School of Medicine

The association between Vitamin D and clinical IVF outcomes, and the in vitro effects of Vitamin D on granulosa cell steroidogenesis and metabolism

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Declaration

To the best of my knowledge, this thesis titled "The association between Vitamin D and clinical IVF outcomes, and the in vitro effects of Vitamin D on granulosa cell steroidogenesis and metabolism" contains no material previously published by any other person, except where due acknowledgement has been made. This thesis does not contain material which has been accepted for the award of any other degree or diploma in any other university.

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Dedication

I dedicate this work to my parents, family, and all my friends who offered me unconditional love and support throughout. Your support was instrumental in my success and I am forever grateful.

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Statement of Contribution by Others

I hereby declare that the work presented in this thesis was primarily designed, experimentally executed, interpreted, and written by the first author of the individual manuscripts (Nikita Walz). Contributions by colleagues are described in detail below. The signed statements by co-authors is in Appendices IV and VII.

Chapter 1

Nikita Walz wrote the manuscript and Prof Philip Newsholme, Dr Kevin N. Keane, and Prof. John L. Yovich critically reviewed, revised, and approved the final manuscript. Nikita Walz drafted the outline and generated the figures.

Chapter 2

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Conference Presentations

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Combined Biological Sciences Meeting	Oral presentation
Scientists in Reproductive Technology	Oral presentation
Australian Society of Medical Research	Oral presentation
2017	
Mark Liveris Seminar	Oral and poster presentation
Curtin Cell Signalling Meeting	Oral presentation
Australian Society of Medical Research	Oral presentation
Scientists in Reproductive Technology	Oral presentation
Science on the Swan	Poster presentation
2018	
Mark Liveris Seminar	Oral and poster presentation
Curtin Cell Signalling Meeting	Oral presentation
Australian Society of Medical Research	Oral presentation
Science on the Swan	Poster presentation
Scientists in Reproductive Technology	Oral presentation
Fertility Society of Australia	Oral presentation
2019	
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Publications

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List of Abbreviations

1,25-hydroxyvitamin D3	1,25-(OH)2D3
25-hydroxyvitamin D	25-(OH)D
2-DG	2-deoxyglucose
2PN	Two pronuclei
3β-HSD	3β-hydroxysteroid dehydrogenase
7-DHC	7-Dehydrocholesterol
AA	Antimycin A
AACEP	Agonist antagonist conversion with estrogen priming
ACC	Acetyl-coenzyme A carboxylase
Acetyl-CoA	Acetyl-coenzyme A
AFC	Antral follicle count
AMH	Anti-Mullerian hormone
AMHR	Anti-Mullerian hormone receptor
AMPK	AMP-activated protein kinase
ANZCTR	Australian New Zealand Clinical Trial Registry
ART	Assisted reproductive technology
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BIA	Bioelectrical impedance analysis
BMI	Body mass index
BMR	Basal metabolic rate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CPR	Clinical pregnancy rate
DET	Double embryo transfer

DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E1	Estrone
E2	Estradiol
E3	Estriol
ECAR	Extracellular acidification rate
ECLIA	electro-chemiluminescence immunoassay
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ERβ1	Estrogen receptor β1
ET	Embryo transfer
EUR	Embryo utilisation rate
FA	Fatty acid
FAI	Free androgen index
FAS	Fatty acid synthase
FBS	Foetal bovine serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FET	Frozen embryo transfer
FF	Follicular fluid
FSH	Follicle stimulating hormone
GC(s)	Granulosa cell(s)
GnRH	Gonadotropin releasing hormone
GPx	Glutathione peroxidase
hCG	Human chorionic gonadotropin
HGMCR	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase

HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
ICSI	Intracytoplasmic sperm injection
IGF1	Insulin-like growth factor 1
IOM	Institute of Medicine
IVF	*In vitro* fertilisation
KIDScores	Known Implantation Data Scores
KL	Kit ligand
LBR	Live birth rate
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LH	Luteinising hormone
Malonyl-CoA	Malonyl-coenzyme A
MAPK	Mitogen-activated pathway kinase
MII	Metaphase two
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
OCR	Oxygen consumption rate
Oligo	Oligomycin
OPU	Oocyte pick-up
OUR	Oocyte utilisation rate
P4	Progesterone
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PER	Proton efflux rate
PGD	Preimplantation genetic diagnosis

PKA	Protein kinase A
RCT	Randomised control trial
rFSH	Recombinant follicle stimulating hormone
rhCG	Recombinant human chorionic gonadotropin
RIA	Radioimmunoassay
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SER	Smooth endoplasmic reticulum
SET	Single embryo transfer
SHBG	Sex hormone binding globulin
SOD	Superoxide dismutase
StAR	Steroid acute regulatory protein
TBS-T	Tween 20 Tris-buffered saline
TCA	Tricarboxylic acid cycle
TVOA	Transvaginal oocyte aspiration
TVS	Transvaginal scan
UV	Ultraviolet
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VitD	Vitamin D
βhCG	β Human chorionic gonadotropin

Abstract

Vitamin D (VitD) deficiency is a worldwide health issue linked to several chronic diseases/disorders including diabetes, obesity, cancer, and infertility. In recent years, clinical studies have investigated the clinical importance of VitD levels regarding IVF success (for clinical pregnancy and live birth). However, very little is known about how VitD is related to embryological measures such as blastocyst development. Additionally, whilst the VitD receptor (VDR) has been found in numerous reproductive cells and tissues, the molecular function of VitD in granulosa cells (GCs) is currently unknown. The overall aim of this thesis was to investigate the impact of VitD status on clinical outcomes in women undergoing IVF with a fresh embryo transfer (ET) and on human GC steroidogenesis and cell metabolism.

A cross-sectional observational study was conducted at a private IVF clinic in Perth, Western Australia. In this study 290 women were included, who underwent 290 IVF cycles and received a fresh ET. In the whole cohort analysis, a multivariable analysis demonstrated VitD sufficiency ($\geq$ 20 ng/mL) was significantly associated with increased chance of developing at least one blastocyst (OR=2.90, p=0.001). A subset analysis of 113 women had their serum and follicular fluid (FF) 25-hydroxyvitamin D (25-(OH)D) levels measured. Serum and FF 25-(OH)D levels were highly correlated in this cohort (r=0.798, p=<0.001). In a different subset analysis, 107 patients had their biometrics (BMI, body fat percentage, and muscle mass) measured and it was found that only BMI and VitD sufficiency were significantly associated (OR=0.94, p=0.017).

In a characterisation study of the human GC lines COV434 and KGN, combination treatment for 24 hours with recombinant follicle stimulating hormone (rFSH) and androstenedione significantly enhanced the secretion of estradiol (E2) and progesterone (P4), and enzyme expression in several biochemical pathways, related to cholesterol biosynthesis/transport and fatty acid synthesis. The combination treatment also enhanced intracellular lipid deposition, and cellular bioenergetics in these cell lines. Treatment with the biologically active form of VitD (1,25-hydroxyvitamin D3 (1,25-(OH)2D3)) in these cell lines significantly enhanced steroid hormone secretion (E2/P4), lipid deposition, and cellular bioenergetics, while decreasing intracellular

reactive oxygen species (ROS). Furthermore, the addition of 1,25-(OH)2D3) with the combination treatment further enhanced these parameters in COV434 and KGN cell lines. Additionally, the VDR was established in KGN cells for the first time.

The major strength of the present clinical study is that it provides an in-depth investigation into the association between VitD status and embryological measures for the first time, using well designed multivariable statistical models. Sample size was the main limitation of the present clinical study, since several key outcomes (clinical pregnancy and live birth) were improved but did not achieve conventional statistical significance. Furthermore, follow up VitD measurement closer to live birth would elucidate this association more accurately as VitD is known to change largely over seasons. In the *in vitro* cell work, the major strength of this work is that many aspects important to both cellular functioning and signalling at the molecular level were investigated. The main limitation of this work was it was conducted in human GC lines, which may not accurately reflect primary human GCs.

This is the first clinical study to investigate the relationship between VitD and blastocyst development chance. While a strong relationship was observed, it is unclear if this has downstream implications on subsequent clinical pregnancy and live birth rates, which were increased but did not reach significance. The *in vitro* cell work detailed here was the first to characterise the human GC lines COV434 and KGN within one study, particularly regarding the use of hormonal pre-stimulation and cellular bioenergetics, and the direct effects of 1,25-(OH)2D3 .

This clinical work identifies a key variable that should be examined in well-designed randomised control trials, which will help further elucidate the role of VitD in IVF. Whilst the cell work provides additional characterisation of commonly used human GC lines with important contributions on several aspects of cell function including steroid hormone output, lipid metabolic flux and bioenergetics.

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Chapter One

Introduction and Literature Review

1.0 Introduction

Vitamin D (VitD) is a group of steroid-based hormones which have a well-established role in calcium and phosphate metabolism (1). Classically, VitD affects intestinal duodenal calcium absorption and renal calcium reabsorption (2, 3), as well as promoting bone mineralisation by altering chondrocyte and osteoblast differentiation (4). It is estimated that 1 billion people worldwide have insufficient serum 25-hydroxyvitamin D (25-(OH)D) levels (5). Currently the recommended classifications for VitD status published by the Institute of Medicine (IOM) consider a 25-(OH)D level < 12 ng/mL as VitD insufficiency, 12-20 ng/mL as VitD deficiency and $\geq$ 20 ng/mL as VitD sufficiency (6). Additionally, the IOM also recommends adults under the age of 70 receive 600 to 800 IU of VitD daily (6). However, these recommendations are based on bone health, specifically on data which shows reduced fracture risk associated with increased VitD supplementation (7). While the benefits of serum concentrations of 25-(OH)D $\geq$ 20 ng/ml on endpoints other than bone health have not been well documented in randomised control trials (RCT), many studies have reported dysregulation of VitD homeostasis is associated with various immunological conditions, metabolic pathologies, cardiovascular disease, certain types of cancers, and infertility (8-19). Consequently, interest in the non-skeletal actions of VitD has increased in recent years, particularly following evidence from VitD receptor (VDR) null mice (5, 20).

Two of the major forms of VitD are cholecalciferol or 25-hydroxyvitamin D3 (25-(OH)D3) and ergocalciferol or 25-hydroxyvitamin D2 (25-(OH)D2, Table 1.1) (21). 25-(OH)D3 is the major source of VitD, accounting for approximately 90% of total VitD, while 25-(OH)D2 makes up the remaining 10%. 25-(OH)D3 is produced by exposure to sunlight, while 25-(OH)D2 is obtained from dietary sources of VitD such as oily fish, dairy products, cereals fortified with VitD and supplements (22). The circulating form of VitD is 25-hydroxyvitamin D (25-(OH)D), which consists of the

combination of 25-(OH)D2 and D3, and therefore is often referred to as total 25-(OH)D (23). This is clinically relevant for determining VitD status as this form of VitD accurately reflects body stores of VitD, and has a longer half-life than other forms of VitD, such as 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3), the active metabolite of VitD (23). In the VitD metabolism pathway, 1,25-(OH)2D3 is generated through a series of complex biochemical reactions which occur in the liver and the kidney (24). 1,25-(OH)2D3 elicits its functions via its interaction with its receptor, known as the VitD receptor (VDR), through both genomic and non-genomic signalling (25). The VDR is located in nearly all tissues and cells in the human body, suggesting an active role of VitD in numerous body systems outside of the classical skeletal functions, including in reproductive tissues and cells (26).

1.1 VitD and fertility

Over the last decade accumulating evidence from animal & human studies suggests that VitD is important in many aspects of reproduction in both genders, including in conception, pregnancy, lactation, and sperm function (27). While the precise molecular functions of VitD in relation to male and female fertility remains elusive, there is an evident increase in the incidence of VitD deficiency in sub-fertile populations compared to the general adult population (28). This may suggest that VitD deficiency is a factor contributing to human infertility. Many studies have attempted to assess the association between VitD status and outcomes in patients undergoing *in vitro* fertilisation (IVF) (29).

Recent clinical reports have suggested that VitD is positively correlated with IVF success, including improved fertilisation rates, implantation rates, clinical pregnancy rate (CPR) and live birth rate (LBR) (19, 30-43). In contrast others have found no association (44-52), or even a negative association (53-55) between VitD status and clinical outcomes in IVF. However, comparison of these studies is difficult for several reasons. For example, the varied inclusion/exclusion criteria between studies which can include age, BMI, and infertility aetiology. Additionally, the clinical protocols between clinics can vary greatly, especially considering reproductive laws and regulations differ between countries on issues such as single vs. double embryo transfer (ET). An important aspect that should be considered when assessing these studies is the timing of serum and/or follicular fluid (FF) levels of 25-(OH)D, which

should be measured close to the outcomes of interest to account for potential seasonal variation (56). Finally, many studies which report findings only examine crude outcomes and do not use adjusted multivariable analyses accounting for confounding factors which could further elucidate these associations.

In addition to the clinical studies investigating the association between VitD status and outcomes in IVF, several studies have also examined VitD in the context of fertility, but at a molecular level. The VDR and its associated metabolising enzymes have been reported to be distributed across various parts of the male and female reproductive tracts including in the ovary, endometrium, placenta, trophoblasts, epididymis, prostate, and in spermatozoa (58-64). While the precise function of VitD in fertility beyond mineral homeostasis is largely unknown, there are some interesting reported findings from reproductive cells and tissues which demonstrate VitD can modulate steroidogenesis and folliculogenesis (57).

Briefly, in sperm treatment with 1,25-(OH)2D3 can modulate calcium signalling and improve sperm motility, indicating a role of VitD in mitochondrial functioning (58). Additionally, acute 1,25-(OH)2D3 exposure stimulates steroidogenesis and can alter expression of the anti-Mullerian hormone (AMH) receptor (AMHR) in granulosa cells (GC) (29, 59-64). These reproductive biological processes such as steroidogenesis and folliculogenesis are crucial in human fertility, and their disruption or dysregulation is closely related to infertility (29). Furthermore, a key aspect which drives these functions is the modulation of cellular metabolism (65). While data from other non-reproductive cell types demonstrate VitD can alter cellular metabolism, the effect of VitD on cellular metabolism and bioenergetics of reproductive cells is currently unknown (66-69).

While data in this area is promising, there is still a large gap in knowledge, in terms of how VitD status is associated with embryological measures (such as blastocyst development) which are crucial and predictive of IVF success. Furthermore, understanding the molecular aspects of VitD in reproductive cells such as GCs may provide insight into how VitD is able to improve clinical outcomes in IVF. Based on these reported findings it can be hypothesised that VitD can improve fertility outcomes in patients undergoing IVF by modulating key steroidogenic hormones driven by

changes in cellular bioenergetics, which, in turn, influences folliculogenesis and oocyte maturation.

1.2 Thesis overview

The overall aim of the present thesis was to investigate the impact of VitD on the clinical outcomes in women undergoing IVF and human GC bioenergetics and metabolism.

1.2.1 Objectives

Several specific objectives and the chapter in which they are addressed in this thesis are outlined below.

Objective 1: Introduce and review the literature on VitD in the context of human fertility (with an emphasis on IVF) and the molecular actions of VitD in reproductive cells and tissues. This work is covered in **Chapter 1.**

Objective 2: Outline the materials and methods utilised throughout this thesis for the relevant experimental procedures. This work is covered in **Chapter 2.**

Objective 3: Perform a retrospective analysis of clinical data obtained from a private University-affiliated IVF clinic and investigate the association between serum VitD status and clinical outcomes of women undergoing IVF treatment. This work is covered in **Chapter 3.**

Objective 4: Conduct a cross-sectional analysis of clinical data obtained from a private University-affiliated IVF clinic and investigate the association between serum VitD status and FF VitD status, patient biometrics and clinical outcomes of women undergoing IVF treatment. This work is covered in **Chapter 4** and includes analysis of the whole cohort and two subset analyses (FF and biometrics).

Objective 5: Characterise the cell viability, hormone secretion, lipid metabolism/deposition, and cellular bioenergetic response of COV434 and KGN human GC lines to follicle stimulating hormone (FSH) and androstenedione stimulation. This work is covered by **Chapter 5.**

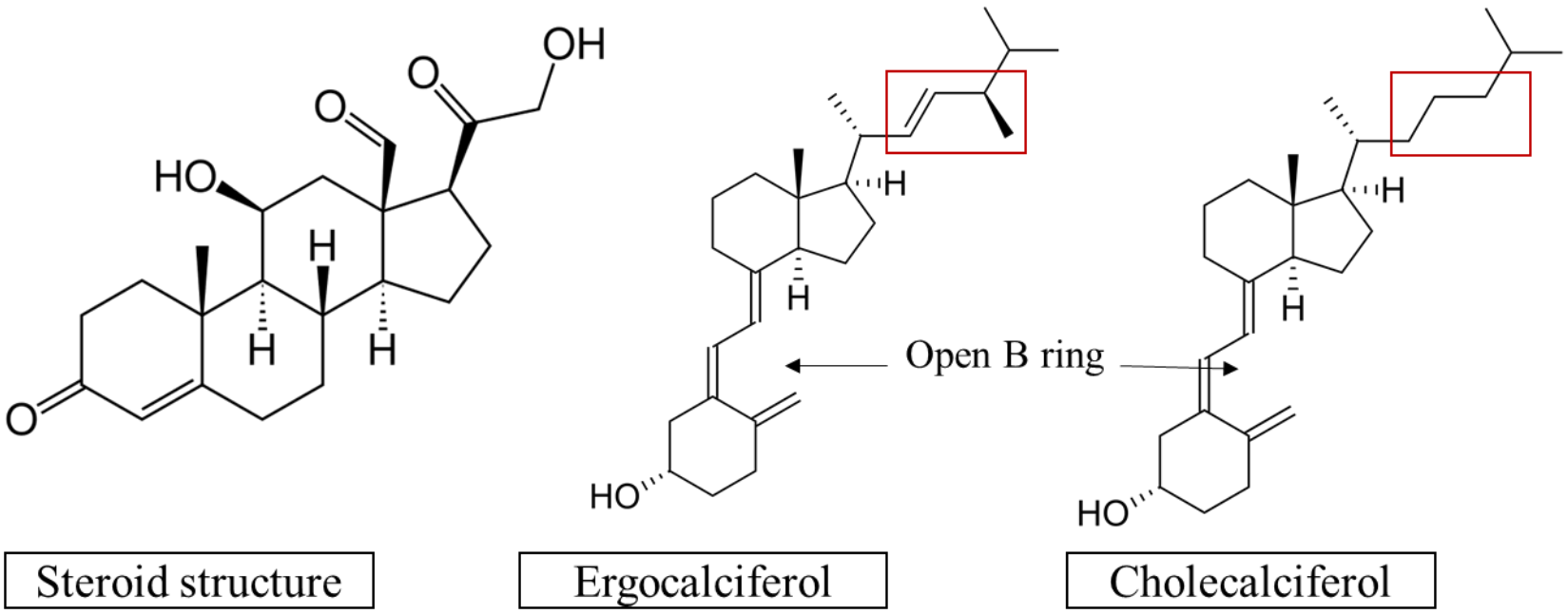
Objective 6: Investigate the effect of VitD treatment on cell viability, hormone secretion, lipid deposition, and cellular bioenergetics on the COV434 and KGN human GC lines. This work is covered by **Chapter 6.**

Objective 7: Summarise and discuss the findings from Chapters 3-6, including hypothesising how these are interrelated, their significance in the field of research and the future directions. This work is covered by **Chapter 7.**

1.3 VitD Literature Review

1.3.1 VitD history

VitD is a group of fat-soluble vitamins and which were first identified in the early 20th century, making it the fourth vitamin to be discovered (70). The biological role of the active form of VitD known as 1,25-dihydroxycholecalciferol (referred throughout this chapter as 1,25-(OH)D3, also known simply as cholecalciferol, Table 1.1) was first described in 1922 when it was observed that exposing children with rickets to summer sunlight or artificial ultraviolet (UV) light, was effective in curing their rickets (71). Following this, researchers irradiated rats and their environment/food with UV light and found this also cured rickets (72). This led to the discovery that an inactive lipid found in the diet and skin could be converted upon exposure to UV light into a biological active substance (73). In 1932, 25-hydroxivitamin D2 (referred throughout this chapter as 25-(OH)2, also known simply as ergocalciferol, Figure 1.1 & Table 1.1) was isolated from an irradiation mixture of ergosterol and identified, making this the first isoform of total 25-(OH)D (this refers to 25-(OH)D2 and 3 in combination, also known as calciferol, Table 1.1) to be identified (74). A few years later, 7-dehydrocholesterol (7-DHC) and 25-(OH)D3 (Figure 1.1 & Table 1.1) were identified, and it was found that 25-(OH)D3 is formed following UV-B irradiation of 7-DHC, found in the dermis of the skin (75-77). In 1937, 1,25-(OH)D3 was reported to be essential to utilise calcium obtained from the diet (78). Decades later, the receptors associated with biologically active 1,25-(OH)D3 (known as vitamin D receptors; VDR, Table 1.1) were discovered to be located in the intestinal tract, stomach, kidney, skin, pituitary and parathyroid glands (79). Since this earlier research, the receptors for 1,25-(OH)D3 have been found to be expressed in several extra-skeletal tissues and cells, including immune cells, pancreatic, respiratory, brain and reproductive cells (80-83). Furthermore, it is currently known that there are several sub-cellular locations of the VDR including in the caveolae (which are small invaginations in the plasma membrane which act as lipid rafts), the inner membrane of the mitochondria and the nucleus (29, 84, 85).



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189

190 **Figure 1.1: Molecular structure of ergocalciferol (25-(OH)D2) and cholecalciferol (25-(OH)D3).** In comparison to a classical steroid chemical
191 structure (left) which has a closed B ring, both ergocalciferol and cholecalciferol (middle and right, respectively) have an open B ring, classifying
192 them as secosteroids. Ergocalciferol is structurally different from cholecalciferol as it features a double bond between carbon 22 and 23, as well
193 as having an additional methyl group at carbon 24 (highlighted in red boxes).

194

1.3.2 VitD metabolism

VitD is a secosteroid hormone, meaning it has the classic steroid skeleton structure, but with an open B-ring between carbon 9 and 10 (Figure 1.1) (86). VitD enters the body from dietary sources as either 25-(OH)D2 or as biologically inactive 25-(OH)D3 (86). 25-(OH)D3 is primarily produced in the dermis of the skin, upon exposure to UV-B radiation and is the most potent source of VitD, accounting for approximately 80-90% of total VitD intake (87). As previously mentioned, both 25-(OH)D2 and D3 can also be obtained from dietary sources and synthetic supplementation (10-20%)(16, 17). 25-(OH)D3 is synthesised within the epidermis of the skin from the precursor 7-DHC, a normal intermediary in the cholesterol pathway (Figure 1.2). 7-DHC is converted to the intermediate pre-VitD1,25-(OH)2D3, which spontaneously isomerises to form 25-(OH)D3 (Figure 1.2) (17). Following this initial synthesis in the skin, 25-(OH)D3 is transported via the blood bound to VitD-binding protein (VDBP) or albumin and hydroxylated in the liver at the carbon 25 position by the micro-stromal enzyme 25-hydroxylase (encoded by CYP2R1, Table 1.1), and forms 25-(OH)D (also known as calcifediol, Figure 1.2 & Table 1.1) (17). Next, 25-(OH)D is converted into its active metabolite, 1,25-(OH)2D3 by the enzyme 1-α-hydroxylase present in the distal tubules of the kidney (Figure 1.2 & Table 1.1) (18). Finally, 1,25-(OH)2D3 is transported from the kidney via plasma bound to VDBP or albumin until it reaches its target tissues, where it can enter the cell facilitated by membrane proteins like caveolin, cubilin, and megalin or via simple diffusion (88). Here, 1,25-(OH)2D3 binds with high-affinity to the VDR and elicits its physiological function, through rapid non-genomic signalling or slower genomic signalling (Figure 1.2) (19). Other forms of VitD such as 25-(OH)D3 bind with lower affinity to the VDR, as does the secondary bile acid lithocholic acid (89, 90).

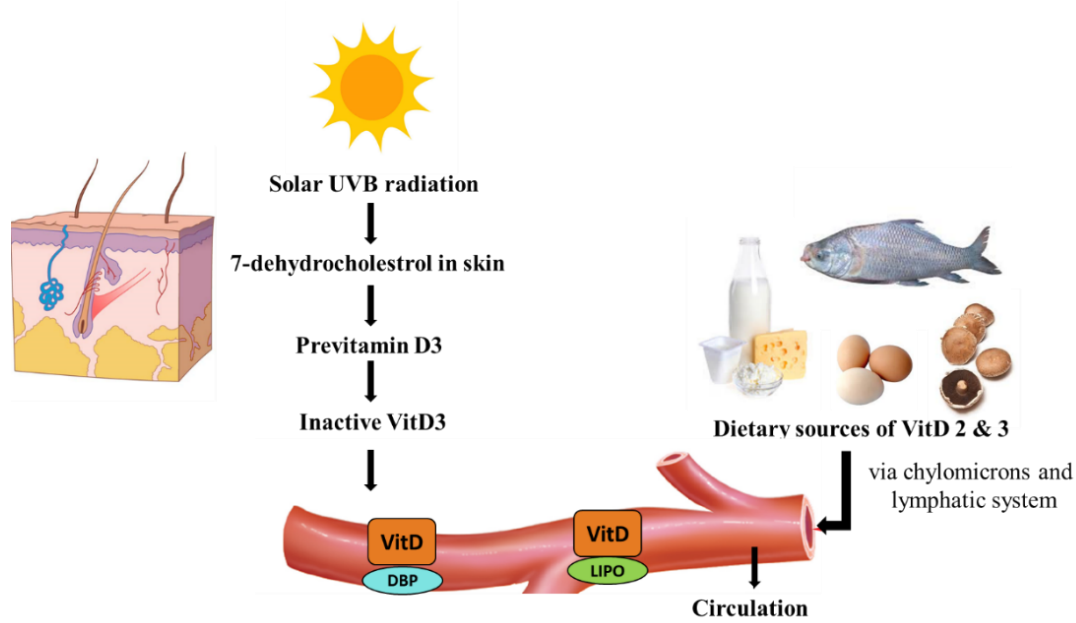
Table 1.1: Terminology involved in the VitD metabolism pathway, including key metabolites and enzymes.

Molecular name	Abbreviation(s)	Alternative name(s)
1,25-dihydroxyvitamin D3 or 1α,25-dihydroxyvitamin D3	1,25-(OH)2D3 or 1α,25-(OH)2D3	Calcitriol
25-hydroxyvitamin D	25-(OH)D	Calcidiol
25-hydroxivitamin D_2	25-(OH)D_2	Ergocalciferol
25-hydroxivitamin D_3	25-(OH)D_3	Cholecalciferol
Total 25-hydroxyvitamin D	Total 25-(OH)D (25-(OH)D_2 + 25-(OH)D_3)	Vitamin D or Calciferol
24,25-dihydroxyvitamin D_3	24,25-$(OH)_2D_3$	(24*R*)-hydroxycalcidiol
1α-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D_3	1a-hydroxycalcioate	Calcitroic acid
Pre-vitamin D_3	-	Precholecalciferol
3-epi-25-hydroxyvitamin D3	3-epi-25(OH)2D3	-
7-dehydrocholesterol reductase	DHCR7	-
Sterol 27-hydroxylase	CYP27A1	Cytochrome P450 oxidase
25-OHD 1α-hydroxylase	CYP27B1	Cytochrome P450 27B1 or 1α-hydroxylase
1,25-OHD_3 24-hydroxylase	CYP24A1	Cytochrome P450 2A1 or Vitamin D3 24-hydroxylase
Vitamin D receptor	VDR	Calcitriol receptor
Retinoid X receptor	RXR	-
Vitamin D response element	VDRE	-

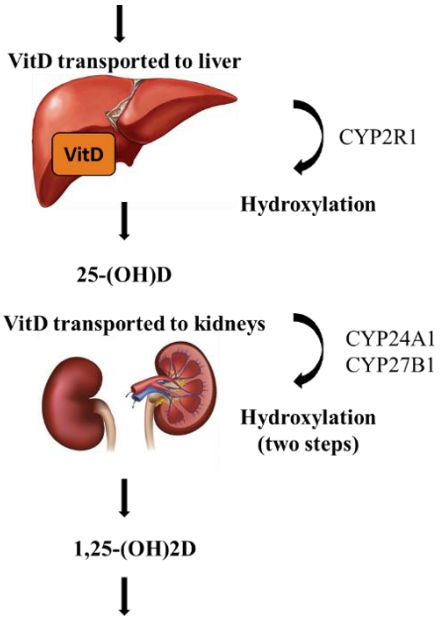
1.3.3 VitD response pathways

The biological activity of VitD occurs through two pathways: a slow genomic signalling pathway (mediated through the nuclear VDR; nVDR) and a rapid, non-genomic pathway (mediated by the mitochondrial and caveolae VDRs, mVDR & cVDR, respectively) (91). It is well established that VitD elicits its biological actions through the interaction with the VDR, in both response pathways (92). The VDR, which was first reported in 1969 as a chromatin-associated protein, is found in most human tissues and cells, notably bone, intestines, parathyroid glands and reproductive tissues such as the ovary, endometrium and placenta (85). Currently, it is known that VitD regulates about 3% of the 26,000 genes in the coding human genome (93).

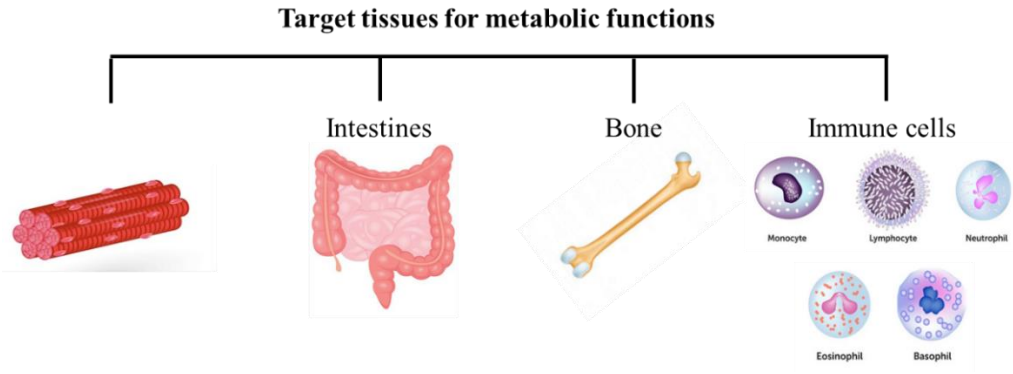
In the genomic signalling pathway, binding of active 1,25-(OH)2D3 with the VDR elicits conformational alterations in the nuclear aspect of VDR which subsequently heterodimerises with the retinoid X receptor (RXR) (Figure 1.2) (94). The VDR-RXR heterocomplex then binds to short sequences of DNA referred to as VitD response elements (VDREs), which are located in the promoter regions of VitD target genes (95). The VDR contains two globular domains, a DNA-binding domain and a ligand-binding domain (96). The DNA-binding domain has two zinc-finger motifs responsible for recognition and binding to VDREs (97). The ligand-binding domain binds to 1,25-OH2D3 with high affinity and is involved in dimerisation and transcription activation (97). Once bound, the VDR–RXR complex induces transcriptional changes via the recruitment of coactivators and corepressors. The steroid receptor coactivator complex 1-3 and VDR interacting complex (DRIP) act as coactivators to enhance gene transcription. In contrast, corepressors (such as those encoded by the hairless gene) bind to VDR in the absence of a ligand, and block VDR-mediated transcription. The corepressors rapidly detach from the VDR upon presence of 1,25-OH2D3. In the non-genomic response pathway, 1,25-OH2D3 binds to VDR associated with caveolae and the ligand-bound VDR then activates one or more signalling cascades, including protein kinase C, mitogen-activated protein kinases, phospholipase A2, and phospholipase C.


Solar UVB radiation
7-dehydrocholestrol in skin
Previtamin D3
Inactive VitD3
Dietary sources of VitD 2 & 3
via chylomicrons and lymphatic system
VitD
DBP
VitD
LIPO
Circulation

254


VitD transported to liver
VitD
CYP2R1
Hydroxylation
25-(OH)D
VitD transported to kidneys
CYP24A1
CYP27B1
Hydroxylation
(two steps)
1,25-(OH)2D

255


Target tissues for metabolic functions
Intestines
Bone
Immune cells
Monocyte
Lymphocyte
Neutrophil
Eosinophil
Basophil

256

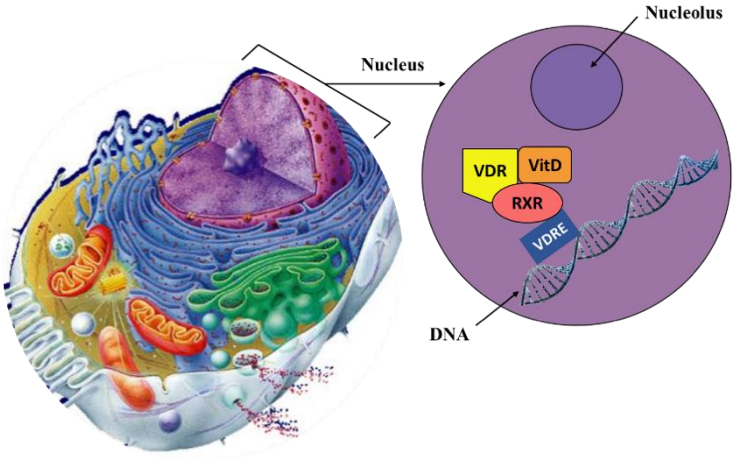


Figure 1.2: VitD metabolism pathway from cutaneous synthesis to nuclear translocation.

1.3.4 VitD sources, measurement, status, and supplementation

25-(OH)D can be obtained from exposure to natural or artificial UV light, in dietary sources (both as naturally occurring and fortified) and in the form of supplementation (5). As very few foods naturally contain 25-(OH)D, the major source of VitD is from the sun in the form of 25-(OH)D3, responsible for approximately 90% of VitD found in the body (98). The major dietary sources of 25-(OH)D are salmon and other oily fish, cod liver oil, yeast, and mushrooms (which can be exposed to UV-B radiation to increase their 25-(OH)D content) (24). Dairy products, such as yoghurt and cheese, cereals and some fruit juice drinks are fortified with 25-(OH)D3 (99). The fortified 25-(OH)D3 added to dietary foods/drinks and supplements is generated from lanolin (24). In recent years, VitD supplementation has been reported to be a widespread source of VitD in adults (100). Despite the large incidence of VitD insufficiency in Australian adults (approximately 31%), only 5% of the population were consuming VitD supplements (101, 102).

Clinically, VitD status is determined by measuring the blood serum concentration of total 25-(OH)D (21). Currently, circulating 25-(OH)D is acknowledged as the most reliable indication of VitD status, and accounts for both dietary intake and VitD produced from sun exposure (103). The half-life of 25-(OH)D in the bloodstream is estimated to be 2-3 weeks, which is significantly longer than alternative VitD metabolites (104, 105). For example, the biologically active 1,25-(OH)D3 has a half-life of approximately 4-6 hours only (21). Additionally, the concentration of 1,25-(OH)D3 are a thousand fold less than that of total 25-(OH)D (106). There are currently several laboratory methods commonly used to measure 25-(OH)D, including liquid chromatography-tandem mass spectrometry (LC-MS), competitive protein binding assays, high-performance liquid chromatography, radioimmunoassay, and enzyme-linked immunoassays (107-112). The gold standard for the radioimmunoassay method is the use of a kit developed by DiaSorin S.p.A (Saluggia, Italy) (113). This assay kit involves a two-step process that entails rapid extraction of 25-(OH)D in serum, followed by a competitive radioimmunoassay procedure using an antibody with specificity for 25-(OH)D (113). The most accepted gold standard method for VitD measurement is the use of LC-MS, which allows the detection of both 25-(OH)D2 and

D3, as well as the D3 epimer (separately) and from this total 25-(OH)D is reported (114).

The cut-offs for VitD status differ between countries and is often confused due to a lack of consensus on the cut-offs and the use of interchangeable terminology referencing VitD status. Currently, the main references are deficiency, insufficiency and sufficiency or inadequacy and adequacy. These discrepancies and inconsistencies make comparisons between countries and studies difficult (115). The Institute of Medicine (IOM) recommendations are the most widely accepted guidelines to determine serum VitD status (116). Here, it is stated that the optimal level of serum 25-(OH)D for good bone health for most of the population is 20 ng/mL (which equates to 50 nmol/L). However, many clinical and research experts argue that optimal VitD status for other health concerns is better off set at 30 ng/mL (which equates to 75 nmol/L) (103, 117, 118).

VitD supplementation is used to achieve and maintain the optimal 25-(OH)D concentrations, with no resulting adverse side effects or hypervitaminosis D (119). Data from previous studies have shown the tolerable upper limit of VitD supplementation is up to 4000 IU/day (which equates to 100 µg/day) for adolescents/adults, 2000 IU/day (which equates to 50 µg/day) for children under ten and 1000 IU/day (which equates to 25 µg/day) for neonates under 1 month (7). A small population of individuals are hypersensitive to VitD, which can cause adverse side effects and therefore VitD supplementation is not recommended (120). This includes idiopathic infantile hypercalcaemia, granulomatous disorders and Williams-Beuren syndrome (121-123). VitD toxicity is poorly understood and not well defined in the literature. VitD toxicity is clinically rare and has been reported in multiple age groups, but results in serious health issues if not identified and treated efficiently (124). Several causes of VitD toxicity have been reported, including manufacturing errors, errors in milk fortification, incorrect dosing from liquid preparations (more commonly seen in children), and ingestion of megadoses of VitD supplements (for example 50,000 IU) (125). The Endocrine Society guidelines state a serum 25-(OH)D concentration of at least 150 ng/mL (which equates to 375 nmol/L) is required before VitD toxicity is evident (124).

It is important to note the IOM guidelines are generalised for a vast population relevant to the United States and Canada, for public health purposes and not

specifically for use in clinical practice. Additionally, these recommendations do not account for a specific age group, BMI, ethnicity, or demographical location (latitude and longitude, as well as altitude). In Australia, the current average estimated intake of VitD for men is 2.6-3.0 g/day and 2.0-2.2 g/day for women, whereas the recommended amount is 5.0 g/day for adults (126). To date, no guidelines specific to an Australian population have been developed to classify VitD status.

Enzyme kinetic data suggests that only 50% of the maximal CYP27B1 activity (which hydroxylates 25-OHD) is achieved when the 25-OHD serum concentration is approximately 40 ng/mL (equates to 100 nmol/L) (119). This suggests the requirement for optimal VitD is significantly higher than the 20 ng/mL cut off derived from studies on bone health outlined by the IOM (7). Considering this, values $\geq$ 30 ng/mL may be more suitable in certain subpopulations (5, 127-131).

1.3.5 Causes of VitD insufficiency and deficiency

Nearly half of the global population is estimated to be VitD insufficient with serum 25-(OH)D levels falling below 20 ng/mL (22). There are numerous causes of VitD insufficiency/deficiency, and one of the major contributing factors to serum 25-(OH)D levels is season. As 90% of the body's circulating VitD is attributed to the exposure to sunlight, countries and seasons with minimal sunlight are associated with higher rates of VitD insufficiency and deficiency (5). There is an evident sinusoidal pattern between VitD levels and month, with the highest concentrations in summer and autumn months, and the lowest in spring and winter months (132). This pattern is seen in Australia, where the incidence of VitD insufficiency/deficiency increases from approximately 31% in summer months to close to 50% in winter months (126). Along with season, several other factors increase the risk of VitD insufficiency/deficiency through reduction in the production of VitD in the skin. These include minimal sun exposure (this may be attributed to religious clothing even in summer months), time of day the sample was taken, latitude, darker skin pigmentation, skin grafts, age related changes in the skin structure/function and sun avoidance behaviour which may be attributed to risk reduction for melanoma (104).

