The effect of Vitamin D (1,25-$(OH)_2$-D3) on human theca and granulosa cell function.

Abstract

Numerous observational and interventional studies have investigated the link between Vitamin D (VD) deficiency and reproductive outcomes, with contradictory results. VD is known to regulate steroidogenic enzymes crucial for human granulosa and cumulus cell function and genes that play a critical role in folliculogenesis have a vitamin D response element (VDRE) on their promoters. This study investigated whether deficient levels of 1,25-$(OH)_2$-D3 altered ovarian cell function; and if the ovary could obtain bioactive 1,25-$(OH)_2$-D3 via local enzymatic expression of *CYP27B1*, to counteract systemic deficiency. A variety of cells and tissues were used for the *in vitro* experiments as practicable.

We have shown for the first time an increase in VDR expression in theca of larger compared to smaller follicles, which along with the ability of 1,25-$(OH)_2$-D3 to decrease Anti-Mullerian hormone (AMH) expression, supports a role for 1,25-$(OH)_2$-D3 in theca and granulosa cell function. Conversely, we have shown that very levels of 1,25-$(OH)_2$-D3 equivalent to hypovitaminosis, inhibited thecal production of androstenedione and cAMP-driven E2 production. Human thecal and unluteinised GC are incredibly hard to obtain for research purposes, highlighting the uniqueness of our data set. For the first time we have demonstrated that deficient levels of 1,25-$(OH)_2$-D3 also down-regulated insulin receptor expression, potentially reducing insulin sensitivity. We have shown that the ovary expresses *CYP27B1* allowing it to make local bioactive 1,25-$(OH)_2$-D3 which along with the upregulation in VDR expression in all ovarian cellular compartments, could be protective locally in counteracting systemic VD deficiency. To conclude a severely deficient VD environment

(<2nM or <1ng/ml) could contribute to impaired ovarian cell function and hence potentially affect folliculogenesis/ovulation, but levels associated with mild deficiency may have less impact, apart from in the presence of hyperinsulinemia and insulin resistance.

Introduction

Vitamin D (VD), is a fat soluble prohormone which has both well-established actions such as its classical role in calcium homeostasis and skeletal integrity (Dusso, 2005), and putative ones such asanti-inflammatory and immunosuppressive roles(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 enzyme 1α-hydroxylase (encoded for by *CYP27B1*) (Schuster, 2011); (Luk J, 2012).

46 1-α-hydroxylase has also been found in extra-renal tissue indicating local conversion of 25-
47 (OH)-D3 to the active 1α,25-$(OH)_2$-D3. *CYP27B1* expression has been detected in follicles from
48 macaque and porcine ovaries (Xu J et al, 2018; Grzesiak M et al, 2020) and these studies
49 showed that 1,25-$(OH)_2$-D3 regulated follicle growth. Expression of 1,25-$(OH)_2$-D3 mRNA and
50 protein was also demonstrated in human granulosa luteal cells (GLCs) and in both benign and
51 malignant ovarian tissue (Fischer D et al, 2009). Thus, it is clear that ovarian expression of 1-
52 α-hydroxylase is capable of producing active 1,25-$(OH)_2$-D3 locally within the ovary from
53 circulating precursors.

54 The ability of active 1α,25-$(OH)_2$-D3 to exert its profound actions is mediated exclusively by
55 VDR, a member of the nuclear hormone receptor super-family. In addition to its non-genomic
56 actions outside of the nucleus, VDR acts as a ligand-inducible transcription factor (Haussler
57 MR, 2013), enabling it to regulate 3% of the human genome (Holick, 2007). Normally 1α,25-
58 $(OH)_2$-D3 enters the cell by diffusion where it binds to and activates VDR to form a
59 heterodimer complex with retinoid X receptor (RXR): this complex interacts with VD response
60 elements (VDREs) found in the promoter regions of both positively and negatively controlled
61 genes (Cheskis & Freedman, 1994). The localization of VDR to numerous cell types highlights
62 the importance of 1,25-$(OH)_2$-D3 as a regulatory hormone in many systems; consequently, VD
63 deficiency is recognised to be associated with numerous pathological conditions which is
64 usually reversed upon supplementation (Holick MF *et al*, 2011;2012).

65 Current guidelines define VD deficiency as 25-(OH)-D3 levels <20ng/ml (or <50nmol/L); with
66 levels of 21-29ng/ml (52.5-72.5nmol/L) characterized as VD insufficiency and >30ng/ml
67 (>75nmol/L) considered as sufficient (Holick MF *et al*, 2011; 2012) (Pilz S, 2019). Due to
68 avoidance of direct exposure to sunlight and/or nutrient-deficient diets, global deficiency of

69 VD is commonplace in both men and women, but this is exacerbated in northern climates due
70 to lack of sunshine.

71 The first indication that VD affected female fertility was shown by VD-deficient rats with
72 reduced fertility rates and litter sizes (reviewed in Lorenzen *et al*, 2017). VD has been shown
73 to interact and regulate steroidogenic enzymes that are crucial for human granulosa and
74 cumulus cell function (Merhi Z *et al*, 2014). Interestingly, genes for insulin receptor *(InsR)*,
75 anti-Müllerian hormone *(AMH)* and *CYP19A1* (encoding aromatase) that play a critical role in
76 folliculogenesis have VDRE on their promoters (Tiejun S *et al*, 1998); (Maestro B *et al*, 2003);
77 (Krishnan AV *et al*, 2007 & 2010), suggesting a role for VD in female fertility. This is supported
78 by studies showing that *in vitro* supplementation of VD promoted oocyte growth in *in-vitro*
79 developed macaque antral follicles (Xu J et al, 2016). Furthermore, there is a growing body of
80 evidence that VD has a role to play in fertility outcomes in assisted reproductive technology
81 and in women with polycystic ovary syndrome (PCOS), but the results are conflicting
82 (reviewed Lorenzen M. B., 2017).

83 PCOS is well-recognised as one of the most common endocrine disorders in women,
84 manifesting with increased androgen production, irregular menstrual cycles, and
85 dysregulated ovarian function. (Pellatt L *et al*, 2010). Additionally, there is a clear link between
86 vitamin D, glucose clearance and insulin secretion, such that insulin sensitivity is reduced in
87 vitamin D-deficient women with PCOS, sometimes independently of obesity (Łagowska *et al*,
88 2018). The compensatory hyper-insulinaemia is known to further amplify the increased thecal
89 androgen secretion in these ovaries (Nestler J.E., 1998) and vitamin D replacement has been
90 shown to reduce serum androgens in some cases (Menichini D., 2020). Interestingly this
91 reduction in hyperandrogenemia can occur in the absence of changes in insulin sensitivity,

92 suggesting a possible direct mechanism of action of VD (reviewed in Menichini D., 2020),
93 though it has to be acknowledged that obesity is a common confounding factor.

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

101 The aim of our study was to investigate the function of ovarian cells in a low vitamin D
102 environment (i.e., ≤20nM), thereby providing mechanistic insight to account for the variable
103 reproductive outcomes observed clinically in women with deficient serum levels of VD. Ideally
104 we would have performed all experiments using human primary ovarian cells and tissues but
105 the scarcity of this source meant we had to use a variety of model systems. In addition, whole
106 ovaries were never given for research, as part were retained for pathology.

107 Our collaboration with St. Lukes Hospital, Malta gave us access to human primary ovarian
108 cells and tissues, which was a valuable and scarce resource. Follicles were dissected from
109 partial human ovarian specimens to yield granulosa and theca cells for culture, and pieces of
110 cortex and stroma that were snap frozen. The ovarian follicles (preantral and early antral) are
111 in the cortical region. The stroma refers to components of the ovary that are not follicles and
112 include general components such as immune cells, blood vessels, stromal cells and extra-
113 cellular matrix. Over time pre-menopausal oophorectomy was justifiably phased out.

114 Where feasible we have utilised our stored specimens and material, along with human
115 luteinised granulosa cells (GLCs) from women undergoing IVF for functional experiments. For
116 more complex experiments requiring large number of cells, we have utilised the KGN
117 granulosa cell line. For preliminary immunohistochemistry (IHC) to determine VDR
118 distribution in various ovarian compartments, fixed mouse ovaries were used as no
119 appropriate human samples were available.

