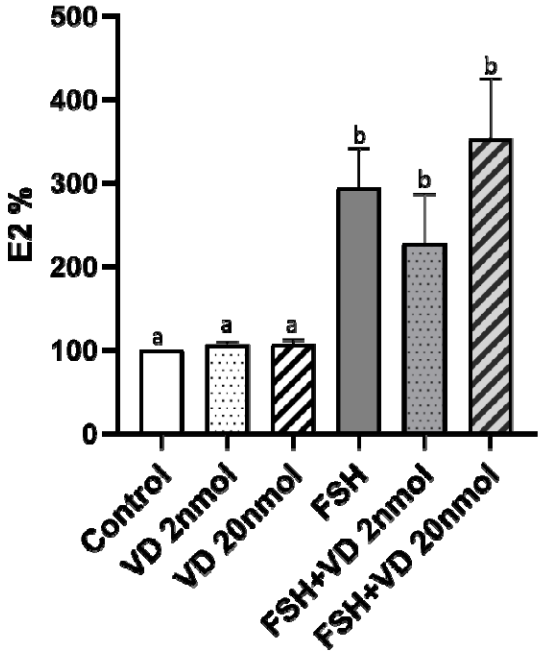
Control
0.2nM
2nM
20nM

Fig 3c



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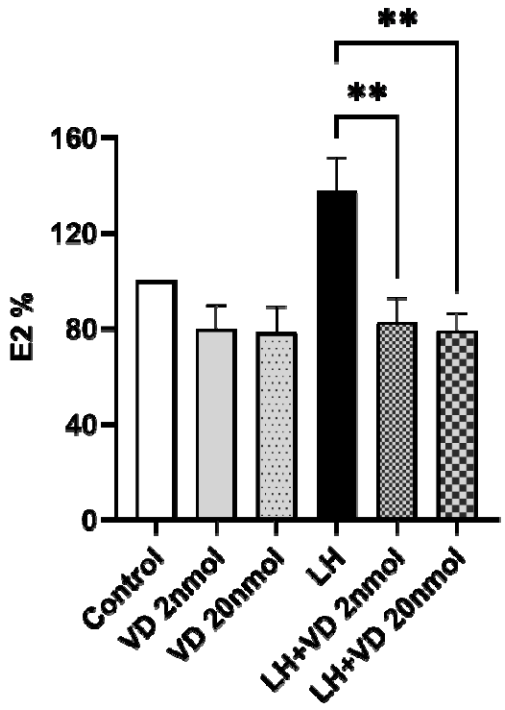

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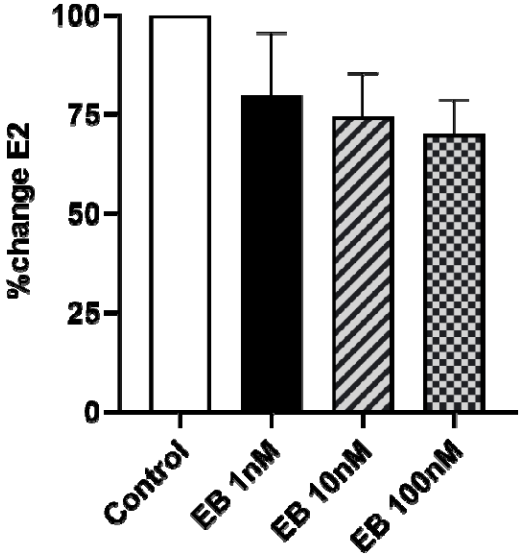