Another cause of VitD insufficiency and deficiency is due to decreased bioavailability of VitD within the gastrointestinal tract (133). Many diseases are associated with malabsorption of VitD, including cystic fibrosis (134, 135), Crohn's disease (136, 137), celiac disease (138, 139), inflammatory bowel disease (140, 141) and certain types of bariatric surgery (gastric bypass) (142). In most of these diseases (Crohn's, celiac and inflammatory bowel disease), immune-mediated enterocyte destruction with atrophic intestinal epithelium results in decreased epithelial surface area for absorption of fat-soluble vitamins such as VitD (143-145) . In contrast, in cystic fibrosis the malabsorption results from exocrine pancreatic enzyme insufficiency with profound steatorrhea, rather than a defect in intestinal epithelial function (146). The mechanism in bariatric surgery patients is currently unknown, but decreased food intake is thought to be a contributing factor (147). Furthermore, there is some evidence to suggest patients who have had bariatric surgery are at a higher risk of developing pancreatic insufficiency, as seen in the pathogenesis of cystic fibrosis (148).

Additionally, certain medications reduce cholesterol absorption, which is a key aspect in the VitD synthesis pathway (149). Other medications can also increase catabolism linked to VitD insufficiency/deficiency, including anticonvulsants, glucocorticoids, antiviral (for the treatment of AIDS) and antirejection drugs following organ transplantation. Lastly, obesity is very commonly associated with reduced serum VitD levels, a concept which is known largely accepted, although the mechanisms driving this are still unclear. In early studies of VitD deficient rats, radio-labelled VitD was found to accumulate in adipose tissue and simultaneously had the slowest release rate (150). A similar trend was observed in humans, where obese subjects had lower 25-OHD levels than their 'healthy' weight counterparts, due to a modulation of uptake and clearance of VitD from adipose tissue (151). More recent studies have suggested two major theories to explain the association between obesity and VitD insufficiency/deficiency. Firstly, it is hypothesised that VitD is sequestered in adipose tissue but not released, leaving less circulating and bioactive VitD available (152). Secondly, it is hypothesised that volumetric dilution could explain the lower levels of VitD in obese patients with larger body mass (153). It remains unclear which of these two theories (or if it is a combination of both) accurately explains this phenomenon.

Since the liver and kidney hydroxylate VitD, liver failure and nephrotic syndrome can reduce the synthesis and increase the urinary loss of 25-(OH)D. Additionally, chronic renal failure reduces the synthesis of 1,25-(OH)2D, the biologically active form of VitD. There are also heredity and genetic factors which effect VitD status. Hereditary VitD-resistant rickets (HVDRR) is an autosomal recessive disease caused by abnormality of the VDR gene and leads to VitD deficiency. Inherited variants in the genes *CYP2R1* (encoding the CYP2R1 protein) and group-specific **c**omponent (encoding the VDBP) have consistently been shown to modify serum 25-(OH)D concentrations. Several acquired disorders have also been implicated in VitD insufficiency/deficiency, including primary hyperparathyroidism, hyperthyroidism, and oncogenic osteomalacia (154, 155). Maternal and newborn 25-(OH)D concentrations are highly correlated, therefore maternal VitD deficiency is a risk factor for foetal/neonatal VitD deficiency (156). Furthermore, infants who are exclusively breastfed are at an increased risk of VitD insufficiency/deficiency because breastmilk is a poor source of VitD (157). Neonates of women with VitD deficiency during foetal development are at a higher risk of being born small for gestational age and developing rickets and hypocalcaemia (158).

1.4 VitD in the context of human fertility

1.4.1 VitD and pregnancy

VitD metabolism during pregnancy undergoes key alterations whereby the active form of VitD (known as calcitriol) increases 3-fold in the first month of pregnancy (159). The kidney is the major production site of serum 1,25(OH)2D but given that PTH concentrations are lower in pregnant compared to non-pregnant women, there are still some gaps in knowledge regarding the regulation of serum 1,25-(OH)2D concentrations in pregnancy (160). The placenta was one of the first extra-renal tissues shown to be capable of synthesising 1,25(OH)2D3, with detectable 25-(OH)D 1-alpha-hydroxylase (CYP27B1) activity in both maternal decidua and foetal trophoblasts (161, 162). Maternal 25-(OH)D crosses the placental barrier and is the main source

of VitD for the developing foetus, while 1,25-(OH)2D does not but is produced by the foetal kidneys (159, 163).

Maternal VitD insufficiency is associated with a higher incidence of complications such as placental insufficiency, spontaneous abortion, impaired foetal development, premature birth, and preeclampsia (164, 165). Furthermore, supplementation with VitD can significantly reduce the risks of foetal complications associated with low maternal VitD (166, 167). Considering this, establishing maternal VitD sufficiency prior to conception is vital to improving pregnancy outcomes in women. Interestingly, the positive correlation between serum 1,25-(OH)2D and 25-(OH)D concentrations is stronger in pregnant compared to non-pregnant women suggesting that 1,25-(OH)2D synthesis is more substrate dependent, i.e., more determined by serum 25-(OH)D concentrations, in pregnancy (168). Several observational studies reported an increase of VDBP during pregnancy with a peak of approximately 40–50% higher serum VDBP concentrations compared to non-pregnant women at the beginning of the third trimester and a decline at term (169).While it is not clearly established currently, the decline in free 25-(OH)D during pregnancy could be partially explained by increased serum VDBP concentrations in pregnant women (170). From a classical physiological point of view VitD is critical for bone and calcium (mineral) homeostasis, and pregnancy/lactation are biological settings that require an adequate VitD status to maintain optimal bone and mineral metabolism (163). Importantly, maternal VitD supplementation can prevent neonatal hypocalcaemia, that may result in softening of bones (e.g., craniotabes and various pathologies of rickets) and in severe cases there is an increased risk of seizures and dilated cardiomyopathy (171-174). Many questions remain regarding the non-classical role of VitD in pregnancy, with growing evidence demonstrating VitD plays a critical role in immunomodulation at the maternal-foetal interface (163, 168, 169, 175).

The expression of the VDR in immune cells has highlighted an interesting role of VitD in both innate and adaptive immune systems (176).VitD displays a local immune effect via activation of intracellular VDRs, which have been shown to be present in monocytes/macrophages, T cells, B cells, natural killer cells, and dendritic cells (177). The maternal immune system is crucial during pregnancy to ensure there is not a immune-cell driven rejection of the conceptus (178). Firstly, during implantation immune suppression via Treg (specialised T cells which act to supress immune

response) is required to negate an immune response against the embryo, whereby several immune cells become quiescent (including cytotoxic T cells, Th1 cells, macrophages, dendritic cells and natural killer cells) (179). Several mechanisms support this immunomodulatory effect at the foetal–maternal interface. Firstly, the adaptive immune response is curtailed by immune suppressive pathways or skewed toward immune tolerance. Secondly, the immune system contributes to the tissue remodelling necessary for placental development and function. In this context, uterine natural killer cells have a special role facilitating trophoblast migration and the consequent development of the spiral arteries in contrast with peripheral cytotoxic natural killer cells (180). This unique immunological environment present at the maternal–foetal interface is sustained during pregnancy by soluble molecules such as cytokines, chemokines, hormones, and prostaglandins (181). Foetal programming is a process through which during the foetal development period certain stimuli and insults (such as nutrition status and immune response) have long-term health effects (182). Permanent changes in many physiological processes which occur during this period can result in epigenetic changes and be significant determinants of future health (183).

1.4.2 VitD, season and fertility

While there is an evident selection bias when it comes to pregnancy and season, whereby women tend to plan their pregnancies around season and in some cases corresponding cultural aspects, with summer being the preferred time to conceive (184-186), there are also numerous additional environmental factors that play a role. For example, extreme weather conditions, high temperatures and sun exposure (187, 188). The correlation between fertility and season have been well documented, in particular in regard to the role of melatonin secretion (189). In more recent years, the relationship between VitD and season (in the context of fertility) has been investigated, given its cutaneous synthesis following UV-B radiation is the major source of VitD in humans.

A study by Currie and Schwandt, followed women over multiple pregnancies to further elucidate the effect of seasonal variation on pregnancy outcomes, specifically within the USA (188). Interestingly, their study of 1,435,213 births found conceptions

in the month of May have the shortest gestational period, which coincides with increased prevalence of influenza in January and February (188). They hypothesise that this may be a mechanism to reduce the risk of contracting influenza during the late third trimester(188). Additionally, they reported that maternal weight gain was highest in summer months, which translated to increased birth weights (188). There is an apparent discrepancy between results for Caucasian and African American individuals when it comes to seasonality and fertility. A large study from the USA, which compared daily weather data to birth outcomes between 1989-2004, found sun exposure had positive effects on birth weight for African American women but not for Caucasian women (190). In contrast, previous findings have demonstrated increased UV exposure reduces folic acid which is vital for the primary prevention of neural tube defects and suppresses the immune system (191-193). This may suggest the benefits of sun exposure could be ambiguous and vary based on race/ethnicity.

Wesselink et al., conducted a large preconception cohort study of 14,331 women in North America and Denmark who were attempting to conceive without fertility treatment and had been attempting pregnancy for ≤ 6 menstrual cycles at enrolment (194). This analysis was adjusted for seasonal patterns during the conception period. Pregnancy attempts peaked during the month of September, with seasonality more prominent in North America compared to Denmark (194). Furthermore, fecundability was highest in autumn and lowest in spring months, with a greater effect seen based on lower latitudes (194). A large prospective study of 1050 participants in Ireland examined VitD metabolites (total 25-(OH)D, 25-(OH)D3, 25-(OH)D2, and 3-epi-25-(OH)D3) in cord serum via liquid chromatography tandem mass spectrometry (LC-MS/MS) (195). The VitD metabolites of total 25-(OH)D, 25-(OH)3, and 3-epi-25-(OH)D3 were significantly lower in Winter months compared to Summer months (mean decrease of 15.5, 15.5, 1.8 nmol/L, respectively) (195). To date, very little, large scale studies on VitD status and fertility have been conducted in the southern hemisphere.

1.4.3 VitD and IVF

Currently, numerous studies have investigated VitD in the context of infertility, particularly populations of women undergoing IVF, with inconclusive findings. These are further complicated as a number of these only use subgroups based on aetiology (such as PCOS), have varying inclusion criteria (age, BMI etc.), while others assess serum total 25-OHD levels at different times of the IVF cycle (disregarding FF concentrations), and there is variation in the determination of VitD status cut-offs (i.e.: 20 ng/mL vs. 30 ng/mL for sufficiency, Table 1.2). Additionally, studies from different countries have vastly different clinical protocols and ethnic diversity and therefore may not be relevant to other studies conducted in other parts of the world (Table 1.2). This makes it difficult to draw comparisons between the many studies within the area.

Table 1.2: Summary of publications examining VitD in the context of IVF patients undergoing fresh ET. Retrospective publications are denoted by (*), while the remaining publications are prospective studies. *Abbreviations: VitD, vitamin D; FF, follicular fluid; VDBP, vitamin-D binding protein; LB, live birth; LBR, live birth rate; CP, clinical pregnancy; CPR, clinical pregnancy rate; hCG, human chorionic gonadotropin; ET, embryo transfer; MII, metaphase two; P4, progesterone; E2, estradiol; PCOS, polycystic ovarian syndrome; GCs, granulosa cells; VDR, vitamin D receptor; TQ, top quality.*

Publication	Location	N=	Grouping	Main Outcome(s)
Potashnik et al., 1992 (196)	Israel	10	20 ng/mL	E2 levels were associated with increased serum and FF 1,25-(OH)2D3.
Estes et al., 2009 (54)	USA	20	LB vs. no LB	VDBP in FF was decreased in the group with successful LB.
Anifandis et al., 2010 (53)*	Greece	101	30 ng/mL	Unadjusted FF VitD levels were negatively correlated to the mean score of embryo quality.
Ozkan et al., 2010 (39)	USA	84	Tertiles	Serum and FF levels of VitD were highly correlated, and higher levels were associated with improved CPR.
Rudick et al, 2012 (31)*	USA	188	30 ng/mL	Among non-Hispanic whites, CPR declined with progressively lower levels of VitD, while in Asians, the reverse was true. Adjusting for age and number and quality of embryos transferred among non-Hispanic whites, the odds of pregnancy were 4x higher in VitD sufficient group.
Firouzabadi et al., 2013 (50)	Iran	221	30 ng/mL	The serum and FF VitD levels were correlated. No significant correlation was seen between CPR and VitD status.

Garbedian et al., 2013 (35)	Canada	173	30 ng/mL	Multivariable logistic regression showed serum VitD was a predictor of CP.
Fabris et al., 2014 (44)*	Spain	267	30 ng/mL	No difference in implantation rates or CPR.
Rudick et al, 2014 (197)*	USA	99	30 ng/mL	VitD sufficiency was associated with higher CPR.
Paffoni et al., 2014 (19)	Italy	335	20 ng/mL	Women with sufficient VitD levels had improved CPRs.
Polyzos et al., 2014 (30)*	Belgium	368	20 ng/mL	Single ET, P4 elevation & endometrial thickness on the day of hCG and VitD insufficiency were all independently associated with CPR (VitD insufficiency is associated with lower CPR).
Franasiak et al., 2015 (45)*	USA	517	30 ng/mL	No significant differences reported for CPR or implantation in oocyte recipients.
Farzadi et al., 2015 (34)	Iran	80	CP vs. no CP	FF conc of VitD was significantly higher in women with successful CP.
Trably et al., 2015 (52)	France	198	20 ng/mL	No correlation was found between serum VitD fertility outcomes.
Abadia et al., 2016 (32)	USA	100	30 ng/mL	VitD sufficiency was positively related to fertilisation rate.
Deriquehem at al., 2016 (49)	Brazil	199	30 ng/mL	Patients with insufficient FF VitD levels had a greater mean weight than those with sufficient levels.
Neville et al., 2016 (51)	USA	64	20 ng/mL	No association between VitD status and fertility outcomes.
Aghadavod et al., 2017 (198)	Iran	80	30 ng/mL	VitD levels of FF were decreased in PCOS patients. VitD serum levels were lower in overweight patients. The gene expression data of VDR in GCs were significantly lower in the PCOS/overweight group.
Banker et al., 2017 (47)	India	192	Donor and Recipient	No association between VitD status and implantation rate or CPR in either the donor or recipient group.
Paffoni et al., 2017 (199)	Italy	103	20 ng/mL	Serum VitD correlates within the partners of infertile couples.

Antunes et al., 2018 (200)	Brazil	197	30 ng/mL	Lower VitD group had more follicles and higher serum E2 concentrations on the day of hCG administration.
Ciepela et al., 2018 (201)	Poland	198	20 ng/mL	FF 25-(OH)D was negatively correlated with zygote formation, day 2 and 3 embryo quality, CPR and LBR.
Mitra et al., 2018 (38)	India	220	30 ng/mL	Number of retrieved MII oocytes and CPR were higher in the VitD sufficient group.
Chu et al., 2019 (33)	UK	500	30 ng/mL	Crude LBR were higher in women with VitD status > 30 ng/mL.
Cunningham et al., 2019 (43)	UK	59	Non-PCOS vs. PCOS	VitD metabolites did not differ between PCOS and controls. 25(OH)D3 correlated with embryo fertilisation rates in PCOS patients alone. For all subjects, 3-epi-25(OH)D3 correlated with fertilisation rate and negatively with HOMA-IR. 25(OH)D2 correlated with cleavage rate, top quality embryos and blastocysts.
Jiang et al., 2019 (46)*	China	1883	Quartiles	Number of transferred embryos and endometrial thickness were associated with increased chance of CPR.
Liu et al., 2019 (37)	China	848	Quartiles	VitD sufficient women had the highest fertilisation rates.
Masjedi et al., 2019 (202)	Iran	100	Non-PCOS vs. PCOS	There were significant positive correlations between FF levels of 25-(OH)D with total antioxidant capacity, estradiol and progesterone concentrations, SOD, GPx, and CAT activities. Negative correlations were found between 25-(OH)D with free and total testosterone, and ROS levels.
Shehadeh et al., 2019 (40)	Israel	71	CP vs. no CP	Derivatives of VitD were highly accumulated in positive-outcome FF patients.

Skowronska et al., 2019 (55)	Poland	50	TQ vs. non-TQ Day 5 blastocysts	Statistically significant negative correlation between the mean day 5 embryo score and the concentration of serum VitD.
Sufen et al., 2019 (48)	China	2569	Quartiles	Total 25-(OH)D correlated with free 25(OH)D, but neither were correlated with any fertility outcomes (implantation rate and CPR).
Zhao et al., 2019 (41)	China	305	Quartiles	Implantation rate and CPR were higher in the VitD sufficient group.
Alavi et al., 2020 (203)	Iran	287	20 ng/mL	Regression analysis showed no association between AMH and VitD levels.
Jiang et al., 2020 (36)	China	1232	Tertiles	Generalised linear regression showed serum VitD levels were related to fertilisation rates.

A small number of these prospective studies focus on VitD in the context of PCOS, drawing conclusions between the role of VitD in PCOS compared to non-PCOS women (43, 198, 202). Firstly, Aghadavod et al., conducted a prospective study of 80 women between the ages of 20-35 years, who were divided in 4 groups PCOS normal and overweight and non-PCOS normal and overweight. From this, GCs were isolated and the VDR was assessed. PCOS women who were overweight had significantly lower levels of 25-(OH)D within their FF and downregulated gene expression of the VDR, compared to non-PCOS normal weight women (198). Secondly, Cunningham et al., examined VitD metabolites in 59 non-obese women, 29 with PCOS and 30 without (who were also age matched) (43). While most metabolites were comparable between the two groups (25-(OH)D2, 25-(OH)D3, 1,25-(OH)D3 and 3-epi-25-(OH)2D3), 24,25-(OH)2D3 was significantly higher in the PCOS group compared to the non-PCOS group and correlated to AMH and AFC. 24,25-(OH)2D3 is an active metabolite which is converted to 1,24,25-trihydroxyvitamin D3 through the C24 oxidation pathway (204). However, the role of 24,25-(OH)2D3 in the ovary is currently unknown. Masjedi et al., compared the association between FF 25-(OH)D levels, sex steroids and oxidative status in GCs from non-obese PCOS and non-PCOS women $\leq$ 36 years old (202). The PCOS women had significantly lower levels of 25-(OH)D, key reproductive hormones such as estradiol (E2) and progesterone (P4), and several antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), compared to controls (202). Additionally, there were significant positive correlations between FF levels of 25-(OH)D with E2,P4 concentrations, and the activity of SOD, GPx, and CAT (202). This suggests that PCOS women have higher oxidative status and reduced sex steroid secretion within the ovary. Lastly, Zhao et al., measured serum 25-(OH)D levels of 305 women with PCOS and stratified these women in quartiles based on their respective 25-(OH)D levels (41). Crude implantation and CPRs were significantly higher in the women who had a sufficient ($\geq$ 20 ng/mL) VitD status, although this was not confirmed in a multivariable logistic regression analysis to account for various confounding factors, such as embryo quality and age (41).

Only two studies to date have investigated VitD status in the context of donor-recipient cycles, which helps elucidate the role of oocyte vs. endometrium and its association with VitD status (42, 47). Firstly, a retrospective analysis examined the

cycles of 99 oocyte donor recipients who had their serum 25-(OH)D levels measured (42). In a multivariable logistic regression analysis, which accounted for transferred embryo quality, patient age BMI and ethnicity, both CPR and LBR were positively associated with VitD sufficiency (42). Secondly, a prospective study compared VitD status of 192 recipients and donors (who were further subdivided into VitD deficient and sufficient) (47). Interestingly, while the proportion of patients in the donor group who were sufficient and deficient was comparable (45.5% vs, 54.5%, respectively), in the recipient group the proportion of deficient women was significantly higher than the sufficient group (64.1% vs. 35.9%) (47). There was no difference in the donor groups in terms of fertilisation rates or embryo utilisation rate or in the recipient groups for implantation rate or CPR (47).

Several retrospective (30, 205) and prospective (19, 32-39, 41) studies have reported an association between VitD sufficiency and improved clinical outcomes in women undergoing IVF, including fertilisation rates, CPR, and LBR (Table 1.2). A retrospective analysis of 188 women found improved CPR in non-Hispanic whites who were VitD sufficient ($\geq$ 30 ng/mL) (205). Furthermore, in an adjusted multivariable analysis accounting for age, number of and quality of embryos, the chance of CP was 4 times higher in the VitD sufficient group for non-Hispanic whites (205). Another retrospective report (where VitD sufficiency was classified as > 20 ng/mL) assessed the VitD status of 368 women undergoing IVF with single ET (30). From this it was demonstrated that P4 elevation & endometrial thickness on the day of hCG trigger and VitD sufficiency were all independently associated with CPR (30). In a prospective study of FF from 80 infertile women, it was found that FF 25-(OH)D was significantly higher in women who achieved CP compared to those who did not, and was positively correlated with implantation rate (34). However, there were no differences in embryological measure related to VitD status, including the number of metaphase two (MII) oocytes collected, oocyte/embryo quality, or serum hormone levels (34). Another prospective analysis of 173 women demonstrated crude CPR's were 8.9% higher in women with serum 25-(OH)D $\geq$ 30 ng/mL. Additionally, following a multivariable logistic regression analysis adjusting for age, BMI and day of embryo transfer, VitD sufficiency was a significant predictor of implantation and CP success (35). Using the same cut-off values, Mitra et al., showed a similar finding of improved CPR related to VitD sufficiency, specifically in an Asian population of

220 women (38). Women who had sufficient serum 25-(OH)D levels had crude CPRs 12.3% higher than those who were deficient, as well as a higher yield of MII oocytes collected (38). However, no multivariable analysis was conducted to account for various confounding factors which may be related to CP outcome or MII oocyte generation, such as age or transferred embryo quality. In contrast, Paffoni et al., used a cut-off value of $\geq$ 20 ng/mL for VitD sufficiency when assessing the serum VitD status of 335 'healthy' weight (BMI 18-25 kg/m^2) women enrolled for IVF (19). The authors found crude CPR were 11.1% higher in the sufficient group, and further subgroup analysis showed women with serum 25-(OH)D $\geq$ 30 ng/mL had the highest CPRs (19). However, as above, no multivariable logistic regression was utilised to further confirm these findings. Given that the current classifications for VitD status are based on bone health and it is unknown what the recommended optimal level for women of reproductive age, some researchers opt for the use of tertiles or quartiles when examining VitD (206). For example, Ozkan et al. prospectively assessed serum and FF 25-(OH)D levels and based on this stratified patients into tertiles (39). Significantly higher crude implantation and CPR were observed across the tertiles (39). Furthermore, in a multivariable logistic regression adjusting for age, BMI, ethnicity, and number of embryos transferred, each ng/mL increase of FF 25-(OH)D increased the likelihood of achieving a CP by 7% (39).

Several prospective studies have shown improved fertilisation rates, although this has not translated into improved CPRs (32, 36, 37). In an investigation whereby100 women undergoing IVF were stratified based on serum 25-(OH)D levels (32). Women in the fourth quartile with the highest serum 25-(OH)D levels had the highest fertilisation rates (9.2% higher), although there was no difference across the quartiles for implantation rate, CPR or LBR (32). In a large prospective study of 1232 women stratified into tertiles (based on serum 25-(OH)D levels), it was observed that female 25-OHD levels were related to improved crude fertilisation rates (6.3% increase) (36). This was further confirmed in a binary logistic regression analysis which showed the lowest 25-(OH)D tertile were 4.8 times less likely to achieve fertilisation when compared to the third tertile with the highest serum 25-(OH)D (36). Finally, another study similarly grouped patients into tertiles based on serum 25-(OH)D in their study of 848 women undergoing IVF treatment and found crude fertilisation rates were 5.8% higher in the fourth quartile which had the highest serum 25-(OH)D (37). However,

there was no significant difference between the quartiles for implantation rate, CPR or LBR (37).

While several other retrospective and prospective studies which investigated serum or FF 25-(OH)D levels found no association with clinical outcomes in IVF (Table 1.2), including fertilisation rate, implantation rate, CPR, and LBR (44-46, 48-52), very few studies show a negative association between VitD status and fertility in the context of IVF (53, 55, 200). A small study of 101 women where serum and FF concentrations of 25-(OH)D were measured and fertility outcomes were assessed (53). They showed the group with FF 25-(OH)D < 20 ng/mL had a CPR 18.0% higher than those in the ≥ 30 ng/mL group (53). Interestingly, this group also had significantly higher mean FF glucose levels (17.6 mg/dl higher than the > 30 ng/mL group) (53). Lastly, a significant negative correlation (r=-0.25) was found between the mean embryo score and FF 25-(OH)D levels, although glucose was not accounted for in this analysis (53). Another report analysed 197 patients and found lower FF 25-(OH)D concentration is related to a higher number of large ovarian follicles in women undergoing IVF, although there was no difference in the number of MII oocytes retrieved from these follicles (200). Lastly, a prospective study of 50 women investigated the effect of the concentration of fat-soluble vitamins including VitD in individual follicles on oocyte quality (55). Patients were stratified based on the presence or absence of a top-quality (TQ) day-5 blastocyst, and FF 25-(OH)D was significantly negatively correlated with day-5 embryo culture quality (55). Furthermore, serum or FF 25-(OH)D concentrations were not associated with fertilisation rates, CPR or LBR (55). However, none of these investigations utilised multivariable logistic regression analysis accounting for confounding factors to confirm and further elucidate the negative associations reported.

A small amount of published data has found interesting associations between VitD status and other aspects of human fertility, outside of clinical outcomes but in the context of IVF. For example, a prospective study which assessed 287 women found no association between serum 25-(OH)D levels and AMH levels or AFC. However, the authors did not state if serum hormonal levels were measured on the same day between participants or at what point in the IVF cycle these measurements occurred. Another prospective study examined 11 potential protein candidates from the FF from 20 age-matched IVF patients, including VDBP, and stratified patients based on

successful or unsuccessful LB. Interestingly, VDBP expression was decreased in the group of patients who had a successful LB outcome, although it was not reported what the serum or FF levels of 25-(OH)D were in these groups and whether this was related to VDBP. One report investigated if there was concordance between 103 infertile couples in relation to serum 25-(OH)D levels. Surprisingly 71% of couples demonstrated shared VitD status, which was 10% higher than what was predicted and the Pearson coefficient of correlation R2 was 0.52 ($p<0.05$) in these couples. However, Paffoni et al., did not report any fertility outcomes within this publication and how male serum 25-(OH)D is associated with outcomes in IVF remains unknown.

An early publication by investigated the relationship between serum E2 and several VitD metabolites (25-(OH)D3, 24,25-(OH)2D3 and 1,25-(OH)2D3) within FF (for the first time) and serum in a small cohort of 10 women undergoing IVF (196). All the FF VitD metabolites measured were highly correlated with serum levels ($r=0.738$, $r=0.751$ and $r=0.787$, respectively) (196). Of interest, throughout the IVF cycle, as serum E2 levels increased, a similar increase was observed in serum 1,25-(OH)2D3, which peaked at oocyte pick up (OPU) and decreased alongside E2 at the mid-luteal phase (196). Numerous other studies have demonstrated serum and FF 25-(OH)D levels are highly correlated in IVF patients on the day of OPU (Table 1.3). Furthermore, a recent report by Shehadeh et al., examined the concentrations of various lipids (including cholesterol esters, sterols, membrane lipids) within the FF of 71 patients with a successful CP compared to women without a successful CP (40). Several lipids were significantly increased in women who had a successful CP, including VitD derivatives (25-(OH)D3, 24,25-(OH)2D3 and 1,25-(OH)2D3), phospholipids, lysophospholipids and total sphingolipids (40). This suggests VitD levels and the lipid environment within the ovary are important for IVF success.

1.4.4 Serum VitD and FF concentrations in IVF patients

Currently, several studies have assessed serum and FF levels of 25-(OH)D in women undergoing IVF (summarised in Table 1.3). Potashnik et al., were the first to demonstrate the presence of VitD metabolites in FF collected from a small cohort of 10 IVF patients (196). Serum was collected on the day of hCG trigger and OPU (along

with FF collected at OPU) and levels of VitD were measured using mass spectrometry and correlated (196). All of the metabolites measured in FF (25-(OH)D3, 24,25-(OH)2D3, and 1,25-(OH)2D3) were highly correlated with serum levels (r=0.74, 0.75, 0.79, respectively) (196). In another report, serum and FF levels were measured by chemiluminescence in a small population of women undergoing IVF treatment and a strong positive correlation (r=0.88, n=14) was found (49). However, the timing of serum collection and statistical significance was not reported by the authors (49). In one investigation of 198 women who had both their serum and FF measured on the day of OPU via chemiluminescence, a strong positive correlation was also demonstrated (r=0.72, p<0.001) (201). A study of 101 women who also had their serum and FF measured on the day of OPU, although by electrochemiluminescence immunoassay, also reported a similar finding with a strong positive correlation between serum and FF 25-(OH)D (r=0.79, p<0.001) (53). Ozkan et al., reported the strongest correlation in a study of 84 women where serum levels on the day of hCG trigger were highly correlated to FF collected on the day of OPU (r=0.94, p=<0.001). Finally, the largest study to date used an enzyme linked immunosorbent assay to analyse serum and FF obtained on the day of OPU from 221 women undergoing IVF (50). As previously demonstrated, there was a significant positive correlation between serum and FF 25-(OH)D levels (r=0.83, p<0.001).

Table 1.3: Summary table of publications which assessed the correlation between serum and FF levels of total 25-(OH)D. *Abbreviations: OPU, oocyte pick-up; NR, not reported; hCG, human chorionic gonadotropin; ECLIA, electrochemiluminescence immunoassay; ELISA, enzyme linked immunosorbent assay.*

Publication	N=	Timing of serum measurement	Assay method	r value	P value
Anifandis et al., (53)	101	OPU	ECLIA	0.79	<0.001
Ciepiela et al., (207)	198	OPU	Chemiluminescence	0.72	<0.001
Deriquehem et al., (49)	14	NR	Chemiluminescence	0.88	NR
Firouzabadi et al., (50)	221	OPU	ELISA	0.83	0.001
Ozkan et al., (39)	84	Day of hCG trigger	N.R	0.94	<0.001
Potashnik et al., (196)	10	Day of hCG trigger & OPU	Mass spectrometry	0.74	<0.001

1.4.5 VitD and oocyte and embryo quality in IVF

Mammalian oogenesis occurs concomitantly with folliculogenesis in a highly coordinated manner in the ovaries. Folliculogenesis is orchestrated by a complex series of cellular and molecular interactions that are evoked by the autocrine, paracrine and endocrine functions of ovarian growth factors, chemokines and steroids (208). Oocyte-GC communication is essential for normal growth and development of both the oocyte and the follicle (209). Briefly, communication in the oocyte-granulosa complex occurs via paracrine signalling and gap-junctional exchange of small regulatory molecules and ions (209). This is a bi-directional communication axis, which is vital for the regulation of early ovarian follicle development, this has been reviewed in detail elsewhere (210). Paracrine signalling molecules and growth factors secreted by oocytes and somatic cells can activate dormant primordial follicles as well as play a role in the selection of secondary follicles. Additionally, these signalling molecules can impact the growth and differentiation with oocytes and GCs (211). As previously mentioned, (Table 1.3), VitD levels within the FF are highly correlated with serum levels, suggesting VitD present in the FF may play a direct role in the cellular function of the theca and GCs.

While many publications (Table 1.2) have investigated the association between VitD status and outcomes in IVF patients, but clinical focus tends to measure fertilisation rate, implantation rate, CPR and LBR. Henceforth, very little is known about the association between VitD status, and embryological measures (such as oocyte/embryo quality). A prospective study by Ciepiela et al., assessed FF levels of 25-(OH)D at OPU in 198 women undergoing IVF with single ET (SET) (207). The authors stratified several outcomes by the corresponding FF 25-(OH)D level and found patients who developed a zygote and a high quality day 2 or 3 embryo had significantly lower FF VitD level, compared to patients who did not (207). However, patients who developed a top-quality blastocyst had slightly higher FF 25-(OH)D than those who did not, although this was not statistically significant (207). Finally, when stratified by VitD status (< 20 ng/mL vs. ≥ 20 ng/mL, insufficient and sufficient, respectively) there was no difference in embryological measures (207). In contrast, a small prospective study of 40 women, examined FF 25-(OH)D levels and their correlation with embryological measures, and found positive correlations between 25-

(OH)D levels and the percentage of oocyte maturity, fertilisation rate and the percentage of high-quality embryos developed (212). As previously mentioned, Cunningham et al., found 25(OH)D2 levels correlated with cleavage rate, top quality embryos and blastocysts (Table 1.2, see section 1.3.3) (43). However, none of these studies assessed serum or FF 25-(OH)D status in a multivariable logistic regression model alongside embryological outcomes (such as embryo quality), to assess if such associations remained when confounding factors were considered.

Additionally, a double-blinded RCT assessed the effect of a 6-week 'Mediterranean' dietary intervention on *in vitro* human embryo development (213). A total of 102 couples undergoing IVF were included in the study, with 55 receiving a 6-week intervention of a supplement drink enriched with omega-3 fatty acids (eicosapentaenoic and docosahexaenoic acids, 800 mg and 1200 mg, respectively) and VitD (10 µg), while the remaining 56 couples received the control intervention (213). The main outcomes the authors assessed was the time taken for the second cell cycle to be completed following fertilisation, the completion time and synchrony for the third and fourth cell cycles, and the day 3 and 5 embryo Known Implantation Data Scores (KIDScores) (213). Embryological morphokinetic used to determine the KIDScores were calculated based on previously published methods (214). While there were no differences between the control and intervention group in the number of oocytes retrieved or the number of normally fertilised embryos developed (213). In the intervention group, the time to complete the second and fourth cell cycle was significantly shorter compared to the control group (213). Additionally, there was a significant reduction in the KIDScore on day 3, but no difference for day 5, in the intervention group compared to the control group (213). A lower KIDScore is indicative of improved embryo quality and enhanced likelihood for successful implantation (214). While this study provides promising evidence that pre-conception nutritional status of VitD alongside omega-3 fatty acids can affect embryo development in women undergoing IVF, very little is known about the mechanism which may be driving these observed changes.

1.5 Molecular action of VitD in reproductive cells and tissues

1.5.1 Granulosa and theca cells

The ovary is a complex, specialised tissue with several important roles in female reproduction, including the secretion of several key steroid hormones involved in female sexual development (estrogens), secretion of gonadotropins from the hypothalamic–pituitary axis (FSH and LH), and regulate the function throughout the female reproductive tract (179). In the ovary the follicles are the compartment in which the major endocrine and reproductive functions occur (215). The follicles consist on two key somatic cell types known as the GC and theca cells, which work closely together via paracrine signalling to support the oocyte during folliculogenesis (215).

GCs are subdivided into two functionally distinct phenotypes: cumulus GCs and mural GCs (Figure 1.3) (211). Cumulus GCs directly surround the oocyte, while mural GCs line the preovulatory follicle walls (211). The cumulus cells have cytoplasmic extensions which penetrate the zona pellucida and forms the cumulus-oocyte complex (211). Cumulus-oocyte complex interactions involve a combination of gap-junctions and paracrine signalling, with oocyte secreted factors alongside GC and theca cell outputs (216). Human GCs examined under light microscopy are contained in clusters in a loosely packed manner, while individual cells are irregularly rounded or polyhedral in appearance and vary between 18-25 μm in diameter (217). In most GCs, rough endoplasmic reticulum were minimal but there were abundant intracellular lipid droplets (217). The major function of GCs of the maturing ovarian follicle is to produce E2 which triggers the LH surge due to feedback regulation via the hypothalamus-pituitary-gonad axis (218).

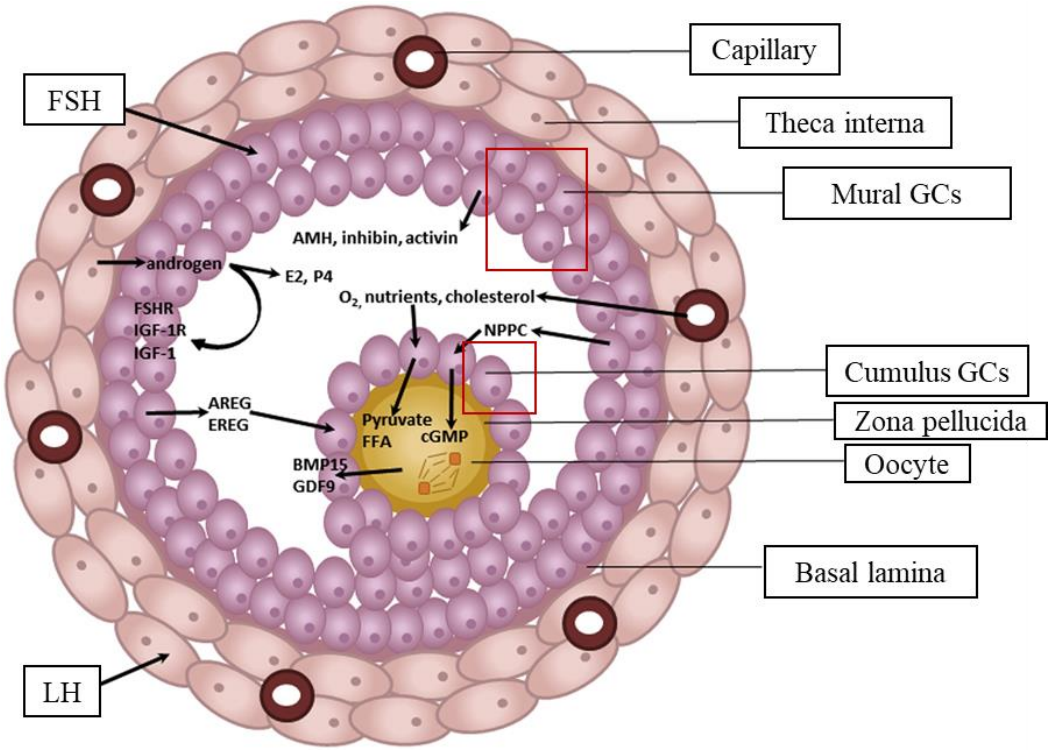


Figure 1.3: Schematic demonstrating the location of GCs (cumulus and mural) and the surrounding theca cells. Adapted from Dumesic et al., 2015 (219).

Human theca cells are subdivided into two distinct phenotypes: theca interna and theca externa cells, both originating from mesenchymal cells (220). Theca interna cells appear epithelial-like in their in shape with a high density of smooth endoplasmic reticulum (SER), while theca externa cells have cytological features characteristic of smooth muscle cells (220). Electron microscopy images of theca cells in small follicles demonstrate theca cells sit on a basal lamina and appear flattened and closely packed with capillaries and bundles of collagen fibres (221). During follicle maturation, theca cells undergo hypertrophy, become rounded, the density of intracellular lipid droplets and SER increases and pseudopodia (temporary cytoplasmic projections which consist primarily of actin filaments) form on the cell membrane (221). Theca cells have several functions during folliculogenesis, including synthesis of androgens, interact/communication with GCs and the developing oocyte, and provide structural support of the maturing follicle as it progresses to produce a mature oocyte for fertilisation (222).