120 Materials and Methods

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

123 Tissue samples for *in vitro* experiments

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

126 *Human Ovarian Tissues:* Informed written consent was obtained from women undergoing
127 trans-abdominal hysterectomy with bilateral oophorectomy for benign gynaecological
128 conditions at St George's Hospital, London and St Luke's Hospital, Malta. Ethical approval was
129 granted by the relevant committee in each institution and informed consent obtained. Clinical
130 details were obtained including age, gynaecological history, menstrual frequency, and day of
131 cycle. The ovaries removed from each patient were seen by a pathologist before a portion of
132 each was taken to the laboratory for dissection. Morphology and ovulatory status were
133 assigned as previously published and were based on ovarian size, follicle sizes and numbers,
134 the presence of a dominant follicle or corpus luteum and the amount and density of stroma
135 as determined by dissection, in conjunction with patient history (Mason HD *et al*, 1994; Gilling
136 Smith C *et al*, 1994). The timing of surgery was random. Patient details, number of follicles

137 obtained, and tissue used in each experiment are shown in Table 1. Follicles were isolated
138 from the surrounding stroma, the diameter measured, and granulosa cells (GC) collected as
139 previously described (Mason H. D. *et al*, 1990; 1996). Finally, the theca cell layer was carefully
140 peeled off and digested in an enzyme cocktail for 30mins at 37C with gentle agitation (Mason
141 H. D. *et al*, 1990; 1996). GC and theca cells were cultures as outlined in subsequent sections,
142 other ovarian tissue samples were immersed in RNA-later or flash-frozen and archived at -
143 80C for further analysis.

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

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

155 **Immunohistochemistry of AMH and VDR in mouse ovarian sections.**

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

160 antibodies (Abcam, Cambridge, UK. Details listed in Table 2) as per manufacturers' protocol
161 with modifications and appropriate controls.

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

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

177 The mRNA expression of VDR in human ovarian cortex, stroma and theca tissue
178 compartments was assessed using archived frozen tissue collected as described above. All
179 tissue was stored and used under HTA regulations. Tissues were defrosted on ice and
180 homogenised in lysis buffer (RLT lysis buffer, Qiagen, Netherlands) using the FastPrep®
181 TissueLyser 24 and the FastPrep® Lysing Matrix A 2mL tubes containing a garnet matrix and a
182 ceramic sphere (MP Biomedicals). After homogenisation RNA was extracted, reverse

183 transcribed and real-time quantitative PCR (qPCR) performed for VDR relative to L19 (the
184 reference gene), as previously described (Rice S *et al*, 2006). In addition, the expression of
185 VDR in the KGN-GC was established using standard PCR as well as qPCR (see Table 3 for details
186 of primers and cycling conditions). For protein extraction the tissue was homogenised in RIPA
187 buffer with a cocktail of phosphatase inhibitors (Sigma-Aldrich, Merck, Gillingham, Dorset),
188 using a sonicator three times for 30s each time. Samples were maintained on ice throughout
189 to prevent overheating and denaturation of proteins. The lysed samples were micro-
190 centrifuged at 4C for 10min at maximum speed. Pelleted debris was discarded, and the
191 protein lysate stored at -80C prior to Bradford assays (for measurement of protein
192 concentration) and western blotting.

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

194 The granulosa or theca cells were pooled from several follicles based on the experimental
195 protocol and cultured in McCoys 5A medium supplemented with penicillin/streptomycin, L-
196 glutamine and amphotericin B (all purchased from Invitrogen, UK). Granulosa cells were
197 plated in 96-well plates at $5x10^4$ and theca cells at $5x10^5$ cells per well as previously described
198 (Mason *et al*, 1990; Willis *et al*, 1996). To increase the adhesion of theca cells to the plate,
199 plates were first coated in ECM gel (McCaig, 2002).

200 Cells were incubated in culture medium (plus experimental treatments) for 48 hours with a
201 range of 1,25-$(OH)_2$-D3 concentrations (0.2, 2 and 20nM serially diluted down from a stock
202 concentration of 2µM). Testosterone ($5x\ 10^{-7}$ M) was used as a substrate for conversion to
203 oestradiol in the granulosa cells and steroid levels measured in the medium by
204 radioimmunoassay (Hillier SG *et al*, 1980); (Gilling-Smith C *et al*, 1994); (Willis DS *et al*, 1996).
205 To confirm that cells were healthy, 10ng/ml LH was used as a positive control in luteinised
206 GCs and theca, and 5ng/ml FSH in cells dissected from small follicles (LH and FSH supplied by

207 Endocrine Services, Biddeford-upon-Avon, UK). Isolation of GLC was performed as previously
208 described (Wright R *et al*, 2002). Cells were plated in 96-wells at $5x10^4$ cells per well in 200µl
209 of serum (5%) supplemented medium for 48 hours, after which the medium was removed
210 and replaced with experimental treatment as detailed above. GLCs were also exposed to the
211 vitamin D analogue ED1089 (kindly donated by Dr. Kay Colston, SGUL), to examine
212 comparative efficacy and demonstrate specificity.

213 **KGN-GC culture experiments**

214 To model the effect that VD deficiency may have on ovarian function, KGN-GC were grown
215 and passaged in 10% DMEM-F12 supplemented with L-glutamine and penicillin/streptomycin
216 (Invitrogen), at 37◦C in 95% air/CO_2. Cells were plated in 12-well plates (3×10^3 cells/well) and
217 cultured in 1% DMEM-F12 (charcoal-stripped) overnight. Cells were serum-starved for 2h and
218 then treated with forskolin (25µM), to reproduce the effect of LH on cAMP stimulation, ±
219 1,25-$(OH)_2$-D3 at 0.02, 0.2, 2 and 20nM and cultured for a further 48h. Testosterone ($5x10^{-}$
220 ^{7}M) was added to all cultures as the aromatase substrate for conversion to oestrogen.
221 Similarly, the effect of VD on AMH mRNA expression was investigated in cells treated with
222 forskolin and VD as described above, using only the upper and lower doses of 1,25-$(OH)_2$-D3
223 from the range above at 0.02 and 20nM. At the end of the relevant culture time, RNA was
224 extracted, reverse transcribed and real-time quantitative PCR (qPCR) performed for *CYP19A1*
225 (encoding aromatase) as described previously (Rice S *et al*, 2013). We have previously
226 established that *CYP19A1/aromatase* mRNA, protein expression and enzyme activity are all
227 tightly correlated in this model and hence any of these techniques can be used
228 interchangeably to assess changes (Rice *et al*, 2006; Rice *et al*, 2013).
229 To investigate the effect of VD on aromatase promoter II (PII) activity, cells were plated at a
230 density of $2x10^4$ cells/well in 96-well plates. After overnight incubation, they were transfected

231 with a *CYP19A1 PII-516* reporter construct expressing firefly Luciferase, along with a control
232 plasmid expressing Renilla luciferase from a constitutive promoter, and a transcription
233 enhancing element, PVAi, as previously described (Rice S *et al*, 2013). After 2h serum-
234 starvation, cells were treated as described above for 24 hours with quadruplicate
235 replicates/treatment, and luciferase reporter assays were carried out using the Dual-Glo
236 Luciferase Assay System (Promega).
237 To mimic a situation of chronic insulin exposure, cells were treated with insulin for 48h at
238 post-prandial (10ng/ml) and hyperinsulinemic (100ng/ml) levels in the presence and absence
239 of the lowest [0.02nM] and highest [20nM] 1,25-$(OH)_2$-D3 levels tested in the experiments.
240 The effect of 1,25-$(OH)_2$-D3 on aromatase and insulin receptor (InsR) mRNA expression was
241 measured by qPCR.
242 **Radioimmunoassay (RIA) for P, E2 and Androstenedione**
243 Conditioned media was collected from the cells at 48 hours, frozen at -20C and E2, P and
244 androstenedione measured using a modified 'in-house' RIA, with tritiated steroids and
245 charcoal separation (Hillier SG *et al*, 1980); (Gilling-Smith C *et al*, 1994); (Willis DS *et al*, 1996).
246 The components of the assay were: tritiated steroid label (Amersham Pharmaceuticals, Bucks,
247 UK), steroid antiserum (sheep anti-human from Guildhay, Guildford, UK) and standards and
248 quality controls (QCs) (approximately 1, 6 and 35% of the top standard), prepared from
249 powdered steroid (Sigma Co Ltd, Poole, UK). Conditioned medium was diluted to be within
250 the midrange values for each assay and therefore on the linear part of the standard curve.
251 Each sample was tested in duplicate.
252 **Immunoprecipitation (IP)**
253 To investigate the effect of 1,25-$(OH)_2$-D3 on VDR:RXR dimer formation, KGN cells were plated
254 at a density of $5x10^5$ cells/well in a 6-well plate and treated as described above with and

255 without forskolin, testosterone and 1,25-$(OH)_2$-D3 at 0.02 and 20nM for 48h. At the end of
256 culture time, media was removed, cells washed with ice-cold PBS and lysed with ice-cold RIPA
257 buffer as previously described (Dilaver N *et al*, 2019). The protein concentration of the lysate
258 was measured by Bradford assay and prior to immunoprecipitation, equal amounts of protein
259 lysate from each treatment group were pre-cleared on A/G-coupled Sepharose beaded
260 (Pierce) support (binding capacity 27mg mouse IgG or ≥ human IgG/ml settled resin) for one
261 hour at 4C to reduce non-specific ligand binding. Immunoprecipitation of the cleared lysates
262 was performed overnight at 4C with 5µg of anti-VDR antibody or anti-RXRα monoclonal
263 antibodies (Abcam – Table 2). The beaded complexes were washed several times with NP-40
264 lysis buffer, after which protein complexes were dissociated by boiling for 10 mins with 1X
265 SDS-DTT reducing buffer (protocol adapted from Luderer HF *et al*, 2011). The collected
266 supernatant was stored at -80C for subsequent Western blotting analysis. Negative controls
267 included retention of pre-cleared beads which were eluted and run on western blots,
268 alongside the total lysates.