Fig 4a

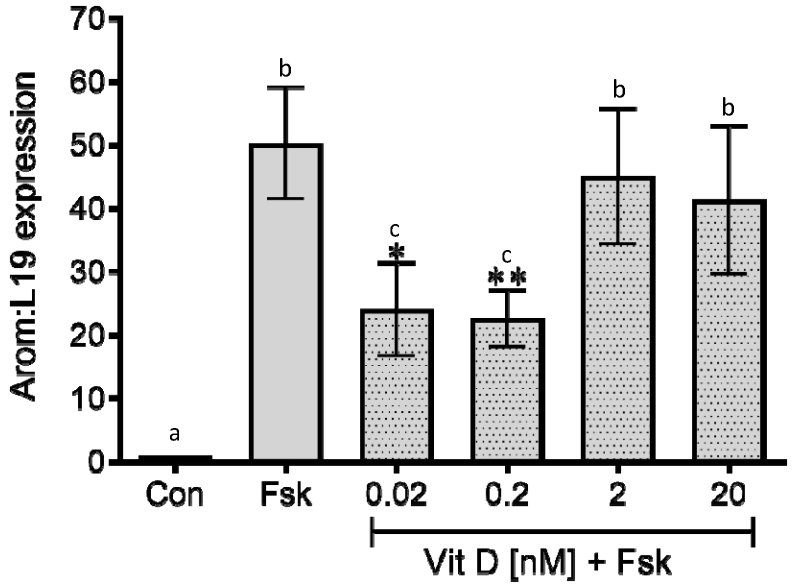

Arom:L19 expression
70
60
50
40
30
20
10
0
a
b
c
*
c
**
b
b
Con
Fsk
0.02
0.2
2
20
Vit D [nM] + Fsk

Fig 4b

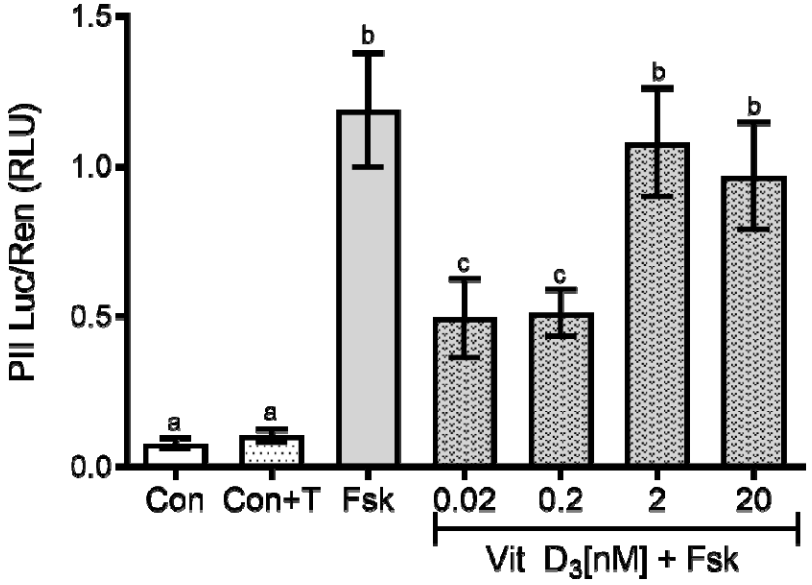

PII Luc/Ren (RLU)
1.5
1.0
0.5
0.0
a
a
b
c
c
b
b
Con
Con+T
Fsk
0.02
0.2
2
20
Vit D3[nM] + Fsk

Fig 5a

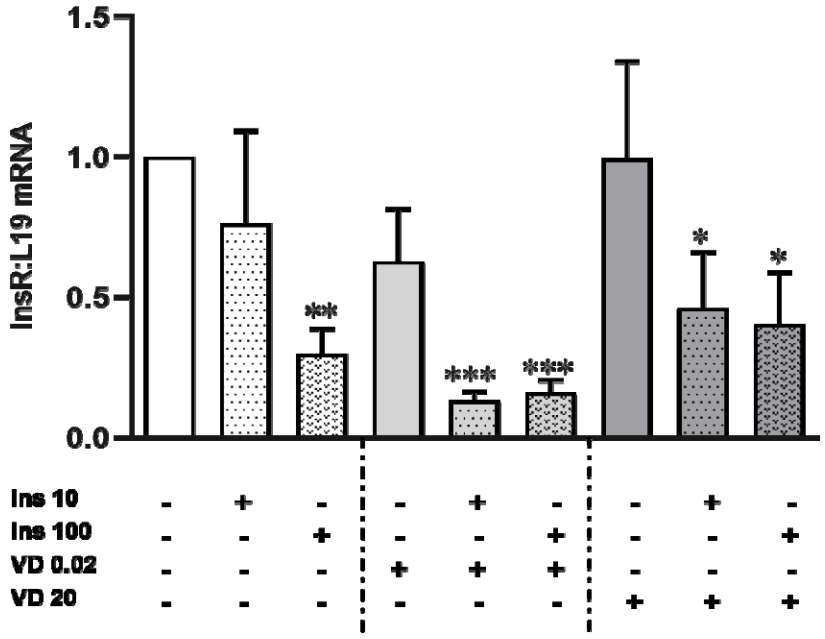

InsR:L19 mRNA
1.5
1.0
0.5
0.0
**
***
***
*
*
Ins 10 - + - - + - - + -
Ins 100 - - + - - + - - +
VD 0.02 - - - + + + - - -
VD 20 - - - - - - + + +