Early studies established GC-theca cell interactions are important in the modulation of their morphology, structure, growth characteristics, functional outputs and gonadotropin responsiveness (223). Kotsuji et al., were the first to investigate the paracrine activity of GCs and theca cells using a co-culture system in which these two cells were attached to opposing sides of a collagen membrane (223). Firstly, the authors established GCs cultured *in vitro* alone appeared flattened and polygonal in shape and formed a monolayer, whilst GCs co-cultured with theca cells form multilayer sheets (as seen in the *in vivo* ovary) (223). In the co-culture study , numerous intracytoplasmic projections protruding from cellular surfaces and connecting cells were observed, where the monoculture showed theca cells to thin, flat and spindle shaped, which was unchanged when co-incubated with GCs (223). However, the apical cell surface changed morphology during co-incubation to a convex shape (223). Additionally, co-culturing the two cells had a significant effect on the rate of cell division with cell numbers increased two-fold compared to cells culture individually (223). In the co-culture system, P4 secretion in the GCs was reduced, while the androstenedione secretion of the theca cells was enhanced (223). For the gonadotropin responsiveness of the cells in co-culture, FSH treatment (1 µg/ml) promoted cellular growth, while LH treatment (1 µg/ml) suppressed cellular growth and augmented P4production. LH induced E2 secretion by GCs both cultured

alone and cocultured with theca cells, although FSH did not induce E2 production (223). These results demonstrate crucial cellular-cellular communication between theca and GCs, which is modified via paracrine signalling of steroid hormones.

Factors secreted from the GCs (insulin-like growth factor (IGF1) and kit ligand (KL)) stimulate the recruitment and androgen production in theca cells from cortical stromal cells, while oocyte-derived growth differentiation factor-9 (GDF-9) is involved in theca cell differentiation during early stages of follicular growth (224). This causes an increase in mRNA expression of 17α-hydroxylase and LH receptor (LHR) (224). In contrast, factors secreted by the theca cells (epidermal growth factor (EGF), transforming growth factor-α (TGFα), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), and bone morphogenetic protein-7 (BMP-7)) promote GC proliferation and supress GC apoptosis (224). This causes an increase mRNA expression of the follicle stimulating hormone receptor (FSHR), as well as enhanced FSH action (summarised below in Figure 1.4) (224).

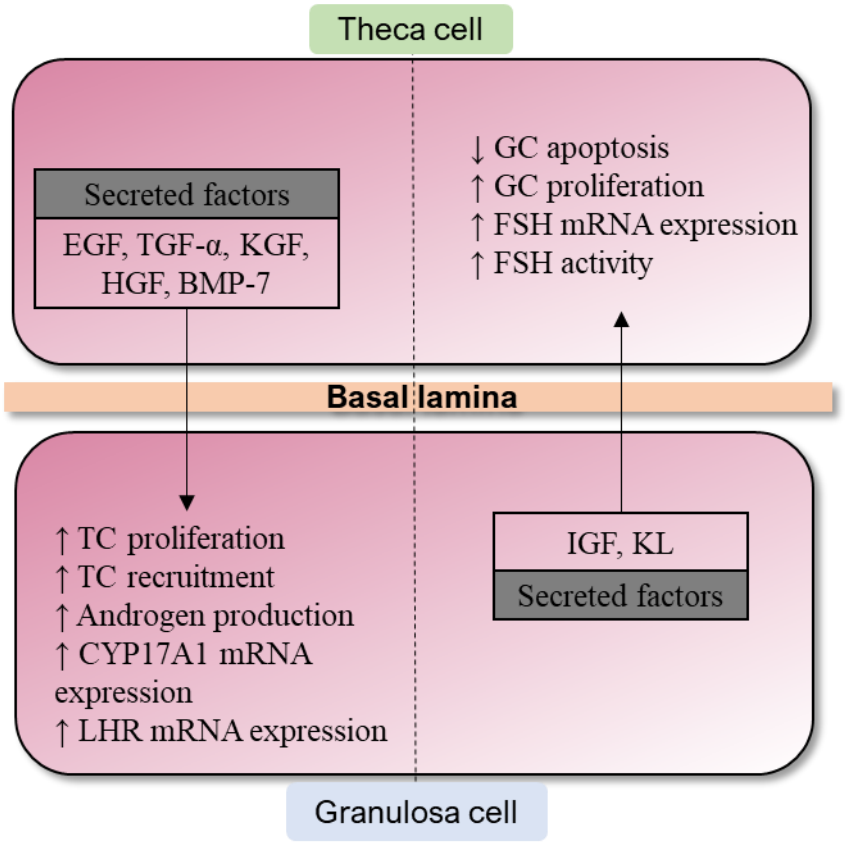
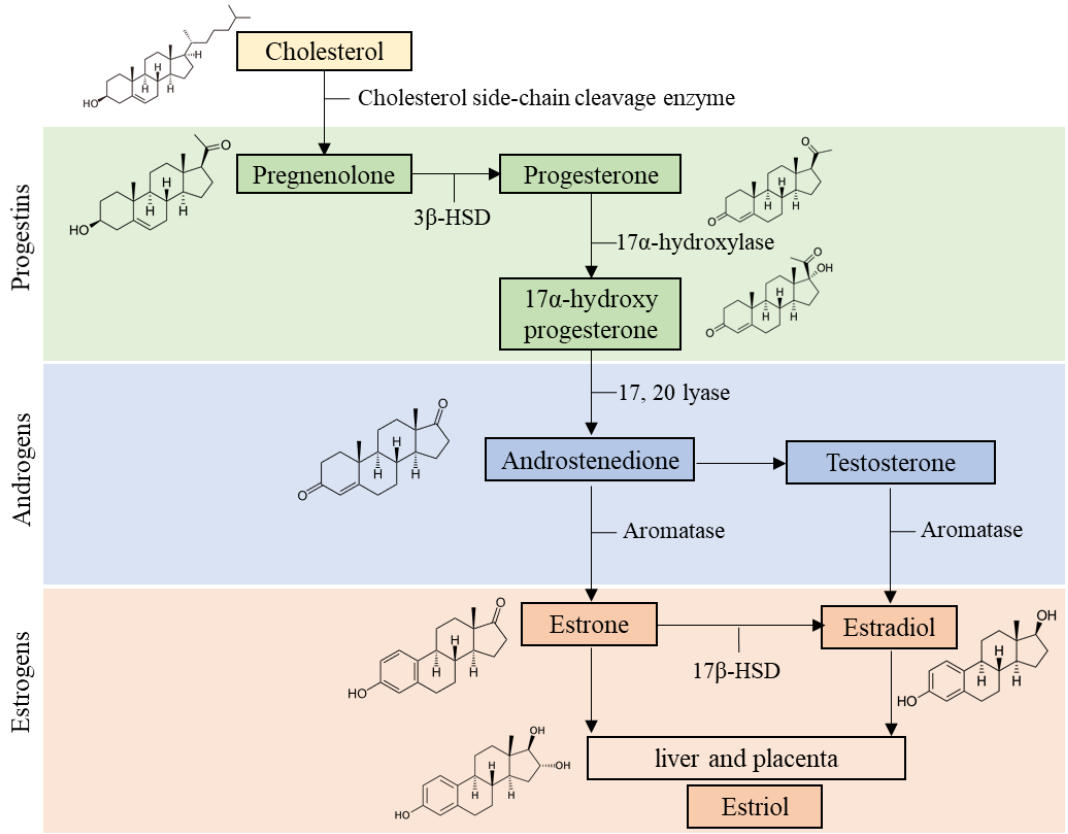


Figure 1.4: Theca-GC bi-directional interactions via secreted factors. *Abbreviations: GC, granulosa cell; TC, theca cell; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; KGF, keratinocyte growth factor; HGF, hepatocyte growth factor; BMP-7, bone morphogenetic protein-7; IGF, insulin-like growth factor; KL, kit ligand.*

The interactions of theca and GCs (in their luteinised forms) drives the secretion of ovarian progestins, androgens and estrogens via the process of steroidogenesis (Figure 1.5) (224). Steroidogenesis is the process by which steroid hormone biosynthesis occurs, with cholesterol as the initial substrate (225). The first step of steroidogenesis is mediated by the transport protein steroidogenic acute regulatory (StAR) which facilitates the transport of cholesterol into the mitochondria (226). Once in the mitochondria, cholesterol is cleaved by the side-chain cleavage enzyme (CYP11A1) to produce pregnenolone (226). Following this, pregnenolone can be converted to P4 under the action of the 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme (227). P4 can then be converted to 17α-hydroxyprogesterone via the 17α-hydroxylase (CYP19A1) (227). 17α-hydroxyprogesterone can also be converted to the androgen androstenedione via the enzyme 17,20 lyase (228). Androstenedione can either be converted to the androgen testosterone by the 17β-HSD enzyme or to the estrogen estrone (E1), under the action of aromatase enzyme (228). Finally, E1 is converted to E2 by 17β-HSD and both E1 and E2 can be further converted to estriol (E3) by the liver and placenta (229).



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983 **Figure 1.5: Steroidogenic pathway of progestins, androgens and estrogens.**

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1.5.2 Molecular signalling of VitD in reproductive cells

Scientific research into the mechanisms of ovarian physiology is largely well understood in modern day. Key findings have been made in many aspects of ovarian physiology including (but not limited to) folliculogenesis, oogenesis and steroidogenesis (230). Despite the many advances in our knowledge of such processes, there is still limited knowledge regarding the effect of VitD on the molecular mechanisms and cell signalling pathways involved in GC functioning. The presence of proteins and enzymes involved in VitD signalling have been demonstrated in several aspects of both the male and female reproductive tracts (58, 63, 81, 231-236). This indicates VitD signalling is intact within reproductive tissues and cells. However, the purpose of VitD in human oocytes and embryos remains elusive.

Previous studies examining the VitD-VDR axis in female reproductive cells have established a regulatory role in several aspects of sex steroid hormone production/secretion and apoptosis, which are critical biological processes modulated during folliculogenesis (29). Briefly, treatment of GCs with 1,25-(OH)2D3 can significantly increase AMH expression/production, E2, P4 and cAMP levels, as well as important steroidogenic enzymes such as StAR, aromatase, and 3β-HSD (62-64, 237, 238).

VitD deficiency has been well established in PCOS women, who have significantly lower levels of serum and FF 25-(OH)D (239). Additionally 25-(OH)D levels are significantly negatively correlated with insulin resistance, and positively correlated with insulin sensitivity (240). Although it is unclear if this relationship between VitD status and PCOS is casual or causative. VitD influences AMH production and signal transduction, which is a key characteristic in PCOS aetiology, alongside insulin resistance and increased androgens (61). In primary GCs treated with testosterone, aromatase expression and 17β-estradiol secretion were elevated, and the addition of 1,25-(OH)2D3 attenuated these effects and enhanced 17β-estradiol (241). Cultured primary human GCs obtained from women undergoing IVF and treated with 1,25-(OH)2D3 significantly increased aromatase activity and 3β-HSD expression (202). In PCOS women with enhanced ROS levels and 1,25-(OH)2D3 attenuated this elevation

to a level seen in non-PCOS women (202). A study on human GCs treated with 1,25-(OH)2D3-alone and in combination with insulin demonstrated a significant increase in E2, P4 and estrone levels, with the addition of insulin enhancing the effects of VitD (63). Interestingly, one report found the FF levels of 25-(OH)D were negatively correlated with AMH and AMHR mRNA expression levels in GCs obtained from small follicles (59). Furthermore, treatment with 1,25-(OH)2D3 decreased AMHR and FSHR mRNA expression, while simultaneously increasing 3β-HSD expression levels (59). Interestingly, in whole primate follicles, 1,25-(OH)2D3 supplementation promoted survival and growth of antral follicles, whilst increasing E2 and AMH production, resulting in improved oocyte maturation (242).Taken together, these findings suggest VitD may aid the terminal maturation of follicles by decreasing the sensitivity to AMH (via decreased AMHR expression), which in turn would prevent the inhibitory action of AMH on the transition from the primordial follicle pool to the primary follicle (59). The lesser AMH sensitivity allows follicles to reach terminal maturation and ovulation (243).

The Kit system, which is composed of KL and its tyrosine kinase receptor (cKit), has been shown to be essential in the process of folliculogenesis (244). Data from hen GCs found 1,25-(OH)2D3 increased the mRNA expression of KL (245). This may indicate 1,25-(OH)2D3 plays a crucial role in the Kit system, driving folliculogenesis. Evidence demonstrates that 1,25-(OH)2D3 also regulates steroidogenesis in GCs through AMP-activated protein kinase (AMPK) activation, a key signalling molecular upstream of steroidogenic, cholesterol biosynthesis and fatty acid synthesis pathways (209). A study conducted across two infertility units gave women undergoing IVF a single dose of 25-(OH)D (600 000 IU) or placebo, 2-12 weeks prior to OPU (246). Following this, GCs collected during OPU were pooled and the transcriptome was assessed. Ingenuity pathway analysis (IPA) was used to identify the top canonical pathways and upstream regulators mediating the action of VitD. Real time-PCR demonstrated upregulation of the VDR, glutathione S-transferase A3, which is involved in the biosynthesis of steroid hormones, and interleukin 21 receptor, which is involved in the proliferation and differentiation of immune cells such as T cells and B cells (246). These findings demonstrate roles of VitD in antioxidant defence and steroidogenesis.

Evidence from porcine derived primary GCs show 1,25-(OH)2D3 effects the biosynthesis of estrogens and P4 via modulation of steroidogenic enzymes (64, 247). Treatment of GCs with 1,25-(OH)2D3 (100 nmol/L) up regulated the expression of AMH, FSHR, and Foxl2, which plays an important role in follicle growth and ovarian differentiation (247, 248). Additionally, there was a significant increase in E2 biosynthesis related enzymes (CYP17A1, HSD17B1, and CYP19A1), alongside a 3-fold increase in E2 secretion compared to control (247). In another study on porcine GCs, P4 levels were increased following stimulation with 1,25-(OH)2D3 alone (100 nmol/L), in combination with insulin (5 µg/mL) or rFSH (1 µg/mL) (64). However, this study did not investigate any changes to steroidogenic enzyme expression to explain these findings. While there is substantial evidence that VitD modulates steroidogenic mechanisms in GCs, there is still a lack of information of what alternative pathways VitD may regulate such as cholesterol biosynthesis, fatty acid synthesis and cellular bioenergetics.

1.5.3 Human GC lines

Currently human GCs are easily obtainable during IVF programs where oocytes and FF are collected, containing luteinised GCs. Several methods have been developed to isolate and purify luteal GCs from IVF patients, such as antibody binding methods, flask method, cell strainer and positive selection of granulosa aggregates following density gradient centrifugation (249). However, the isolation of such cells is often limited due to low GC number yield, contamination with red and white blood cells, and potential cellular damage during the isolation or cryopreservation procedures (250). Additionally, freshly isolated human GCs are unable to be cultured *ex vivo* for prolonged time points unlike commercially available cell lines, which are modified to allow long-term culture (250). To overcome these difficulties several human GC lines have been established, such as those originating from ovarian tumours and oncogenic transformation (251). These cell lines include HTOG, COV434, KGN, HGL5, HO-23, GC1α and HGP53, HGrC1 and HSOGT (Table 1.4) (252-261).

There are numerous advantages and disadvantages, or limitations of these cell lines based on published data. Firstly, some do not produce key hormones related to GC

function including E2 (HOTG) and P4 (GC1α) (252, 257). Secondly, several of these GC lines are non-responsive to FSH (HGL5, GC1α) (255, 257), and LH (COV434, KGN, HGL5, HO-23, and GC1α) stimulation or have yet to be shown to be responsive to cAMP (HOTG, GC1α, HGrC1, and HSOGT) (254-256, 258-260). Thirdly, while several GC lines have been shown to exhibit aromatase activity (HOTG, COV434, KGN, HGL5 and HGrC1) (252, 254, 255, 259, 260), some have not yet been investigated (HO-23 and HGP53) or do not show aromatase activity (GC1α) (257). Finally, most of these human GC lines have been shown to express key steroidogenic enzyme expression such as StAR and 3β-HSD, the HOTG and HSOGT cell lines have not yet been assessed for these properties. Based on these reported findings, it appears several of these human GC lines may not accurately reflect GC functioning.

Thill et al showed VDR was present in both COV434 and HGL5 cell lines using real-time PCR and Western blot analysis to show mRNA and protein expression, respectively (262). HGL5 cells had significantly higher gene and protein expression compared to COV434 cells, although while freshly isolated human GCs had the highest mRNA expression, HGL5 cells had the highest protein expression levels of the VDR (262). Currently the VDR has yet to be reported in the literature for the HOTG, KGN, HO-23, GC1α, HGP53, HGrC1 and HSOGT human GC lines (Table 1.4).

While many of these cell lines have been used throughout the literature to assess GC signalling and function, there is a largely consistent issue- many researchers assess the steroid hormone output for E2 and P4 under the action of FSH-alone and neglect the role of androgens which would accurately reflect the *in vivo* granulosa-theca cell interaction. In circumstances whereby co-culture of granulosa and theca cells is not possible, the addition of androgens is essential to appropriately stimulate human GC lines.

Table 1.4: Summary of the steroidogenic characteristics of human GC lines. *Abbreviations: E2, estradiol; P4, progesterone; FSH, follicle stimulating hormone; LH, luteinising hormone; cAMP, cyclic adenosine monophosphate. NR= Not reported, (+)= Positive result has been established, (-)= Negative result has been established.*

Cell line	E2 synthesis	P4 synthesis	FSH responsive	LH responsive	cAMP responsive	A
HOTG (252)	-	+	NR	NR	NR	
COV434 (260)	+	+	+	-	+	
KGN (254)	+	+	+	-	+	
HGL5 (255)	+	+	-	-	+	
HO-23 (256)	NR	+	NR	-	+	
GC1α (257)	NR	-	-	-	NR	
HGP53 (258)	NR	+	+	NR	+	
HGrC1 (259)	+	+	+	NR	NR	
HSOGT (261)	+	NR	NR	NR	NR	

1.5.4 VitD and cell metabolism

A state of negative energy balance has been proposed as a cause or contributing factor of subfertility in dairy cows during lactation whereby increased demand for milk production requires a large energy output and ovarian function declines (263). A study investigated the impact of negative energy balance (as determined by blood concentrations of β-hydroxybutyrate) on the gene expression of GCs derived from dairy cows at 60 days postpartum (263). Genes associated with many key aspects of ovarian function were impaired during severe negative energy balance, including cellular organisation (KRT4 and PPL), cellular proliferation (TACSTD2), and fatty acids metabolism (VNN2) (263). Currently, very little is known about negative energy balance in the subfertility human population, or how VitD impacts cellular metabolism in reproductive cells. However, it is known that GCs are dependent on mitochondrial respiration and glycolysis for energy provision and certain aetiologies such as PCOS are associated with dysregulated cellular metabolism (264). In a murine model of PCOS, isolated GCs treated with 1,25-(OH)2D3 increased the levels of mitochondrial biogenesis through the mitogen-activated pathway kinase-extracellular signal-regulated kinases (MAPK-ERK1/2) pathway, while simultaneously reducing intracellular ROS levels (265).

Evidence from other (non-reproductive) cell types suggests VitD may play a role in regulation of cellular metabolism and bioenergetics. For example, Consiglio et al., demonstrated a novel VitD signalling pathway in which 1,25-(OH)2D3 modulates mitochondrial activity in human keratinocytes (266, 267). When differentiated keratinocytes were treated with 1,25-(OH)2D3 (100 nmol/L) transport chain (ETC) transcription was impaired and increased lipid deposition was also observed (267). Additionally, silencing of the VDR decreased de novo synthesis of cholesterol and enhanced respiratory chain activity via oxidation of metabolic intermediates, preventing their utilisation in biosynthetic pathways (266). A study conducted in circulating peripheral blood mononuclear cells (PBMCs) derived from humans, found that VitD status modulated the bioenergetic profile of these cells (67). PBMCs derived from individuals with a VitD status < 20 ng/mL had significantly higher basal respiration, non-mitochondrial respiration, ATP production, proton leak, glycolysis, and glycolytic reserve, as measured using extracellular flux analysis (which can

measure mitochondrial and glycolytic metabolism in real time) (67). In another report on PBMCs, seasonal improvements in 25-(OH)D status was associated with reduced systemic inflammation, PBMC bioenergetic profiles and whole body energy metabolism (66). PBMC mitochondrial and glycolytic parameters were significantly reduced in summer compared to winter, including basal respiration, non-mitochondrial respiration, ATP production, proton leak, maximal respiration, glycolysis, and glycolytic capacity (66). Further evidence from skeletal muscle cells demonstrated that 1,25-(OH)2D3 treatment significantly increased oxygen consumption rate (OCR) and ATP production, indicating enhanced mitochondrial bioenergetics (69). 1,25-(OH)2D3 has been shown in many studies to have potent immunomodulatory effects in cells of both the innate and adaptive immune systems (268). In particular, immune cells such macrophages and dendritic cells undergo a metabolic switch when treated with 1,25-(OH)2D3 (this has been reviewed extensively elsewhere) (269). Further studies are required to examine whether 1,25-(OH)2D3 exerts similar effects on cellular metabolism in reproductive cells such as human GCs.

1.6 Conclusion

While there are many studies that show a positive association between VitD and IVF outcomes such as fertilisation rate, CPR and LBR, very little is known about how VitD status in women undergoing IVF is related to embryological measure such as embryo development and quality. Furthermore, many clinical studies in this area only report crude values for these outcomes and do not implement more complex statistical methodologies to assess these associations in more detail. Whilst the research surrounding VitD and IVF is promising, there is still very little known at a molecular level of how VitD may be able to influence reproductive processes (such as cellular metabolism and signalling) that occur in key reproductive cells (such as the GCs) which support oocyte maturation and subsequently impact embryological development. Considering this, more research is required to investigate these areas and further elucidate the association between VitD and clinical IVF outcomes, and the *in vitro* effects of VitD on GC steroidogenesis and metabolism.

Chapter Two

Materials and Methods

2.0 Introduction

For this thesis, the materials and methods detailed here are separated in two distinct sections. Section 2.1 examines materials and methods relevant to the clinical work in Chapters 3 and 4, while section 2.2 covers the *in vitro* work throughout Chapters 5 and 6.

2.1 Clinical studies

2.1.1 Ethics approval

Human ethics for the present study was granted by the Curtin Human Research Ethics Committee (ethics approval number RD-26-10) and complies with the National Statement on Ethical Conduct in Human Research (2007). PIVET is accredited with self-regulatory National Australian Reproductive Technology Committee (RTAC) and the Reproductive Technology Council (RTC) of Western Australia, established under the Western Australian Human Reproductive Technology Act, 1991.

2.1.2 Clinical trial registration

The clinical trial detailed here throughout was registered through the Australian New Zealand Clinical Trial Registry (ANZCTR; Trial number ACTRN12617001221347). The trial was registered from the 21/08/2017 as an uncontrolled, cross-sectional observational clinical study. The trial was registered retrospectively after ethics was approved, although patients were prospectively recruited.

2.1.3 Research consents and confidentiality

Following consent for their IVF procedures and during the recruitment phase, patients received consent forms and were required to read and discuss the current research with a qualified member of scientific/clinical staff. Patients were given the opportunity to ask questions and were well informed of all the details of the current project. Patient data which was recorded was not identifiable by name, only patient ID as determined via the clinics system. Data was recorded within the PIVET Medical Centre database and only accessible via staff. Researchers were blind to the patient treatment at the time of obtaining consent.

2.1.4 Patient recruitment and characteristics assessment

Informed consent was obtained from 392 patients in total. The final data presented here was derived from 287 eligible patients, who underwent conventional ovarian stimulation and received a fresh embryo transfer (ET) between 1 April 2016 to 30 November 2018, and who had serum 25-(OH)D2 and D3 status (total Vitamin D 2/3, both forms referred to as VitD throughout this Chapter) measured on site. The flow chart describing the cycle selection process is described in Figure 2.1. For both the retrospective and prospective studies detailed throughout our exclusion criteria involved cycles with no fresh ET (such as cancelled cycles, failed fertilisation/oocyte retrieval, freeze all cycles, no live birth outcome, preimplantation genetic diagnosis), PCOS diagnosis based on the Rotterdam criteria (270), excessive follicle recruitment and inadequate serum sampling. Additionally, oocyte and embryo donor recipients were excluded from recruitment to minimise participant complexity in terms of the relevance of donor vs. recipient VitD status. To reduce potential patient and cycle selection bias, only the first chronological IVF cycle, where a VitD status was obtained for each individual patient following consent sign-off within the study time frame was examined. Therefore, patients could have been IVF naïve or non-naïve. No cycles were excluded based on age at cycle, BMI, stimulation protocol, ethnicity, or medical history, except for those with PCOS, which has been shown in numerous studies to be associated with VitD inadequacy (271).

2.1.5 Patient biometrics

Body weight measurements were obtained at the initial consult and updated every 3 months upon visitation to the clinic for all patients. Height (cm) was measured using a stadiometer fixed to a wall. In a subset of prospective patients, body composition was measured using a BC-545N segmental body composition monitor (Tanita, Australia), which utilises Bio-electrical Impedance Analysis (BIA) technology. Biometrics parameters obtained via BIA included: weight (kg), body mass index (BMI, kg/m^2), body fat percentage (%), muscle mass (kg), bone mass (kg), and basal metabolic rate (BMR, kJ/day).

2.1.6 Blood sample collection and analysis

For all patients, a non-fasting whole venous blood sample was collected via venepuncture between 7am-11am) on site at our integrated Clinipath clinic (West Leederville, Perth, Western Australia) by an experienced phlebotomist immediately following initial consult with a clinician. Blood parameters measured included: total 25-(OH)D, E2, P4, LH, FSH, AMH, prolactin, sex-hormone binding globulin (SHBG), testosterone, free testosterone, free androgen index (FAI), and dehydroepiandrosterone (DHEA).

Blood sample collection for VitD was performed on the day of OPU or in the case of haemolysis or lipemia, an additional sample from the day of fresh ET was collected. Blood separation was performed by centrifugation at 2700 rpm for 10 mins at room temperature. Serum was aliquoted in 1 mL aliquots, frozen and stored at -20°C (for up to 3 months) and batch tested. Total 25-(OH)D was determined by chemiluminescence assay using an ADVIA Centaur XP Immunoassay System (Siemens, Australia). The assay detection and the linearity limits were 4.0 and 150.3 ng/mL, while the intra- and inter-assay coefficients of variation were 4.2 and 11.9%, respectively. The ADVIA Centaur VitD assay has a similar limit of detection and cross reactivity as compared to the gold standard Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), and has one of the lowest total error percentages (following LC-MS/MS), compared to other commercially available automated VitD assays (270). VitD

inadequacy/insufficiency was grouped and defined as serum 25-(OH)D levels < 20 ng/mL and adequacy/sufficiency as ≥ 20 ng/mL, in accordance with the Institute of Medicine (IOM) and the Endocrine Society clinical practice guidelines (23, 271).

2.1.7 Ovarian stimulation

Our clinic utilises validated in-house developed algorithms, based on age and antral follicle count (AFC), for individualised recombinant follicle stimulating hormone (rFSH) dosing in IVF cycles (272). These algorithms allow for adjustment for various parameters (including AMH level, AFC, BMI, day-2 FSH level, and smoking history), as well as producing 10 ± 2 mature oocytes and reducing the risk of developing ovarian hyperstimulation syndrome (272). Ovarian reserve was measured by transvaginal ultrasound to determine AFC, which is defined as the number of follicles (between 2-10 mm in diameter) in both ovaries, which were present on day 5 of the preliminary assessment prior to recombinant FSH (rFSH) stimulation.

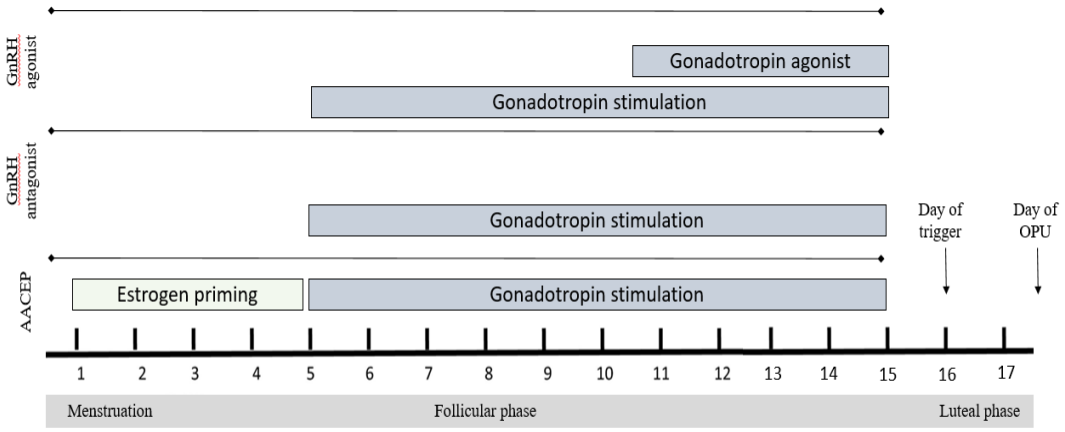
Selection of the appropriate ovarian stimulation protocol was at the discretion of the clinician and independent of the researchers, but protocol type (antagonist vs. agonist/other cycles) was examined as a confounder during data analysis. Women with high AFC ratings (all A categories and some B if AFC > 15 antral follicles) were treated preferentially by an antagonist regimen. Those with low categories (D and E) were treated by a flare regimen with some very poor responders treated by an agonist antagonist conversion with estrogen priming (AACEP) regimen. Women with AFC category B <15 antral follicles and category C could be treated by either regimen or even a long downregulation protocol, which was also preferred for patients who have underlying adenomyosis and endometriosis. AFC grading and ovarian stimulation protocol is summarised below in Table 2.1.

Table 2.1: Ovarian stimulation protocols based on AFC grading.

AFC Grading	Ovarian Stimulation Protocol
A	Antagonist regime
B	AFC $\geq$ 15 follicles- Antagonist regime AFC < 15 follicles- Antagonist or agonist regime
C	Antagonist, agonist, or long downregulation regime
D E	Flare or agonist antagonist conversion with estrogen priming (AACEP) regime

2.1.8 Ovulation induction and luteal support

Detailed descriptions regarding the procedures in place at our clinic have been published previously (273). This includes ovarian stimulation and induction protocols, luteal support, embryo culture and transfer procedures, CP testing and LB follow up. Oocyte maturation was initiated using a single 13,000 IU dose of Ovidrel rhCG (Merck, Australia) for patients with at least 2 leading follicles (≥ 18 mm in diameter), as visualised by transvaginal ultrasound. For patients with ≤ 4 follicles, Ovidrel (19,500 IU) was used. OPU was performed 35-37 hours post-trigger via transvaginal oocyte aspiration (TVOA). Luteal support for fresh IVF-ET cycles was based on the number of oocytes recovered at OPU as previously described (272). More specifically, luteal support included administration of rhCG 500–1,000 IU on days 4, 7, 10, and 13 following TVOA for cases where < 12 follicles and < 12 oocytes were developed/obtained, all other cases received P4 400 mg thrice daily.



Trigger types= hCG trigger (Synarel or Decapeptyl) or GnRH agonist (Ovidrel or Pregnyl (200-750 µg)

Gonadotropin types= Gonal-F, Elonva or Puregon (62.5-450 IU)

Luteal support= Clomid, Progestins, Progesterone, hCG or GnRH agonists

Figure 2.1: Ovarian stimulation clinical protocols.

2.1.9 Collection of primary oocytes and follicular fluid

On the day of oocyte retrieval, FF containing oocytes were aspirated from ovarian follicles using a single lumen needle (Cook Medical, Australia). Prior to flushing of ovarian follicles, a sample of follicular fluid is obtained, processed, and stored as previously described for serum 25-(OH)D. Flushing was performed using Flushing medium (Origio, Australia), to ensure remove of all oocytes within the aspirated follicles). Two embryologists independently assess the FF and flushing medium, distinguishing between oocytes and granulosa cells. Oocytes were removed and placed in Fertilisation culture medium (Catalogue # 83020060, Origio, Australia).

2.1.10 Embryological procedures (insemination, grading, culture, and transfer)

Retrieved oocytes were cultured for 4–5 hours post-collection prior to insemination with spermatozoa (100,000/mL) for IVF or denuded with hyaluronidase and mature oocytes were injected using ICSI. The majority of patients within our clinic receive ICSI only or an ICSI/IVF split (the rationale behind opting for primary utilisation ICSI cycles has been previously published and discussed elsewhere (274)). Following fertilisation all two pronuclei (2PN) zygotes were placed into specialised cleavage-stage medium (Quinn's Advantage Sequential medium) and cultured to Day-3 in benchtop microprocessor controlled, gassed, and humidified MINC^{TM} incubators (Cook, Australia). Experienced embryologists examined these under an inverted ICSI microscope (IX71 Model, Olympus, USA) under the highest magnification (60x magnification) and graded day-3 cleavage stage embryos on a 1–4-point scale (Table 2.2). If there were fewer than 3 high-grade embryos progressing at the 6–8 cell stage at this point, ET was considered.

Table 2.2: Day-3 cleaved embryos grading system.

Embryo grade	Number of cells	Fragmentation (%)	Compaction (%)
1.0	≥ 4	≥ 60	0
1.5	> 4	30-60	0
2.0	≥ 5	20-30	< 10
2.5	> 5	10-20	10-30
3.0	≥ 6	< 10	≥ 10
3.5	7-10	0	≥ 30
4.0	Morula	0	100

In most cases, there were a minimum of 3 high-grade cleavage embryos, and these were transferred to blastocyst culture medium (Quinn's Advantage Sequential medium) and maintained (as described above) and cultured through to Day-5 or Day-6 (whilst assessed daily) when ET or cryopreservation via vitrification (using the Cryotop method) was considered. Embryos not at the blastocyst stage by Day-5, were left one more day to Day-6 and re-assessed. Day 5/6 embryos were examined (as described above for cleavage embryos) and graded using the Gardeners blastocyst grading system for day 5/6 embryos (Table 2.3). The highest graded embryo(s) are selected for fresh ET and remaining embryos are cryopreserved (if deemed suitable quality), these include fully developed blastocysts (score 3-6) with a 'good' number of cells in the inner cell mass and trophectoderm (both grade A & B).

Table 2.3. Blastocyst grading system based on Gardener's grading system.

Grade	Specific descriptions of each grading
Expansion	**Developmental stage (expansion and hatching)**
1	Blastocoel cavity is less than 25% of the total inner embryo volume
2	Blastocoel cavity is more than 25% of the total inner embryo volume
2/3	Blastocoel cavity is more than 50% of the total embryo volume and an inner cell mass is forming
3	Blastocyst, cavity is completely filling the inner embryo
4	Blastocyst expanded, the cavity is larger than the embryo and the shell is thinning
5	Hatching out of the shell has begun
6	Fully hatched out of the shell
Inner cell mass	**Inner cell mass quality**
A	There are many tightly packed cells
B	Several cells and loosely grouped together
C	Very few cells, not grouped together
Trophectoderm	**Trophectoderm quality**
A	There are many cells, forming a cohesive trophectoderm layer
B	There are few cells, forming a loose epithelial layer
C	Very few large cells

Single embryo transfer (SET) is used in most cases; although exceptions are made upon written request for patients who have had ≥3 unsuccessful IVF attempts, at the discretion of the clinician. Embryo transfers were conducted in the lithotomy position with moderate Trendelenburg (head down) tilt under ultrasound guidance with non-empty bladder. Depending upon uterine position (anteverted, axial, or retroverted) the bladder can be partially fill to the degree enabling a satisfactory transvesical ultrasonic view of the endometrial cavity, ideally with minimal cervico-uterine angulation. The single blastocyst was transferred using either the K-JETS catheter system (K-Jets-7019-SIVF; Cook Australia) or the Wallace Classic Catheter (Gytech Pty Ltd, Australia for Smiths Medical, UK) and a clear mid-fundal flash was identified on ultrasound signifying an appropriately conducted embryo transfer procedure.

2.1.11 Clinical outcomes measured

Several clinical outcomes related to embryological measures and CP and LB were assessed in the present study. For embryological measures: fertilisation rate, oocyte utilisation rate (OUR), proportion of high-quality blastocysts, blastocyst development rates, embryo utilisation rate (EUR) were all outcomes of interest (definitions outlined below in Table 2.4). For crude CPR and LBR, fresh and cumulative CPR and LBR, as well as miscarriage rates were the main clinical outcomes of interest (definitions outlined below in Table 2.4).

Table 2.4: Definitions of embryological measures and clinical outcomes assessed.

Clinical Outcome	Definition
Fertilisation rate per insemination (%)	(Total 2PNs/# of total oocytes inseminated) x 100
Oocyte utilisation rate (%)	(# of oocytes transferred, or cryopreserved/total # of oocytes collected) x 100
Proportion of high-quality blastocysts generated per cycle (%)	(# of high-quality blastocyst/total blastocysts (high, medium, and low quality)) x 100
Blastocyst rate per oocytes collected (%)	(# of total blastocysts generated/# of MII oocytes) x 100
Blastocyst rate per MII oocytes collected (%)	(# of total blastocysts generated/# of MII oocytes) x 100
Blastocyst development rate (%)	(# of cycles producing a minimum of 1 blastocyst/total number of cycles) x 100
Embryo utilisation rate (%)	(# of oocytes transferred or cryopreserved/total # of 2PNs generated) x 100
Fresh CPR (%)	(# of fresh cycles with a CP/total # of cycles) x 100
Fresh LBR (%)	(# of fresh cycles with a LB/total # of cycles) x 100
Fresh miscarriage rate (%)	(# of fresh cycles with a miscarriage/total # of cycles with a CP) x 100
Cumulative CPR, per ET (%)	(# of fresh & frozen cycles with CP/s/total # of cycles) x 100
Cumulative LBR, per ET (%)	(# of fresh & frozen cycles with LB/total # of cycles) x 100

 # = Number.

Pregnancy blood tests measuring βhCG (both free βhCG and hCG), E2, and P4 were performed at approximately four weeks gestation or 19 days post-trigger for IVF-ET as previously described (273). Weekly blood tests for the same hormone parameters were performed until 8 weeks' gestation to monitor the pregnancy progression and provide hormonal support if necessary (275). CP was confirmed at week 8 post-trigger via ultrasound to confirm the presence of a gestational sac and foetal heartbeat. Transvaginal scan (TVS) was also performed at the first trimester screenings at weeks 11-13 to monitor progress.

From the second trimester and onwards, antenatal care was managed by external obstetricians. Pregnancy loss were recorded at the 7-week TVS, and includes miscarriage, ectopic pregnancy, and terminations (< 20 week's gestation, these were excluded from analysis). After the expected delivery date, live birth outcomes were retrospectively obtained via telephone follow up with the relevant hospital by a clinical nurse within our private IVF centre.

2.1.12 Statistical analysis

All data was subjected to a Kolmogorov-Smirnov analysis to test for normality and determine the presence or absence of normal distribution. Normally distributed continuous data was represented as mean ± standard deviation (SD) and analysed for statistical significance using independent sample t-test (two groups). Categorical data was represented as number of patients [percentage] and analysed via chi-squared contingency tables. Non-normally distributed data was expressed as median (interquartile range, IQR) and analysed non-parametrically by applying Mann-Whitney U tests. Clinical definitions of VitD inadequacy (< 20 ng/mL) and adequacy ($\geq$ 20 ng/mL) are generally based on bone health as per the Institute of Medicine (IOM) and the Endocrine Society clinical practice guidelines (23, 271).

Binary logistic regression models were used to assess associations between predictors of IVF success and clinical outcomes. Two main approaches were utilised: (1) Univariate models- clinical outcome of interest was set as the dependant variable, while patient demographics & characteristics, cycle characteristics and embryological measures were set as the independent variables (Table 2.5). From the univariate

analysis, individual predictors of IVF success were determined and utilised in approach 2. (2) Multivariate models- clinical outcome of interest was set as the dependant variable, while significant predictors determined in the initial univariate model were applied in a single adjusted logistic regression model (Table 2.5). Data pertaining to logistic regression models was expressed as odds ratio (OR), 95% confidence interval (95% CI) and p-value. All statistical calculations were performed using SPSS statistic version 25 (IBM Corporation, USA). For all analysis statistical significance was defined as $p<0.05$.

Table 2.5: Logistic regression model dependant and independent variables of interest.