269 **Western Blotting of VDR and RXR**

270 Protein concentrations were measured using the Bradford assay (BioRad, Hertfordshire) and
271 equal amounts of protein obtained from lysing ovarian tissues or from immunoprecipitation
272 experiments, were resolved with Western blotting as previously described (Dilaver N *et al*,
273 2019). PVDF(fl) (Immobilon, Millipore Merk, Massachusetts) membranes with transferred
274 proteins were incubated with rat anti-human VDR or rabbit anti-human RXR (both used at
275 1:1000) and/or mouse anti-human β-actin (1:2000). Fluorescently conjugated relevant
276 secondary antibodies (1:5000) (see Table 2 for details of antibodies) were used for
277 visualisation using the Odyssey Imaging System (Li-Cor Biosciences) (Dilaver N *et al*, 2019).

278

279 **Experimental Quantification and Statistical Analysis**

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

297

298 ## Results

299 We analysed expression (via qPCR, IHC and WB) of key elements (VDR and *CYP27B1)* involved
300 in the synthesis and signalling pathway of VD in various ovarian cellular compartments. We
301 then proceeded to investigate the effect of a range of doses of VD on steroid output (RIA)

302 from primary theca and unluteinised granulosa cells dissected from antral follicles of varying
303 sizes cultured *in vitro*. The effect of VD on oestradiol (E2) production (RIA and qPCR) was also
304 analysed in luteinised GLCs and the KGN cell-line. Since VD is known to interact with other
305 factors involved in granulosa cell proliferation, we chose two of particular interest, namely
306 AMH and insulin to investigate via qPCR. Finally, we examined the relationship via IP between
307 varying doses of VD and the degree of binding to VDR and RXR receptors, which then complex
308 to VDREs to modulate its actions.

309

310 **Expression of VDR and *CYP27B1* in ovarian tissue and cellular compartments**

311 IHC was used to detect the extent of VDR protein expression in sequential mouse ovary
312 sections, and the anti-VDR antibody revealed strong and extensive staining throughout all
313 mouse ovarian tissue compartments including the cellular components of the follicles (figure
314 1a (negative control) and 1b). Closer examination revealed more intense staining in thecal
315 cells of LAF compared to SAF (inset figures 1b). The general uniformity and ubiquity of VDR
316 expression was in marked contrast to staining with anti-AMH antibody in the sequential
317 section, which showed a distinctive pattern of expression i.e., greater staining in GC of SAF
318 compared to LAF, with no staining in theca or ovarian compartments (figure 1c).
319 This finding in the mouse ovary was extended by measuring VDR mRNA levels in human theca
320 cells dissected from human follicles of varying sizes to ascertain whether expression levels
321 changed with follicle size. VDR mRNA levels were significantly higher in theca from larger
322 antral follicles (LAF) and those approaching preovulatory sizes (7-12mm, n=11), compared to
323 those from smaller antral follicles (SAF) (5-6mm, n=4) (unpaired t-test, *p=0.013; figure 2a)
324 which supported the observations in the mouse ovary (fig 1b).

325 In addition, VDR mRNA (fig 2b) and protein (fig 2c) expression were also detected in human
326 cortex and stroma... There was no statistically significant difference between the two
327 compartments. *CYP27B1* mRNA was expressed in human ovarian stroma as assessed by qPCR,
328 though there was considerable variation in expression levels between different biological
329 samples (fig 2d).
330
331 **Effect of 1,25 (OH)2-D3 on steroid production in ovarian cells.**
332 ***VD and theca steroidogenesis:*** Overall, in theca from LAF (15-22mm), exposure to all doses
333 of 1,25-$(OH)_2$-D3 consistently suppressed Androstenedione production (mean 22%
334 suppression) but had no effect on androstenedione production from SAF (n=4-8, Two-way
335 ANOVA, p=0.048 for treatment and p=0.002 for follicle size) (fig 3a). Treatment with 1,25-
336 $(OH)_2$-D3 had no effect on either P or 17-OH-P production (supplemental figures S1 and S2).
337
338 ***VD and unluteinised GC steroidogenesis***: There was no effect of 1,25-$(OH)_2$-D3 at any dose
339 on E2 production in GCs taken from follicles <10mm diameter (n=4; figure 3b); however, in
340 follicles >10mm there appeared to be a trend to increasing E2 with 1,25-$(OH)_2$-D3 though this
341 was not significant (figure 3b). In addition, 1,25-$(OH)_2$-D3 did not alter the normal
342 responsiveness to FSH in SAF (n=4, ANOVA p<0.0001) (figure 3c).
343
344 ***VD and E2 production in GLC:*** The effect of VD on E2 production from luteinised granulosa
345 cell culture was then investigated. Luteinisation of GC occurs once follicles have been exposed
346 to the mid-cycle ovulatory LH surge. As expected, exposure of the cells to LH produced a
347 substantial stimulation in the production of E2, surprisingly this was considerably attenuated
348 by 1,25-$(OH)_2$-D3 at both 2nM and 20nM doses (figure 3d) (ANOVA p<0.0001; post-hoc t-test

349 **p<0.005). This contrasts with the results from unluteinised GC from small follicles in which
350 1,25-$(OH)_2$-D3 had no effect on FSH-stimulated E2 production (figure 3c). Interestingly
351 culturing GLC with the VD analogue EB1089 also appeared to suppress E2 production in a
352 dose-related manner (figure 3e), though the data did not reach statistical significance (ANOVA
353 n/s). There was, however, no effect on progesterone production in the cells by any dose of
354 VD (supplemental figure S3).
355
356 **Effect of VD on aromatase expression in KGN-GC**: To determine the mechanism by which
357 1,25-$(OH)_2$-D3 was altering E2 production in primary cells, KGN cells were cultured with
358 forskolin to increase cAMP and hence aromatase activity (equivalent to stimulation by either
359 gonadotrophin, which both act via cAMP). Very low 1,25-$(OH)_2$-D3 levels [0.02 & 0.2nM]
360 significantly down-regulated Fsk-stimulated aromatase mRNA expression (n=6, One-way
361 ANOVA**p=0.0018; post-hoc t-tests *p<0.05, **p<0.005), but when doses approached
362 sufficiency levels [2 and 20nM] this attenuation was reversed. An identical pattern was seen
363 in PII transfected KGN cells demonstrating that 1,25-$(OH)_2$-D3 affected aromatase activity as
364 well as expression (figures 4a & 4b) (n=3; ANOVA****p<0.0001; post-hoc t-tests *p=0.02,
365 **p=0.0095).
366
367 **Effect of VD on other factors implicated in regulation of follicle growth.**
368 *Insulin:* There is compelling evidence that systemic VD levels are correlated with insulin
369 sensitivity (Łagowska *et al*, 2018); however, there is little supportive evidence at a cellular
370 level. Chronic exposure of granulosa cells to high (100ng/ml) insulin significantly
371 downregulated expression of total InsR mRNA (p=0.001), and this reduction was surprisingly
372 potentiated by both doses of 1,25-$(OH)_2$-D3: 20nM (<50% of basal) and 0.02nM (<25% of

373 basal) (n=5-8; One-way ANOVA *p=0.048, one-sample t-tests to the control ***p<0.0005,
374 **p<0.005, *p<0.05) (figure 5a). This profound attenuation of insulin receptor expression by
375 1,25-$(OH)_2$-D3 in the presence of chronic insulin exposure did not have a concomitant
376 reduction on aromatase expression in the same cells. In fact, the presence of insulin reversed
377 the attenuation of basal aromatase levels brought about by low dose VD (figure 5b).
378 *AMH:* Forskolin down-regulated AMH expression compared to basal (<50%), which was
379 further potentiated in the presence of 1,25-$(OH)_2$-D3, with a more potent attenuation by
380 20nM 1,25-$(OH)_2$-D3 compared to 0.02nM (figure 5c) (n=3, ANOVA *p=0.02; multiple
381 comparison t-test *p<0.05).
382
383 **Investigating the differential effects of varying doses of VD ligand.**
384 Data presented so far indicate that those doses of 1,25-$(OH)_2$-D3 equivalent to extremely
385 deficient levels have differential effects compared to those approaching sufficiency. To
386 determine a possible mechanism, the relationship between levels of VD ligand and the
387 proportion of either VDR or RXR was investigated. KGN-GC treated with extremely low or
388 sufficient doses of 1,25-$(OH)_2$-D3 in the presence of Fsk, were immunoprecipitated with either
389 anti-VDR or anti-RXR antibodies. The VDR-IP pulls down all forms of VDR:homodimers,
390 heterodimers and solo receptor as does the RXR-IP. The immuno-precipitated samples were
391 then immunoblotted using either anti-VDR or anti-RXR antibodies to assess just the
392 heterodimer association between RXR and VDR in the relevant IP samples (figure 6). The
393 subsequent western blot membranes were analysed in a variety of ways (tables 4a and
394 4b).There was a significant reduction in levels of VDR associated with RXR-IP in the presence
395 of VD [20nM] combined with Fsk compared to basal, FSk alone or Fsk+ 0.02nM 1,25-$(OH)_2$-D3
396 (table 4a) However, there was no change in RXR levels associated with VDR-IP (table 4b).