Fig 5b

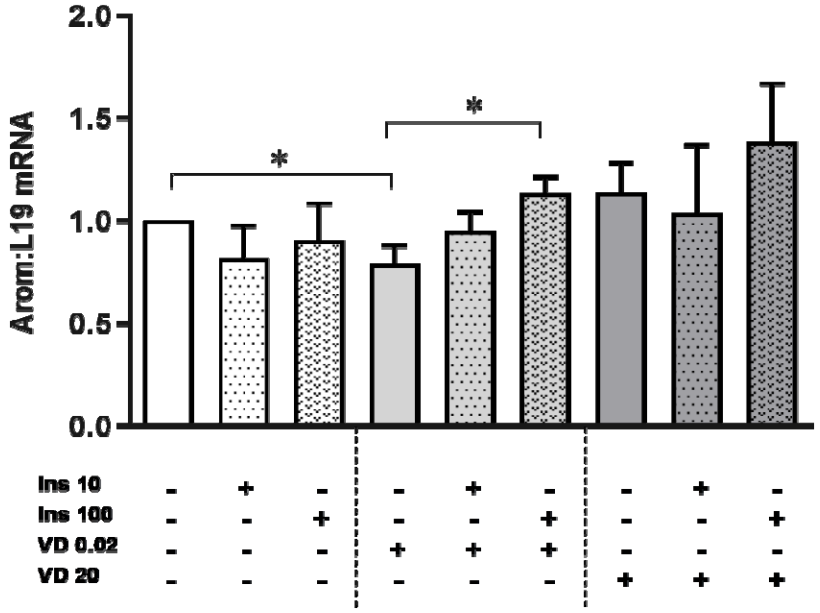

Arom:L19 mRNA
2.0
1.5
1.0
0.5
0.0
*
*
Ins 10 - + - - + - - + -
Ins 100 - - + - - + - - +
VD 0.02 - - - + + + - - -
VD 20 - - - - - - + + +

Fig 5c

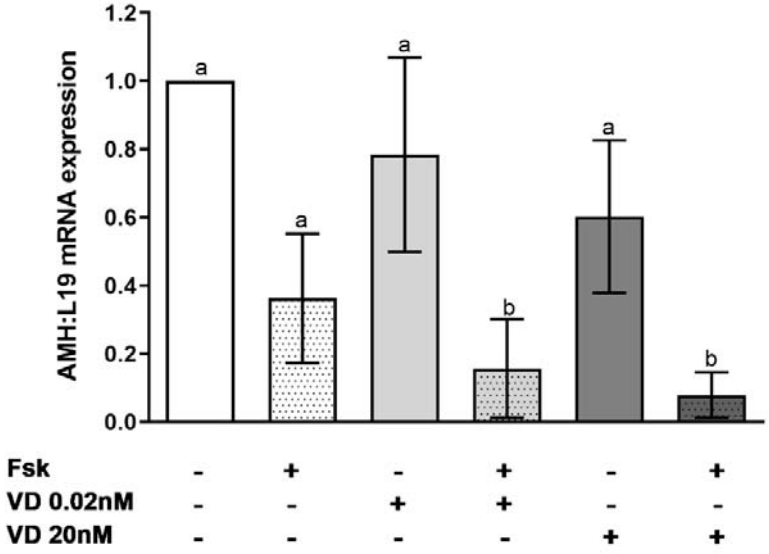

AMH:L19 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
b
a
b
Fsk - + - + - +
VD 0.02nM - - + + - -
VD 20nM - - - - + +

Fig 6

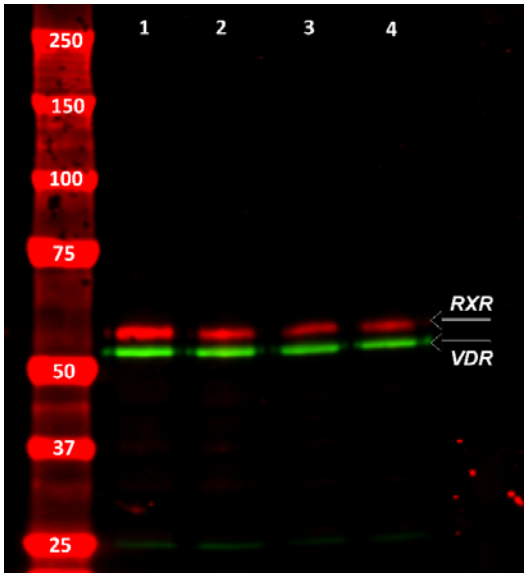

1
2
3
4
250
150
100
75
50
37
25
RXR
VDR

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

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

Table 2: List of antibodies used and conditions.