Dependant variables	
Vitamin D sufficiency	Clinical pregnancy
Blastocyst development	Live birth
Independent variables	
Patient demographics:	
Female age at cycle	
Body composition (BMI, muscle mass, fat mass, BMR)	
Ethnicity (Caucasian vs. Non-Caucasian)	
VitD grouping (< 20 ng/mL vs. ≥ 20 ng/mL)	
AMH level	
Uterine receptivity (progesterone at trigger and endometrial thickness)	
Infertility aetiology (tubal, endometriosis or unexplained)	
Cycle characteristics:	
Stimulation protocol (antagonist vs. agonist/other)	
AFC grouping (A vs. B/C vs. D/E)	
Season of ET/VitD testing (Winter/Spring vs. Summer/Autumn)	
ART attempt number/Previous IVF (0 events vs. 1 event vs. 2 events vs. ≥ 3 events)	
Insemination type (IVF only vs. ICSI only vs. IVF/ICSI split)	
rFSH/trigger dose and trigger drug	
Embryological measures:	
Number of total & MII oocytes collected	
Number of fertilised 2PNs generated per cycle	
Fertilisation rate (%) per insemination	
Number of day 3 embryos in culture	
Number and percentage of blastocysts generated per cycle	
Proportion of high-quality blastocysts generated per cycle	

2.2 *In vitro* cell work

2.2.1 Cell culture and reagents

COV434 and KGN human GC lines were used for the studies detailed in this section. The culture media used for all experiments was Dulbecco's Modified Eagles Medium (DMEM)/Nutrient Mixture F12 Ham with L-glutamine, 15 mM HEPES and sodium bicarbonate (Sigma, Australia) and supplemented with 10% foetal bovine serum (FBS), 100 u/mL penicillin and 0.1 mg/mL streptomycin (Life Technologies, USA). Cells were maintained in T75 flasks in a humidified incubator with 5% CO_2 at 37°C prior to subculturing. For harvesting cells, 5 mL trypsin was added to cell flasks and incubated for 5 mins in a 5% CO_2 at 37°C. Additionally, for KGN cells, a sterile cell scraper was used to aid the collection of cells following trypsination. To stop trypsinisation, 5 mL of DMEM/F12 (+ 10% FBS) media was added to the cell culture flask and then the cell suspension was centrifuged for 3 mins at 300x(g) to collect the cell pellet.

1,25-OH2D3 was obtained from Astral Scientific, Australia (Item ID: C-1026-100ug) and solubilised in 100% ethanol to create a 100 mM stock. Aliquots were covered with foil to reduce exposure to artificial light sources. Androstenedione (4-Androstene-3,17-dione) was obtained from Merck, Australia and solubilised in 100% dimethyl sulfoxide (DMSO) to create a 100 mM stock. Human rFSH Gonal-f® follitropin alfa 900 IU/1.5mL (Merck, Australia) & Rekovelle follitropin delta 36 micrograms/1.08 mL (Ferring Pharmaceuticals Ltd) pre-filled injection pens were obtained from supernumerary patient pens from PIVET Medical Centre (Australia). All reagent stocks were stored in 5 µl aliquots to avoid multiple freeze-thaw cycles, at -20°C prior to experimental use or -80°C for long term storage (6-12 months depending on drug stability).

2.2.2 Cell culture treatment strategy & BCA protein normalisation

Several main treatment strategies were implemented (summarised in Table 2.6). Briefly, treatment strategies included: (1) Hormonal stimulation for human granulosa cell line characterisation. (2) 1,25-(OH)2D3 treatment to investigate the impact of 1,25-(OH)2D3 on human granulosa cell lines in unstimulated conditions (no addition of hormonal stimulation). (3) Combination of hormonal stimulation and 1,25-(OH)2D3 to investigate any additional effect or interaction between hormonal stimulation and 1,25-(OH)2D3 treatment. Firstly, for all experiments cells were seeded on day 1 (Table 2.6) in DMEM/F12 media and left overnight to adhere and maintained in a 5% CO2 at 37°C. On day 2 the appropriate cell treatment strategy was performed according to Table 2.6 and maintained as above, for either 24 or 48 hours (depending on experimental design).

Protein concentration as a means of normalisation for several experimental procedures was determined using the Pierce™ BCA Protein Assay Kit (Thermofisher, USA). Briefly, an appropriate volume of Radioimmunoprecipitation assay (RIPA) buffer (Astral Scientific, Australia) containing phosphatase and protease inhibitors (Cell Signalling Technology, USA) was added to directly to the cells in a transparent cell culture microplate depending on the cell density (Table 2.5). Following cell lysis, 25 µL/sample was transferred to a sterile transparent 96 well cell culture microplate kept on ice. For each well, 200 µL of BCA working reagent was added and mixed with the lysed cell solution. The cell culture plate was covered in foil and incubated in a 37 °C incubator for 30 mins in the dark. Following incubation, the cell plate was cooled to room temperature and the absorbance was measured at 562 nm using an EnSpire multimode plate reader (Perkin Elmer).

Table 2.6: Cell culture seeding densities and treatment strategies.

Cell culture plate	Experimental Procedure	Cell seeding density (cells/well)			RIPA volume (µL)
		Cell line	24-hour treatment	48-hour treatment	
96 well plate	MTT cell viability, Seahorse XF, Oil Red O,	COV434	20,000	10,000	25
		KGN	20,000	20,000	
24 well plate	Glucose uptake, Cell cycle analysis, ROS	COV434	150,000	75,000	50
		KGN	150,000	150,000	
6 well plate	Western Blot, Immunofluorescence, Mitochondrial density	COV434	500,000	250,000	100
		KGN	500,000	500,000	
Treatment strategy					
Day 1: Cells seeded as above, according to the experimental design and left overnight to adhere.					
Day 2: Media is removed and replaced with fresh DMEM/F12 media containing treatments (as detailed below) depending on the experimental procedures.					
Hormonal stimulation					
Media & Solvent Controls					
rFSH (125 ng/mL)					
Androstenedione (5 µM)					
Combo rFSH & Androstenedione (125 ng/mL & 5 µM, respectively)					
1,25-(OH)2D3*					
Media & Solvent Controls					
1,25-(OH)2D3 (10, 20, 30, 40 and 80 ng/mL)					
Combination of hormonal stimulation & 1,25-(OH)2D3					
Media & Solvent Controls					
rFSH (125 ng/mL)					
Androstenedione (5 µM)					

1,25-(OH)2D3 (40 ng/mL)

Combo rFSH & Androstenedione (125 ng/mL & 5 µM, respectively)

Combo rFSH, Androstenedione & 1,25-(OH)2D3 (125 ng/mL, 5 µM, 40 ng/mL, respectively)

**1,25-(OH)2D3 conversion: 10, 20, 30, 40, 50 ng/mL=25, 50, 75, 100, 200 nmol/L (respectively).*

2.2.3 MTT cell viability

The MTT assay assesses cell dehydrogenase (primarily mitochondrial) metabolic activity therefore is utilised as an indirect measure of cell viability and proliferation. To determine the effect of our various treatments on cell viability and proliferation, MTT assays were performed. Cells were seeded and treated as per Table 2.5. On day-3 (for a 24-hour treatment) and day-4 (for a 48-hour treatment), 4-hours before the respective time point is reached the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (drug stock= 5 mg/mL) was added to the culture media at a 1:10 dilution. After a 4-hour incubation at 37°C, cell supernatant was carefully aspirated as to not disrupt the formazan crystals formed. Formazan crystals were then solubilized with 100 µL of DMSO (Merck, Australia). Optical density (absorbance) was measured using the EnSight Multimode Plate Reader (Perkin Elmer, USA) at a wavelength of 550 nm.

2.2.4 Metabolic flux analysis

Metabolic flux analysis assesses metabolic activity of various biochemical pathways, such as mitochondrial oxidative activity and capacity and glycolytic activity and capacity. The Seahorse XFe96 Extracellular Flux analyser (Seahorse Biosciences, USA) was used to determine the effect of our various treatments on cellular bioenergetics. Specifically, oxygen consumption rate (OCR; which is an indicator of mitochondrial respiration) and proton efflux rate (PER; which is an indirect measure of glycolysis which correlates with lactate production) were assessed.

Seahorse 'base' media was prepared by dissolving one bottle of DMEM powder (Sigma, Australia) in 950 mL of double distilled water (ddH_2O) and supplemented with 3 mL of phenol red, 10 mL of sodium pyruvate, 10 mL of L-glutamine (pH= 7.35 at 37 °C), then adjusted to a final volume of 1 L. This 'base' media was then sterile filtered using a Stericup vacuum filtration system (Merck Millipore, USA) and stored at 4 °C until the day of assay. Seahorse 'assay' media was prepared by adding 1 mL of 45% glucose to 1 L of 'base' media. The buffering capacity of the base or assay media was measured by aliquoting 10 mL of media, and measuring the initial pH at 37

°C. Following the initial pH reading, 20 µL of freshly prepared 1 M sodium hydroxide (NaOH) and recording the final pH. The following equation was used to calculate the buffering capacity of the seahorse media: $\frac{(\text{Conc of NaOH in M}) \times (20\times10^{-6}\ \text{L})}{(\text{pH Change}) \times (10^{-3}\ \text{L})}$.

Individual stocks of the mitochondrial modulators- oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), Antimycin A and rotenone, were prepared in DMSO at 20 mM concentrations (All Sigma, Australia). Aliquots of 100 µL were stored at -20 °C and thawed as required, where they were Aliquots were further diluted with 300 µL DMSO to make a 5 mM working reagent which was aliquoted into 20 µL aliquots (1 aliquot per assay) and stored at -20 °C. The optimised injection strategy consisted of seahorse 'base' media (Injection A), 2 µM oligomycin (Injection B), 0.75 µM FCCP (Injection C), and 1 µM of each Antimycin A and Rotenone in combination (Injection D). Mito Stress reagents used are summarised below in Table 2.7.

Table 2.7: Seahorse reagents for Mito Stress Test (Seahorse Biosciences, USA).

Seahorse drug	Molecular weight (g/mol)	Stock solution (mM)	Working solution (mM)	Final concentration (µM)
Oligomycin	791.06	20	5	2
FCCP*	254.17	20	5	0.75
Antimycin A	548.63	20	5	1
Rotenone	394.42	20	5	1

*Drug is light sensitive and should be prepared in the dark and covered in foil for storage and on the day of assay when diluted.

OCR was assessed using the Agilent Seahorse XF Cell Mito Stress Test Kit (Seahorse Bioscience, USA). The principle of the assay is an initial measurement of basal respiration is obtained based on OCR, followed by the injection of a series of mitochondrial modulators and measuring the real time cellular responses to these modulators (Figure 2.2). Firstly, the ATP synthase inhibitor oligomycin is injected, followed by FCCP, which is a mitochondrial uncoupling agent. Lastly, a combination of Antimycin A and Rotenone is injected, these are inhibitors of mitochondrial complexes I and III (respectively). From these, several parameters related to cellular mitochondrial bioenergetics can be calculated as visually depicted in Figure 2.2, and described in Table 2.8.

Table 2.8: Parameters and their equations related to Mito Stress Test (Seahorse Biosciences, USA).

Parameter	Equation
Basal respiration	(Last rate measurement before first injection) - (non-mito respiration rate)
Maximal respiration	(Maximum rate measurement after FCCP injection) – (non-mito respiration rate)
Proton leak	(Minimum rate measurement after oligomycin injection) – (non-mito respiration rate)
ATP production	(Last rate measurement before oligomycin injection) - (minimum rate measurement after oligomycin injection)
Spare respiratory capacity (%)	(Maximal respiration) / (basal respiration) x 100
Coupling efficiency (%)	(ATP production rate) / (basal respiration) x 100
Non-mitochondrial OCR (non-mito OCR)	Minimum rate measurement after injection of rotenone & Antimycin A

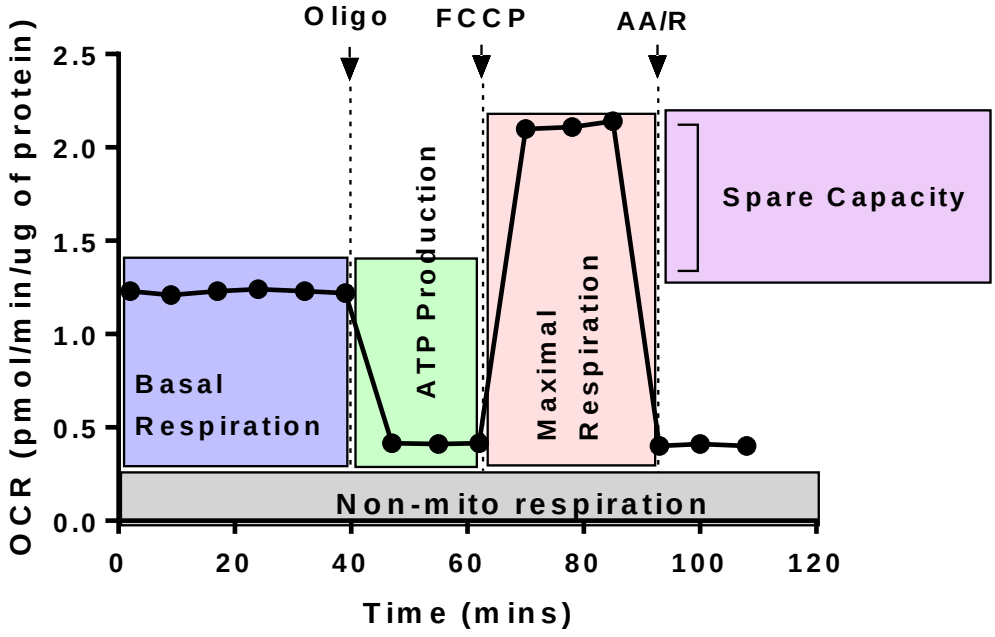


Figure 2.2: Seahorse graph demonstrating an example of a trace obtained from Mito Stress Test with mitochondrial modulators and parameters labelled.

To determine PER, cells were seeded and treated as per Table 2.6, in an XFe96 cell culture plate. PER was assessed using the Agilent Seahorse XF Glycolysis Rate Assay Kit (Seahorse Bioscience, USA). The principle of the assay is an initial measurement of basal PER, followed by an injection of Antimycin A and Rotenone to inhibit any resulting mitochondrial-produced acidification (Figure 2.3). Secondly, an excess of 2-deoxyglucose (2-DG) is injected which acts as a competitive inhibitor of glucose-6-phosphate production from glucose at the phosphoglucoisomerase level (step 2 of the glycolytic pathway, Figure 2.3). Individual stocks of Antimycin A and rotenone were prepared as previously described above. Additionally, a 1 M working reagent of 2-DG (Sigma, Australia) was prepared in 'base' media (Molecular weight =164.16 g/mol). Aliquots of 250 µL were stored at -20 °C, and 1x 250 µL aliquot was used per assay. The injection strategy consisted of 1 µM of each drug Antimycin A and Rotenone in combination (Injection A), followed by 200 mM 2-DG (Injection B, Figure 2.3). From these, several parameters related to cellular glycolytic bioenergetics can be calculated as visually depicted in Figure 2.3, and described in Table 2.9.

Table 2.9: Parameters and their equations related to Glycolytic Rate Test (Seahorse Biosciences, USA).

Parameter	Equation for calculation
Basal glycolysis	Last glycoPER measurement before first injection
Basal PER	Last PER measurement before first injection
% PER from glycolysis	(Basal glycolysis)/(Basal PER) x 100%
Compensatory glycolysis	Maximum glycoPER measurement after Rotenone/antimycin A injection.
Post 2-DG acidification	Minimum glycoPER measurement after 2-DG injection

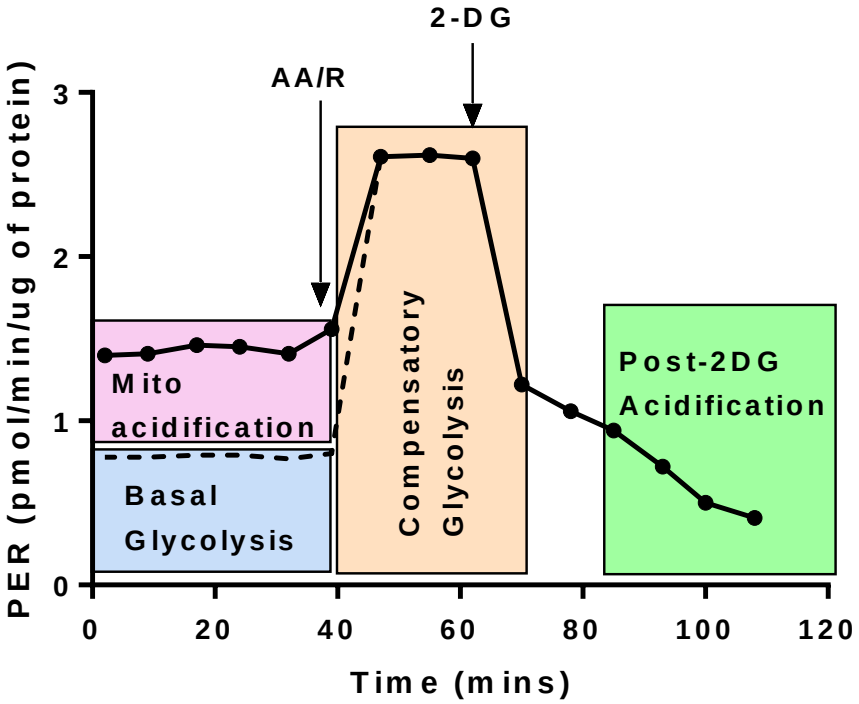


Figure 2.3: Seahorse graph demonstrating an example of a trace obtained from Glycolytic Rate Test with glycolytic modulators and parameters labelled.

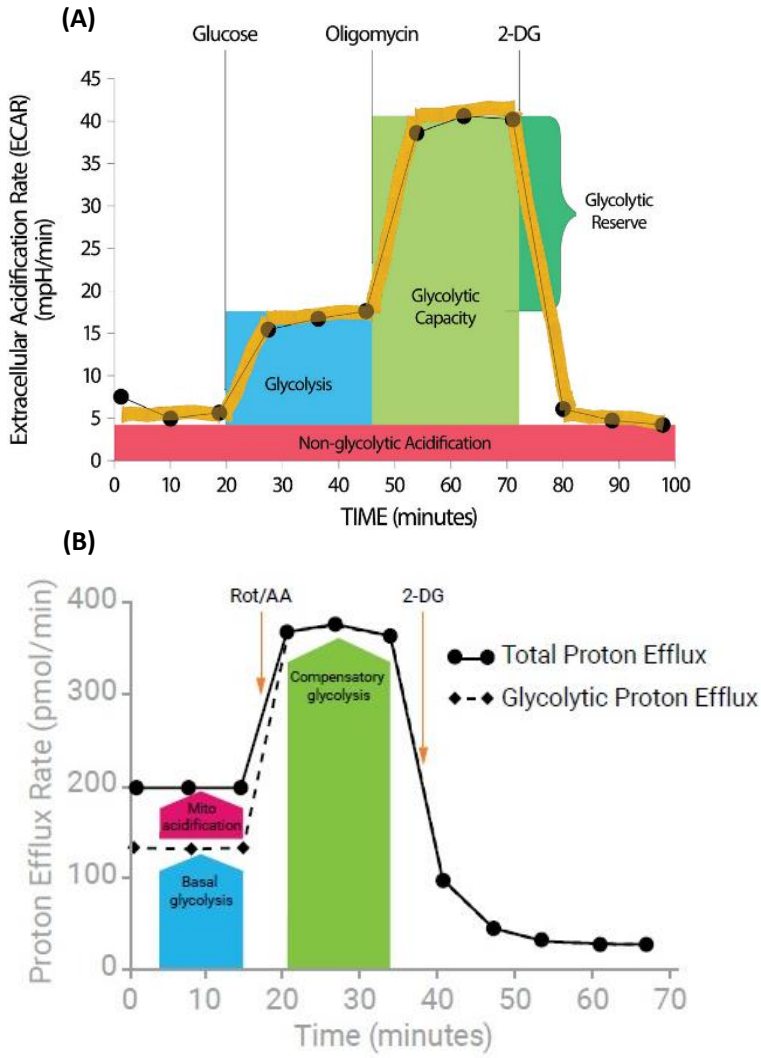


Figure 2.4: Representative Agilent seahorse traces for injection strategies and measurements of glycolytic assays. (A) Glycolytic stress test. (B) Glycolytic rate assay. Images adapted from https://www.agilent.com. *Abbreviations- 2-DG, deoxyglucose; Rot, rotenone; AA, antimycin A.*

2.2.5 Mitochondrial density

To assess if any alterations in mitochondrial bioenergetics was due to increasing density of mitochondria, mitochondrial density was assessed using quantitative PCR (qPCR). Cells were seeded and treated as per Table 2.6. On day 3 cell supernatant was removed and cells were washed once with PBS and then 300 µL/well of cell lysis buffer (10 mM Tris pH 8.0, 400 mM NaCl, 2 mM EDTA, 1% SDS (w/v), proteinase K 0.5% (v/v)) was added. The plate was incubated on a plate shaker for 2 minutes to ensure complete homogenisation of cellular material. The cellular material was then transferred to micro centrifuge tubes and incubated on a heat block for 1-hour at 37 °C. Samples were placed on ice to cool, then 100 µL of 6 M sodium chloride (NaCl) was added per sample and vortexed for 30 secs. Samples were then centrifuged at 14,000x(g) for 2 mins at 4 °C. Supernatant containing mitochondrial and nuclear DNA (mtDNA and nDNA, respectively) was removed to a new tube and this process was repeated until no visible salt pellet was formed (a minimum of three times).

To precipitate DNA following protein precipitation, 350 µL of ice-cold isopropanol was added to each sample and inverted several times. Samples were then centrifuged at 14,000x(g) for 2 mins and the supernatant, containing insoluble cell components, was discarded. Next, 1 mL of ice cold 70% ethanol was added to each sample and vortexed for 30 secs to wash DNA. The samples were centrifuged and washed as previously described. The supernatant, containing ethanol and remanent of insoluble cell components, was carefully aspirated by vacuum then the pellet containing mtDNA and nDNA was left to dry by leaving the micro centrifuge tube open for 10 mins at room temperature. The pellet was then resuspended in 100 µL of ddH_2O and incubated for 1-hour at 65 °C to rehydrate. DNA was then quantified using a nanodrop (Thermofisher, USA). DNA was diluted to 10 ng/µL for each sample and 1 µL of DNA was added to 3.5 µL of SYBR green (Thermofisher, USA), 0.25 µL of each primer (forward and reverse) and 2 µL of ddH_2O, giving a final reaction mix volume of 7 µL per sample for a qPCR 384 well plate format. Data analysis was using the 2-ΔΔCT method. Details of primer sequences are described below in Table 2.10.

Table 2.10: Primer sequences for mitochondrial and nuclear DNA.

Gene name	Primer sequence (5'-3')	Temp (°C)	Size (base pairs)
nDNA Forward	CGAGGGATACCTGTGAGCAGCTT	65	152
nDNA Reverse	GTCACTTCTTGTGCTGCCATCGT	65	152
mtDNA Forward	GCTCCTGATATAGCATTCCCACGA	61	151
mtDNA Reverse	CATGAGCAATTCCAGCGGATAAA	61	151

2.2.6 Estradiol production

To determine the functional secretion of E2, levels of E2 were detected in cell culture supernatant using a human 17 β-estradiol competitive ELISA kit, catalogue number ab108667, with the manufactures reporting 0.02% cross-reactivity with testosterone (0.02%) (Abcam, USA), although no cross-reactivity with androstenedione has been demonstrated. Cells were seeded and treated as per Table 2.5. On day 3, cells supernatant was removed, centrifuged at 300x(g) and kept on ice. Assaying was performed based on manufacturer's instructions. Standards included in the kit were: 0, 20, 120, 300, 600, and 2000 pg/mL. Briefly, 25 µL of standard, control and undiluted sample were pipetted into their respective pre-coated wells. Then 200 µL of 17 β-estradiol-HRP linked conjugate was added to each well (minus the blank wells). Wells were covered in foil and left to incubate with slow agitation on a plate shaker for 2 hours at 37°C. Following incubation, well contents were aspirated, and wells were washed 3 times with 300 µL/well of washing solution. Next, 100 µL TMB substrate solution was added into all wells and incubated for 30 mins in the dark at RT. Finally, 100 µL/well stop solution was added into all wells, and the absorbance was read at 450 nm using an EnSpire multimode plate reader (Perkin Elmer, USA). Data was normalised to protein concentration (as detailed previously in section 2.2.2).

2.2.7 Progesterone production

P4 production was detected in cell culture supernatant using a human P4 competitive ELISA kit, catalogue number KA0299, with the manufactures reporting 0.28% cross-reactivity with androstenedione (Abnova, USA). Cells were seeded and treated as per Table 2.5. On day 3, cells supernatant was removed, centrifuged at 300x(g) and kept on ice. Assaying was performed based on manufacturer's instructions. To prepare the standard curve, a solution of ethanolic 100,000 pg/mL was serial diluted using cell culture media (Table 2.11).

Table 2.11: Standard curve preparation for progesterone ELISA kit.

Standard	Cell culture media (µL)	Volume added (µL)	Progesterone concentration (pg/mL)
1	1990	10 (P4 stock)	500
2	500	500 (Standard 1)	250
3	500	500 (Standard 2)	125
4	500	500 (Standard 3)	62.5
5	500	500 (Standard 4)	31.25
6	500	500 (Standard 5)	15.62

Briefly, 100 µL of standard diluent (cell culture media) was pipetted into the no substrate blank (NSB) wells, while 100 µL of sample was added to the appropriate wells. Next, 50 µL of Assay Buffer was added into the NSB wells, while sample wells received 50 µL of blue conjugate and 50 µL of yellow conjugate/well. Wells were covered in foil and left to incubate with slow agitation on a plate shaker for 2 hours at RT. Following incubation, well contents were aspirated, and wells were washed 3 times with 400 µL/well of washing solution. After the final wash was aspirated, 5 µL of blue conjugate was added to the total activity wells. Next, 200 µL pNpp substrate solution was added into all wells and incubated for 45 mins in the dark at RT with no agitation. Finally, 50 µL stop solution was added into all wells, and the absorbance was read at 405 nm using an EnSpire multimode plate reader (Perkin Elmer, USA). Data was normalised to protein concentration (as detailed previously in section 2.2.2).

2.2.8 Lipid deposition

To investigate lipid deposition, Oil Red O staining of intracellular lipids was performed. Cells were seeded and treated as per Table 2.5. A working solution of Oil Red O stain was prepared by adding 30 mL of 0.5% Oil Red O in 100% isopropanol (Sigma, Australia) with 20 mL ddH_2O. After exposure to the various treatments, the cells were washed once with PBS and fixed with 4% paraformaldehyde for 30 minutes. Fixed cells were then washed three times with PBS and stained with the Oil Red O working solution for 30 minutes. Cells were then washed with PBS once, observed for the stained intracellular lipid droplets, and photographed using bright field inverted Nikon microscope. Quantification of the stained area was performed by eluting the stain off the plate by incubation with 50 µL of 100% isopropanol/well for 1 hour. Absorbance was measured at 510 nm using an EnSpire multimode plate reader (Perkin Elmer).

2.2.9 Western Blot analysis

To assess changes in protein expression Western blot analysis was used. Cells were seeded and treated as per Table 2.6. On day 3, cell supernatant was removed, and cells were washed three times with 37 °C PBS (Hyclone, USA). For cell lysis 100 µL of ice cold RIPA buffer (Astral Scientific, Australia) with phosphatase and protease inhibitors, containing a proprietary mix of Aprotinin, Bestatin**,** *trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64), and Leupeptin (Cell Signalling Technology, USA) were added per well and protein concentrations were quantified (as detailed in section *2.2.2*).

Following protein determination, samples were diluted appropriately and NuPAGE® LDS buffer (6 µL/sample) and dithiothreitol reducing agent (2.4 µL/sample) was added. Samples were heated using a heat block at 70 °C for 10 minutes, centrifuged at 14,000x(g) and stored at -20 °C. Proteins were separated using SDS-page and then transferred onto nitrocellulose membranes. Antigen detection of membranes was investigated by blocking membranes in 3% bovine serum albumin (BSA) or 5% skim milk in Tween 20 Tris-buffered saline (TBS-T) for 1-hour at room temperature. Primary antibodies (detailed below in Table 2.12) were incubated overnight in 1% BSA or 0.1% skim milk in TBS-T at 4 °C followed by washing three times (5 mins each wash) with TBS-T. Secondary antibodies (detailed below in Table 2.12) were incubated at room temperature in 1% BSA for 30 mins followed by washing three times (5 mins each wash) with TBS-T. To detect bands, 500 µL of Clarity™ Enhanced chemiluminescent substrate (ECL; Bio-Rad) was added per membrane and chemiluminescence was assessed using the Chemidoc™ XRS+ system (Bio-Rad).

Table 2.12: Primary and secondary antibody details for proteins assessed using Western Blot analysis.

Antibody	Molecular weight (kDa)	Serum	Supplier & catalog number	Host species	Dilution factor
Anti-Fatty acid synthase (FAS)	273	3% BSA	Cell signalling technology 3189S	Rabbit	1:1000
Anti-Acetyl CoA Carboxylase (ACC)	280	3% BSA	Cell signalling technology 3676S	Rabbit	1:1000
Anti-HMG-CoA reductase (HMG-CR)	97	3% BSA	Abcam ab174830	Rabbit	1:1000
Anti-Vitamin D Receptor (VDR)	48	3% BSA	Santa Cruz sc-13133	Mouse	1:500
Anti-Steroid acute regulatory (StAR)	30	3% BSA	Santa Cruz sc-166821	Mouse	1:1000
Anti-Beta actin (β actin)	45	1% BSA	Cell signalling technology 4970S	Rabbit	1:1000

2.2.10 Immunofluorescence staining

Immunofluorescence staining was used to visualise the presence and density of specific receptors and proteins. Cells were seeded onto sterile coverslips and treated as per Table 2.6. On day 3, cells were fixed with 4% paraformaldehyde (pH=7.4) for 10 mins at RT, then washed three times with PBS. Cells were then permeabilised by incubation with TBS-T for 10 mins at room temperature. Cells were incubated in 3% BSA diluted in TBS-T to reduce non-specific binding of antibodies. Primary antibodies (Table 2.13) diluted in 1:300 in TBS-T containing 1% BSA were added to each well (except the secondary control) and incubated overnight at 4 °C in the dark, on a rocking station. On day 4, the primary antibodies were removed, and wells were washed three times in PBS for 5 mins/wash. The secondary antibody Anti-mouse IgG Fab specific diluted (1:500) in TBS-T containing 1% BSA was added to each well and left to incubate for 1 hour at room temperature in the dark on a rocking station. Lastly, the counter stain DAPI (1 µg/mL) was used to visualise the nuclear location following incubation for 10 mins at room temperature. Coverslips were mounted onto glass microscope slides using DPX mounting medium (Thermofisher, USA) and left overnight in a cool dark place to cure. On day 5 cell immunofluorescence staining was imaged using the Nikon A1 confocal microscope (Nikon, USA) and NIS-elements software (Nikon).

Table 2.13: Primary and secondary antibody details for proteins assessed using immunofluorescence analysis.

Antibody	Molecular weight (kDa)	Serum	Supplier & catalog number	Host species	Dilution factor
Anti-Vitamin D Receptor (VDR)	48	3% BSA	Santa Cruz sc-13133	Mouse	1:300
Anti-Beta actin (β actin)	45	1% BSA	Cell signalling technology 4970S	Rabbit	1:1000
Anti-mouse IgG Fab Alexa Fluor® 594 Conjugate	-	1% BSA	Cell signalling technology 8890S	Goat	1:1000

2.2.11 Cell cycle analysis

Flow cytometry was used to assess any changes in the stages of the cell cycle. Cells were seeded and treated as per Table 2.5. On day 3 cells were collected via trypsination (as described above in section 2.2.1) then transferred to Eppendorf tubes and centrifuged for 3 mins at 300x(g) to collect the cell pellets. Cells were resuspended and washed in warm PBS three times. After the final wash step, cells were resuspended in a small volume of PBS (50 µL) and 1 mL of ice cold 70% ethanol was added dropwise and left overnight at -20ºC. On day 4, cells were centrifuged at 1000x(g), resuspended, and washed in PBS three times in preparation for flow cytometry. After the final wash, cells were resuspended in 150 µL of PBS containing propidium iodine (40 µg/mL) and RNase (10 µg/mL) and incubated in the dark for 40 mins at room temperature. Staining of DNA content was assessed using the BD FACSFortessa (BD Biosciences, USA) flow cytometer and phases of the cell cycle were determined using FlowJo v10.6.2 software (FlowJo LLC, USA).

2.2.12 ROS generation

Flow cytometry was used to assess any alterations to the levels of generalised intracellular ROS generation. One bottle of DMEM powder (Sigma, Australia) was dissolved in ddH2O to the final volume of 1 L (with no supplementation with FBS or phenol red), sterile filtered in a laminar flow hood and stored at 4 °C. A stock solution of the ROS probe CM-H2DCFDA (Invitrogen, USA) was prepared in DMEM by dissolving 346 uL of DMSO (concentration= 50 µg/vial), and 30 µL aliquots were prepared with a final concentration of 250 µM and stored at -20 °C. Cells were seeded and treated as per Table 2.5.On day 3 cells were washed once with 37 °C PBS. Cells were then either exposed to the staining solution containing 1 µM of CM-H2DCFDA diluted in DMEM media or DMEM media alone (for unstained control samples) and incubated for 30 mins at 37 °C. Cells were washed three times with warm PBS and

collected via trypsinisation as described in section 2.2.1. In the final step cells were resuspended in 150 µL of DMEM containing 1 µg/mL of propidium iodine (PI) to stain dead cells and fluorescent intensity was assessed using the BD LSRFortessa (BD Biosciences, USA) flow cytometer and levels of ROS were quantified by assessing the fluorescent intensity in FlowJo v10.6.2 software (FlowJo LLC, USA).

2.2.13 Statistical analysis

All statistical calculations were performed using SPSS statistic version 25 (IBM Corporation, USA). Data is represented as mean or percentage ± standard deviation (SD) and independent experiments were replicated a minimum of 3 times. The statistical differences were analysed using one-way ANOVA with multiple comparisons and Tukey post-hoc tests. The statistical significance was defined as $p<0.05$. Graphs were generated using GraphPad Prism version 8 software (GraphPad Software Inc., USA).

Chapter Three

VitD status and outcomes in patients undergoing IVF: Retrospective study.

3.0 Introduction

VitD inadequacy is progressively being recognised as an emerging global public health issue, with the mean levels of VitD in adult populations falling below 20 ng/mL (50 nmol/L) (276). Current clinical implications of VitD cut-offs are largely based on early studies relating to bone health (5), with the importance of VitD in extra-skeletal tissues emerging in recent years (20). More specifically, the importance of VitD in the context of fertility is a relatively new area of interest, ttherefore the precise function of VitD in female infertility remains elusive.

Data from a large population report of Australian adults (n= 11,247) demonstrated that despite the relatively high sun-exposure in Australia, 31% of the study participants were VitD insufficient (serum VitD < 20 ng/mL), with women being at a higher risk of insufficiency (102). Interestingly, a large study investigating the influence of season on IVF outcomes have shown the number of rainy days in the month prior to IVF treatment is negatively correlated with LB outcomes, but not CP (277). In another investigation which assessed season independently of VitD status, the incidence of eclampsia was nearly doubled during winter months, leading researchers to hypothesis a potential link between VitD and the preeclampsia risk (278).

Currently, several retrospective studies have attempted to identify the relationship between VitD status and clinical outcomes of women undergoing IVF (30, 31, 42, 44-46, 53). There are numerous variations in these studies, particularly the method of analysis to investigate the relationship between VitD and clinical outcomes. One of the major limitations of these studies is they do not report the number of days between serum/FF collection and cycle OPU or ET (31, 42, 44, 46). Furthermore, given the nature of these types of studies, a sophisticated statistical analysis approach using

adjusted logistic regression models is necessary for retrospective studies. Using logistic regression models accounting for important variables that impact the clinical outcome of interest, allows researchers to scrutinise the association between VitD directly, rather than indirect influences of other variables. While several of these studies do apply logistic regression methods (31, 42, 45, 46, 279), important confounding factors (such as AMH level and transferred embryo quality) are not included. Furthermore, there are issues with multicollinearity where variables that are related are included in the same model namely AMH level and AFC grouping (which are both indicative of ovarian reserve). Lastly, in some reports the full models (inclusive of confounding factors) are not reported, making it difficult to draw conclusions or interpret results. Additionally, to date, none of the aforementioned studies investigated blastocyst development as a clinical outcome or analysed the association between VitD with patient demographics, cycle characteristics, embryological measures, or clinical outcomes.

The objective of this chapter covering results, is to address these gaps in knowledge- particularly regarding blastocyst development. This will be accomplished using robust, well-designed multivariate logistic regression models to retrospectively analyse data from our private university affiliated IVF clinic.

3.0.1 Chapter Objectives:

1) Investigate how patient and cycle characteristics are associated with VitD sufficiency (using a 20 ng/mL cut off).
2) Investigate how serum VitD is associated with embryological measures and clinical outcomes, including blastocysts development, CP, and LB chance,
3) Identify study limitations and strengths in this retrospective cohort, to inform the design and analysis of a future robust, prospective observational study (presented in Chapter 4).

3.1 Results

3.1.1 Patient recruitment

A total of 4012 autologous cycles were extracted from the PIVET clinic database, consisting of 1157 FET cycles and 1235 'other' cycles (inclusive of hormone replacement therapy and intrauterine insemination cycles) and the remaining 1620 cycles were IVF cycles (Figure 3.1). An additional 1190 IVF cycles were excluded based on the following selection criteria: cancelled or failed cycle/OPU (n=331), cycle converted to a freeze all or PGD (n=371, or embryo or oocyte donors were utilised (n=488) (Figure 3.1).

A total of 263 patients who underwent an IVF cycle with a single fresh ET cycle were included in the final analysis (Figure 3.1). There was no significant difference between the two VitD groups regarding the proportion of excluded cases or the reason for exclusion in the final analysis. Based on the IOM guidelines, 45.2% of patients had insufficient VitD levels (< 20 ng/mL, n=119), and 54.8% had sufficient levels (≥ 20 ng/mL, n=144), while only 6.8% of patients had a deficient VitD level of < 10 ng/mL (n=18) and 17.5% of patients had a serum VitD level > 30 ng/mL (n= 46, Fig. 3.1). The range of serum VitD measurements for the whole cohort was 4.0-59.7 ng/mL (Table 3.1).