397

398 **Discussion**

399 We have discovered widespread distribution of VDR in the human ovary, pointing to a likely
400 critical role for VD in female reproduction. We have demonstrated for the first time, a clear
401 relationship between increasing VDR expression in theca and follicle size, highlighting a
402 possible important functional role for Vitamin D in antral follicle progression.

403 Our findings in human theca were supported by the results of histological analysis of mouse
404 ovarian follicles which showed an increase in the intensity of VDR expression in theca of LAFs.
405 That activation of these receptors is playing a role in follicle growth was demonstrated by the
406 fact that VD3 supplementation promoted the survival and growth of *in vitro* cultured
407 preantral follicles to the antral stage in the primate ovary (Xu J, 2018). Our finding of protein
408 expression of VDR in human cortex and stroma was also matched by extensive and uniform
409 expression of VDR protein in granulosa cells, cortex, and stroma of mouse ovaries, further
410 indicating an important role for VD throughout the ovary. This is also supported by the fact
411 that female VDR knockout mice have impaired folliculogenesis with no progression beyond
412 primary and secondary stages and low oestradiol levels (Yoshizawa T, 1997).

413 The detection of *CYP27B1* mRNA expression in human ovarian stroma was intriguing as it
414 indicated that the human ovary is capable of being an extra-renal site of active 1,25-$(OH)_2$-D3
415 synthesis, as supported by studies in other species (Xu J *et al*, 2018). The contribution of any
416 locally produced bioactive 1,25-$(OH)_2$-D3 is difficult to determine, but could play an important
417 role in counteracting systemic VD deficiency. Active 1,25-$(OH)_2$-D3 negatively regulates
418 expression of CYP27B1, hence regulating its own circulating concentrations (Bouillon R *et al*,
419 2008).

420 This increasing expression of VDR in the growing follicle at the LAF stage corresponds to
421 increasing steroidogenesis and the acquisition of LH receptors. It had been documented that
422 incubation of human cumulus GC with VD at normal levels affects steroid output (Merhi Z *et*
423 *al*, 2014), but we wished to investigate the extent of and mechanisms by which exposing cells
424 to a 1,25-$(OH)_2$-D3-deficient environment affected steroidogenesis. To that end we incubated
425 cells with 20, 2, 0.2 or 0.02nmol/L (since serum levels <50nmol/L is defined as severe
426 deficiency) (Holick MF *et al*, 2011). A recent study of women of childbearing age in rural
427 northern China found the prevalence of severe VD deficiency was 16% in the 1151 women
428 studied, with a median serum level of only of 5.63ng/ml (equivalent to 14nmol/L) (Lin S *et al*,
429 2021); indicating that severe hypovitaminosis of this magnitude occurs in the population.
430 Interestingly, 1,25-$(OH)_2$-D3 is established to act at nanomolar, or even picomolar,
431 concentrations, as a direct regulator of specific target genes in VDR-expressing cell and
432 tissues. It also binds to VDR with an affinity of 0.1nM (reviewed in Carlsberg, 2022).

433 In GC, gonadotrophins stimulate oestrogen synthesis via cAMP-mediated signalling, and it is
434 well-established that a cAMP-response element (CRE) sequence has been identified within
435 promoter II (PII); the predominant aromatase promoter used in the ovary (Michael MD, 1997).
436 Changes in the 1,25-$(OH)_2$-D3 environment had no effect on E2 production from GC of either
437 SAFs/LAFs, in the presence or absence of FSH and FSH responsiveness was retained.
438 Conversely in <u>luteinised</u> granulosa cells (GLCs), 1,25-$(OH)_2$-D3 did prevent the LH-mediated
439 stimulation of E2 production. It is without doubt that 1,25-$(OH)_2$-D3 can decrease E2
440 production as the same effect was observed with the VD analogue in GLCs.

441 This would imply that attenuation by VD only occurred when GC acquired LHR or else is
442 dependent on levels of cAMP generated, since LH stimulates more cAMP activity than FSH

443 (Aharoni D *et al*, 1995). This is supported by the observation that extremely low doses of 1,25-
444 $(OH)_2$-D3 down-regulated forskolin-stimulated aromatase expression and activity in KGN cells
445 (which do not have LHR), an effect that was lost once dosing levels were increased.

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

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

464 <u>Unliganded</u> VDR-RXR heterodimers are initially bound to a VDRE and recruit co-repressor
465 complexes, which prevents basal transcription through the activity of histone deacetylase.

466 Once sufficient ligand has bound, the repressors are substituted by co-activator complexes,
467 allowing gene transcription to commence (Carlsberg C, 2022; Dwivedi PP *et al*, 1998; Perissi
468 V *et al*, 2010). The results of our IP experiments showed a difference in the levels of VDR
469 associated with immunoprecipitated RXR, at different concentrations of VD ligand in the
470 presence of forskolin. In the presence of higher concentrations of VDR ligand there appears
471 to be less VDR associated with immunoprecipitated RXR. However, it must be pointed out
472 that it was not possible from our experiments to determine the proportion of heterodimers
473 in the cytoplasmic or nuclear compartments (and hence non-genomic/genomic forms) as we
474 used whole cell lysates. To answer this, we would need to carry out further experiments
475 (beyond scope of this study) to explore VDR sub-cellular trafficking between nuclear and
476 cytoplasmic compartments and components of the repressor/activator complexes bound to
477 chromatin. Interestingly, the ability of forskolin to alter the association of the intracellular
478 dimers would indicate the presence of a ligand-independent cAMP activated pathway outside
479 the nucleus (Luk J *et al*, 2012); (Haussler MR *et al*, 2013) which could account for the different
480 outcomes observed when using either FSH or LH.

481 As stated earlier, women with PCOS commonly present with increased serum levels of AMH
482 (reflecting the pool of stalled SAF) and hyperinsulinemia, both of which are also linked to VD
483 status (Luk J, 2012; Lorenzen M. B., 2017). To investigate this link, we replicated
484 hyperinsulinemia and insulin resistance *in vitro* by chronically exposing KGN cells to insulin at
485 either post-prandial (10ng/ml) or hyperinsulinemic (100ng/ml) doses. The HI dose
486 downregulated expression of insulin receptor mRNA by more than 50% reproducing insulin
487 resistance, whereas there was no significant effect noted at post-prandial treatment levels.
488 To our surprise, culture of cells in an extremely low and deficient VD environment caused an
489 even further attenuation of insulin receptor expression in the presence of both doses of

490 insulin. This effect only occurred in the presence of insulin, as 1,25-$(OH)_2$-D3 alone had no
491 effect on insulin receptor expression. Systemic VD deficiency is clearly linked to a reduction
492 in insulin sensitivity (independent of BMI) as others have shown (Muscogiuri G *et al*, 2017),
493 and this insulin receptor reduction may be a contributory mechanism. Interestingly, in the
494 same cells a combination of insulin and 1,25-$(OH)_2$-D3 had no effect on aromatase, unlike in
495 the forskolin experiments, again indicating that this was a cAMP-driven process, possibly
496 linked to overlap between VDRE and CRE on PII as described previously.

497 It is well established that follicle progression in the normal human ovary requires down-
498 regulation of AMH expression to permit FSH-driven activity (Pellatt L *et al*, 2010). The IHC
499 analysis of mouse ovaries also revealed high AMH expression in SAF which was substantially
500 reduced in pre-ovulatory follicles. This is thought to normally occur via LH down-regulating
501 AMH expression (Pierre A *et al*, 2013). Using forskolin instead of LH to achieve down-
502 regulation, we demonstrated that this reduction in AMH was potentiated by 1,25-$(OH)_2$-D3
503 [0.02nM] with a further reduction occurring with higher doses of 1,25-$(OH)_2$-D3 [20nM].
504 Interestingly treatment with 1,25-$(OH)_2$-D3 alone had no significant effect on AMH mRNA
505 levels, indicating again that this only occurs when the cAMP pathway is activated.