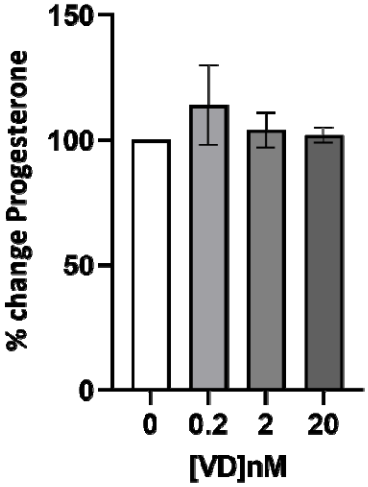
Antibody	Conc	Species raised	Source	Secondary used
Anti-VDR 53kDa	1:1000	Rat monoclonal	Abcam ab8756	Biotinylated monoclonal anti-rat (Vector) 0.1% [IHC] Goat anti-rat (Licor) 1:5000 conjugated 780nm IR dye [WB]
Anti-AMH	1:50	Mouse monoclonal	Abcam ab84952	Biotinylated monoclonal anti-mouse (Vector)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

Table 3: List of Primer used and conditions

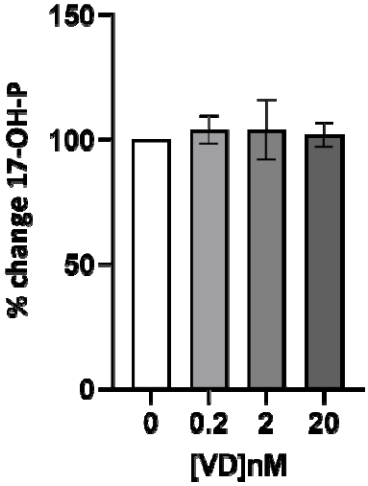
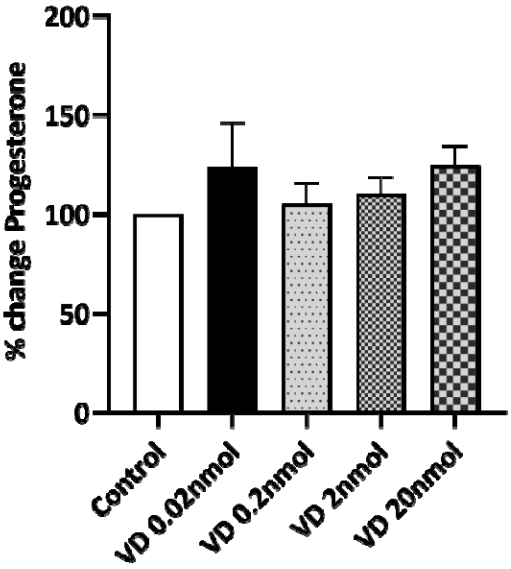
Gene	**Forward sequence 5'-3' Reverse sequence 5'-3'**	**Annealing temp °C**	**Primer conc [nM]**	**Accession number**
CYP19A1 (Aromatase)	F – GACTCTAAATTGCCCCCTCTG R – CAGAGATCCAGACTCGCATG	60	100	NM_000103.4
VDR	F-GACTTTGACCGGAA R-CATCATGCCGATGTCCACACA	55	300	NM_001017535.2
AMH	F- GCATGTTGACACATCAGGC R- GAGTGGCCTTCTCAAAGAGC	60	100	NM_000479.5
L19	F – GCGGAAGGGTACAGCCAAT R - GCAGCCGGCGCAAA	60	100	NM_000981.4
CYP27B1	F-CACTGTCCCAAAGCTGGCTA R-GCTTTCTGGCCGAACTTTTC	60	300	NM_000785.4
Insulin Receptor	F-GGTGCAGCCGTGTGACTTAC R-GTCATCAACGGGCAGTTTG	60	300	NM_000208.4

Supplemental Figures

S1. Progesterone production from theca



S2. 17-OH-P production from theca



S3. Progesterone production from GLC