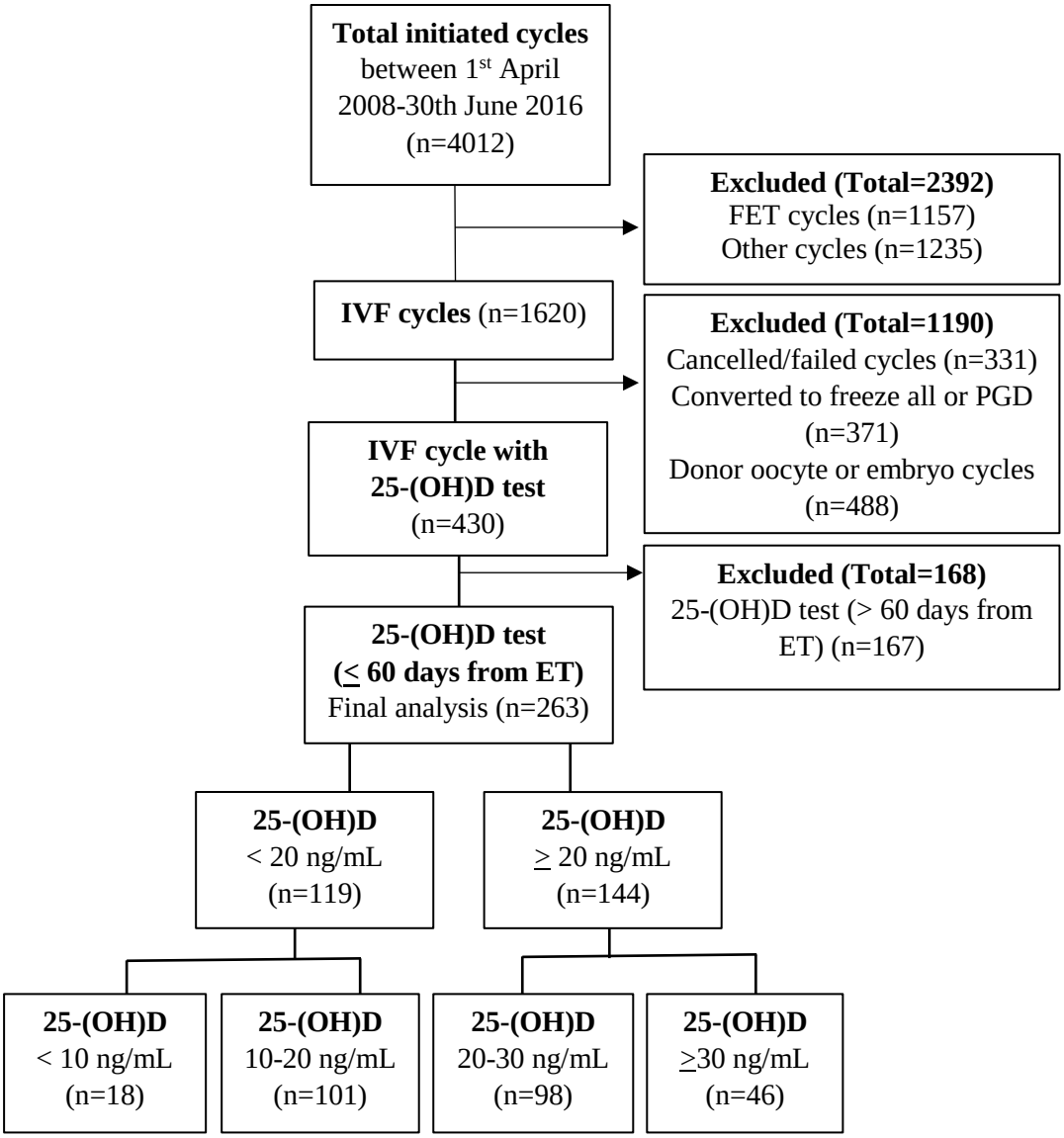


Figure 3.1: Flow diagram detailing the extraction of eligible cycles and final data analysis cohort for retrospective analysis. Data was extracted from the PIVET database from the period 1st April 2008-30th June 2016. Cycles were removed if there was no fresh ET, the cycle was cancelled or failed, donor embryos/oocytes were used, or where there were multiple cycles for one patient (only first cycles with a successful ovum pick-up, a VitD measurement closest to the cycle and fresh ET during the specified period were included in the final analysis). *25-(OH)D, 25-hydroxyvitamin D.*

3.1.2 Patient demographics and characteristics

A significantly higher proportion of patients who were VitD sufficient had their levels tested during summer or autumn months (57.6%) compared to the deficient group (35.3%, p<0.001, Table 3.1). The median BMI of patients in the ≥ 20 ng/mL group was lower than the < 20 ng/mL group but was not statistically significant (22.7 vs. 24.3 kg/m^2, p=0.073, Table 3.1). The proportion of patients with unexplained infertility, was 10.6% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group but was not statistically significant (69.4% vs 58.8%, p=0.073). There were no significant differences in the < 20 ng/mL group compared to the ≥ 20 ng/mL group for infertility aetiology, including endometriosis (4.2% vs. 2.8%, tubal defect (6.7% vs. 6.3%), male factor infertility (25.2% vs. 18.1%) or unexplained infertility (74.8% vs. 80.6%). The groups were comparable in terms of median female age (p=0.220) and partner age (p=0.788) at the time of cycle, AFC (p=0.685) and previous IVF cycles (p=0.986, Table 3.1).

Table 3.1: Patient characteristics stratified by serum status (20 ng/mL cut off).

Variable	< 20 ng/mL	≥ 20 ng/mL	p value
Initiated cycles, n	119	144	-
Mean Serum 25-(OH)D level, ng/mL	14.5 ± 3.8	27.9 ± 6.6	**<0.001**
Median Serum 25-(OH)D level, ng/mL	15.2 (4.8)	26.4 (8.0)	**<0.001**
Serum 25-(OH)D level range, ng/mL	4.0-19.6	20.0-59.6	-
Season of 25-(OH)D testing, n/total [%]	-	-	**<0.001**
Winter/Spring	77/119 [64.7]	61/144 [42.4]	-
Summer/Autumn	42/119 [35.3]	83/144 [57.6]	-
Female age at cycle, years	35.5 (8.0)	35.0 (7.0)	0.220
Partner age at cycle, years	37.5 (11.0)	37.0 (9.0)	0.788
BMI, kg/m^2	24.3 (6.3)	22.7 (5.8)	0.073
AFC, n/total [%]	-	-	0.685
Group A (≥ 20 follicles)	37/119 [31.3]	44/144 [30.6]	-
Group B/C (9-19 follicles)	39/119 [32.8]	54/144 [37.5]	-
Group D/E (≤ 8 follicles)	43/119 [36.1]	46/144 [31.9]	-
Infertility aetiology, n/total [%]	-	-	-
Endometriosis	5/119 [4.2]	4/144 [2.8]	0.527
Tubular defect	8/119 [6.7]	9/144 [6.3]	0.877
Male factor	30/119 [25.2]	26/144 [18.1]	0.158
Unexplained	89/119 [74.8]	116/144 [80.6]	0.262
Multiple factors	13/119 [10.9]	11/144 [7.9]	0.357
Previous IVF cycle, n/total [%]	-	-	0.986
First cycle	78/119 [65.5]	92/144 [63.9]	-
Second cycle	17/119 [14.3]	22/144 [15.3]	-
≥ Third cycle	24/119 [20.2]	30/144 [20.8]	-

Mean ± SD; Median (IQR); number/total cases [%]. *Abbreviations- 25-(OH)D, 25-hydroxyvitamin D; BMI, body mass index; AFC, antral follicle count.*

3.1.3 Patient cycle characteristics

The patient cycle characteristics were highly comparable between the VitD groups in several aspects. There were no differences in the proportion of patients receiving antagonist or agonist/other stimulation protocols, insemination technique (ICSI v IVF v ICSI/IVF split), median rFSH dose, endometrial thickness, day of ET, and number of or quality of transferred embryos (Table 3.2). The only observed difference for cycle characteristics between the groups was regarding trigger dosage, with the VitD deficient group receiving a higher median trigger dose (750.0 µg) compared to the VitD sufficient group (500.0 µg, p=0.027, Table 3.2).

Table 3.2: Patient cycle characteristics stratified by serum VitD status (20 ng/mL cut off).

Variable	< 20 ng/mL	≧ 20 ng/mL	p value
Initiated cycles, n	119	144	-
Median Serum 25-(OH)D level, ng/mL	15.2 (4.8)	26.4 (8.0)	**<0.001**
Stimulation protocol, n/total [%]	-	-	0.660
Antagonist	87/119 [73.1]	107/144 [74.3]	-
Agonist/Other	32/119 [26.9]	37/144 [25.7]	-
Insemination type, n/total [%]	-	-	0.684
ICSI	106/119 [89.1]	124/144 [86.1]	-
IVF	7/119 [5.9]	9/144 [6.3]	-
ICSI/IVF split	6/119 [5.0]	11/144 [7.6]	-
rFSH dose, IU	350.0 (400.0)	187.8 (162.5)	0.604
Trigger dose, μg	750.0 (9500)	500.0 (9500)	**0.027**
Endometrial thickness, mm	9.3 (2.9)	9.3 (2.4)	0.892
ET Day, n/total [%]	-	-	0.204
Day 2/3	77/119 [64.7]	80/144 [55.6]	-
Day 4	2/119 [1.7]	1/144 [0.7]	-
Day 5	40/119 [33.6]	63/144 [43.8]	-
Transferred Embryo quality, n/total [%]	-	-	0.750
High quality blastocyst	22/119 [18.5]	32/144 [22.2]	-
Medium quality blastocyst	18/119 [15.1]	20/144 [13.9]	-
Low quality blastocyst/Day 3	79/119 [66.4]	92/144 [63.9]	-
Embryos at transfer, n/total [%]	-	-	0.931
Single ET	98/119 [82.4]	118/144 [81.9]	-
Double ET	21/119 [17.6]	26/144 [18.1]	-

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- 25-(OH)D, 25-hydroxyvitamin D; ICSI, intracytoplasmic sperm injection; rFSH, recombinant follicle stimulating hormone; ET, embryo transfer*.

3.1.4 Patient hormonal profile

There were several significant differences between the two groups regarding patient hormonal parameters. Median levels of prolactin (510.0 vs. 380.0 mIU/L, p=0.002) and luteinising hormone (0.70 vs. 0.25 IU/L, p=0.016) at OPU were significantly higher in the < 20 ng/mL group compared to the ≥ 20 ng/mL (Table 3.3). Similarly, during the mid-luteal phase, median levels of P4 were significantly higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL (287.0 vs. 224.0 ng/mL, p=0.015, Table 3.3). There were no significant differences in AMH level, P4 at trigger or OPU, E2 at OPU or at mid luteal phase, or any of the androgen panel (SHBG, testosterone, free androgen index, free androgens or DHEA; Table 3.3).

Table 3.3: Patient hormonal profile stratified by serum 25-(OH)D status in subset of patients (20 ng/mL cut off).

Variable *VitD range*	**< 20 ng/mL** (4.0-19.6)	**≥ 20 ng/mL** (20.0-59.7)	**p value**
Initiated cycles, n	119	144	-
Median Serum 25-(OH)D level, ng/mL	15.2 (4.8)	26.4 (8.0)	**<0.001**
AMH, pmol/L	11.0 (21.7)	14.4 (20.2)	0.375
P4 at trigger, ng/mL	1.9 (2.0)	1.9 (2.0)	0.960
OPU			
E2, pmol/L	3050.0 (3825.0)	2800.0 (3100.0)	0.930
P4, ng/mL	19.0 (23.1)	22.0 (16.0)	0.527
Prolactin, mIU/L	380.0 (542.5)	510.0 (430.0)	**0.002**
Luteinising hormone, IU/L	0.25 (1.0)	0.70 (1.2)	**0.016**
Mid Luteal phase			
E2, pmol/L	4200.0 (3100.0)	4200.0 (4100.0)	0.890
P4, ng/mL	224.0 (151.0)	287.0 (278.0)	**0.015**
Androgens			
SHBG, nmol/L	64.4 (86.5)	77.9 (54.7)	0.745
Testosterone, nmol/L	0.4 (1.0)	0.6 (0.7)	0.891
Free Androgen Index, ratio	0.5 (3.9)	0.7 (1.0)	0.693
Free Androgens, ng/mL	3.6 (22.1)	5.9 (7.0)	0.520
DHEA, ng/mL	4.6 (9.6)	4.4 (4.7)	0.542

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- AMH, anti-Mullerian hormone; OPU, oocyte pick-up; SHBG, sex homone binding globulin; DHEA, Dehydroepiandrosterone.*

3.1.5 Embryological and clinical outcomes

There were no significant differences observed between the two VitD groups (< 20 ng/mL vs. ≥ 20 ng/mL) in relation to the median number of total oocytes collected (8.0 vs. 9.0, p=0.459), or number of MII oocytes collected (6.0 vs. 6.0, p=0.909, both Table 3.4). Similarly, there were no difference observed between the two VitD groups (< 20 ng/mL vs. ≥ 20 ng/mL) in the median number of 2PNs generated per cycle (5.0 vs. 5.0, p=0.913, both Table 3.4).

The median fertilisation rate (when expressed as the number 2PN's/the number of MII oocytes inseminated per cycle) was not significantly increased in the ≥ 20 ng/mL compared to the < 20 ng/mL (83.3% vs. 80.0%, p=0.587, Table 3.4). However, when expressed as overall fertilisation rate (the number of MII oocytes/insemination per whole group), the ≥ 20 ng/mL fertilisation rate was 3% higher compared to the < 20 ng/mL (90.5% vs. 87.2%, p=0.030, Table 3.4).

Blastocyst development rate (%) did not differ between the two VitD groups when expressed as a percentage of total oocytes or MII oocytes collected. However, when expressed as a percentage of 2PNs generated, blastocyst development rate was slightly but significantly higher in the < 20 ng/mL group compared to the ≥ 20 ng/mL group (26.8% vs. 22.4%, respectively, p=0.049, Table 3.4). Conversely, the number of blastocysts generated per cycle or overall blastocyst development rate (expressed as mean or median %), and the percentage of cycles developing at least one blastocyst were not statistically different between the two VitD groups (Table 3.4).

There was no difference in the number of embryos transferred per cycle in the < 20 ng/mL vs. ≥ 20 ng/mL, when expressed as mean (1.2 vs.1.2, p=0.864) or median (1.0 vs. 1.0, p=0.940, Table 3.4). Similarly, there was no difference in the number of cryopreserved embryos per cycle in the < 20 ng/mL vs. ≥ 20 ng/mL, when expressed as mean (1.2 vs.1.4, p=0.228) or median (1.0 vs. 1.0, p=0.900, Table 3.4).

Median oocyte utilisation rate (which accounts for the embryos utilised fresh and frozen from the oocytes collected) was 2.3% higher in the > 20 ng/mL group compared

to the < 20 ng/mL group, but was not statistically significant (27.3% vs. 25.0, p=0.228, Table 3.4). Median embryo utilisation rate (which accounts for the embryos utilised fresh and frozen from the total embryos generated) did not differ between the < 20 ng/mL vs. > 20 ng/mL (50.0 vs. 50.0, p=0.784, Table 3.4).

Fresh CPR was 5.4% higher in the < 20 ng/mL group compared to the > 20 ng/mL group, however this was not statistically significant (35.3% vs. 29.9%, p=0.348, Table 3.4). Similarly, fresh LBR was 6.8% higher in the < 20 ng/mL group compared to the > 20 ng/mL group, however this was not statistically significant (31.1% vs. 24.3%, p=0.0.219, Table 3.4). Despite the difference in LBR, there was no statistically significant increase in miscarriage rate in the $\geq$ 20 ng/mL group compared to the < 20 ng/mL (18.6% vs. 11.9%, respectively, p=0.461, Table 3.4).

Cumulative CPR (when expressed per ET) was 4.6% higher in the < 20 ng/mL group compared to the $\geq$ 20 ng/mL group, however this was not statistically significant (37.2% vs. 32.6%, p=0.323, Table 3.4). Similarly, cumulative CPR (when expressed per initiated cycle), was 6.7% higher in the < 20 ng/mL group compared to the > 20 ng/mL group, again this was not statistically significant (58.8% vs. 52.1%, p=0.274, Table 3.4).

Interestingly, cumulative LBR (when expressed per ET) was comparable between the < 20 ng/mL group compared to the $\geq$ 20 ng/mL group (28.7% vs. 29.6%, p=0.851, Table 3.4). Cumulative LBR (when expressed per initiated cycle) was 1.8% higher in the $\geq$ 20 ng/mL group compared to the < 20 ng/mL group, however, again this was not statistically significant (47.2% vs. 45.4%, p=0.0.765, Table 3.4).

Table 3.4: Embryological measures stratified by serum VitD status (20 ng/mL cut off).

Variable	< 20 ng/mL	> 20 ng/mL	p-value
Initiated cycles, n	119	144	-
Oocytes			
Total oocytes collected, n	1136	1389	-
Median Oocytes collected per cycle, n	8.0 (9.0)	9.0 (7.0)	0.456
Total MII oocytes collected, n	748	936	-
Median MII oocytes collected per cycle, n	6.0 (6.0)	6.0 (5.0)	0.909
Fertilisation			
Total fertilised (2PN), n	652	847	-
Overall fertilisation rate, n/inseminated [%]	652/748 [87.2]	847/936 [90.5]	0.030
Median fertilised (2PN) per cycle, n	5.0 (5.0)	5.0 (5.0)	0.913
Median fertilisation rate % per cycle, %	80.0 (42.3)	83.3 (33.3)	0.587
Cleavage Embryos			
Total embryos cultured beyond day 2, n	495	615	-
High quality day 3 embryos	320	420	-
Blastocysts			
Total blastocysts formed, n	175	190	-
High quality blastocysts	85	82	-
Blastocysts rate per oocytes collected, n/total [%]	175/1136 [15.4]	190/1389 [13.7]	0.219
Blastocysts rate per MII collected, n/total [%]	175/748 [23.4]	190/936 [20.3]	0.125
Blastocyst rate per 2PN, n/total [%]	175/652 [26.8]	190/847 [22.4]	0.049
Median blastocyst number generated per cycle, n	1.0 (2.0)	1.0 (2.0)	0.475
Mean blastocyst number generated per cycle, n	1.3 ± 1.7	1.5 ± 1.9	0.208
[A]Median blastocyst development rate, %	11.1 (33.0)	12.5 (33.0)	0.918

[A]Mean blastocyst development rate, %	17.9 ± 23.1	18.2 ± 20.7	0.687
Cycles developing at least 1 blastocyst, n/total [%]	61/119 [51.3]	77/144 [53.5]	0.960
Transfer & Cryopreservation			
Total embryos transferred, n	140	170	-
Median embryos transferred per cycle, n	1.0 (0.0)	1.0 (0.0)	0.940
Mean embryos transferred per cycle, n	1.2 ± 0.4	1.2 ± 0.4	0.864
Total embryos cryopreserved, n	148	204	-
Median embryos cryopreserved per cycle, n	1.0 (2.0)	1.0 (2.0)	0.900
Mean embryos cryopreserved per cycle, n	1.2 ± 1.5	1.4 ± 1.6	0.228
[B]Median oocyte utilisation rate, %	25.0 (22.6)	27.3 (20.3)	0.228
[B]Median embryo utilisation rate, %	50.0 (46.7)	50.0 (36.7)	0.784
Pregnancy, Miscarriage & Live Births			
Fresh CPR, n/total [%]	42/119 [35.3]	43/144 [29.9]	0.348
Fresh LBR, n/total [%]	37/119 [31.1]	35/144 [24.3]	0.219
Fresh miscarriage rate, n/total CP [%]	5/42 [11.9]	8/43 [18.6]	0.461
Cumulative CPR (Fresh + Frozen per ET), [%]	70/188 [37.2]	75/230 [32.6]	0.323
Cumulative CPR (Fresh + Frozen, per initiated cycle), n/total [%]	70/119 [58.8]	75/144 [52.1]	0.274
Cumulative LBR (Fresh + Frozen per ET), n/total [%]	54/188 [28.7]	68/230 [29.6]	0.851
Cumulative LBR (Fresh + Frozen per initiated cycle), n/total [%]	54/119 [45.4]	68/144 [47.2]	0.765

Mean ± SD; Median (IQR); n/total [%]. [A]Group median/mean % derived from the individual total number of blastocysts developed in a cycle as a function of the total number of normally fertilisaed oocytes (i.e. 2PN) in that cycle. [B]Group median utilisation rates derived from the individual calculated utilisation rate for each case. Oocyte utilisation rate equals the sum of embryos transferred and frozen, divided by the number of oocytes collected in that cycle. Embryo utilisation rate equals the number of embryos transferred and frozen, divided by the number of 2PN generated in that cycle. MII, metaphase II oocytes; 2PN, two pronucleate zygotes; CPR, clincal pregnancy rate; LBR, live birth rate.

3.1.6 Univariate logistic regression model for VitD sufficiency

Patient demographics, cycle characteristics, embryological measures and clinical outcomes variables were explored in a univariate logistic regression model to investigate if changes in these variables were associated with an increased chance of a patient having a sufficient VitD status (≥ 20 ng/mL) which was assessed as the dependent variable. As predicted, summer and autumn months were highly associated with having a sufficient VitD status (≥ 20 ng/mL) when compared to winter and spring months (OR=2.50, CI=1.51-4.12, p=<0.001, Table 3.5).

Female age at cycle was not associated with patient VitD sufficiency (OR=0.98, 0.93-1.03, p=0.341, Table 3.5). BMI was not significantly associated with VitD sufficiency when expressed as a continuous (OR=0.96, p=0.084), or as grouped variable comparing patients within the obese BMI range (≥ 30 kg/m^2) to those in the overweight (25.0-29.9 kg/m^2, healthy (18.5-24.9 kg/m^2) or underweight (< 18.5 kg/m^2) ranges (OR=0.84, p=0.676; OR= 1.48, p=0.299; OR=1.75, p=0.431, respectively, Table 3.5). Height (as a pseudo marker for ethnicity) was not associated with patient VitD sufficiency (OR=0.97, p=0.137, Table 3.5).

AMH level was not associated with patient VitD sufficiency (OR=1.01, 1.00-1.02, p=0.213, Table 3.5). Patients within the AFC group A (≥ 20 follicles) or group B/ C (9-19 follicles) were not more likely to have a sufficient VitD status when compared to patients in the group D/E (OR=1.29, p=0.388; OR=1.11, p=0.731, respectively, Table 3.5).

No specific type of infertility aetiologies was associated with VitD sufficiency, including unexplained infertility, endometriosis or tubal (OR=1.59, p=0.074); (OR=0.93, p=0.877), OR=0.65, p=0.530, respectively, Table 3.5). ART attempt number, when expressed as a continuous variable, was not associated with VitD sufficiency (OR=1.00, p=0.997, Table 3.5). Similarly, when grouped as previous IVF events, and compared to ≥ 3 IVF events, having no, 1 or 2 previous IVF cycles was not associated with the likelihood to be VitD sufficient (OR=0.89, p=766; OR= 0.97, p=0.952; OR=0.88, p=0.808l, respectively, Table 3.5).

Having an agonist or other ovarian stimulation protocol compared to an antagonist cycle, was also not associated with increased likelihood of VitD sufficiency (OR=0.94,

p=0.826, Table 3.5). Similarly, insemination via ICSI only or IVF/ICSI split were not associated with VitD status when compared to IVF only (OR= 0.91, p=0.856; OR=1.43, p=0.620, respectively, Table 3.5). Patients who received a 'moderate' (200-400 IU) or 'high' (400-600 IU) dose of rFSH compared those who received a 'low' (< 200 IU) were not significantly more likely to be VitD sufficient (OR= 1.39, p=0.285; OR=0.81, p=0.495, respectively, Table 3.5). In contrast, patients who received a higher trigger dose (≥ 10,000 µg) were 50% significantly less likely to be in the VitD sufficient group (OR=0.50, p=0.021, Table 3.5).

P4 level at trigger and endometrial thickness were not related to VitD status (OR=1.07, p=426; OR= 0.96, p=0.611; respectively, Table 3.5). No embryological measures were significantly associated with VitD sufficiency including but not limited to- collected oocyte number (total and M2), fertilised 2PN's, fertilisation rate, day 3 embryos, ET day and quality, utilisation rates (oocyte and embryo), or CPRs and LBRs (fresh & cumulative) (p=>0.05, Table 3.5).

Table 3.5: Univariate regression model for fertility confounding factors associated with VitD sufficiency. Associations are presented as odds ratio (OR) and 95% confidence interval (CI).

Variable		Likelihood to be VitD sufficient (≥ 20 ng/mL) (OR, 95% CI)	p-value
Season of VitD Test	Winter/Spring	1.00	-
	Summer/Autumn	2.50 (1.51-4.12)	**<0.001**
Female age, years		0.98 (0.93-1.03)	0.341
Height*, cm		0.97 (0.94-1.01)	0.137
BMI, kg/m^2		0.96 (0.91-1.01)	0.084
BMI Group	≥ 30 kg/m^2	1.00	-
	< 18.5 kg/m^2	1.75 (0.44-7.04)	0.431
	18.5-24.9 kg/m^2	1.48 (0.71-3.07)	0.299
	25.0-29.9 kg/m^2	0.84 (0.38-1.88)	0.676
AMH, pmol/L		1.01 (1.00-1.02)	0.213
AFC Group	D/E (≤ 8 follicles)	1.00	-
	B/C (9-19 follicles)	1.11 (0.61-2.03)	0.731
	A (≥ 20 follicles)	1.29 (0.72-2.32)	0.388
Infertility aetiology	Tubal	0.93 (0.35-2.48)	0.877
	Endometriosis	0.65 (0.17-2.48)	0.530
	Male factor	0.65 (0.36-1.18)	0.160
	Unexplained	1.40 (0.78-2.51)	0.263
	Multiple factors	0.67 (0.29-1.57)	0.359
ART attempt, n		1.00 (0.83-1.21)	0.997
Previous IVF	≥ 3 Events	1.00	-
	0 Events	0.89 (0.40-1.98)	0.766
	1 Event	0.97 (0.36-2.59)	0.952
	2 Events	0.88 (0.30-2.56)	0.808

Stimulation Type	Antagonist	1.00	-
	Agonist/Other	0.94 (0.54-1.63)	0.826
Insemination type	IVF Only	1.00	-
	ICSI Only	0.91 (0.33-2.53)	0.856
	IVF-ICSI Split	1.43 (0.35-5.79)	0.620
rFSH dose, IU Group	< 200	1.00	-
	200-400	1.39 (0.76-2.54)	0.285
	400-600	0.81 (0.44-1.48)	0.495
P4 at trigger, ng/mL		1.07 (0.90-1.28)	0.426
Trigger dose, μg	< 10,000	1.00	-
	≥ 10,000	0.50 (0.30-0.92)	**0.021**
Endometrial thickness, mm		0.96 (0.80-1.14)	0.611
ET day, n		1.22 (0.95-1.56)	0.126
Transferred embryo quality	Low quality/Day 3	1.00	-
	High quality	1.25 (0.67-2.32)	0.482
	Medium quality	0.95 (0.47-1.93)	0.896
Embryos transferred, n		1.03 (0.55-1.94)	0.931
Oocytes collected per cycle, n		1.01 (0.96-1.05)	0.801
MII oocytes collected per cycle, n		1.01 (0.96-1.07)	0.652
Fertilised (2PN) per cycle, n		1.03 (0.97-1.10)	0.347
Fertilisation rate per insemination, %		1.01 (1.00-1.02)	0.184
D3 embryos in culture per cycle, n		1.03 (0.94-1.13)	0.516
Blastocyst number generated per cycle, n		0.95 (0.83-1.09)	0.463
Blastocyst percentage generated per cycle, %		1.01 (0.99-1.01)	0.898
Proportion of high-quality blastocysts generated per cycle, %		0.99 (0.97-1.02)	0.689
Embryos cryopreserved, n		1.07 (0.92-1.26)	0.374
Oocyte utilisation rate per cycle, %		0.99 (0.98-1.01)	0.318
Embryo utilisation rate per cycle, %		1.00 (0.99-1.01)	0.242

Cycles with at least one Blastocyst	No	1.00	-
	Yes	0.99 (0.61-1.61)	0.960
Cycles with a Clinical Pregnancy	No	1.00	-
	Yes	0.78 (0.47-1.31)	0.349
Cycles with a Live Birth	No	1.00	-
	Yes	0.71 (0.41-1.23)	0.220

ET, embryo transfer; BMI, body mass index; AMH, anti Mullerian hormone; AFC, antral follicle count; 2PN, two pronuceli zyogtes. *Height (cm) was used as a pseudo marker for ethnicity.

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3.1.7 Univariate logistic regression model for blastocyst development

Patient demographics, cycle characteristics, embryological measures and clinical outcomes were explored in a univariate logistic regression model to investigate if increases in these variables were associated with an increased chance of developing a blastocyst (assessed as the dependent variable).

There was no association between patients with a sufficient VitD status and the development of at least one blastocyst (OR=0.99, CI=0.61-1.61, p=0.960, Table 3.6). Summer and autumn months were not associated with blastocyst development when compared to winter and spring months (OR=1.54, CI=0.94-2.51, p=0.084, Table 3.6).

Interestingly, female age at cycle was not associated with blastocyst development (OR= 0.98, CI=0.93-1.02, p=0.305, Table 3.6). Similarly, BMI was not significantly associated with blastocyst development when expressed as a continuous (OR=0.99, p=0.796), or as grouped variable comparing patients within the obese BMI range (> 30 kg/m2) to those in the overweight (25.0-29.9 kg/m2, healthy (18.5-24.9 kg/m2) or underweight (< 18.5 kg/m2) ranges (OR=0.90, p=0.786; OR= 1.12, p=0.770; OR=1.07, p=0.918, respectively, Table 3.6).

AMH level was not associated with blastocyst development (OR=1.01, 1.00-1.02, p=0.213, Table 3.6). Patients within the AFC group A ($\geq$ 20 follicles) or group B and C (9-19 follicles) when compared to the AFC groups D and E ($\leq$ 8 follicles) were not more likely to develop a blastocyst (OR=1.51, p=0.188; OR=0.88, p=0.656, respectively, Table 3.6).

With every increasing ART attempt there was a 22% reduction in the likelihood of at least one blastocyst developing within that cycle (OR=0.78, CI=0.64-0.96, p=0.016, Table 3.6). Patients with a tubal defect had an 89% decreased likelihood of developing a blastocyst compared to those with no tubal defect (OR=0.11, CI=0.03-0.51, p=0.004, Table 3.6), however there was only 1 case of tubal defect. Patients with unexplained infertility were 69% more likely to develop at least one blastocyst compared to patients with a diagnosed cause of infertility (endometriosis or tubal defect, OR=1.69, CI=1.02-2.82, p=0.043, Table 3.6).

Patients who underwent an agonist or AACEP ovarian stimulation regime (utilised for women diagnosed as poor ovarian responders) were 55% less likely to develop a

blastocyst compared to patients who received an antagonist regime (OR=0.45, CI=0.26-0.79, p=0.005, Table 3.6). Patients who received an ovarian stimulation rFSH dose of < 200 or 200-400 IU compared those with a dose between 400-600 IU were 4.2 and 2.6 times (respectively) more likely to develop at least one blastocyst (OR= 4.20, CI=2.21-7.97, p=<0.001; OR=2.63, CI=1.35-5.15, p=0.005, respectively, Table 3.6).

For every single unit increase in the total oocytes and MII oocytes collected, the likelihood of developing a blastocyst increased by 8% and 7%, respectively (OR=1.08, CI=1.03-1.14, p=0.002; OR=1.07, CI=1.01-1.14, p=0.025, Table 3.6). Furthermore, for every additional 2PN generated, the likelihood of developing at least one blastocyst increased by 16% (OR=1.16, CI=1.08-1.25, p=0.025, Table 3.6). Lastly, for every increase in the number of day 3 embryos in culture the likelihood of developing a blastocyst increased by 17% (OR=1.17, CI=1.05-1.30, p=0.004, Table 3.6).

For embryological procedures, insemination via ICSI when compared to IVF decreased the likelihood of developing a blastocyst by 77%. However, there were only 16 cases of IVF-only insemination, as clinic protocol mostly opts for ICSI-only insemination (OR=0.23, CI=0.06-0.82, p=0.023, Table 3.6). Furthermore, when compared to IVF/ICSI split there was no significant change in the likelihood of developing a blastocyst, although as above there were only 17 cases of split insemination (OR=1.08, CI=0.18-6.32, p=0.935, Table 3.6).

Table 3.6: Univariate regression model for fertility confounding factors associated with blastocyst development. Associations are presented as odds ratio (OR) and 95% confidence interval (CI).

Variable		Likelihood to develop at least one blastocyst (OR, 95% CI)	p-value
Serum VitD Group, ng/mL	< 20 ng/mL	1.00	-
	≥ 20 ng/mL	0.99 (0.61-1.61)	0.960
Season of ET/VitD Test	Winter/Spring	1.00	-
	Summer/Autumn	1.54 (0.94-2.51)	0.084
Female age, years		0.98 (0.93-1.02)	0.305
BMI, kg/m^2		0.99 (0.94-1.05)	0.796
BMI Group	≥ 30 kg/m^2	1.00	-
	< 18.5 kg/m^2	1.07 (0.28-4.16)	0.918
	18.5-24.9 kg/m^2	1.12 (0.54-2.32)	0.770
	25.0-29.9 kg/m^2	0.90 (0.40-2.00)	0.786
AMH, pmol/L		1.01 (1.00-1.02)	0.213
AFC Group	D/E (≤ 8 follicles)	1.00	-
	B/C (9-19 follicles)	0.88 (0.49-1.57)	0.656
	A (≥ 20 follicles)	1.51 (0.82-2.78)	0.188
Infertility aetiology	Tubal	0.11 (0.03-0.51)	**0.004**
	Endometriosis	0.42 (0.10-1.72)	0.227
	Male factor	0.81 (0.45-1.45)	0.473
	Unexplained	1.69 (1.02-2.82)	**0.043**
	Multiple factors	0.62 (0.27-1.45)	0.270
ART attempt, n		0.78 (0.64-0.96)	**0.016**
Previous IVF	≥ 3 Events	1.00	-
	0 Events	2.21 (0.98-5.00)	0.058
	1 Event	1.19 (0.45-3.21)	0.725

	2 Events	1.55 (0.53-4.55)	0.429
Stimulation Type	Antagonist	1.00	-
	Agonist/Other	0.45 (0.26-0.79)	**0.005**
Insemination type	IVF Only	1.00	-
	ICSI Only	0.23 (0.06-0.82)	**0.023**
	IVF-ICSI Split	1.08 (0.18-6.32)	0.935
rFSH dose, IU Group	400-600	1.00	**-**
	200-400	2.63 (1.35-5.15)	**0.005**
	≤ 200	4.20 (2.21-7.97)	**<0.001**
P4 at trigger, ng/mL		0.96 (0.82-1.13)	0.651
Trigger dose, μg	< 10,000	1.00	**-**
	≥ 10,000	0.43 (0.26-0.72)	**0.001**
Endometrial thickness, mm		1.01 (0.85-1.20)	0.900
Oocytes collected per cycle, n		1.08 (1.03-1.14)	**0.002**
MII oocytes collected per cycle, n		1.07 (1.01-1.14)	**0.025**
Fertilised (2PN) per cycle, n		1.16 (1.08-1.25)	**<0.001**
Fertilisation rate per insemination, %		1.01 (1.00-1.02)	0.191
D3 embryos in culture per cycle, n		1.17 (1.05-1.30)	**0.004**
Blastocyst number generated per cycle, n		0.95 (0.83-1.09)	0.463
Blastocyst percentage generated per cycle, %		1.01 (0.99-1.01)	0.898
Proportion of high-quality blastocysts generated per cycle, %		0.99 (0.97-1.02)	0.689

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3.1.8 Multivariate logistic regression model for blastocyst development

Based on the individual factors identified in Table 3.6, and to reduce multicollinearity between related variables, the final adjusted stepwise, backwards elimination multivariate model for blastocyst development consisted of VitD group (our main study interest), female age at cycle, stimulation type, tubal factor infertility, rFSH and trigger dose, insemination type, and the number of oocytes collected. Two logistic regression models are displayed below in Table 3.7. Model 1 is adjusted for all significant univariate variables identified in Table 3.6 and listed above. Model 2 excluded the variables tubal defect and insemination type from this list, in which the number of cases were limited and could potentially skew the data (Table 3.7).

In model 1, tubal factor infertility reduced the likelihood of developing a blastocyst by 79% (OR=0.21, CI=0.06-0.82, p=0.025, Table 3.7). In the adjusted model, insemination via ICSI or IVF/ICSI split were not significantly associated with blastocyst development, compared to IVF alone (OR=0.38, CI=0.10-1.41, p=0.150 & OR=5.04, CI=0.62-41.13, p=0.128, respectively, Table 3.7). However, given there were only 16 total cases of tubal factor infertility, 16 cases of IVF only and 17 cases of IVF/ICSI split, these variables were removed in model 2. Patients with a 'low' dose of rFSH (≤ 200 IU) and a 'moderate' dose (200-400 IU) were 3.77 and 2.58 times (respectively) more likely to develop a blastocyst, compared to those who received a 'high' dose (400-600 IU) of rFSH (OR=3.77, CI=1.87-7.63, p=<0.001; OR=2.58, CI=1.25-5.34, p=0.016, respectively, Table 3.7). Finally, for every oocyte collected, the chance of developing at least one blastocyst significantly increased by 23% (OR=1.23, CI=1.14-1.32, p=<0.001, Table 3.7). As there were several variables related to the number of oocytes collected, these variables could also be used as alternative control variables for the total number of oocytes collected. These variables include the number of MII oocytes collected, the number of 2PNs generated or the number of day 3 embryos in culture. Inclusion of these variable showed similar statistical results.

For model 2, patients with a 'low' dose of rFSH (≤ 200 IU) and a 'moderate' dose (200-400 IU) were 3.74 and 2.58 times (respectively) more likely to develop a blastocyst, compared to those who received a 'high' dose (400-600 IU) of rFSH

(OR=3.74, CI=1.91-7.36, p=<0.001; OR=2.56, CI=1.26-5.21, p=0.009, respectively, Table 3.7). Patients who received a trigger dose of > 10,000 (µg) were 2.36 times more likely to develop a blastocyst than those who received a trigger of dose ≤ 10,000 (µg), however this was not statistically significant (OR=2.36, CI=0.82-6.79, p=0.111, Table 3.7). Finally, for every increasing number of oocytes collected, the chance of developing at least one blastocyst increased by 20% (OR=1.20, CI=1.12-1.29, p=<0.001, Table 3.7).

Table 3.7: Final multi-variable logistic regression model for fertility measures significantly associated with blastocyst development. Associations are presented as odds ratio (OR) and 95% confidence interval (CI).

Model 1 Variable		**Likelihood to develop at least one blastocyst (OR, 95% CI)**	**p-value**
Tubular defect	No	1.00	-
	Yes	0.21 (0.06-0.82)	**0.025**
Insemination type	IVF	1.00	-
	ICSI	0.38 (0.10-1.41)	0.150
	IVF/ICSI split	5.04 (0.62-41.13)	0.128
rFSH dose, IU	400-600	1.00	-
	200-400	2.58 (1.25-5.34)	**0.016**
	≤ 200	3.77 (1.87-7.63)	**<0.001**
Total oocytes collected		1.23 (1.14-1.32)	**<0.001**

Model 2 Variable		**Likelihood to develop at least one blastocyst (OR, 95% CI)**	**p-value**
rFSH dose, IU	400-600	1.00	-
	200-400	2.56 (1.26-5.21)	**0.009**
	≤ 200	3.74 (1.91-7.36)	**<0.001**
Trigger dose, µg	< 10,000	1.00	-
	> 10,000	2.36 (0.82-6.79)	0.111
Total oocytes collected		1.20 (1.12-1.29)	**<0.001**

Model 1- Step 1: VitD & stimulation group is removed from the model. Step 2: Trigger dose (grouped) and female age is removed from the model. Step 3: Final model remains. **Model 2**- Step 1: Female age, VitD & stimulation group is removed from the model. Step 2: Final model remains.

3.1.9 Univariate logistic regression model for clinical pregnancy

Patient demographics, cycle characteristics, embryological measures and clinical outcomes were explored in a univariate logistic regression model to investigate if changes in these variables were associated with an increased chance of a successful CP (assessed as the dependent variable).

There was no association between patients with a sufficient VitD status and CP chance (OR=0.78, CI=0.47-1.31, p=0.349, Table 3.8). Summer and autumn months were not associated with CP chance when compared to winter and spring months (OR=1.29, CI=0.77-2.16, p=0.342, Table 3.8).

For ever increasing year of female age at cycle the chance of CP significantly decreased by 10% (OR= 0.90, CI=0.86-0.96, p=<0.001, Table 3.8). In contrast, BMI was not associated with CP chance when expressed as a continuous (OR=1.02, p=0.442), or as grouped variable comparing patients within the obese BMI range (> 30 kg/m2) to those in the overweight (25.0-29.9 kg/m2, healthy (18.5-24.9 kg/m2) or underweight (< 18.5 kg/m2) ranges (OR=1.04, p=0.922; OR= 0.92, p=0.833; OR=0.75, p=0.706, respectively, Table 3.8).