506 In hen GC from 3-5mm and 6-8mm follicles, vitamin D3 was shown to substantially decrease
507 AMH mRNA expression, with a more robust effect seen in the LAF (Wojtusik J, 2012). In
508 addition, Mehri *et al* showed that in AF (<14mm) from women with insufficient/deficient
509 follicular fluid levels of 25-OH-D3, there was significantly higher AMHR-II mRNA level
510 compared to those with normal VD levels (Merhi Z *et al*, 2014). In the same study, 25-OH-D3
511 was shown to decrease AMH-mediated pSMAD 1/5/8 nuclear localisation in cumulus GC,
512 indicative of reduced AMH signalling. Hence at a local ovarian level, our data and other

513 studies, clearly showed that VD is involved in AMH regulation and expression, which could
514 impact on follicle progression particularly in conditions such as PCOS. At a systemic level
515 however, evidence for correlations between serum vitamin D and serum AMH levels are
516 contradictory and dependent on a woman's ovulatory status (Moridi I *et al*, 2020).

517 To summarise, we have shown that Vitamin D clearly has a role to play in the theca and
518 granulosa cell function and hence growth of ovarian follicles, as shown by the increased
519 expression of VDR in follicles of increasing size. In addition, we have shown that Vitamin D
520 may promote follicle progression by downregulating the expression of AMH, thereby reducing
521 AMH's well-documented inhibitory effect on follicle growth (Pellatt L *et al*, 2010). Vitamin D
522 is known to affect steroidogenesis and we have demonstrated that levels of 1,25-$(OH)_2$-D3
523 equivalent to hypovitaminosis, inhibited thecal production of androstenedione. In addition,
524 extremely low levels of vitamin D had an attenuating effect on cAMP-driven aromatase
525 expression, which translated to decreased E2 production. Encouragingly this reduction in E2
526 is reversed as levels of 1,25-$(OH)_2$-D3 increased; apart from in the presence of LH in luteinised
527 GC, which could have consequences for regular ovulation in women with severe Vitamin D
528 deficiency. For the first time we have demonstrated that deficient levels of 1,25-$(OH)_2$-D3 also
529 down-regulated insulin receptor expression, potentially reducing insulin sensitivity. This could
530 have serious implications for women with hyperinsulinemia and insulin resistance, typically
531 seen in PCOS; indicating that insulin resistant women should try and maintain sufficient levels
532 of systemic vitamin D for regular ovarian function. The ability of the ovary to make local
533 bioactive 1,25-$(OH)_2$-D3, was demonstrated by the expression of *CYP27B1.* This together with
534 the upregulation of VDR expression in all ovarian cellular compartments, could potentially
535 counteract the effect of systemic VD deficiency and protect the local ovarian environment.
536 To conclude a severely deficient VD environment (<2nM or <1ng/ml) could contribute to

537 impaired ovarian cell function and hence potentially affect folliculogenesis/ovulation, but
538 levels associated with mild deficiency may have less impact, apart from in the presence of
539 hyperinsulinemia and insulin resistance.

540

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686 ***Statement regarding use of archived tissue***: Research and the Human Tissue Act 2004 -
687 Consent IS REQUIRED to use and store tissues for research; UNLESS: The relevant material is
688 classed as an existing holding i.e., held prior to 1st September 2006 and/or the relevant
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692 Prof. Mark Brincat. Additionally, we would like to thank Mr Michael Lacey for his technical
693 assistance with various aspects of the project; along with Ms Yvette Bland for her help with
694 the IHC of mouse ovaries. Particular thanks must go to Erin Vang who helped with the retrieval
695 and conversion of Statview Data files to Excel. We also acknowledge the experimental
696 contribution of intercalated and BSc students at St. George's University of London – Angus
697 Mansfield, Andrew Standing and Ella Jameson.

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

700

701 **Figure Legends:**

702

703 **Fig 1a:** Negative control (no VDR primary antibody) of the sectioned mouse ovary with a
704 variety of small antral follicles (SAF) and large antral follicles LAF).

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

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

714 **Fig 2a:** mRNA expression of VDR quantified by qPCR, in <u>human theca</u> taken from human
715 ovarian follicles (both normal and polycystic ovaries), showed significantly lower expression
716 in SAF (5-6mm ●) compared to LAF (7-12mm ▲). *(Unpaired t-test, two-tailed*p=0.0128; 5-*
717 *6mm (n=4); 7-12mm (n=11)).*

718 **Fig 2b:** VDR mRNA expression quantified by qPCR in human cortex (grey bar ●, *n=6*) and
719 stroma (white bar ■, *n=5*). There was no significant difference in expression.

720 **Fig 2c:** VDR protein expression levels quantified by western blotting, in human ovarian cortex
721 (*n=5; grey bar ■)* and stroma (*n=4; white bar* ●), also showed no significant difference in
722 expression. *Densitometry values on y-axis multiplied by 1000 for scaling purposes*.

723 **Fig 2d:** CYP27B1 mRNA expression quantified by qPCR, in human ovarian stroma samples –
724 both normal and PCO *(n=8).*

725 **Fig 3a:** Effect of 1,25$(OH)_2D_3$ on androstenedione production from theca taken from small
726 (<10mm, white bars, *n=4 subjects, total follicles=15*) and large follicles (15-22mm, grey bars,
727 *n=5 subjects, total follicles=5*) from normal ovaries. 1,25$(OH)_2D_3$ suppressed androstenedione
728 production significantly but only in theca from large follicles. Results expressed as a
729 percentage change from control, where the control was taken as 100%. *(Two-way ANOVA*
730 *p=0.05, source of variation follicle size **p=0.002, treatment*p=0.048; Tukeys post-hoc*
731 *multiple comparison *p<0.05).*

732 **Fig 3b**: 1,25$(OH)_2D_3$ had no effect on E_2 production from granulosa cells taken from small
733 antral follicles (<10mm, white bars; *n=3 subjects, total follicles=11*) and large antral follciles
734 (>10mm, grey bars; *n=6 subjects, total follicles=8*) from normal ovaries. Results showed no
735 effect of 1,25$(OH)_2D_3$ (vd 0.2-20nM) on E_2 production. The results are expressed as mean
736 percentage change from control where the control is taken as 100%.

737 **Fig 3c:** 1,25$(OH)_2D_3$ at 2 (spotted bar) & 20nM (hashed bar) had no effect on E_2 production
738 from granulosa cells in the absence (white bars)/presence(dark grey bars) of FSH (5ng/ml),
739 which as expected significantly simulated E2 production. The results are expressed as mean

740 percentage change from control where the control is taken as 100%. *(n=4 subjects; total*
741 *follicles=49. ANOVA ****p<0.0001; **p<0.005 Sidak's multiple comparison test).*

742 **Fig 3d:** Effect of 1,25(OH)$_2$D$_3$ (at 2 & 20nM) on E$_2$ production from granulosa-lutein cells in the
743 presence of LH (10ng/ml). LH significantly stimulated E2 production, which was significantly
744 attenuated in the presence of VD *(n=4; ANOVA **p<0.005; Post-hock Sidak's multiple*
745 *comparison test. Alphabetical annotations are used to denote differences in statistical*
746 *significance).*

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

750 **Fig 4a:** Aromatase mRNA expression levels were measured in KGNs cultured with forskolin
751 (Fsk) to stimulate aromatase and different doses of VD (0.02-20nM). Testosterone (5x10^{-7}M)
752 was used as an aromatase substrate. VD at the lowest two doses significantly down-regulated
753 Fsk-stimulated aromatase expression, but as the doses increased this attenuation was lost.
754 *(One-way ANOVA ***p=0.0002; Tukey's multiple comparisons test con vs fsk **p<0.005; con*
755 *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).*
756 *Alphabetical annotations are used to denote differences in statistical significance).*

757 **Fig 4b**: KGN cells were transfected with PII-specific-Luc reporter construct and treated as for
758 the qPCR experiments. The luciferase assay showed that the effect of VD doses on aromatase
759 expression was direct action on aromatase transcription, with very low doses down-
760 regulating Fsk-stimulated PII activity. As VD concentrations increased this attenuation was
761 lost. *(One-way ANOVA****p<0.0001; Tukey's multiple comparison- C/C+T vs Fsk***; Con vs*

762 *2***; Con vs 20***; Fsk vs 0.02**; Fsk vs 0.2*; 0.02 vs 2* (n=3). Alphabetical annotations are*
763 *used to denote differences in statistical significance).*

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

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

776 **Fig 5c:** mRNA expression of AMH in the presence of VD (at 0.02 and 20nM) and forskolin (Fsk)
777 in KGNs. Fsk reduced AMH expression below basal (white spotted bars) and this was further
778 attenuated in the presence of 1,25$(OH)_2D_3$ with the strongest reduction seen in the presence
779 of 20nM VD (dark grey spotted bars) *(ANOVA*p=0.02; Tukey's multiple comparison *p<0.05).*