For every increase in AMH level (pmol/L) the likelihood of achieving a CP increased by 2% (OR=1.02, 1.01-1.03, p=0.008, Table 3.8). Patients within the AFC group A ($\geq$ 20 follicles) or groups B/C (9-19 follicles) when compared to groups D/E ($\leq$ 8 follicles) were 2.8 and 1.4 times (respectively) more likely to achieve a CP (OR=2.80, CI=1.43-5.48, p=0.003; OR=1.84, CI=0.95-3.58, p=0.072, Table 3.8).

Patients who underwent an agonist or AACEP ovarian stimulation regime were 39% less likely to achieve a CP compared to patients who received an antagonist regime; but this was not statistically significant (OR=0.61, CI=0.33-1.13, p=0.114, Table 3.8).

For every increase in the number of total and MII oocytes collected the likelihood of developing a CP increased by 9% and 13%, respectively (OR=1.09, CI=1.04-1.15, p=0.001; OR=1.13, CI=1.06-1.20, p=<0.001, Table 3.8). Additionally, for every 2PN generated the likelihood of developing at least one blastocyst increases by 12% (OR=1.12, CI=1.04-1.20, p=0.002, Table 3.8). For every increasing percentage of blastocysts generated the likelihood of achieving a CP increased by 2% (OR=1.02,

CI=1.01-1.03, p=0.001, Table 3.8). Lastly, for every increase in the number of cryopreserved embryos, the chance of CP significantly increased by 26% (OR=1.26, CI=1.07-1.49, p=0.005, Table 3.8).

Taken together, the main statistically significant univariate variables associated with CP chance were female age at cycle, ovarian reserve (AMH and AFC), number of oocytes/MII oocytes collected, number of 2PN's, percentage of blastocysts generated per cycle, transferred embryo quality and the number of embryos cryopreserved.

Table 3.8: Univariate regression model for fertility confounding factors associated with clinical pregnancy chance. Associations are presented as odds ratio (OR) and 95% confidence interval (CI).

Variable		Likelihood of CP chance (OR, 95% CI)	p-value
Serum VitD Group, ng/mL	< 20 ng/mL	1.00	-
	≥ 20 ng/mL	0.78 (0.47-1.31)	0.349
Season of ET/VitD Test	Winter/Spring	1.00	-
	Summer/Autumn	1.29 (0.77-2.16)	0.342
Female age, years		0.90 (0.86-0.96)	**<0.001**
BMI, kg/m^2		1.02 (0.97-1.08)	0.442
BMI Group	≥ 30 kg/m^2	1.00	-
	< 18.5 kg/m^2	0.75 (0.17-3.35)	0.706
	18.5-24.9 kg/m^2	0.92 (0.42-2.00)	0.833
	25.0-29.9 kg/m^2	1.04 (0.45-2.44)	0.922
AMH, pmol/L		1.02 (1.01-1.03)	**0.008**
AFC Group	D/E (≤ 8 follicles)	1.00	-
	B/C (9-19 follicles)	1.84 (0.95-3.58)	0.072
	A (≥ 20 follicles)	2.80 (1.43-5.48)	**0.003**
Infertility aetiology	Tubal	1.51 (0.55-4.11)	0.422
	Endometriosis	0.59 (0.12-2.90)	0.514
	Male factor	1.48 (0.80-2.73)	0.211
	Unexplained	0.74 (0.44-1.27)	0.278
ART attempt, n		0.84 (0.67-1.05	0.122
Previous IVF	≥ 3 Events	1.00	-
	0 Events	1.64 (0.66-4.07)	0.290

	1 Event	1.50 (0.51-4.44)	0.463
	2 Events	0.71 (0.20-2.61)	0.611
Stimulation Type	Antagonist	1.00	-
	Agonist/Other	0.61 (0.33-1.13)	0.114
Insemination type	IVF Only	1.00	-
	ICSI Only	2.18 (0.60-7.88)	0.234
	IVF-ICSI Split	1.81 (0.35-9.24)	0.478
rFSH dose group, IU	400-600	1.00	-
	200-400	1.10 (0.56-2.15)	0.788
	≤ 200	0.76 (0.40-1.45)	0.409
P4 at trigger, ng/mL		0.88 (0.72-1.08)	0.235
Trigger dose group, μg	< 10,000	1.00	-
	≥ 10,000	1.00 (0.58-1.71)	0.987
Endometrial thickness, mm		1.00 (0.84-1.21)	0.968
Oocytes collected per cycle, n		1.09 (1.04-1.15)	**0.001**
MII oocytes collected per cycle, n		1.13 (1.06-1.20)	**<0.001**
Fertilised (2PN) per cycle, n		1.12 (1.04-1.20)	**0.002**
Fertilisation rate per insemination, %		1.00 (0.99-1.01)	0.758
D3 embryos in culture per cycle, n		1.05 (0.95-1.16)	0.322
Blastocyst number generated per cycle, n		1.07 (0.93-1.23)	0.324
Blastocyst percentage generated per cycle, %		1.02 (1.01-1.03)	**0.001**
Proportion of high-quality blastocysts generated per cycle, %		1.00 (0.99-1.01)	0.638
Embryos transferred, n		0.67 (0.30-1.37)	0.274
Embryos cryopreserved, n		1.26 (1.07-1.49)	**0.005**
Oocyte utilisation rate per cycle, %		0.99 (0.98-1.01)	0.414
Embryo utilisation rate per cycle, %		0.99 (0.98-1.00)	0.058
Transferred embryo quality	Low quality/Day 3	1.00	-
	High quality blastocyst	2.45 (1.24-4.83)	**0.010**
	Medium quality blastocyst	1.63 (0.74-3.60)	0.227

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3.1.10 Multivariate logistic regression model for clinical pregnancy

Based on the individual factors identified in Table 3.8, and to reduce multicollinearity between related variables, the final adjusted stepwise, backwards elimination multivariate model for CP included the following variables for analysis; VitD group (our main study interest), female age at cycle, AMH level and transferred embryo quality.

In the final adjusted model, for every increasing year of female age at cycle, the likelihood of achieving a CP was reduced by 8% (OR=0.92, CI=0.86-0.99, p=0.016, Table 3.9). Additionally, patients who received a high-quality blastocyst at transfer were 2.21 times more likely to have a CP than those who received a medium quality (OR=1.55, CI=0.69-3.49, p=0.293), when compared to a low-quality blastocyst or day 3 embryo (Table 3.9).

Table 3.9: Final multi-variable logistic regression model for fertility measures significantly associated with clinical pregnancy chance. Associations are presented as odds ratio (OR) and 95% confidence interval (CI).

Variable		Likelihood for CP (OR, 95% CI)	p-value
Female age. years		0.92 (0.86-0.99)	0.016
Transferred embryo quality	Low quality/Day 3	1.00	-
	High quality blastocyst	2.21 (1.10-4.46)	0.026
	Medium quality blastocyst	1.55 (0.69-3.49)	0.293

Step 1: No variables removed. Step 2: AMH value removed. Step 3: VitD group removed.

3.2.11 Univariate logistic regression model for live birth chance

Parameters related to patient demographics, cycle characteristics, embryological measures and clinical outcomes were explored in a univariate logistic regression model to investigate if changes in these variables were associated with an increased chance of a successful LB (assessed as the dependent variable).

There was no association between patients with a sufficient VitD status and LB chance (OR=0.71, CI=0.41-1.23, p=0.220, Table 3.10). Summer and autumn months were not associated with LB chance when compared to winter and spring months (OR=1.15, CI=0.67-1.97, p=0.622, Table 3.10).

Female age at cycle was significantly associated with LB chance, with every year of increasing female age, the likelihood of LB decreased by 10% (OR= 0.90, CI=0.85-0.96, p=0.001, Table 3.10). In contrast, BMI was not associated with LB chance when expressed as a continuous (OR=1.02, p=0.573), or as grouped variable comparing patients within the obese BMI range ($\geq$ 30 kg/m2) to those in the overweight (25.0-29.9 kg/m2, healthy (18.5-24.9 kg/m2) or underweight (< 18.5 kg/m2) ranges (OR=1.29, p=0.589; OR= 1.09, p=0.835; OR=1.13, p=0.880, respectively, Table 3.10).

For every increase in AMH level (pmol/L) the likelihood of achieving a CP increased by 1% (OR=1.01, 1.01-1.03, p=0.038, Table 3.10). Patients within the AFC group A ($\geq$ 20 follicles when compared to the AFC groups D and E ($\leq$ 8 follicles) were 2.83 times more likely to achieve a LB (OR=2.83, CI=1.40-5.71, p=0.004), while those in the B or C groups were 1.68 times more likely to achieve a live birth, however this was not statistically significant (OR=1.68, CI=0.83-3.41, p=0.153, Table 3.10). Patients who underwent an agonist or AACEP ovarian stimulation regime were 33% less likely to achieve a CP compared to patients who received an antagonist regime; however, this was not statistically significant (OR=0.67, CI=0.35-1.28, p=0.223, Table 3.10).

For every increase in the number of total and MII oocytes collected the likelihood of developing a LB increased by 8% and 12%, respectively (OR=1.08, CI=1.02-1.14, p=0.005; OR=1.12, CI=1.05-1.19, p=0.001, Table 3.10). Additionally, for every 2PN generated the likelihood of LB increases by 10% (OR=1.10, CI=1.03-1.19, p=0.008,

Table 3.10). For every increasing percentage of blastocysts generated (from fertilised oocytes) the likelihood of achieving a LB increased by 2% (OR=1.02, CI=1.01-1.03, p=0.001, Table 3.10).

Patients who received a high-quality blastocyst were significantly more likely to have a successful LB than those who received a medium quality blastocyst, compared to a low-quality blastocyst of day 3 embryo (OR=2.19, CI=1.10-4.39, p=0.026 vs. OR=1.47, CI=0.63-3.27, p=0.389, respectively, Table 3.10). Lastly for every increase in the number of cryopreserved embryos, the chance of LB significantly increased by 26% (OR=1.26, CI=1.07-1.49, p=0.006, Table 3.10).

In summary, the main statistically significant univariate variables associated with LB chance were female age at cycle, ovarian reserve (AMH and AFC), number of oocytes/MII oocytes collected, number of 2PN's, percentage of blastocysts generated per cycle, transferred embryo quality and the number of embryos cryopreserved.

Table 3.10: Univariate regression model for fertility confounding factors associated with live birth chance. Associations are presented as odds ratio (OR) and 95% confidence interval (CI).

Variable		Likelihood of LB chance (OR, 95% CI)	p-value
Serum VitD Group, ng/mL	< 20 ng/mL	1.00	-
	≥ 20 ng/mL	0.71 (0.41-1.23)	0.220
Season of ET/VitD Test	Winter/Spring	1.00	-
	Summer/Autumn	1.15 (0.67-1.97)	0.622
Female age, years		0.90 (0.85-0.96)	**0.001**
BMI, kg/m^2		1.02 (0.96-1.08)	0.573
BMI Group	≥ 30 kg/m^2	1.00	-
	< 18.5 kg/m^2	1.13 (0.24-5.18)	0.880
	18.5-24.9 kg/m^2	1.09 (0.47-2.53)	0.835
	25.0-29.9 kg/m^2	1.29 (0.52-3.20)	0.589
AMH, pmol/L		1.01 (1.00-1.03)	**0.038**
AFC Group	D/E (≤ 8 follicles)	1.00	-
	B/C (9-19 follicles)	1.68 (0.83-3.41)	0.153
	A (≥ 20 follicles)	2.83 (1.40-5.71)	**0.004**
Infertility aetiology	Tubal	1.49 (0.53-4.18)	0.452
	Endometriosis	0.75 (0.15-3.70)	0.725
	Male factor	1.49 (0.79-2.82)	0.217
	Unexplained	0.81 (0.46-1.42)	0.462
ART attempt, n		0.79 (0.61-1.02)	0.066
Previous IVF	≥ 3 Events	1.00	-
	0 Events	2.57 (0.85-7.79)	0.095

	1 Event	2.67 (0.76-9.38)	0.127
	2 Events	1.43 (0.34-6.03)	0.627
Stimulation Type	Antagonist	1.00	-
	Agonist/Other	0.67 (0.35-1.28)	0.223
Insemination type	IVF Only	1.00	-
	ICSI Only	1.74 (0.48-6.32)	0.397
	IVF-ICSI Split	0.93 (0.16-5.45)	0.935
rFSH dose, IU Group	≤ 200	1.00	-
	200-400	1.16 (0.60-2.25)	0.657
	400-600	1.17 (0.60-2.30)	0.639
P4 at trigger, ng/mL		0.92 (0.75-1.13)	0.409
Trigger dose, μg	< 10,000	1.00	-
	≥ 10,000	1.05 (0.59-1.84)	0.876
Endometrial thickness, mm		1.05 (0.87-1.26)	0.640
Oocytes collected per cycle, n		1.08 (1.02-1.14)	**0.005**
MII oocytes collected per cycle, n		1.12 (1.05-1.19)	**0.001**
Fertilised (2PN) per cycle, n		1.10 (1.03-1.19)	**0.008**
Fertilisation rate per insemination, %		1.00 (0.99-1.01)	0.702
D3 embryos in culture per cycle, n		0.98 (0.88-1.09)	0.688
Blastocyst number generated per cycle, n		1.07 (0.93-1.24)	0.335
Blastocyst percentage generated per cycle, %		1.02 (1.01-1.03)	**0.001**
Proportion of high-quality blastocysts generated per cycle, %		1.00 (0.99-1.01)	0.539
Embryos transferred, n		0.49 (0.22-1.10)	0.084
Embryos cryopreserved, n		1.26 (1.07-1.49)	**0.006**
Oocyte utilisation rate per cycle, %		1.00 (0.99-1.01)	0.821
Embryo utilisation rate per cycle, %		0.99 (0.98-1.00)	0.166
Transferred embryo quality	Low quality/Day 3	1.00	-
	High quality blastocyst	2.19 (1.10-4.39)	**0.026**
	Medium quality blastocyst	1.47 (0.63-3.27)	0.389

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3.1.12 Multivariate logistic regression model for live birth chance

Based on the individual factors identified in Table 3.10, and to reduce multicollinearity between related variables, the final adjusted stepwise, backwards elimination multivariate model for LB consisted of VitD group (our main study interest), female age at cycle, AMH level and the quality of transferred embryo.

In the final adjusted model, for every increasing year of female age at cycle, the likelihood of achieving a LB was reduced by 6% (OR=0.94, CI=0.88-1.01, p=0.009, Table 3.11). Patients with a serum VitD status $\geq$ 20 ng/mL were 45% less likely to have a successful LB compared to patients with a VitD status < 20 ng/mL, however this was not statistically significant (OR=0.55, CI=0.29-1.03, p=0.062, Table 3.11). Lastly, patients who received a high-quality blastocyst at transfer were 2.22 times more likely to have a LB than those who received a medium-quality when compared to a low-quality blastocyst or day 3 embryo (OR=2.22, CI=1.08-4.58, p=0.031 vs. OR=1.42, CI=0.61-3.32, p=0.415, respectively, Table 3.11).

Table 3.11: Final multi-variable logistic regression model for fertility measures significantly associated with live birth chance. Associations are presented as odds ratio (OR) and 95% confidence interval (CI).

Variable		Likelihood for live birth (OR, 95% CI)	p-value
Female age. years		0.94 (0.88-1.01)	0.009
Serum VitD Group	< 20 ng/mL	1.00	-
	≥ 20 ng/mL	0.55 (0.29-1.03)	0.062
Transferred embryo quality	Low quality/day 3	1.00	-
	High quality blastocyst	2.22 (1.08-4.58)	0.031
	Medium quality blastocyst	1.42 (0.61-3.32)	0.415

Step 1: No variables removed. Step 2: AMH level removed.

3.2 Discussion

In many regions worldwide, mean VitD levels fall below the current recommended cut-off of 20 ng/mL. However, large gaps exist in reported data, as many studies measure a limited number of parameters eg: aging populations, exclude pregnant women, VitD is only measured at one time point, or are from population dense cities and do not account for regional populations (276). Based on this, the incidence of the VitD insufficiency and deficiency is likely under reported (276). In the present retrospective study of 263 women undergoing IVF, the observed prevelence of VitD insufficiency (< 20 ng/mL) was 45.2%. In Australian women of reproductive age, the prevalnce of VitD insufficiency is approximately 33% (102). The descrepancy between our data and population data may suggest VitD insufficiency is more prevelant in sub-fertile populations. Similar conclusions have been drawn by other authors (280, 281), with several retrospective studies investigating the association between VitD status in women undergoing IVF reporting largely varied prevelence of VitD insufficiency, between 20.7-65.0% (30, 31, 44, 45, 53, 197). Other factors that may account for these variations could include the ethnic variation of the popluation, the season of VitD measurement, use of supplementation and socio-economic factors of the study cohorts (103). The risk of VitD insufficiency is associated with various demographic characteristics including (but not limited to) gender (with women having a higher risk), ethnicity, advanced age, obesity, season and socioeconomic status (102). The major source of VitD for both children and adults is exposure to UVB radiation from sunlight (2) therefore the most common cause of VitD insufficiency is inadequate sun exposure of the skin (19).

As expected, patients with a sufficient level of VitD were more likely to have had their ET performed in summer or autumn months, a relationship well established in numerous studies (33, 282). Seasonal variations in VitD levels have previously been shown to not be evident in older women (283), however this was not observed in this cohort as the age range was limited (23-44 years of age at cycle initiation). Seasonal variations have also been shown to impact maternal glucose homeostasis, independent of VitD status (284). Furthermore, improved metabolic profiles of women in summer months, suggests a favorable cardiometabolic profile for pregnancy (285). This evidence suggest the relationship between season and pregnancy outcomes are more

complex than VitD status alone. Unfortunately for this study no data was available regarding metabolic status.

It has previously been demonstrated that ethnicity is not only related to pregnancy in fertile women (286, 287) but also to CPRs in IVF (31, 39). Unfortunately in the current study no data pertaining to ethnicity or country of birth was obtained, therefore ethnicity was not included as a confounding factor within our statistical models. In an attempt to investigate ethnic variations and their impact on VitD status and clinical outcomes height was used as a pseudo-marker for ethnicity.(288). However height was not found to be associated with any clinical outcomes or VitD sufficiency, suggesting height was not an accurate pseudo-marker in our cohort (289, 290). Previous reports have demonstrated ethnic minority groups have lower CPRs and LBRs in IVF and ICSI cycles (291-293), therefore ethnicity is an important confounding factor when assessing VitD status, but also investigating clinical outcomes in ART.

The VitD groups in the current study were well-matched for patient demographics and characteristics, including female and partner age at cycle, ovarian reserve measures (AFC grouping proportions and AMH level), infertility aetiologies and the proportion of previous IVF. Age has been identified as a risk factor for developing VitD insufficiency (< 20 ng/mL) in post-menopausal women over 50 years of age (28), however in the present study no such association was found between female age at cycle and VitD status, likely due to the majority of patients being of pre-menopausal (< 45 years of age) reproductive age. It has been well established previously that advanced maternal age increases the risk of adverse outcomes in pregnancy, which occurs regardless of ART use (294). One study demonstarted levels of AMH (indicative of ovarian reserve (295)) were an important predictor of IVF outcome, but only in women > 35 years of age. In another investigation of women with endometriosis, AMH and P4 levels and transferred embryo quality, were all significantly associated with clinical outcomes (296). In data presented here, patient AMH level was automatically removed (via backwards elimination) from the multivariate models for both CP and LB chance. However, female age at cycle (along with transferred embryo quality) were the only significant variables associated with CP and LB success. Many reports to date have also indicated female age is the major predictor of IVF outcomes, inclusive of women > 40 years of age (297-300).

Obesity is a serious health risk in pregnancy in both natural and ART cycles, with an increased risk of hypertension, gestational diabetes, and cesarian deliveries (301). Many studies have found an inverse relationship between BMI and VitD status, where as serum VitD levels decrease, BMI status increases. Furthermore, clinical trials have shown VitD supplementation can reduce BMI level, as well as metabolic profiles in overweight and obese women (18). Additionally, weight loss and reduction of BMI status can improve pregnancy rates (302). In one investigation, researchers reported overweight and obese PCOS women produced a similar number of retrieved oocytes, 2PN zygotes, and high quality embryos to PCOS women with a normal BMI status, however the latter had higher implantation and CPRs (303). Additionally, a meta analysis of LBRs in obese women undergoing IVF establish prognosis is poorer in obese PCOS patients compared to obese non-PCOS women (304). Data presented here demonstrated no significant difference in median BMI status between the two VitD groups. However, there was a trend towards an inverse relationship between BMI and VitD status. It is important to note, in the PIVET clinic there is a maximum BMI cut-off of 35.0 kg/m^2 for women undergoing IVF treatment. A significant difference between the two groups may have been evident in a larger sample size. Additionally, the effect may be slightly reduced due to the inclusion of only women in obese class 1 (BMI 30-35.0 kg/m^2), where the highest level of VitD deficiency is evident in women in obese classes ≥ 2 (≥ 35 kg/m^2) (305).

In the present study of non-PCOS patients, prolactin and P4 levels were significantly higher in the ≥ 20 ng/mL group. In contrast, an investigation of metabolic syndrome in PCOS patients, reported no such relationship between VitD status and prolactin levels (306). Furthermore, basal prolactin levels (> 30 ng/mL) have previously been associated with an increase in cumulative CPR in IVF (307). Prolactin increase in pregnancy has also been shown to be negatively correlated to BMI status, and is associated with more favorable long-term metabolic health (308). This, in conjunction with the trend towards a relationship between BMI and VitD sufficiency, suggest higher levels of prolactin in the VitD sufficient group may actually be related to BMI status and not VitD directly.

Decidualisation of the human endometrium does not require embryo implantation, unlike in the majority of other mammals (309). Instead, a post-ovulatory rise in P4 levels drives this process (309). For this reason, P4 levels in women trying to conceive,

are a good predictor of endometrial thickness and receptivity (310). Molecular research has found VDR expression in endometrial cells is significantly decreased in the secretory phase, compared to the proliferative phase (233). Furthermore, treatment of endometrial stromal cells (obtained from biopsies) with biologically active 1,25-(OH)2D3 increases P4 receptor expression (311). This evidence suggests VitD may play an important role in implantation through the modification of the P4 receptor. Data presented here indicated median P4 levels were higher in the VitD sufficient group. To date very few clinical studies have found an association between increased P4 levels or endometrial thickness and VitD status. Lower P4 and VitD levels have been associated with repeated implantation failure (312). Evidence from donor-oocyte recepient cycles found adjusted CPRs were lower among VitD deficient recipients compared to those with sufficient VitD status (197). Additionally, data from FET cycles with thawed blastocysts have reported no improvement in CPRs, when comparing deficient and sufficient VitD patients (313). Given our cohort excluded oocyte donation and FET cycles, investigating the association between VitD and endometrial receptivity, without the impact of confounding ooycte-related factors, is limited and inconclusive.

Interestingly, despite there being a positive relationship in the current study between serum VitD and LH at OPU, there are no studies to date which support this finding. In fact, in animal and human studies there appears to be an opposite trend with VitD supplementation reducing the levels of LH (314, 315). Given our inconsistent timing of VitD testing in the present study, the VitD level obtained may not be relevant to the patient hormonal profiles analysed here. In the future, more meaningful conlusions can be made from such findings if VitD was measured consistently at the same point of the cycle, and closer to the day of OPU or ET.

Cycle and embryological charactertistics were also well-matched between the VitD groups. This includes ovarian stimulation protocol, insemination method, day of ET, transferred embryo quality and the number of embryo's transferred. The majority of women in this study received single ET, due to the Australian guidelines limiting the use of double ET (316). In our clinic, we opt for insemination via ICSI over conventional IVF or ICSI/IVF split as detailed elsewhere (272, 274). Conventional IVF has been shown to be less effective in cases of male factor infertility (317, 318), while the benefit of widespread use of ICSI in non-male factor infertility is still under

debate (319, 320). Although the multivariate model for blastocyst development suggests insemination via ICSI has a negative impact on blastocyst development, this is likely due to the small number of cases of IVF only (n=16) and ICSI/IVF split (n=17). Data presented here suggests no additional beneficial effect of ICSI in a mixed population of infertile women. However, previous data from our clinic has indicated higher ICSI rates minimise complete failed fertilisations, whilst having no detrimental effect on foetal outcomes (274).

In this study there was no association in adjusted multivariate models between VitD status and the clinical outcomes of blastocyst development, CPRs and LBRs. Female age at cycle and transferred embyro quality were the only significant predictors of both CP and LB chance. Two multivariate models were presented here for blastocyst development. The first model included tubular defect, unexplained fertility, rFSH/trigger dose, insemination method and total number of oocytes collected at OPU. The second model was the same as model one, but excluded tubal defect (n=17) and insemination method due to small numbers and the high chance of type II error. Following this exclusion in model two, all the remaining variables were significant predictors of blastocyst development in the adjusted analysis.

Currently, there is no data supporting or refuting an association between serum VitD status and blastocyst development in IVF. It is well known that blastocyst quality is a strong indicator of CP outcome in euploid transfer cycles (321). Identifying an association between VitD status and blastocyst development may indicate a more direct relationship than what has been peviously eluded to with VitD status and clinical pregnancy. There is data to support the quality of transferred embryo or the day of EThas a significant impact in the likelihood of achieving a CP, alongside serum VitD status (279).

Data presented here indicated rFSH dosage was a significant predictor of blastocyst development. Interestingly, despite previous data suggesting age-related decline of oocyte quality (322), female age was not predictive of the development of at least one blastocyst in our cohort. Our clinic utilises an in-house designed rFSH dosing alogirthm which is specialised to produce an optimal number of well-developed oocytes, whilst reducing the risk of ovarian hyperstimulation (58, 64, 65). The PIVET algorithm for individualised rFSH dosing is based on female age and AFC grading and adjusted for AMH level, BMI, day-2 level of FSH, and smoking history (64). In clinics

where rFSH dosing is not individualised in the same manner, a relationship between female age at cycle and blastocyst development may be evident.

One of the major challenges in IVF is the management of poor ovarian response (POR). The widely accepted definition of POR is based on the Bologna criteria (323), however several criticisms of this criteria are evident. The Bologna criteria fails to address the influence of oocyte quality and the relevant factos which impact embryo quality. Our clinic uses several approaches to address POR, including: the use of our FSH dosing alogirthms, preferential single ET, blastocyst culture (with best quality emrbyos cyropreserved), strong luteal support regimes, FET cycles conducted either under natural conditions or hormonal replacement therapy (with preferential single ET and P4 pessaries), and growth hormone (GH) adjuvent therapy (for women deficient in GH) (272, 324-326). As expected, in the adjusted multivariate models, rFSH and trigger dosage were significantly associated with blastocyst development. This was an inverse relationship, meaning when rFSH dosage was increased, the likelihood of developing at least one blastocyst decreased significantly. This relationship is likely due to POR patients being less responsive to conventional IVF stimulation protocols, therefore receiving higher doses of rFSH during ovarian stimulation (327, 328).

Many studies to date have investigated the relationship between VitD status and clinical outcomes such as implantation, clinical preganancy, and LBRs, both retrospectively (30, 31, 42, 44-46, 53) and prospectively (19, 32, 33, 35, 38, 39, 48, 50, 51). Several of these have reported no association between serum VitD status and clinical outcomes in women undergoing IVF with fresh ET (31, 32, 44, 46, 48). However, the majority of these have found positive relationships between VitD status and clinical outcomes. A few studies found VitD sufficiency ($\geq$ 30 ng/mL) was related to crude fertilisation rates (35, 50) and oocyte/embryo quality (38, 53), as well as clinical pregnancy, and LBRs (33, 35, 38, 39). However, the majority of these do not utilise adjusted multivariate regression models (38, 50, 53) or statistical significance was lost upon adjustment for confounding factors such as female age and embryological parameters (33, 35).

One investigation in oocyte recipients demonstrated serum VitD sufficiency ($\geq$ 30 ng/mL) was associated with increased CP chance in an adjusted multivariate model (42). However, the multivariate model was not adjusted for number or quality of transferred embryo/s (42). Similarly, Paffoni et al., reported women with normal

ovarian reserve and serum VitD sufficiency (≥ 30 ng/mL) had higher CPRs, no adjustment for embryo quality was performed (19). Another study which assessed follicular fluid levels of VitD, also reported VitD sufficiency was related to increased implantation and CPRs (39). While the authors did adjust for ET day, statistical significance supporting the relationship of VitD status and clinical outcomes was lost in the adjusted model (39). Lastly, Polyzos et al., conducted a robust retrospective study of women who had a single ET with a day 5 blastocyst (30). Here, researchers found VitD sufficiency was significantly associated with clinical pregancy chance in an adjusted model accounting for numerous confounding factors, inclusive of embryo quality (30). There is an evident lack of well-designed statistical models in the literature which utilise important confounding factors that significantly impact clinical outcomes, namely transferred embryo number and quality. In our robust, well-designed, multivariate adjusted logistic regression models, no positive association between VitD sufficiency and clinical outcomes was observed. Future studies (both retrospective or propective in nature) should aim to utilise such models and account for embyro quality as an important predictor of IVF success.

The major limitation of the present retrospective study is the inconsistent and varied timing of serum VitD measurement. Many retrospective studies investigating the relationship between VitD and clinical outcomes in IVF do not report the timing of VitD testing at all (31, 42, 44, 46), while others measure VitD on the day of OPU (53), trigger (45), or 7 days prior to ET (30). Another limitation is the present study is female focused and therefore did not investigate any potential male impact of fertilisation and subsequent blastocyst development, CP and LB. However, data from our clinic suggests that male age at the time of semen collection nor the semen quality influence IVF outcomes (329). Additionally, the exclusion of subsequent frozen cycles (in which there are improved CPRs compared to fresh cycles (273, 275)), may be limiting potential relationships with VitD in frozen cycles. Previous data from PIVET has demonstrated frozen cycles under hormonal control, with an optimal mid-luteal P4 concentration of 70–99 nmol/L, is related to significantly improved CPRs, irrelevant to frozen embryo quality (275). Lastly, the nature of retrospective studies themselves are prone to limitations such as convience sampling and selection bias.

One of the major strengths of this retrospective study is the relatively large sample size of 263 women, who underwent263 initiated IVF cycles. The other major strength

is the robust statistical approach applied to the adjusted multivariate models, which accounts for numerous confounding factors. Firstly, the use of univariate models to identify variables individually associated with the clinical outcomes of interest. Secondly, the application of backwards elimination in the adjusted models to reduce potential selection bias from the researchers. Furthermore, by displaying alternative models in the supplementary data to show the outcomes were not altered when the omitted variables (which were directly related to variables used in the adjusted models), consequently reducing multicolinerality within the adjusted models and potential selection bias. To date the best retrospective analysis of VitD status and women undergoing IVF was conducted by Polyzos et al (2014), as the researchers consistently measured serum VitD 7 days prior to ET, and implemented similar robust statistical analysis methods (30). However, in this study we present blastocyst development as an additional, non-traditional clinical outcome which has not been investigated in regards to its' association with VitD status to the best of our knowledge. Blastocyst development is an important outcome to consider as blastocyst quality is an important predictor of CP and LB outcomes in IVF. Additionally, investigating how VitD influences blastocyst development may identify a more direct link, than downstream outcomes such as CP and LB.

3.4 Conclusion

In conclusion, the present study found no association between VitD and clinical outcomes of women undergoing IVF. Despite the robust statistically analysis methods utilised here, the inconsistent timing of VitD between cycles largely impacts the confidence of the findings. In the future, well-designed prospective clinical studies and randomised control trials are essential to further elucidate the potential impact of VitD status on clinical outcomes in IVF, with robust statistical models (as detailed in the present study) and consistent measurement of serum VitD status to further investigate the potential impact of VitD status on clinical outcomes of women undergoing IVF. Strengths and limitations of both study design and gaps in knowledge identified in the present study were used to inform the prospective study detailed in Chapter 4, which investigated the impact of serum and FF VitD in IVF cycles with a fresh ET.

Chapter Four

VitD status and outcomes in patients undergoing IVF: a cross sectional observational cohort study.

4.0 Introduction

VitD insufficiency is a serious public health issue with an estimated 1 billion people worldwide having sub-optimal VitD levels (5). Classically, VitD was known for its association with optimal bone mineral density (330), however in recent years there has been an increasing interest in non-skeletal effects of VitD, and the association of sub-optimal levels with various metabolic conditions and disorders such as obesity, type 2 diabetes mellitus, autoimmune diseases and infertility (27, 331-333). Difficulties arise in understanding the relationship between VitD and various diseases, which is partially driven by technical limitations in measuring hydroxylated metabolites and the lack of well-designed clinical studies (334, 335). There are numerous factors known to be associated with serum 25-(OH)D levels including (but not limited to) physical characteristics (such as age, gender, and BMI), ethnicity/geographical location, genetic factors, and socio-economic determinants (336-340).

Over the last decade the VDR and VitD metabolising enzymes have been found to be distributed across various animal and human reproductive tissues, including ovarian, endometrium, placental, epididymis, prostate, seminal vesicles, and spermatozoa (58, 82, 232, 341). Although, the precise action of VitD in reproductive tissues remains elusive, these findings suggest an active function of VitD in these tissues which could have implications for VitD in human infertility.

A strong association between VitD status and PCOS patients has been established in numerous studies to date (342-346). In contrast, the relationship between VitD status and women with alternative infertility aetiologies or unexplained infertility is limited and inconclusive (347, 348). Recent reports have suggested 25-(OH)D was positively associated with CPRs in women undergoing IVF (19, 31, 35). However, comparisons between reports are difficult as there is an inconsistency in study design and inclusion

criteria. These include the recruitment/inclusion of only PCOS patients, oocyte donor recipients, women of a certain age, ethnicity, or BMI group.

For example, Garbedian et al. investigated VitD status of women aged 18-41 years old, and did not exclude based on PCOS-status, however patients with a FSH level ≥ 12 IU/L (cycle day 3) were excluded from analysis (35). For their analysis, Paffoni et al. included women aged 18-42 years with adequate ovarian reserve and of a healthy BMI (18.0-25.0 kg/m^2), although it is unclear if PCOS patients were included (19). Lastly, the study by Rudick et al. was a retrospective cohort study of 99 recipients of oocyte donation, and it is unclear if PCOS patients were included in this analysis (31). Discrepancies between study designs (particularly for inclusion/exclusion criteria) and the lack of reporting of key features (such as PCOS-status) are important factors to consider when drawing conclusions from these studies. There is an evident lack of studies in the area which focus on non-PCOS patients.

To the best of our knowledge, Polyzos et al. have the most well-designed prospective study in the area to date (30). In this relatively large study (n=368) of women undergoing IVF with a single ET with a day 5 embryo, 16 variables potentially related to their main outcome of interest (CP) were included as confounding factors for analysis (30). Despite the well-designed nature of this study, the authors did not investigate the relationship between VitD and embryo development. Currently no studies have investigated the association between VitD status in IVF and blastocyst development (as an outcome in multivariate logistic regression analysis). This could be an important link between improved CPRs seen in various studies that has yet to be elucidated (19, 31, 35).

The overall aim of the present prospective study was to identify if and how VitD status is associated with patient characteristics, embryological measures, and clinical outcomes in a mixed cohort of non-PCOS women undergoing IVF with a fresh ET.

4.0.1 Chapter Four Objectives:

4) To examine if patient and cycle characteristics are associated with VitD sufficiency (using a 20 ng/mL cut off),
5) Determine if serum and FF VitD status are correlated.

6) Investigate how serum and FF VitD status are associated with embryological measures and clinical outcomes, including blastocysts development, CP, and LB chance.
7) Assess if patient biometrics (analysed by bioelectrical impendence analysis) is related to VitD sufficiency and clinical outcomes.

4.1 Cross sectional study: Results (Whole cohort)

4.1.1 Patient recruitment

A total of 392 patients who gave informed consent for the present study were prospectively recruited from our private IVF clinic. Of the total 392 patients, 32 patients were excluded from the final analysis prior to OPU (Fig. 4.1) due to PCOS diagnosis (n=21), cycle cancellation due to failed fertilisation or OPU (n=8) and excessive follicle recruitment following stimulation (n=3). Of the total consenting patients, 91.8% (n=360) were scheduled for a fresh ET, and a further 71 patients were excluded from the final analysis (Fig 4.1) due to a conversion to freeze all cycle (n=64), failed OPU or fertilisation (n=2), PGD (n=1) or having insufficient sample for VitD testing (n=4. An additional 2 patients were excluded following successful ET and VitD testing due to no LB outcome (due to pregnancy termination), leaving a total of 287 patients who had a fresh ET with a measurable outcome for our final analysis (Fig. 4.1). Based on the IOM guidelines, 41.8% of patients had inadequate VitD levels (< 20 ng/mL, n=120), and 58.2% had adequate levels (≥ 20 ng/mL, n=167) (Fig. 4.1), while only 3.1% of patients had a serum VitD level of < 10 ng/mL (n=9) and 14.6% of patients had a serum VitD level ≥ 30 ng/mL (n=42, Fig. 4.1).

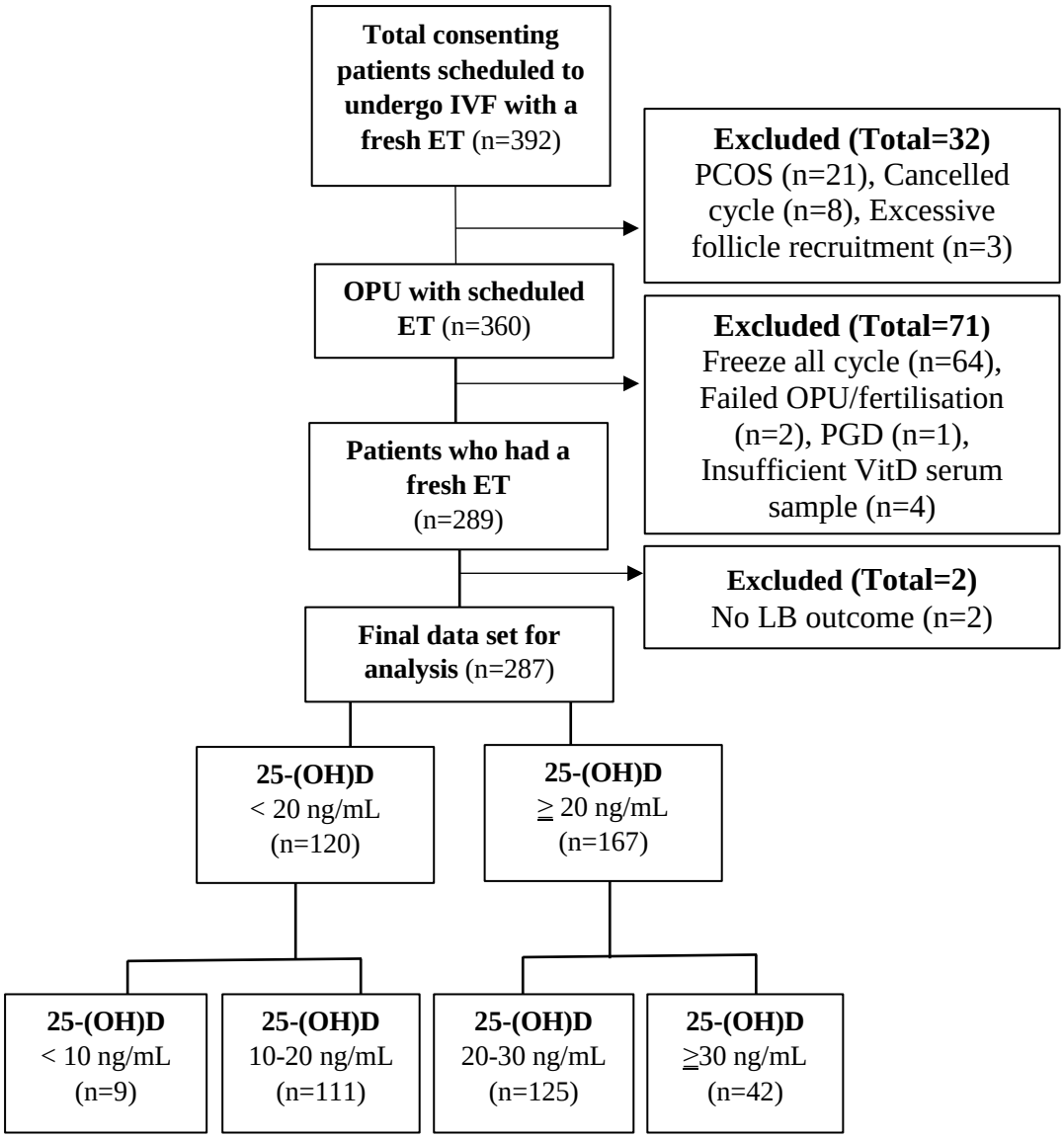


Figure 4.1: Flow diagram detailing the recruitment of consenting patients and final data analysis for whole cohort. Data for consenting patients was extracted from the PIVET database and cases were removed if the patient was diagnosed with PCOS, there was no fresh ET, the cycle was cancelled, failed, or converted to a freeze all, where there was insufficient sample for VitD measurement or no measurable LB outcome. *Abbreviations: ET, embryo transfer; PCOS, polycystic ovarian syndrome; OPU, oocyte pick up; PGD, pre-implantation genetic diagnosis; 25-(OH)D, 25-hydroxyvitamin D.*

4.1.2 Patient demographics and characteristics

A significantly higher proportion of Caucasian patients were in the ≥ 20 ng/mL VitD group compared to the < 20 ng/mL (96.4% vs. 85.8%, respectively, p=0.001, Table 4.1). The median BMI of patients in the ≥ 20 ng/mL group was lower than the < 20 ng/mL group but was not statistically significant (23.2 vs. 24.9 kg/m^2, p=0.088, Table 4.1).

There was no difference in the proportion cases where VitD testing or ET was performed during summer/autumn months in the ≥ 20 ng/mL compared to the < 20 ng/mL group (43.7% vs. 41.7%, respectively, p=0.730, Table 4.1). There was also no difference in the proportion of patients taking VitD supplementation between the ≥ 20 ng/mL group compared to the < 20 ng/mL group (95.8% vs. 94.2%, respectively, p=0.524, Table 4.1). The ≥ 20 ng/mL group and < 20 ng/mL group were comparable in terms of median female age (p=0.455) and partner age (p=0.918) at the time of cycle, AFC grouping (p=0.492) and previous IVF cycles (p=0.797, Table 4.1).

There were no significant differences between the groups for infertility aetiology, including endometriosis (4.2% vs. 4.2%, < 20 ng/mL vs. ≥ 20 ng/mL), tubal defect (11.7% vs. 6.6%, < 20 ng/mL vs. ≥ 20 ng/mL), male factor infertility (23.3% vs. 23.4%, < 20 ng/mL vs. ≥ 20 ng/mL) or unexplained infertility (66.7% vs. 76.0%, < 20 ng/mL vs. ≥ 20 ng/mL, Table 4.1).

Table 4.1: Patient demographics stratified by serum VitD status (20 ng/mL cut off).

Variable	< 20 ng/mL	≥ 20 ng/mL	p-value
Initiated cycles, n	120	167	-
Mean Serum VitD level, ng/mL	15.5 ± 3.4	28.0 ± 8.1	<**0.001**
Median Serum VitD level, ng/mL	16.2 (5.1)	25.6 (7.5)	<**0.001**
Serum VitD level range, ng/mL	4.0-19.8	20.0-72.0	-
Season of ET/VitD Test, n/total [%]	-	-	0.730
Winter/Spring	70/120 [58.3]	94/167 [56.3]	-
Summer/Autumn	50/120 [41.7]	73/167 [43.7]	-
Female age at cycle, years	35.0 (8.0)	35.0 (8.0)	0.455
Partner age at cycle, years	38.0 (7.0)	38.0 (9.0)	0.918
BMI, kg/m^2	24.9 (8.2)	23.2 (5.9)	0.088
Ethnicity, n/total [%]	-	-	**0.001**
Caucasian	103/120 [85.8]	161/167 [96.4]	-
Non-Caucasian[#]	17/120 [14.2]	6/167 [3.6]	-
Consuming VitD supplements, n/total [%]	113/120 [94.2]	160/167 [95.8]	0.524
AFC Group	-	-	0.492
Group A (≥ 20 follicles)	40/120 [33.3]	45/167 [26.9]	-
Group B/C (9-19 follicles)	55/120 [45.8]	82/167 [49.1]	-
Group D/E (≤ 8 follicles)	25/120 [20.8]	40/167 [24.0]	-
Infertility aetiology, n/total [%]	-	-	-
Endometriosis	5/120 [4.2]	7/167 [4.2]	0.992
Tubular defect	14/120 [11.7]	11/167 [6.6]	0.132
Male factor	28/120 [23.3]	39/167 [23.4]	0.997
Unexplained	80/120 [66.7]	127/167 [76.0]	0.080
Previous IVF cycle, n/total [%]	-	-	0.797
First cycle	59/120 [49.2]	92/167 [55.0]	-
Second cycle	32/120 [26.7]	40/167 [24.0]	-
≥ Third cycle	29/120 [24.1]	35/167 [21.0]	-

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- ET, embryo transfer; BMI, body mass index; AFC, anral follicle count. [#]Non-Caucasian include those of Indigenous, African and Asian ethnicity, as self-reported by patients.*

4.1.3 Patient cycle characteristics

The patient cycle characteristics were highly comparable between the VitD groups in various aspects. In the ≥ 20 ng/mL group, the proportion of patients who received an antagonist cycle was 33.7% lower than in the < 20 ng/mL group, however this was not statistically significant (30.5% vs. 64.2% respectively, p=0.346, Table 4.2). Most patients received insemination via ICSI, and the proportion of cases in the ≥ 20 ng/mL and the < 20 ng/mL VitD groups was not significantly different (82.6% vs. 85.0%, p=0.258, Table 4.2). The median rFSH and trigger dose was identical in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (200.0 vs. 200.0, p=0.962, 500.0 vs. 500.0, p=0.897, respectively, Table 4.2). There was no significant difference in the ≥ 20 ng/mL group compared to the < 20 ng/mL group for endometrial thickness (9.5 vs. 9.4 mm respectively, p=0.953, Table 4.2).

The proportion of patients in the ≥ 20 ng/mL group who received a day-5 ET was 6% higher compared to the < 20 ng/mL group, but this was not statistically significant (48.5% vs. 42.5% respectively, p=0.129, Table 4.2). Furthermore, the proportion of patients in the ≥ 20 ng/mL group who received a high-quality blastocyst at ET was only 1% higher compared to the < 20 ng/mL group (59.3% vs. 58.3%, p=0.986, Table 4.2). Most patients received a single ET, and the proportion of cases in the ≥ 20 ng/mL and the < 20 ng/mL VitD groups was not significantly different (86.8% vs. 85.0%, p=0.659, Table 4.2).

Table 4.2: Cycle characteristics stratified by serum VitD status (20 ng/mL cut off).

Variable *VitD Range*	**< 20 ng/mL (4.0-19.8)**	**≥ 20 ng/mL (20.0-72.0)**	**p-value**
Initiated cycles, n	120	167	-
Median Serum VitD level, ng/mL	16.2 (5.1)	25.6 (7.5)	**<0.001**
Stimulation protocol, n/total [%]	-	-	0.346
Antagonist	77/120 [64.2]	51/167 [30.5]	-
Agonist/Other	43/120 [35.8]	116/167 [69.5]	-
Insemination type, n/total [%]	-	-	0.258
ICSI	102/120 [85.0]	138/167 [82.6]	-
IVF	3/120 [2.5]	1/167 [0.6]	-
ICSI/IVF split	15/120 [12.5]	28/167 [16.8]	-
rFSH dosage, IU	200.0 (256.3)	200.0 (212.5)	0.962
Trigger dose, µg	500.0 (0.0)	500.0 (0.0)	0.897
Endometrial thickness, mm	9.4 (2.3)	9.5 (3.0)	0.953
ET Day, n/total [%]	-	-	0.129
Day 2/3	41/120 [34.2]	39/167 [23.4]	-
Day 4	28/120 [23.3]	47/167 [28.1]	-
Day 5	51/120 [42.5]	81/167 [48.5]	-
Transferred Embryo quality, n/total [%]	-	-	0.986
High quality blastocyst	70/120 [58.3]	99/167 [59.3]	-
Medium quality blastocyst	16/120 [13.3]	22/167 [13.2]	-
Low quality blastocyst/Day 3	34/120 [28.3]	46/167 [27.5]	-
Embryos at transfer, n/total [%]	-	-	0.659
Single ET	102/120 [85.0]	145/167 [86.8]	-
Double ET	18/120 [15.0]	22/167 [13.2]	-

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- ICSI, intracytoplasmic sperm injection; rFSH, recombinant follicle stimulating hormone; ET, embryo transfer.*

4.1.4 Patient hormonal profile

There was no significant change in the median hormone levels between the ≥ 20 ng/mL group and the < 20 ng/mL VitD groups including: AMH (14.9 vs. 17.4 pmol/L, p=0.172), progesterone at trigger (2.0 vs. 2.1 ng/mL, p=0.584) or OPU (20.0 vs. 22.0 ng/mL, p=0.141), estradiol at OPU (3400.0 vs. 3350.0 pmol/L, p=0.995) or mid-luteal phase (3600.0 vs. 4450.0 pmol/L, p=0.192), prolactin (270.0 vs. 290.0 mIU/L, p=0.478), or luteinising hormone (0.7 vs. 0.7 IU/L, p=0.634, both at OPU; Table 4.3).

There was also no change in the ≥ 20 ng/mL and < 20 ng/mL VitD groups for any of the androgen panel hormones assessed (expressed as median values) including: SHBG (88.0 vs. 75.8 nmol/L, p=0.345), total (0.7 vs. 0.7 nmol/L, p=0.623) or free (6.1 vs. 6.4 ng/mL, p=0.838) testosterone, free androgen index (0.8 vs. 0.8 [ratio], p=0.970) or DHEA (5.4 vs. 4.6, p=0.202 ng/mL; Table 4.3).

Table 4.3: Patient hormonal profile stratified by serum VitD status (20 ng/mL cut off).

Variable *VitD range*	**< 20 ng/mL** (4.0-19.6)	**≥ 20 ng/mL** (20.0-59.7)	**p value**
Initiated cycles, n	120	167	-
Median Serum VitD level, ng/mL	16.2 (5.1)	25.6 (7.5)	<0.001
AMH, pmol/L	17.4 (20.5)	14.9 (18.3)	0.172
Progesterone at trigger, ng/mL	2.1 (2.0)	2.0 (1.0)	0.584
OPU			
Estradiol, pmol/L	3350.0 (3300.0)	3400.0 (3100.0)	0.995
Progesterone, ng/mL	22.0 (19.0)	20.0 (18.0)	0.141
Prolactin, mIU/L	290.0 (150.0)	270.0 (180.0)	0.478
Luteinising hormone, IU/L	0.7 (1.3)	0.7 (1.7)	0.634
Mid Luteal phase			
Estradiol, pmol/L	4450.0 (4850.0)	3600.0 (3500.0)	0.192
Progesterone, ng/mL	313.5 (250.0)	292.0 (211.0)	0.454
Androgens			
SHBG, nmol/L	75.8 (49.4)	88.0 (68.3)	0.345
Total Testosterone, nmol/L	0.7 (0.8)	0.7 (0.7)	0.623
Free Androgen Index, ratio	0.8 (1.2)	0.8 (1.1)	0.970
Free Testosterone, ng/mL	6.4 (8.4)	6.1 (8.2)	0.838
DHEA, ng/mL	4.6 (4.8)	5.4 (3.4)	0.202

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- AMH, anti Mullerian hormone; OPU, oocyte pick-up; SHBG, sex hormone binding globulin (SHBG); DHEA, dehydroepiandrosterone.*

4.1.5 Embryological measures and outcomes

There were no significant differences observed between the two VitD groups (≥ 20 ng/mL vs. < 20 ng/mL) in relation to the median number of total oocytes collected (9.0 vs. 10.0 p=0.375), or number of MII oocytes collected (7.0 vs. 6.0, p=0.334; Table 4.4). Similarly, there were no difference observed between the two VitD groups (≥ 20 ng/mL vs. < 20 ng/mL) in the median number of 2PNs generated per cycle (5.0 vs. 4.0, p=0.364, Table 4.4).

The median fertilisation rate (when expressed as the number of 2PN's generated/insemination per cycle) was not significantly increased in the ≥ 20 ng/mL compared to the < 20 ng/mL (78.3% vs. 77.8, p=0.835, Table 4.4). The overall fertilisation rate (when expressed as the sum of total number of 2PNs generated per group/total sum of MII oocytes collected of the whole group) was not significantly increased in the ≥ 20 ng/mL compared to the < 20 ng/mL (79.0% vs. 76.7%, p=0.226, Table 4.4).

Blastocyst development rate (BDR, %) was significantly higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, when expressed in several ways (per number of total oocytes/MII oocytes/2PN's generated) (Table 4.4). When expressed as the total number of blastocysts generated/the number of oocytes collected, BDR was 6.0% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (20.7% vs. 14.7%, p=<0.001, Table 4.4). Similarly, when expressed the total number of blastocysts generated/the number of MII oocytes collected, BDR was 6.9% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (28.4% vs. 21.5%, p=<0.001, Table 4.4). Lastly, when expressed as the total number of blastocysts generated/the number of 2PNs generated, BDR was 8.0% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (36.0% vs. 28.0%, p=0.009). Additionally, the percentage of cycles developing at least one blastocyst was 17.2% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (68.0% vs. 50.8%, p=0.004; Table 4.4). Finally, the mean number of blastocysts generated per cycle was significantly higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (2.11 vs. 1.49, p=0.026, Table 4.4).

There was no significant difference in the mean number of embryos transferred per cycle in the < 20 ng/mL vs. ≥ 20 ng/mL, when expressed as mean (1.2 vs.1.1, p=0.661) or median (1.0 vs. 1.0, p=0.940, Table 4.4). However, there was a significant increase

in the mean number of cryopreserved embryos per cycle in the ≥ 20 ng/mL vs. < 20 ng/mL, when expressed as mean (1.9 vs.1.4, p=0.030, Table 4.4). The median oocyte and embryo utilisation rates were 4.4% and 6.0% (respectively) higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, although this was not statistically significant (29.4 vs. 25.0, p=0.314; 56.0 vs. 50.0, p=0.314; Table 4.4).

The fresh CPR was 9.9% higher in the < 20 ng/mL group compared to the ≥ 20 ng/mL group, but this was not statistically significant (40.7% vs. 30.6%, p=0.086, Table 4.4). Similarly, the fresh LBR was 7.1% higher in the < 20 ng/mL group compared to the ≥ 20 ng/mL group, although this was not statistically significant (32.9% vs. 25.8%, p=0.195, Table 4.4). There was no statistically significant difference in miscarriage rate between the ≥ 20 ng/mL and < 20 ng/mL VitD groups (19.1% vs. 16.2%, p=0.712, Table 4.4).

Cumulative CPR (when expressed per ET) was not different between the in the ≥ 20 ng/mL and < 20 ng/mL VitD groups (35.6% vs. 31.1%, p=0.293, Table 4.4). Cumulative LBR (when expressed per ET) was 3.4% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (25.4% vs. 22.0%, p=0.377, Table 4.4).

Table 4.4: Embryological measures and outcomes stratified by serum VitD status (20 ng/mL cut off).

Variable *VitD Range*	**< 20 ng/mL (4.0-19.8)**	**≥ 20 ng/mL (20.0-72.0)**	**p-value**
Initiated cycles, n	120	167	-
Oocytes			
Total oocytes collected, n	1155	1685	-
Median Oocytes collected per cycle, n	10.0 (7.0)	9.0 (8.0)	0.375
Total MII oocytes collected, n	791	1225	-
Median MII oocytes collected per cycle, n	6.0 (5.0)	7.0 (6.0)	0.334
Fertilisation			
Total fertilised (2PN), n	607	968	-
Overall fertilisation rate, n/inseminated [%]	607/791 [76.7]	968/1225 [79.0]	0.226
Median fertilised (2PN) per cycle, n	4.0 (4.0)	5.0 (5.0)	0.364
Median fertilisation rate % per cycle, %	77.8 (38.1)	78.3 (25.9)	0.835
Cleavage Embryos			
Total embryos cultured beyond day 2, n	557	936	-
High quality day 3 embryos	348	634	-
Blastocysts			
Total blastocysts formed, n	170	348	-
High quality blastocysts	98	221	-
Blastocysts rate per oocytes collected, n/total [%]	170/1155 [14.7]	348/1685 [20.7]	**<0.001**
Blastocysts rate per MII collected, n/total [%]	170/791 [21.5]	348/1225 [28.4]	**<0.001**
Blastocyst rate per 2PN, n/total [%]	170/607 [28.0]	348/968 [36.0]	**0.009**
Mean blastocyst number generated per cycle, n	1.49 ± 2.07	2.11 ± 2.50	**0.026**
Mean blastocyst development rate, %	21.8 ± 26.5	30.0 ± 28.3	**0.013**
Cycles developing at least 1 blastocyst, n/total [%]	61/120 [50.8]	113/167 [68.0]	**0.004**

Transfer & Cryopreservation			
Total embryos transferred, n	138	189	-
Mean embryos transferred per cycle, n	1.2 ± 0.4	1.1 ± 0.3	0.661
Total embryos cryopreserved, n	164	320	-
Mean embryos cryopreserved per cycle, n	1.4 ± 1.8	1.9 ± 2.3	**0.030**
Median oocyte utilisation rate, %	25.0 (23.9)	29.4 (25.5)	0.314
Median embryo utilisation rate, %	50.0 (42.0)	56.0 (54.0)	0.314
Pregnancy, Miscarriage & Live Births			
Fresh CPR, n/total [%]	37/120 [30.8]	68/167 [40.7]	0.086
Fresh LBR, n/total [%]	31/120 [25.8]	55/167 [32.9]	0.195
Fresh miscarriage rate, n/total CP [%]	6/37 [16.2]	13/68 [19.1]	0.712
Cumulative CPR (Fresh + Frozen per ET), [%]	65/209 [31.1]	105/295 [35.6]	0.293
Cumulative LBR (Fresh + Frozen per ET), n/total [%]	46/209 [22.0]	75/295 [25.4]	0.377

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- MII, metaphase II oocytes; 2PN, two pronuclear zygotes; CPR; clinical pregnancy rate, CP, clinical pregnancy.* Oocyte utilisation rate equals the sum of embryos transferred and frozen, divided by the number of oocytes collected in that cycle. Embryo utilisation rate equals the number of embryos transferred and frozen, divided by the number of 2PN generated in that cycle.

4.1.6 Univariate model of factors associated with VitD sufficiency

Patient demographics, cycle characteristics, embryological measures and clinical outcomes variables were explored in a univariate logistic regression model to investigate if changes in these variables were associated with an increased chance of a patient having a sufficient VitD status (≥ 20 ng/mL) which was assessed as the binary dependent variable.

Interestingly, summer and autumn months were not associated with having a sufficient VitD status (≥ 20 ng/mL), when compared to winter and spring months (OR=1.10, CI=0.68-1.76, p=0.706, Table 4.5). Female age at cycle was not associated with patient serum VitD sufficiency (OR=0.99, 0.94-1.03, p=0.531, Table 4.5). When expressed as a continuous variable, BMI was significantly associated with VitD sufficiency, with every increasing unit of BMI resulting in a 6% decrease in the likelihood to be in the VitD sufficient group (OR=0.94, CI=0.90-0.99, p=0.017, Table 4.5). Furthermore, when expressed as a categorical variable, patients in the normal BMI range (18.5-24.9 kg/m^2) were 2.46 times more likely to be in the sufficient VitD group compared to those in the obese BMI range (≥ 30 kg/m^2; OR= 2.45, CI=1.28-4.72, p=0.007, Table 4.5). However, there was no significant association between the overweight (25.0-29.9 kg/m^2, OR=1.89, CI=0.91-3.93, p=0.089) or the underweight (<18.5 kg/m^2) group (OR=1.90, CI=0.65-5.54, p=0.240, Table 4.5) with VitD sufficiency. Caucasian women were 4.44 times more likely to be in the VitD sufficient group compared to Non-Caucasian women (OR=4.44, CI=1.70-11.63, p=0.002, Table 4.5).

For every single increase in day-3 embryos in culture, the likelihood of being VitD sufficient increased by 7% (OR=1.07, CI=1.01-1.14, p=0.034, Table 4.5). Additionally, for every blastocyst generated per cycle, the likelihood of being in the VitD sufficient group increased by 13% (OR=1.13, CI=1.01-1.26, p=0.029, Table 4.5). When blastocysts generated was expressed as a percentage (the number of blastocysts generated/the number of MII oocytes collected), for every single percentage increase in blastocysts generated per cycle, the likelihood of being in the VitD sufficient group increased by 2% (OR=1.02, CI=1.01-1.03, p=0.008, Table 4.5). Lastly, for cycles where at least one blastocyst was formed, these patients were 2.03 times more likely to be in the ≥ 20 ng/mL VitD group compared those cycles where no blastocysts were

formed (OR=2.03, CI=1.25-3.28, p=0.004, Table 4.5). Cycles with a CP or a LB were not significantly associated with VitD sufficiency (OR=1.53, CI=0.93-2.51, p=0.092; OR=1.40, CI=0.83-2.36, p=0.204, Table 4.5).

Table 4.5: Univariate logistic regression model for factors associated with VitD sufficiency. Associations are presented as odds ratio (OR) and 95% confidence interval (CI).

Variable		Likelihood to be VitD sufficient (≥ 20 ng/mL) OR (95% CI)	p-value
Season of ET/VitD Test	Winter/Spring	1.00	-
	Summer/Autumn	1.10 (0.68 -1.76)	0.706
Female age, years		0.99 (0.94 - 1.03)	0.513
BMI, kg/m^2		0.94 (0.90 - 0.99)	**0.017**
BMI Group	≥ 30 kg/m^2	1.00	-
	< 18.5 kg/m^2	1.90 (0.65 - 5.54)	0.240
	18.5-24.9 kg/m^2	2.46 (1.28 - 4.72)	**0.007**
	25.0-29.9 kg/m^2	1.89 (0.91 - 3.93)	0.089
AMH, pmol/L		1.00 (0.99 - 1.02)	0.491
AFC Group	D/E (≤ 8 follicles)	1.00	-
	B/C (9-19 follicles)	0.93 (0.51 - 1.71)	0.819
	A (≥ 20 follicles)	0.72 (0.37 - 1.38)	0.716
Ethnicity	Non-Caucasian	1.00	-
	Caucasian	4.44 (1.70 - 11.63)	**0.002**
Infertility aetiology	Tubal	0.53 (0.23 - 1.22)	0.532
	Endometriosis	1.00 (0.31 - 3.24)	0.997
	Unexplained	1.60 (0.95 - 2.68)	0.790
ART attempt, n		0.98 (0.83 - 1.17)	0.840

Previous IVF	≥ 3 Events	1.00	-
	0 Events	1.29 (0.58 - 2.86)	0.537
	1 Event	1.02 (0.43 - 2.42)	0.972
	2 Events	0.91 (0.34 - 2.47)	0.859
Stimulation Type	Antagonist	1.00	-
	Agonist	0.78 (0.48 - 1.29)	0.337
Insemination type	ICSI Only	1.00	-
	IVF Only	0.24 (0.03 - 2.39)	0.226
	IVF-ICSI Split	1.37 (0.70 - 2.70)	0.358
rFSH dose, IU	≥ 400-600	1.00	-
	200-400		
	< 200		
Progesterone at trigger, ng/mL		0.93 (0.78 - 1.11)	0.423
Trigger dose, μg	≥ 10,000	1.00	-
	< 10,000		
Endometrial thickness, mm		1.01 (0.83 - 1.24)	0.909
ET day, n		1.24 (0.981 - 1.58)	0.071
Transferred embryo quality	Low	1.00	-
	High	1.05 (0.61 – 1.79)	0.872
	Med	1.02 (0.47 – 2.22)	0.968
Embryos transferred, n		0.86 (0.44 - 1.68)	0.651
Oocytes collected per cycle, n		1.02 (0.97 - 1.06)	0.463
MII oocytes collected per cycle, n		1.04 (0.98 - 1.10)	0.168
Fertilised (2PN) per cycle, n		1.05 (0.99 - 1.13)	0.117
Fertilisation rate per insemination, %		1.00 (0.99 - 1.02)	0.207
D3 embryos in culture per cycle, n		1.07 (1.01 - 1.14)	**0.034**
Blastocyst number generated per cycle, n		1.13 (1.01 - 1.26)	**0.029**

Blastocyst percentage generated per cycle, %		1.02 (1.01 - 1.03)	**0.008**
Proportion of high-quality blastocysts generated per cycle, %		1.01 (1.00 - 1.03)	0.059
Embryos cryopreserved, n		1.12 (0.99 - 1.26)	0.053
Oocyte utilisation rate per cycle, %		1.01 (1.00 - 1.02)	0.110
Embryo utilisation rate per cycle, %		1.00 (1.00 - 1.01)	0.455
Cycles with at least one Blastocyst	No	1.00	-
	Yes	2.03 (1.25 - 3.28)	**0.004**
Cycles with a Clinical Pregnancy	No	1.00	-
	Yes	1.53 (0.93 - 2.51)	0.092
Cycles with a Live Birth	No	1.00	-
	Yes	1.40 (0.83 - 2.36)	0.204

OR= Odds ratio. CI= Confidence interval. Abbreviations- ET, embryo transfer; BMI, body mass index; AMH, anti-Mullerian hormone; AFC, antral follicle count; 2PN, two pronuceli zygotes. Oocyte utilisation rate equals the sum of embryos transferred and frozen, divided by the number of oocytes collected in that cycle. Embryo utilisation rate equals the number of embryos transferred and frozen, divided by the number of 2PN generated in that cycle.

4.1.7 Multivariate model of embryological measures associated with VitD sufficiency

Each embryological measure was explored to see if increases in these variables were associated with an increased chance of VitD sufficiency (dependent variable). A series of stepwise logistic regression models were developed for each specific embryological measure, controlling for factors known to be associated with IVF success and VitD sufficiency, including female age at cycle, season of ET/VitD test, BMI, ethnicity, AMH, rFSH & trigger dose, progesterone level at trigger, stimulation type and IVF attempt number.

In the adjusted model, for every blastocyst generated per cycle, the likelihood of being in the VitD sufficient group increased by 37% (OR=1.37, CI=1.11-1.69, p=0.003, Table 4.6). Additionally, when blastocysts generated was expressed as a percentage (the number of blastocysts generated/the number of MII oocytes collected), for every increase in the percentage of blastocysts generated per cycle the likelihood of being in the VitD sufficient group increased by 2% (OR=1.02, CI=1.01-1.04, p=0.001, Table 4.6). In the adjusted model, for every increase in the number of high-quality blastocysts, these patients were 1% more likely to be in the ≥ 20 ng/mL VitD group compared to the < 20 ng/mL group (OR=1.01, CI=1.01-1.02, p=0.040, Table 4.6). For every increase in the number of cryopreserved embryos, patients were 37% more likely to be in the ≥ 20 ng/mL VitD group compared to the < 20 ng/mL group (OR=1.37, CI=1.11-1.70, p=0.004, Table 4.6). Lastly, for every increase in the percentage of oocyte utilisation rate, patients were 3% more likely to be in the ≥ 20 ng/mL group (OR=1.03, CI=1.01-1.05, p=0.005, Table 4.6). Conversely, embryo utilisation rate was not significantly associated with VitD sufficiency (OR=1.01, CI=1.00-1.02, p=0.091, Table 4.6),

Table 4.6: Multivariate logistic regression model of embryological measures and their association with VitD sufficiency.

Variable	Likelihood to be VitD sufficient (≥ 20 ng/mL) OR (95% CI)	p-value
↑ embryos transferred, n	0.57 (0.22 - 1.50)	0.253
↑ oocytes collected per cycle, n	0.99 (0.93 - 1.05)	0.643
↑ MII oocytes collected per cycle, n	1.03 (0.95 - 1.12)	0.445
↑ 2PNs per cycle, n	1.07 (0.97 - 1.18)	0.172
↑ fertilisation rate per insemination, %	1.01 (0.99 - 1.02)	0.442
↑ day 3 embryos in culture per cycle, n	1.08 (0.99 - 1.19)	0.091
↑ blastocyst number per cycle, n	1.37 (1.11 - 1.69)	**0.003**
↑ blastocyst development rate, %	1.02 (1.01 - 1.04)	**0.001**
↑ percentage of high-quality blastocysts generated, %	1.01 (1.01 - 1.02)	**0.040**
↑ embryos cryopreserved, n	1.37 (1.11 - 1.70)	**0.004**
↑ oocyte utilisation rate per cycle, %	1.03 (1.01 - 1.05)	**0.005**
↑ embryo utilisation rate per cycle, %	1.01 (1.00 - 1.02)	0.091

OR= Odds ratio. CI= Confidence interval. *Abbreviations- 2PN, two pronuclei zygotes.* Each embryological measure was adjusted for female age at cycle, BMI, season of ET/VitD test, ethnicity, AMH, rFSH & trigger dose, progesterone level at trigger, stimulation type and IVF attempt number in a forced-entry model.

4.1.8 Univariate model of factors associated with blastocyst development

Patient demographics, cycle characteristics, embryological measures and clinical outcomes were explored in a univariate logistic regression model to investigate if increases in these variables were associated with an increased chance of a cycle with at least one developing blastocyst (assessed as a binary dependent variable, yes = cycle with at least one blastocyst or no = a cycle where no blastocysts developed [reference value]).

Patients in the ≥ 20 ng/mL group were 2.02 times more likely have developed at least one blastocyst in their cycle, compared to patients in the < 20 ng/mL VitD group (OR=2.02, CI=1.25-3.28, p=0.004, Table 4.7). For every increasing year of female age at cycle, the likelihood of developing at least one blastocyst decreased by 13% (OR=0.87, CI=0.83-0.92, p=<0.001, Table 4.7). Conversely, for every increasing unit of AMH (pmol/L), the likelihood of developing at least one blastocyst increased by 3% (OR=1.03, CI=1.01-1.04, p=0.004, Table 4.7). Patients in the AFC group A (≥ 20 follicles) or group B and C (9-19 follicles) were 4.57 and 2.38 times (respectively) more likely to develop at least one blastocyst compared to those in the AFC groups D and E (< 8 follicles) (OR=4.57, CI=2.27-9.20, p=<0.001 & OR=2.38, CI=1.30-4.35, p=0.005, Table 4.7).

For every increasing ART attempt, the likelihood of developing at least one blastocyst decreased by 43% (OR=0.57, CI=0.46-0.71, p=<0.001, Table 4.7). When grouped, patients who had no previous IVF attempt were 11.52 times more likely to develop a blastocyst compared to patients who had ≥ 3 IVF attempts (OR=11.52, CI= 4.14-32.1, p=<0.001, Table 4.7). Patients who received an agonist or agonist-type cycle, were 73% less likely to develop a blastocyst compared to an antagonist cycle (OR=0.27, CI=0.16-0.45, p=0.918, Table 4.7).

For every unit increase in the number of total oocytes and MII oocytes collected the likelihood of developing a blastocyst increased by 29% and 52%, respectively (OR=1.29, CI=1.21-1.39, p=<0.001; OR=1.52, CI=1.36-1.69, p=<0.001, Table 4.7). Additionally, for every 2PN generated, the likelihood of developing at least one blastocyst increased by 86% (OR=1.86, CI=1.60-2.16, p=<0.001, Table 4.7). Lastly, for every increase in the number of day-3 embryos in culture the likelihood of

developing a blastocyst increased by 77% (OR=1.77, CI=1.35-2.03, p=<0.001, Table 4.7).

Table 4.7: Univariate logistic regression model for factors associated with blastocyst development.

Variable		Likelihood to develop blastocyst (OR, 95% CI)	p-value
Serum VitD Group, ng/mL	< 20 ng/mL	1.00	-
	≥ 20 ng/mL	2.02 (1.25-3.28)	**0.004**
Season of ET/VitD Test	Winter/Spring	1.00	-
	Summer/Autumn	0.72 (0.45-1.16)	0.174
Female age, years		0.87 (0.83-0.92)	**<0.001**
BMI, kg/m^2		0.98 (0.94-1.03)	0.494
BMI Group	≥ 30 kg/m^2	1.00	-
	< 18.5 kg/m^2	1.14 (0.38-3.40)	0.810
	18.5-24.9 kg/m^2	1.08 (0.56-2.09)	0.811
	25.0-29.9 kg/m^2	0.91 (0.44-1.90)	0.804
AMH, pmol/L		1.03 (1.01-1.04)	**0.004**
AFC Group	D/E (≤ 8 follicles)	1.00	-
	B/C (9-19 follicles)	2.38 (1.30-4.35)	**0.005**
	A (≥ 20 follicles)	4.57 (2.27-9.20)	**<0.001**
Infertility aetiology	Tubal	0.97 (0.42-2.25)	0.946
	Endometriosis	2.00 (0.53-7.55)	0.307
	Unexplained	0.85 (0.51-1.42)	0.537
ART attempt, n		0.57 (0.46-0.71)	**<0.001**
Previous IVF	≥ 3 Events	1.00	-
	0 Events	11.52 (4.14-32.1)	**<0.001**
	1 Event	7.37 (2.52-21.59)	**<0.001**
	2 Events	5.40 (1.67-17.50)	**0.005**
Stimulation type	Antagonist	1.00	-
	Agonist/Other	0.27 (0.16-0.45)	**<0.001**
Insemination type	IVF/IVF-ICSI Split	1.00	-
	ICSI only	0.23 (0.10-0.53)	**0.001**
rFSH dose, IU	≥ 200	1.00	-
	< 200	1.40 (0.87-2.25)	0.165
Progesterone at trigger, ng/mL		0.76 (0.62-0.94)	**0.010**
Trigger dose, μg	≥ 500	1.00	-
	< 500	0.84 (0.44-1.58)	0.582
Endometrial thickness, mm		0.98 (0.80-1.20)	0.844
Oocytes collected per cycle, n		1.29 (1.21-1.39)	**<0.001**
MII oocytes collected per cycle, n		1.52 (1.36-1.69)	**<0.001**
Fertilised (2PN) per cycle, n		1.86 (1.60-2.16)	**<0.001**
Fertilisation rate per insemination, %		1.02 (1.00-1.03)	**0.011**
D3 embryos in culture per cycle, n		1.77 (1.53-2.03)	**<0.001**

OR= Odds ratio. CI= Confidence interval. *Abbreviations- ET, embryo transfer; BMI, body mass index; AMH, anti-Mullerian hormone; AFC, antral follicle count; 2PN, two pronuclear zygotes; ART, assisted reproductive technology; rFSH, recombinant follicle stimulating hormone; MII, metaphase two oocyte; 2PN, two pronuclear zygote.*

4.1.9 Multivariate model of factors associated with blastocyst development

Significant patient and cycle characteristics, and embryological measures identified in Table 4.7, were assessed in an adjusted multivariate logistic regression model to assess which variables were independently associated with blastocyst development (binary dependent variable). In a stepwise backward elimination logistic regression model including VitD group (< 20 ng/mL vs. ≥ 20 ng/mL), female age at cycle, AMH, stimulation type, ART attempt number, and total number of oocytes collected per cycle, only AMH was not retained in the final adjusted model (p=≥0.05).

Patients in the ≥ 20 ng/mL group were 2.90 times more likely to develop a blastocyst than those in the < 20 ng/mL group (OR=2.90, CI=1.54-5.46, p=0.001, Table 4.8). For every increasing year of female age at cycle the likelihood of developing a blastocyst decreased by 7% (OR=0.93, CI=0.87-0.99, p=0.021, Table 4.8). Additionally, for every increasing ART attempt the likelihood of developing a blastocyst decreased by 43% (OR=0.57, CI=0.44-0.74, p=<0.001, Table 4.8). Patients who received an agonist or other cycle were 46% less likely to develop a blastocyst compared to those who underwent an antagonist cycle (OR=0.54, CI=0.27-1.10, p=0.044, Table 4.8). Lastly, for every increasing number of oocytes collected at OPU the likelihood of developing a blastocyst increased by 30% (OR=1.30, CI=1.20-1.41, p=<0.001, Table 4.8).

Table 4.8: Multivariate logistic regression model for factors associated with blastocyst development.

Variable		Likelihood for a blastocyst development OR (95% CI)	p-value
Serum VitD Group, ng/mL	< 20 ng/mL	1.00	-
	≥ 20 ng/mL	2.90 (1.54-5.46)	**0.001**
Female age, years		0.93 (0.87-0.99)	**0.021**
ART attempt, n		0.57 (0.44-0.74)	**<0.001**
Stimulation type	Antagonist	1.00	-
	Agonist/Other	0.54 (0.27-1.10)	**0.044**
Total oocytes collected, n		1.30 (1.20-1.41)	**<0.001**

OR= Odds ratio. CI= Confidence interval. *Abbreviations- ART, assisted reproductive technology. Step 1: All variables entered into the model. Step 2: AMH was removed from the model. Step 3: Final model.*

4.1.10 Univariate model of factors associated with clinical pregnancy chance

Patient demographics, cycle characteristics, embryological measures and clinical outcomes were explored in a univariate logistic regression model to investigate if changes in these variables were associated with an increased chance of a successful CP (assessed as the binary dependent variable).

VitD status was not significantly associated with CP chance (OR=1.54, CI=0.94-2.53, p=0.087, Table 4.9). Additionally, summer and autumn months when compared to winter and spring months were not associated with CP chance (OR=0.83, CI=0.51-1.35, p=0.458, Table 4.9).

For every increasing year of female age at cycle the likelihood of achieving a CP decreased by 13% (OR= 0.87, CI=0.83-0.92, p=<0.001, Table 4.9). In contrast, BMI was not associated with CP chance when expressed as a continuous (OR=1.00, CI=0.95-1.05 p=0.971), or as grouped variable comparing patients within the obese BMI range (≥ 30 kg/m2) to those in the overweight (25.0-29.9 kg/m2, healthy (18.5-24.9 kg/m2) or underweight (< 18.5 kg/m2) ranges (OR=1.38, p=0.403; OR= 1.31, p=0.440; OR=0.76, p=0.647, respectively, Table 4.9).

For every pmol/L increase in AMH level, the likelihood of achieving a CP increased by 3% (OR=1.03, 1.01-1.04, p=0.001, Table 4.9). Patients within the AFC group A (≥ 20 follicles) and the B/C groups (9-19 follicles) were 2.48 and 1.82 times (respectively) more likely to achieve a CP when compared to the AFC groups D and E (< 8 follicles), however only Group A was statistically significant (OR=2.48, CI=1.22-5.03, p=0.012 & OR=1.82, CI=0.94-3.52, p=0.077, Table 4.9).

Patients who underwent an agonist or 'other' (AACEP) ovarian stimulation regime were 56% less likely to achieve a CP compared to patients who received an antagonist regime (OR=0.0.44, CI=0.25-0.76, p=0.003, Table 4.9).