780 **Fig 6:** Representative image of western blot of protein from KGN cells cultured ± Fsk and VD
781 at 0.02 and 20nM; immuno-precipitated with anti-VDR antibody (1:1000) and western blotted
782 with anti-RXR antibody (1:1000) to detect RXR:VDR heterodimers. Equal quantities of protein
783 were loaded onto each lane to allow for comparisons. *Lanes: 1=control, 2=Fsk,*
784 *3=Fsk+VD20,4=Fsk+VD0.02 Red band=anti-RXRα, Green band=anti-VDR (IP-VDR).*

785 **Supplemental Figure Legends:**

786 **Fig S1:** 1,25$(OH)_2D_3$ had no effect on progesterone production from theca taken from LAF (15-
787 22mm, grey bars, *n=5 subjects, total follicles=5*) from normal ovaries. *(ANOVA n/s).*

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

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

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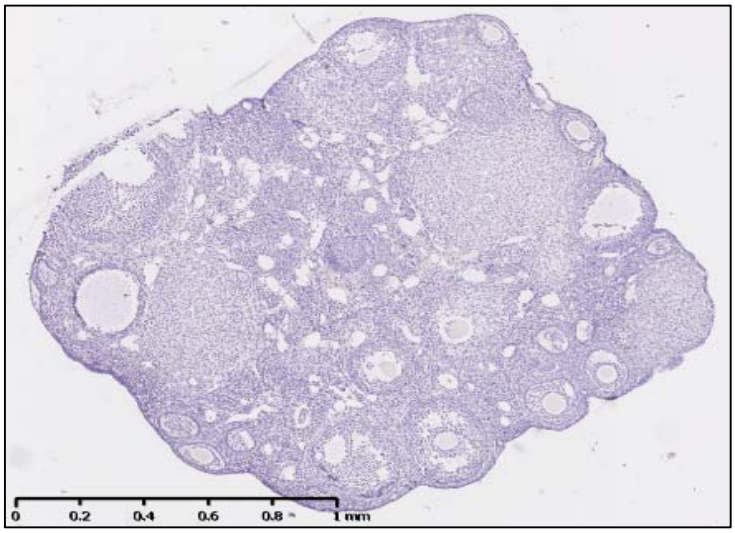
800

Fig 1a



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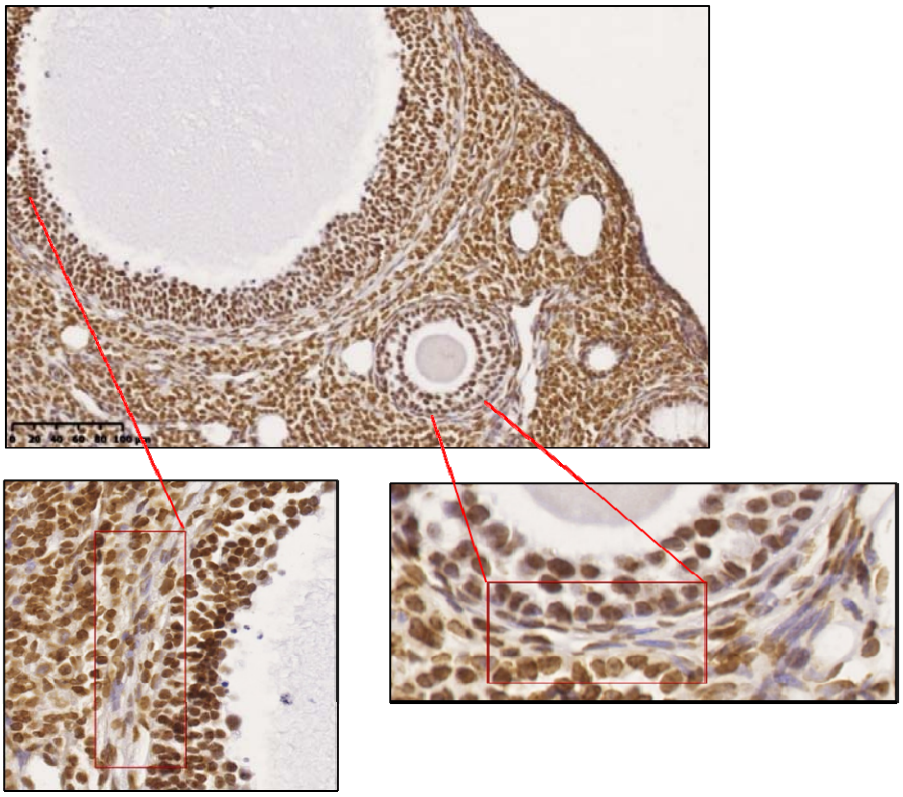

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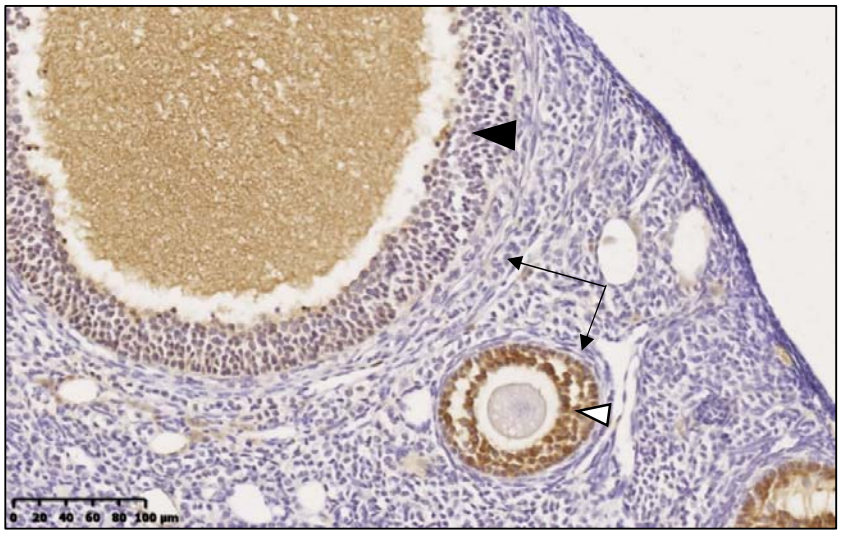
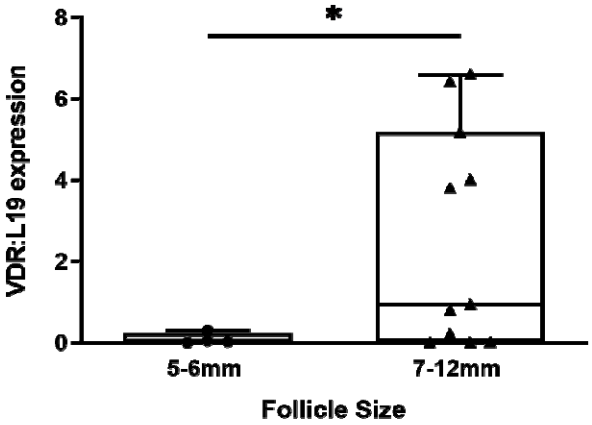


Fig 2a



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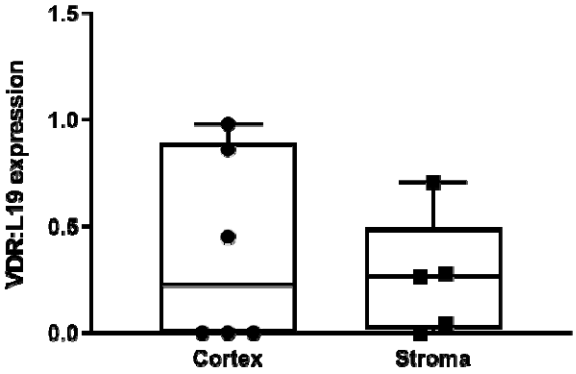

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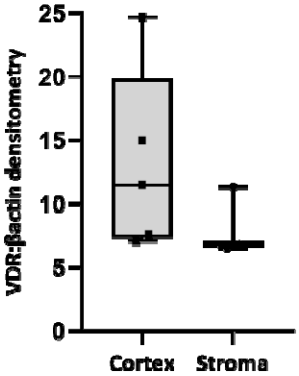

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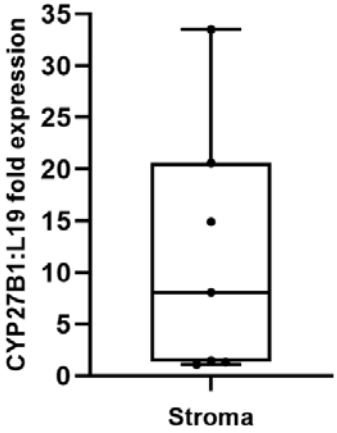

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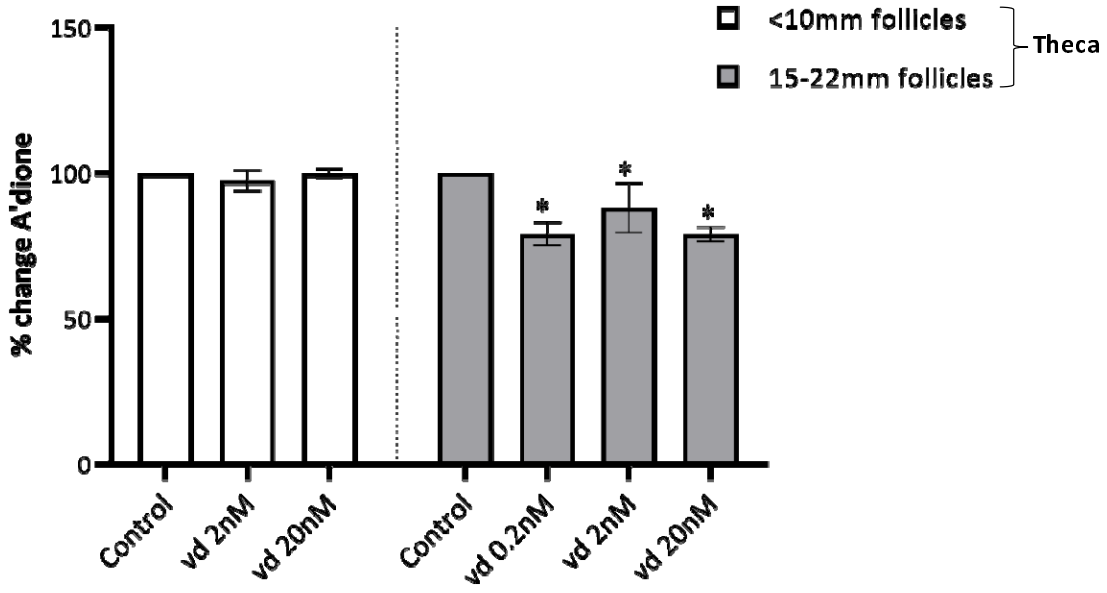

<10mm follicles
15-22mm follicles
Theca
% change A'dione
150
100
50
0
*
*
*
Control
vd 2nM
vd 20nM
Control
vd 0.2nM
vd 2nM
vd 20nM

Fig 3b

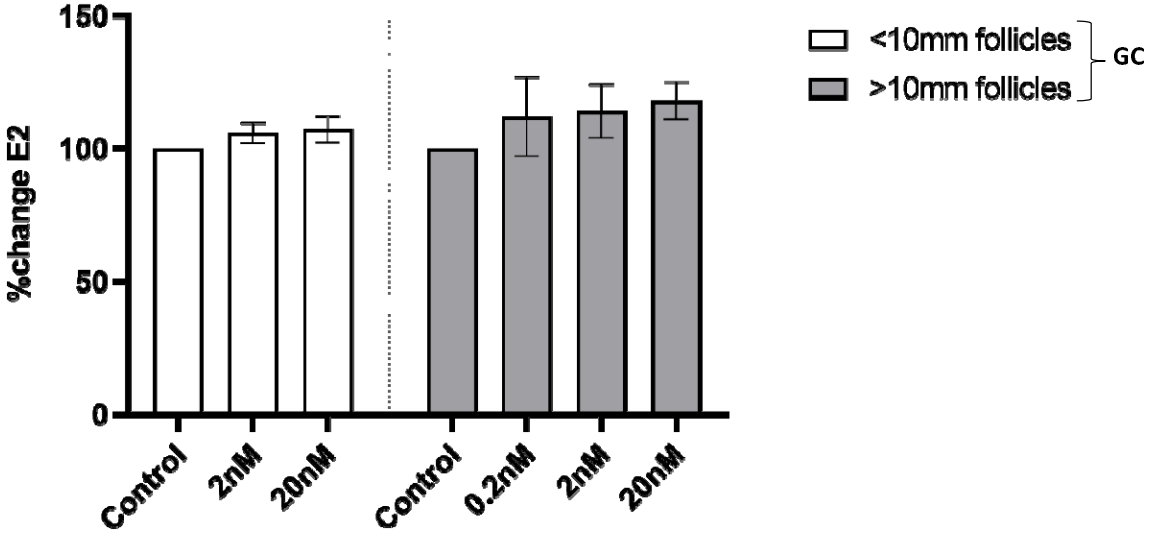
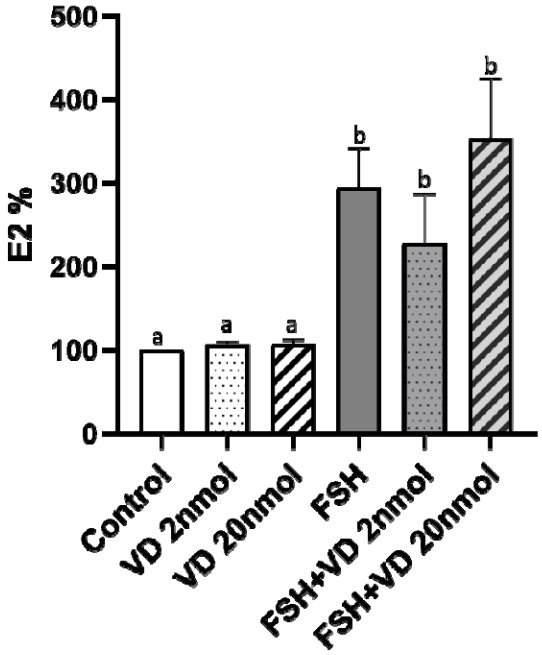

<10mm follicles
>10mm follicles
GC
%change E2
150
100
50
0
Control
2nM
20nM
Control
0.2nM
2nM
20nM

Fig 3c



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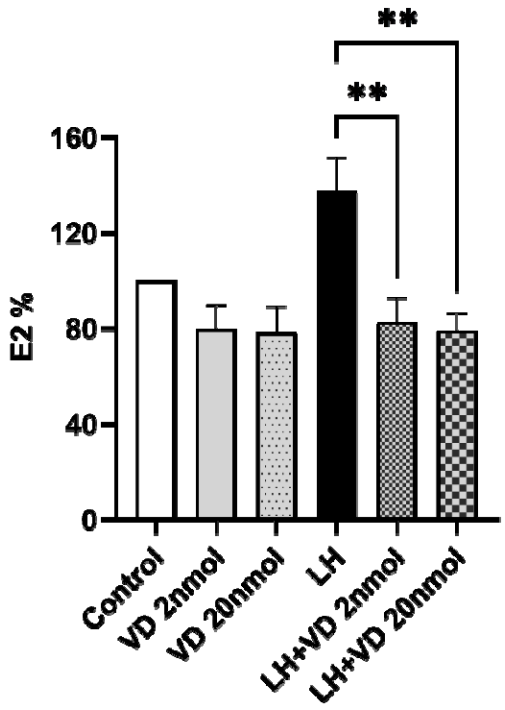

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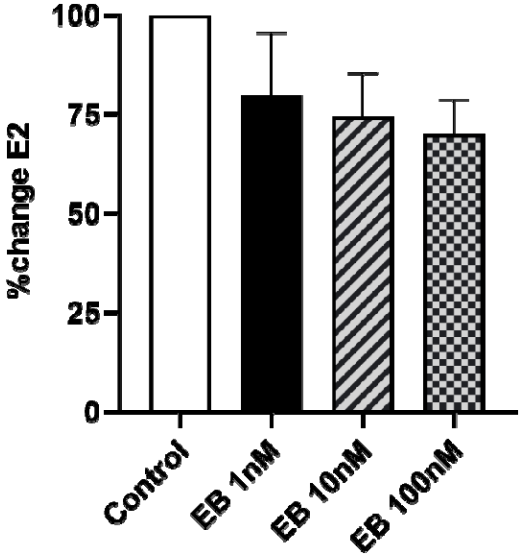


Fig 4a

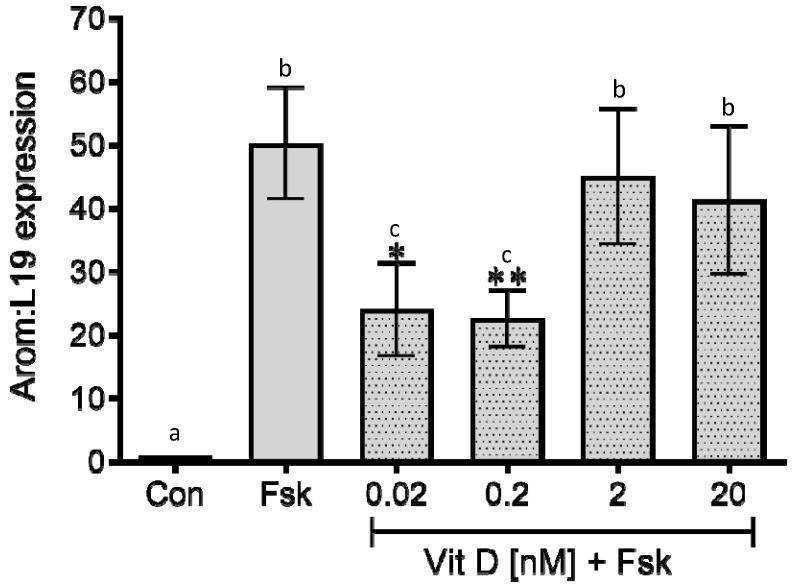

Arom:L19 expression
70
60
50
40
30
20
10
0
a
b
c
*
c
**
b
b
Con
Fsk
0.02
0.2
2
20
Vit D [nM] + Fsk

Fig 4b

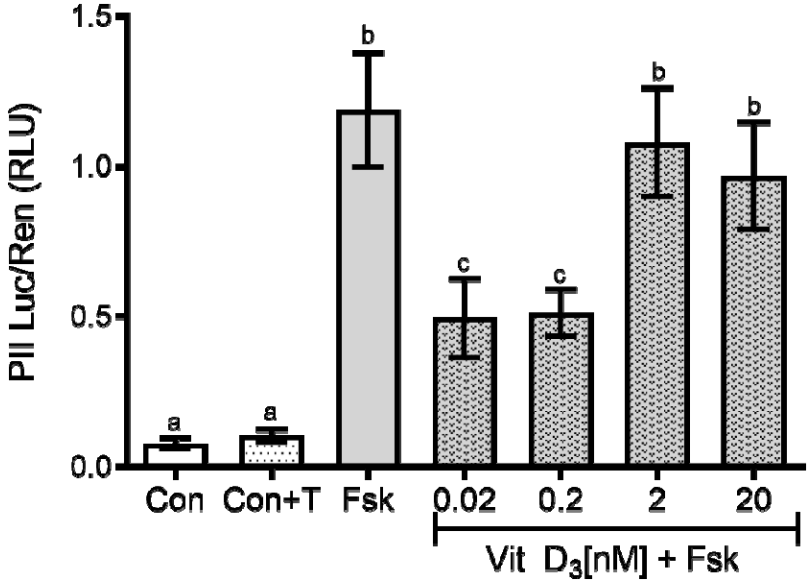

PII Luc/Ren (RLU)
1.5
1.0
0.5
0.0
a
a
b
c
c
b
b
Con
Con+T
Fsk
0.02
0.2
2
20
Vit D3[nM] + Fsk

Fig 5a

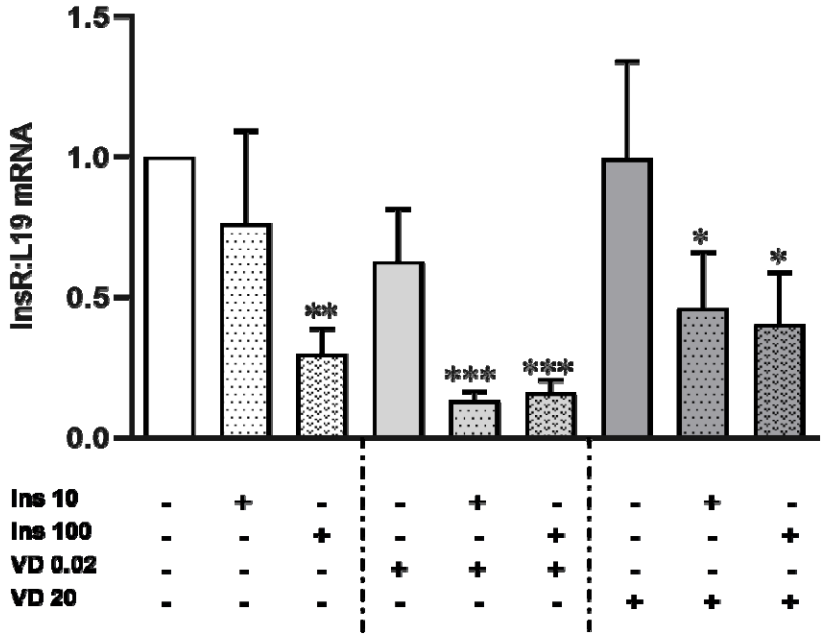

InsR:L19 mRNA
1.5
1.0
0.5
0.0
**
***
***
*
*
Ins 10 - + - - + - - + -
Ins 100 - - + - - + - - +
VD 0.02 - - - + + + - - -
VD 20 - - - - - - + + +

Fig 5b

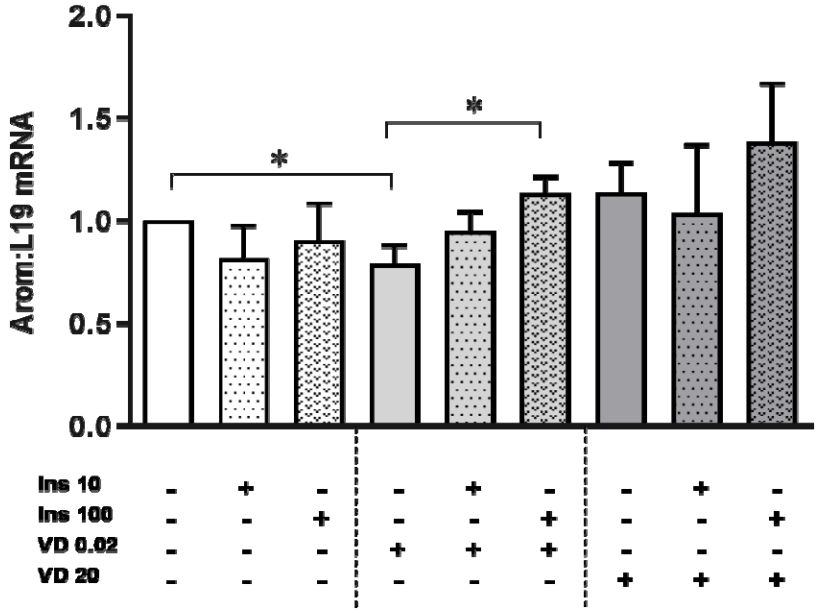

Arom:L19 mRNA
2.0
1.5
1.0
0.5
0.0
*
*
Ins 10 - + - - + - - + -
Ins 100 - - + - - + - - +
VD 0.02 - - - + + + - - -
VD 20 - - - - - - + + +

Fig 5c

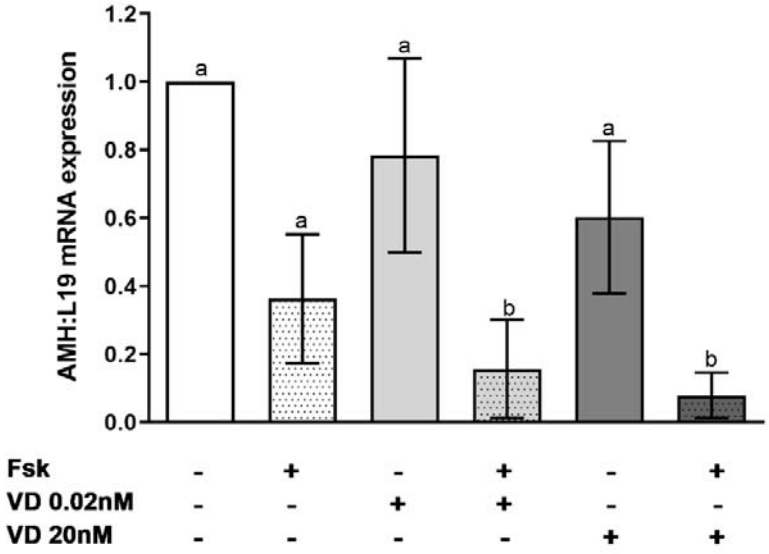
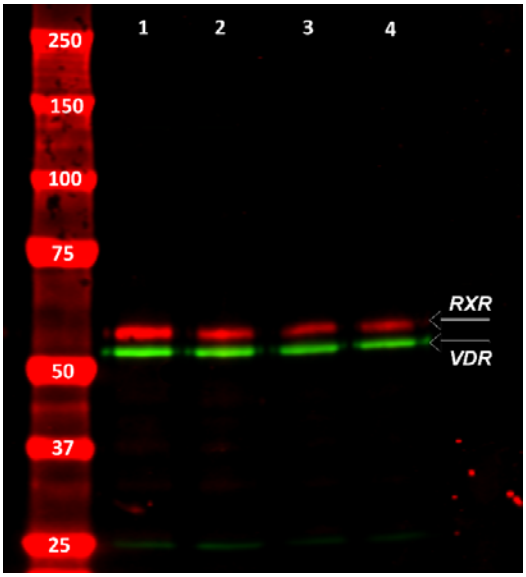

AMH:L19 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
b
a
b
Fsk - + - + - +
VD 0.02nM - - + + - -
VD 20nM - - - - + +

Fig 6


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