For every increase in the number of total and MII oocytes collected the likelihood of achieving a CP increased by 4% and 6%, respectively (OR=1.04, CI=0.99-1.09, p=0.0115; OR=1.06, CI=1.00-1.12, p=0.010, Table 4.9). Additionally, for every 2PN generated, blastocyst developed, and embryo cryopreserved, the likelihood achieving a clinical pregnancy increased by 9% (OR=1.09, CI=1.02-1.17, p=0.010, Table 4.9), 1% (OR=1.01, CI=1.01-1.02, p=0.011, Table 4.9) and 20% (OR=1.20, CI=1.06-1.35,

p=0.003, Table 4.9), respectively. For every increasing number of embryos transferred, the likelihood of achieving a CP decreased by 68% (OR=0.32, CI=0.14-0.76, p=0.009, Table 4.9). Lastly, for every increasing number of cryopreserved embryos, the chance of CP significantly increased by 20% (OR=1.20, CI=1.06-1.35, p=0.003, Table 4.9).

Lastly, patients who had either a high or medium quality embryo at ET were 2.08 and 2.57 times (respectively) significantly more likely to achieve a CP than those who received a low-quality blastocyst or day-3 embryo (OR=2.08, CI=1.19-3.64, p=0.010 & OR=2.57, CI= 1.26-5.22, p=0.009, Table 4.9).

Table 4.9: Univariate logistic regression model for factors associated with clinical pregnancy chance.

Variable		Likelihood of CP chance OR (95% CI)	p-value
Serum VitD Group, ng/mL	< 20 ng/mL	1.00	-
	≥ 20 ng/mL	1.54 (0.94-2.53)	0.087
Season of ET/VitD Test	Winter/Spring	1.00	-
	Summer/Autumn	0.83 (0.51-1.35)	0.458
Female age, years		0.87 (0.83-0.92)	**<0.001**
BMI, kg/m^2		1.00 (0.95-1.05)	0.971
BMI Group	≥ 30 kg/m^2	1.00	-
	< 18.5 kg/m^2	0.76 (0.23-2.47)	0.647
	18.5-24.9 kg/m^2	1.31 (0.66-2.58)	0.440
	25.0-29.9 kg/m^2	1.38 (0.65-2.96)	0.403
AMH, pmol/L		1.03 (1.01-1.04)	**0.001**
AFC Group	D/E (≤ 8 follicles)	1.00	-
	B/C (9-19 follicles)	1.82 (1.94-3.52)	0.077
	A (≥ 20 follicles)	2.48 (1.22-5.03)	**0.012**
Infertility aetiology	Tubal	1.17 (0.51-2.71)	0.711
	Endometriosis	0.86 (0.25-2.93)	0.811
	Unexplained	1.13 (0.68-1.88)	0.648
ART attempt, n		0.76 (0.61-0.95)	**0.013**
Previous IVF	≥ 3 Events	1.00	-
	0 Events	3.07 (1.18-7.96)	**0.021**
	1 Event	2.08 (0.75-5.79)	0.159
	2 Events	0.82 (0.23-2.89)	0.759
Stimulation Type	Antagonist	1.00	-
	Agonist/Other	0.44 (0.25-0.76)	**0.003**
Insemination type	IVF Only	1.00	-
	ICSI Only	5.68 (0.58-55.4)	0.135
	IVF-ICSI Split	1.50 (0.78-2.89)	0.229
rFSH dose, IU		1.00 (1.00-1.00)	**<0.001**
Progesterone at trigger, ng/mL		0.85 (0.69-1.06)	0.140
Trigger dose, μg		1.00 (1.00-1.00)	0.377
Endometrial thickness, mm		0.99 (0.81-1.22)	0.951
Oocytes collected per cycle, n		1.04 (0.99-1.09)	0.115
MII oocytes collected per cycle, n		1.06 (1.00-1.12)	0.050
Fertilised (2PN) per cycle, n		1.09 (1.02-1.17)	**0.010**
Fertilisation rate per insemination, %		1.01 (1.00-1.02)	0.057
D3 embryos in culture per cycle, n		1.08 (1.01-1.15)	**0.023**
Blastocyst number generated per cycle, n		0.97 (0.88-1.07)	0.548
Blastocyst percentage generated per cycle, %		1.01 (1.00-1.02)	**0.011**
Proportion of high-quality blastocysts generated per cycle, %		1.01 (1.00-1.01)	0.329

Embryos transferred, n		0.32 (0.14-0.76)	**0.009**
Embryos cryopreserved, n		1.20 (1.06-1.35)	**0.003**
Oocyte utilisation rate per cycle, %		1.00 (0.99-1.01)	0.798
Embryo utilisation rate per cycle, %		1.00 (0.99-1.00)	0.558
Transferred embryo quality	Low Blastocyst/Day-3	1.00	-
	High Blastocyst	2.08 (1.19-3.64)	**0.010**
	Medium Blastocyst	2.57 (1.26-5.22)	**0.009**

OR= Odds ratio. CI= Confidence interval. *Abbreviations- CP, clinical pregnancy; ET, embro transfer; BMI, body mass index; AMH, anti-mullerian hormone; AFC, antral follicle count; ART, assisted reproductive technology; rFSH, recombinant follicle stimulating hormone; MII, metapahse II oocytes; 2PN, two pronucleate zygotes; D3, day-3.*
Oocyte utilisation rate equals the sum of embryos transferred and frozen, divided by the number of oocytes collected in that cycle. Embryo utilisation rate equals the number of embryos transferred and frozen, divided by the number of 2PN generated in that cycle.

4.1.11 Multivariate model of factors associated with clinical pregnancy chance

Based on the individual factors identified in Table 4.9, and to reduce multicollinearity between related variables, the final adjusted stepwise, backwards elimination multivariate model for CP chance consisted of- VitD group (our main study interest), female age at cycle, AMH level, ART attempt number, stimulation type, rFSH dosage, and the number of/quality of transferred embryo/s.

In this final statistical model, rFSH, stimulation type, VitD group, ART attempt number and number of transferred embryos were all automatically removed from the model, while female age at cycle, AMH level and transferred embryo quality were all retained. VitD group was automatically removed in step 5 of the model demonstrating its insignificance (total 6 step model).

In the adjusted model with the retained factors, for every increasing year of female age at cycle, the likelihood of achieving a CP was reduced by 11% (OR=0.89, CI=0.84-0.94, p=<0.001, Table 4.10). For every increasing unit of AMH level, the likelihood of achieving a CP was increased by 1 %, however this was not statistically significant (OR=1.01, CI=1.00-1.03, p=0.059, Table 4.10). Lastly, patients who had either a high or medium quality embryo at ET were 1.48 and 2.30 times (respectively) more likely to achieve a CP than those who received a low-quality blastocyst or day-3 embryo. However, only the latter was statistically significant (OR=1.48, CI=0.82-2.68, p=0.195 & OR=2.30, CI= 1.12-4.75, p=0.026, Table 4.10).

Table 4.10: Multivariate logistic regression model for factors associated with clinical pregnancy chance.

Variable		Likelihood for a CP OR (95% CI)	p-value
Female age, years		0.89 (0.84-0.94)	<**0.001**
AMH, pmol/L		1.01 (1.00-1.03)	0.059
Transferred embryo quality	Low Blastocyst/Day3	1.00	-
	High Blastocyst	1.48 (0.82-2.68)	0.195
	Medium Blastocyst	2.30 (1.12-4.75)	**0.026**

OR= Odds ratio. CI= Confidence interval. *Abbreviations- AMH, anti-Mullerian hormone. Step 1: All variables were entered into the model and rFSH dose was removed. Step 2: Stimulation was removed from the model. Step 3: Number of embryos transferred was removed from the model. Step 4: ART attempt number and VitD group removed from the model. Step 5: Final model remained.*

4.1.12 Univariate model of factors associated with live birth chance

Patient demographics, cycle characteristics, embryological measures and clinical outcomes were explored in a univariate logistic regression model to investigate if changes in these variables were associated with an increased chance of a successful LB (assessed as the dependent binary variable).

VitD status was not significantly associated with LB chance (OR=1.40, CI=0.83-2.36, p=0.204, Table 4.11). Summer and autumn months were not associated with LB chance when compared to winter and spring months (OR=0.69, CI=0.41-1.16, p=0.165, Table 4.11).

For every increasing year of female age at cycle, the likelihood of achieving a LB decreased by 16% (OR= 0.84, CI=0.80-0.90, p=<0.001, Table 4.11). In contrast, BMI was not associated with LB chance when expressed as a continuous (OR=0.99, CI=0.93-1.04 p=0.522), or as grouped variable and comparing patients within the obese BMI range (≥ 30 kg/m2) to those in the overweight (25.0-29.9 kg/m2, healthy (18.5-24.9 kg/m2) or underweight (< 18.5 kg/m2) ranges (OR=1.81, p=0.161; OR= 1.62, p=0.210; OR=0.95, p=0.932, respectively, Table 4.11).

For every pmol/L increase in AMH level, the likelihood of achieving a LB increased by 3% (OR=1.03, 1.01-1.05, p=<0.001, Table 4.11). Patients within the AFC group A (≥ 20 follicles) and the B/C groups (9-19 follicles) were 3.09 and 2.01 times (respectively) more likely to achieve a LB when compared to the AFC groups D and E (< 8 follicles), however only Group A was statistically significant (OR=3.09, CI=1.42-6.73, p=0.004 & OR=2.01, CI=1.00-4.41, p=0.0051, Table 4.11).

Patients who underwent an agonist or 'other' (AACEP) ovarian stimulation regime were 59% less likely to achieve a LB compared to patients who received an antagonist regime (OR=0.41, CI=0.22-0.74, p=0.003, Table 4.11).

The number of total or MII oocytes collected was not significantly associated with LB chance (OR=1.04, CI=0.99-1.09, p=0.126; OR=1.06, CI=1.00-1.12, p=0.069, Table 4.11). However, for every 2PN generated the likelihood achieving a LB significantly increased by 7% (OR=1.07, CI=1.00-1.15, p=0.045, Table 4.11).

For every increasing number of day-3 embryos in culture or total number of blastocysts generated, the likelihood of having a LB significantly increased by 9% and 16%, respectively (OR=1.09, CI=1.02-1.16, p=0.013 & OR=1.16, CI= 1.04-1.29, p=0.007, Table 4.11). When the number of blastocysts generated was expressed as a percentage (of MII oocytes), the likelihood of having a LB also significantly increased by 2% (OR=1.02, CI=1.00-1.03, p=0.014, Table 4.11).

The number of transferred embryos was not significantly associated with LB chance (OR=0.46, CI=0.20-1.08, p=0.075, Table 4.11). In contrast, for every increase in the number of cryopreserved embryos, the chance of LB significantly increased by 18% (OR=1.18, CI=1.05-1.33, p=0.004, Table 4.11). Lastly, patients who had either a high or medium quality embryo at ET were 2.19 and 2.54 times (respectively) significantly more likely to achieve a LB than those who received a low-quality blastocyst or day-3 embryo (OR=2.19, CI=1.23-3.92, p=0.008 & OR=2.54, CI= 1.24-5.22, p=0.011, Table 4.11).

Table 4.11: Univariate logistic regression model for factors associated with live birth chance.

Variable		Likelihood for live birth (OR, 95% CI)	p-value
Serum VitD Group, ng/mL	< 20 ng/mL	1.00	-
	≥ 20 ng/mL	1.40 (0.83 - 2.36)	0.204
Season of ET/VitD Test	Winter/Spring	1.00	-
	Summer/Autumn	0.69 (0.41 - 1.16)	0.165
Female age, years		0.84 (0.80 - 0.90)	**<0.001**
BMI, kg/m^2		0.99 (0.93 - 1.04)	0.522
BMI Group	≥ 30 kg/m^2	1.00	-
	< 18.5 kg/m^2	0.95 (0.26 - 0.3.44)	0.932
	18.5-24.9 kg/m^2	1.62 (0.76 - 3.44)	0.210
	25.0-29.9 kg/m^2	1.81 (0.79 - 4.15)	0.161
AMH, pmol/L		1.03 (1.01 - 1.05)	**<0.001**
AFC	≤ 8 follicles	1.00	-
	9-19 follicles	2.01 (1.00 - 4.41)	0.051
	≥ 20 follicles	3.09 (1.42 - 6.73)	**0.004**
Ethnicity	Non-Caucasian	1.00	-
	Caucasian	1.57 (0.56 - 4.37)	0.390
Infertility aetiology	Tubal	1.37 (0.58 - 3.24)	0.469
	Endometriosis	1.20 (0.35 - 4.08)	0.776
	Male Factor	0.84 (0.46 - 1.54)	0.569
	Unexplained	1.06 (0.60 - 1.87)	0.835
ART attempt, n		0.75 (0.59 - 0.96)	**0.019**
Previous IVF	≥ 3 Events	1.00	-
	0 Events	3.64 (1.20 - 10.97)	**0.022**
	1 Event	2.40 (0.74 - 7.78)	0.143
	2 Events	1.08 (0.26 - 4.46)	0.918
Stimulation Type	Antagonist	1.00	-
	Agonist	0.41 (0.22 - 0.74)	**0.003**
Insemination type	ICSI Only	1.00	-
	IVF Only	2.57 (0.36 - 18.64)	0.349
	IVF-ICSI Split	1.52 (0.77 - 3.01)	0.223
rFSH dose, IU		0.99 (0.99 - 0.99)	**<0.001**
Progesterone at trigger, ng/mL		0.80 (0.63 - 1.03)	0.080
Trigger dose, μg		0.99 (0.99 - 1.00)	0.500
Endometrial thickness, mm		1.00 (0.81 - 1.23)	0.978
Oocytes collected per cycle, n		1.04 (0.99-1.09)	0.126
MII oocytes collected per cycle, n		1.06 (1.00-1.12)	0.069
Fertilised (2PN) per cycle, n		1.07 (1.00-1.15)	**0.045**
Fertilisation rate per insemination, %		1.01 (0.99-1.02)	0.312
D3 embryos in culture per cycle, n		1.09 (1.02-1.16)	**0.013**
Blastocyst number generated per cycle, n		1.16 (1.04-1.29)	**0.007**
Blastocyst percentage generated per cycle, %		1.02 (1.00-1.03)	**0.014**
Proportion of high-quality blastocysts generated per cycle, %		1.17 (0.96-1.41)	0.114
Embryos transferred, n		0.46 (0.20 - 1.08)	0.075
Embryos cryopreserved, n		1.18 (1.05-1.33)	**0.004**
Oocyte utilisation rate per cycle, %		1.01 (0.99-1.02)	0.417
Embryo utilisation rate per cycle, %		1.00 (0.99-1.01)	0.931
Transferred embryo quality	Low Blastocyst/Day 3	1.00	-
	High Blastocyst	2.19 (1.23 - 3.92)	**0.008**

	Medium Blastocyst	2.54 (1.24 - 5.22)	**0.011**

OR= Odds ratio. CI= Confidence interval. *Abbreviations- CP, clinical pregnancy; ET, embro transfer; BMI, body mass index; AMH, anti-mullerian hormone; AFC, antral follicle count; ART, assisted reproductive technology; rFSH, recombinant follicle stimulating hormone; MII, metapahse II oocytes; 2PN, two pronucleate zygotes; D3, day-3.*
Oocyte utilisation rate equals the sum of embryos transferred and frozen, divided by the number of oocytes collected in that cycle. Embryo utilisation rate equals the number of embryos transferred and frozen, divided by the number of 2PN generated in that cycle.

4.1.13 Multivariate model of factors associated with live birth chance

Based on the individual factors identified in Table 4.11, and to reduce multicollinearity between related variables, the final adjusted stepwise, backwards elimination multivariate logistic regression model for LB chance consisted of- VitD group (our main study interest), female age at cycle, AMH level, ART attempt number, stimulation type, rFSH dosage, and the quality of transferred embryo/s.

In the final logistic regression model, rFSH, stimulation type, VitD group, and attempt number were all automatically removed from the model, while female age at cycle, AMH level and transferred embryo quality were all retained. VitD group was automatically removed in step 4 of the model (total 5 step model).

In this adjusted model, for every increasing year of female age at cycle, the likelihood of achieving a LB was reduced by 14% (OR=0.86, CI=0.81-0.92, p=<0.001, Table 4.12). For every increasing unit of AMH level, the likelihood of achieving a LB increased by 2% (OR=1.02, CI=1.00-1.03, p=0.029, Table 4.11). Lastly, patients who had either a high or medium quality embryo at ET were 1.51 and 2.47 times (respectively) more likely to achieve a LB than those who received a low-quality blastocyst or day-3 embryo, although only the latter was statistically significant (OR=1.51, CI=0.81-2.85, p=0.198 & OR=2.47, CI= 1.15-5.31, p=0.021, Table 4.12).

Table 4.12: Multivariate logistic regression model for factors associated with live birth chance.

Variable		Likelihood for a LB OR (95% CI)	p-value
Female age, years		0.86 (0.81-0.92)	**<0.001**
AMH, pmol/L		1.02 (1.00-1.03)	**0.029**
Transferred embryo quality	Low Blastocyst/D3	1.00	-
	High Blastocyst	1.51 (0.81-2.85)	0.198
	Medium Blastocyst	2.47 (1.15-5.31)	**0.021**

OR= Odds ratio. CI= Confidence interval. *Abbreviations- LB, live birth; AMH, anti-Mullerian hormone. Step 1: All variables were entered into the model. Step 2: Stimulation was removed from the model. Step 3: rFSH dose was removed from the model. Step 4: ART attempt number and number of transferred embryos were removed from the model. Step 5: VitD group was removed from the model. Step 6: Final model remained.*

4.2 Results: Follicular Fluid subset analysis

4.2.0 Introduction

Ovarian follicular fluid (FF) is an important biological fluid composing the external environment for oocytes. FF contains a myriad of growth factors, enzymes, hormones, electrolytes, nutrients, reactive oxygen species and antioxidants, which all play an integral role in oocyte development (349). FF is a by-product of not only the exchange of blood plasma constituents that cross the blood-follicular barrier but also the secretory activity of granulosa and theca cells (350). In recent years, the development of proteomic, lipidomic and metabolomic technologies have shed light on FF constituents and how these may impact female fertility (54, 351-354).

Several studies have reported correlations between FF VitD levels and anti-inflammatory/oxidative stress markers, total antioxidant capacity, AMH, testosterone, estradiol, and progesterone levels (200, 202, 355, 356). Additionally, low levels of FF VitD have been shown to be associated with reduced endometrial thickness and markers of ovarian development, through alterations in FF lipid species (40, 357, 358). Furthermore, recent identify porcine antral follicles as a target tissue for VitD action, where it alters ovarian steroidogenesis (359). Interestingly, another report found FF VitD levels negatively correlate with an oocytes' fertilisation potential and subsequently impacts pre-implantation embryo development (207). The importance of VitD in FF could extend to male fertility too, with biologically active (1,25-(OH)D) levels in FF hypothesised to promote selection of high-quality spermatozoa in women, therefore promoting fertilisation (360). Furthermore, significant decreases in VDR gene expression in granulosa cells and FF derived from PCOS patients has been observed (198).

Many studies have reported strong positive correlations between serum and FF levels of VitD (37, 39, 50, 53, 55, 360, 361). However, the relationship between FF 25-(OH)D and clinical outcomes in IVF is still largely inconsistent in the literature. Some reports have demonstrated a positive relationship between FF VitD status and implantation rates (34, 37, 39), CPR and LBR (39), while others have found no relationship (50, 52, 361). Additionally, the number of mature oocytes and blastocyst

development rate has been shown to be higher in women with a sufficient FF VitD level (362). In contrast, one investigation found oocytes matured in FF with low VitD levels are more likely to produce top quality embryos and are associated with higher CPR and LBR (201, 207). Alternatively, to FF VitD levels, the expression of VitD binding protein in FF has been proposed as a biomarker of ART success, after it was reported to be significantly correlated with LB success in one study (54).

Additionally, the biological relevance of serum levels compared to FF levels of VitD is highly debated and inconclusive. Ozkan et al. documented that high levels of both serum and FF 25-(OH)D were positively related with CP chance, when patients were stratified into tertiles, rather than strict VitD cut offs (39). Interestingly, Rudick et al. reported a similar finding but only in their non-Asian patient population (31). In contrast, Aleyasian et al. found no correlation between FF levels of 25-(OH)D and IVF success (361). In a similar study, researchers found FF 25-(OH)D levels were negatively correlated with FF glucose levels and CPRS (53). Most of the studies in this area only investigate women with PCOS and crude values of the clinical outcomes (such as CPR and LBR), but do not assess these in adjusted analysis using multivariate logistic regression analysis methods. There is an evident lack of adjusted multivariate analyses which investigate how FF 25-(OH)D levels impact clinical outcomes in IVF in non-PCOS women, in conjunction with confounding variables.

Chapter 4.2 aims to (1) Examine if serum and FF 25-(OH)D levels are correlated in our cohort. (2) Assess how patient characteristics and clinical measures are independently related to FF sufficiency (≥ 20 ng/mL). (3) Investigate how FF 25-(OH)D is related to the clinical outcomes: blastocyst development, CP, and LB chance, in multivariate logistic regression models.

4.2.1 Patient recruitment

Patient recruitment for the whole cohort was described in detail in section *4.2.1 Patient recruitment.* Of the 287 patients with a fresh ET analysed above, 113 had both their serum and their FF VitD measured and were included in this analysis (i.e. total excluded with no FF measurement, n=174). As there are currently no guidelines in place for determining VitD sufficiency/insufficiency in FF, VitD groups were

determined based on the IOM guidelines for serum VitD, 45.1% of patients had insufficient/deficient VitD levels (< 20 ng/mL, n=51), and 53.9% had sufficient levels (≥ 20 ng/mL, n=62) (Fig. 4.2), while only 4.4% of patients had an FF VitD level of < 10 ng/mL (n=5) and 12.4% of patients had a FF VitD level ≥ 30 ng/mL (n=14, Fig. 4.2).

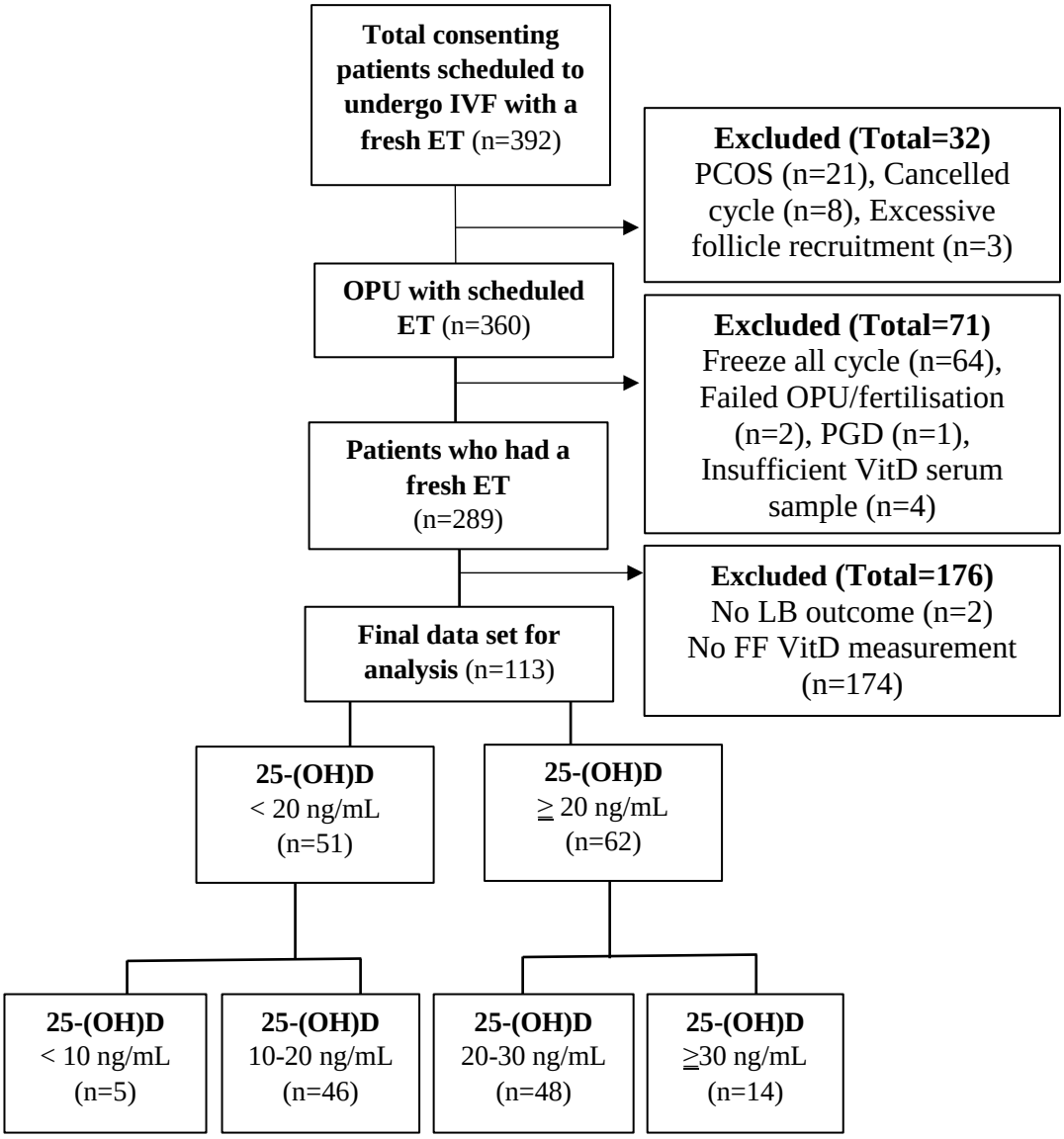


Figure 4.2: Flow diagram detailing the recruitment of consenting patients and final data analysis for FF subset cohort. Data for consenting patients was extracted from the PIVET database and cases were removed if the patient was diagnosed with PCOS, there was no fresh ET, the cycle was cancelled, failed, or converted to a freeze all, where there was insufficient sample or no FF VitD measurement or no measurable LB outcome. *Abbreviations- ET, embryo transfer; PCOS, poly-cystic ovarian syndrome; OPU, oocyte pick up; PGD, pre-implantation genetic diagnosis; FF, follicular fluid; 25-(OH)D, 25-hydroxyvitamin D.*

4.2.2 Patient demographics and characteristics

There was no significant difference in the mean female age at cycle between the < 20 ng/mL VitD group compared to the ≥ 20 ng/mL (35.7 vs. 34.5 years, n=0.228, Table 4.13). Similarly, there was no significant difference in the mean BMI between the < 20 ng/mL VitD group compared to the ≥ 20 ng/mL (24.6 vs. 24.3 years, n=0.697, Table 4.13). The proportion of Caucasian patients and patients consuming VitD supplements was not significantly different in the ≥ 20 ng/mL and < 20 ng/mL VitD groups (88.7% vs. 86.3%, p=0.696; 93.5% vs. 90.2%, p=0.512, respectively, Table 4.13).

The proportion of cases where VitD testing/ET was performed during summer and autumn months was not significantly different in the ≥ 20 ng/mL and < 20 ng/mL VitD groups (23.5% vs. 16.1%, respectively, p=0.323, Table 4.13). Additionally, the proportion of patients in the ≥ 20 ng/mL group who were classified as Group A and B/C AFC were 12.0% and 3.9% (respectively) higher, while Group C were 15.8% lower compared to the < 20 ng/mL group (p= 0.214, Table 4.13).

There were no significant differences in the < 20 ng/mL group vs. the ≥ 20 ng/mL for infertility aetiology, including endometriosis (5.9% vs. 4.8, p=0.806), tubal defect (9.8% vs. 8.1%, p=0.746), male factor infertility (33.3% vs. 25.8%, p=0.381) or unexplained infertility (52.9% vs. 64.5%, p=0.213, Table 4.13).

Table 4.13: Patient demographics stratified by FF VitD status (20 ng/mL cut off).

Variable	< 20 ng/mL	≥ 20 ng/mL	p-value
Initiated cycles, n	51	62	-
Mean Serum VitD level, ng/mL	16.8 ± 5.6	25.9 ± 10.2	**<0.001**
Median Serum VitD level, ng/mL	16.7 (7.3)	23.4 (8.9)	**<0.001**
Mean FF VitD level, ng/mL	14.8 ± 3.4	27.1 ± 8.1	**<0.001**
Median FF VitD level, ng/mL	16.2 (6.0)	25.4 (6.0)	**<0.001**
FF VitD level range, ng/mL	6.8-19.6	20.0-74.8	-
Days between FF test & ET	4.1 ± 1.1	4.3 ± 0.9	0.372
Season of ET/VitD Test, n/total [%]	-	-	0.323
Winter/Spring	39/51 [76.5]	52/62 [83.9]	-
Summer/Autumn	12/51 [23.5]	10/62 [16.1]	-
Female age at cycle, years	35.7 ± 5.3	34.5 ± 5.2	0.228
Partner age at cycle, years	38.9 ± 6.3	37.4 ± 6.1	0.244
BMI, kg/m^2	24.6 ± 5.3	24.3 ± 4.2	0.697
Ethnicity, n/total [%]	-	-	0.696
Caucasian	44/51 [86.3]	55/62 [88.7]	-
Non-Caucasian	7/51 [13.7]	7/62 [11.3]	-
Consuming VitD supplements, n/total [%]	46/51 [90.2]	58/62 [93.5]	0.512
AFC Group	-	-	0.214
Group A (≥ 20 follicles)	12/51 [23.5]	22/62 [35.5]	-
Group B/C (9-19 follicles)	27/51 [52.9]	23/62 [37.1]	-
Group D/E (≤ 8 follicles)	12/51 [23.5]	17/62 [27.4]	-
Infertility aetiology, n/total [%]	-	-	-
Endometriosis	3/51 [5.9]	3/62 [4.8]	0.806
Tubular defect	5/51 [9.8]	5/62 [8.1]	0.746
Male factor	17/51 [33.3]	16/62 [25.8]	0.381
Unexplained	27/51 [52.9]	40/62 [64.5]	0.213
Previous IVF cycle, n/total [%]	-	-	0.702
First cycle	32/51 [62.7]	39/62 [62.9]	-
Second cycle	12/51 [23.5]	13/62 [21.0]	-
≥ Third cycle	7/51 [13.7]	10/62 [16.1]	-

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- FF, follicular fluid; ET, embryo transfer; BMI, body mass index; AFC, antral follicle count.*

4.2.3 Patient cycle characteristics

The patient cycle characteristics were comparable between the FF VitD groups in several aspects. In the ≥ 20 ng/mL group, the proportion of patients who received an antagonist cycle was 3.0% higher than in the < 20 ng/mL group, however this was not statistically significant (67.7% vs. 64.7%, p=0.734, Table 4.14). In both groups most patients received insemination via ICSI (80.4% vs. 80.6%, < 20 ng/mL vs. ≥ 20 ng/mL). Additionally, the ≥ 20 ng/mL group had no cases of IVF only insemination while the < 20 ng/mL group had only 1 case (2.0% of group total) while the proportion of ICIS/IVF split insemination was 1.8% lower in the < 20 ng/mL group compared to the ≥ 20 ng/mL group. (17.6% vs. 19.4%, p=0.533, Table 4.14).

The median rFSH dose was 25.0 IU higher in the < 20 ng/mL group compared to the ≥ 20 ng/mL group, however this was not statistically significant (225.0 vs. 200.0, p=0.311, Table 4.14). The median trigger dose was identical in the < 20 ng/mL group compared to the ≥ 20 ng/mL group (500.0 vs. 500.0, p=0.708, Table 4.14). The mean endometrial thickness was not statistically different between the ≥ 20 ng/mL and < 20 ng/mL VitD groups (9.9 vs. 9.1 mm, p=0.225, Table 4.14).

The proportion of patients who received a day-5 ET, a high-quality blastocyst or a single ET was not significantly different between the ≥ 20 ng/mL and < 20 ng/mL VitD groups (51.6% vs. 45.1%, p=0.0.468; 35.5% vs. 23.5%, p=0.378; 90.3% vs. 84.3%, p=0.334, respectively, Table 4.14).

Table 4.14: Cycle characteristics stratified by FF VitD status (20 ng/mL cut off).

Variable *VitD Range*	**< 20 ng/mL (4.0-19.8)**	**≥ 20 ng/mL (20.0-72.0)**	**p-value**
Initiated cycles, n	51	62	-
Median FF VitD level, ng/mL	16.2 (6.0)	25.4 (6.0)	<0.001
Stimulation protocol, n/total [%]	-	-	0.734
Antagonist	33/51 [64.7]	42/62 [67.7]	-
Agonist/Other	18/51 [35.3]	20/62 [32.3]	-
Insemination type, n/total [%]	-	-	0.533
ICSI	41/51 [80.4]	50/62 [80.6]	-
IVF	1/51 [2.0]	0/62 [0.0]	-
ICSI/IVF split	9/51 [17.6]	12/62 [19.4]	-
rFSH dosage, IU	225.0 (262.5)	200.0 (189.4)	0.311
Trigger dose, µg	500.0 (0.0)	500.0 (0.0)	0.708
Endometrial thickness, mm	9.1 ± 0.9	9.9 ± 2.4	0.225
ET Day, n/total [%]	-	-	0.468
Day 2/3	16/51 [31.4]	21/62 [33.9]	-
Day 4	12/51 [23.5]	9/62 [14.5]	-
Day 5	23/51 [45.1]	32/62 [51.6]	-
Transferred Embryo quality, n/total [%]	-	-	0.378
High quality blastocyst	12/51 [23.5]	22/62 [35.5]	-
Medium quality blastocyst	8/51 [15.7]	9/62 [14.5]	-
Low quality blastocyst/Day 3	31/51 [60.8]	31/62 [50.0]	-
Embryos at transfer, n/total [%]	-	-	0.334
Single ET	43/51 [84.3]	56/62 [90.3]	-
Double ET	8/51 [15.7]	6/62 [9.7]	-

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- ICSI, intracytoplasmic sperm injection; rFSH, recombinant follicle stimulating hormone; ET, embryo transfer*.

4.2.4 Patient hormonal profile

There was no significant change in the median hormone levels between the ≥ 20 ng/mL group and the < 20 ng/mL VitD groups including: AMH (14.7 vs. 17.5 pmol/L, p=0.526), progesterone at trigger (1.9 vs. 2.0 ng/mL, p=0.912) or OPU (20.0 vs. 23.5 ng/mL, p=0.118), estradiol at OPU (2700.0 vs. 3200.0 pmol/L, p=0.164) or mid-luteal phase (3300.0 vs. 4600.0 pmol/L, p=0.104), prolactin (280.0 vs. 235.0 mIU/L, p=0.454), or luteinising hormone (0.7 vs. 0.6 IU/L, p=0.479, both at OPU; Table 4.15).

There was also no change in the ≥ 20 ng/mL and < 20 ng/mL VitD groups for any of the androgen panel hormones assessed (expressed as median values) including: SHBG (94.1 vs. 74.0 nmol/L, p=0.310), total (0.7 vs. 0.7 nmol/L, p=1.000) or free (5.3 vs. 6.3 ng/mL, p=0.612) testosterone, free androgen index (0.7 vs. 0.8 ratio, p=0.484) or DHEA (5.2 vs. 4.5 ng/mL, p=0.128; Table 4.15).

Table 4.15: Patient hormonal profile stratified by FF VitD status (20 ng/mL cut off).

Variable *VitD range*	**< 20 ng/mL (4.0-19.8)**	**≥ 20 ng/mL (20.0-72.0)**	**p value**
Initiated cycles, n	51	62	-
Median Serum FF level, ng/mL	16.2 (6.0)	25.4 (6.0)	<0.001
AMH, pmol/L	17.5 (14.6)	14.7 (24.4)	0.526
Progesterone at trigger, ng/mL	2.0 (2.0)	1.9 (1.0)	0.912
OPU			
Estradiol, pmol/L	3200.0 (4325.0)	2700.0 (2200.0)	0.164
Progesterone, ng/mL	23.5 (21.0)	20.0 (15.0)	0.118
Prolactin, mIU/L	235.0 (188.0)	280.0 (160.0)	0.454
Luteinising hormone, IU/L	0.6 (1.1)	0.7 (0.8)	0.479
Mid-Luteal phase			
Estradiol, pmol/L	4600.0 (4200.0)	3300.0 (2950.0)	0.104
Progesterone, ng/mL	263.0 (292.0)	294.0 (205.0)	0.908
Androgens			
SHBG, nmol/L	74.0 (55.0)	94.1 (55.2)	0.310
Total Testosterone, nmol/L	0.7 (0.6)	0.7 (0.7)	1.000
Free Androgen Index, ratio	0.8 (1.1)	0.7 (1.0)	0.484
Free Testosterone, ng/mL	6.3 (6.9)	5.3 (7.4)	0.612
DHEA, ng/mL	4.5 (2.3)	5.2 (3.2)	0.128

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- AMH, anti-Mullerian hormone; OPU, oocyte pick-up; SHBG, sex hormone binding globulin; DHEA, dehydroepiandrosterone.*

4.2.5 Embryological measures and outcomes

The median number of total and MII oocytes collected was 1 and 2 oocytes (respectively) higher in the ≥ 20 ng/mL compared to the < 20 ng/mL group, however neither of these were statistically significant (10.0 vs. 9.0, p=0.319 & 7.0 vs. 5.0, p=0.101, Table 4.16). Similarly, the median number of total 2PNs generated was 1 2PN higher in the ≥ 20 ng/mL compared to the < 20 ng/mL group, however this was not statistically significant (5.0 vs. 4.0, p=0.319, Table 4.16).

The median fertilisation rate per cycle (when expressed as the number of MII oocytes/insemination) was 2.5% lower in the ≥ 20 ng/mL compared to the < 20 ng/mL, however this was not significant (72.5% vs. 75.0%, p=0.980, Table 4.16). The overall fertilisation rate (when expressed as the sum of total number of 2PNs generated per group/total sum of MII oocytes collected of the whole group) was consistent in the ≥ 20 ng/mL compared to the < 20 ng/mL (76.7% vs. 76.4%, p=0.935, Table 4.16).

The mean blastocyst development rate was 3.7% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (19.1% vs. 15.4%, p=0.264, Table 4.16). When expressed per total oocytes collected blastocyst development rate was 6.7% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (22.0% vs. 15.3%, p=<0.001, Table 4.16). Blastocyst development rate, when expressed per MII oocytes collected, was 8.5% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (30.9% vs. 22.4%, p=0.008, Table 4.16). Lastly, when expressed per 2PNs generated, blastocyst development rate was 11.0% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (40.3% vs. 29.3%, p=0.005, Table 4.16). Additionally, the percentage of cycles developing at least one blastocyst was 11.8% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, however this was not statistically significant (72.6% vs. 60.8%, p=0.184; Table 4.16). Interestingly, the mean number of blastocysts generated per cycle was higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, however this was not statistically significant (2.1 vs. 1.8, p=0.585, Table 4.16).

There was no significant difference in the mean number of embryos transferred per cycle in the < 20 ng/mL vs. ≥ 20 ng/mL, when expressed as mean (1.2 vs.1.1, p=0.339, Table 4.16). The mean number of embryos cryopreserved here was 0.6 higher in the ≥ 20 ng/mL compared to the < 20 ng/mL (2.0 vs.1.4, p=0.128, Table 4.16). The median

oocyte utilisation rate was 6.1% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, although this was not statistically significant (39.4% vs. 33.3%, p=0.479; Table 4.16). There was no significant difference in the median embryo utilisation rate in the < 20 ng/mL compared to the ≥ 20 ng/mL group (66.7% vs. 66.7%, p=0.991; Table 4.16).

Fresh CPR was 4.0% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, however this was not statistically significant (45.2% vs. 41.2%, p=0.671, Table 4.16). Similarly, fresh LBR was 7.7% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, however this was not statistically significant (37.1% vs. 29.4%, p=0.390, Table 4.16). The miscarriage rate was 10.7% higher in the < 20 ng/mL group compared to the ≥ 20 ng/mL, however this was not statistically significant (28.6% vs. 17.9%, p=0.374, Table 4.16).

Cumulative CPR (when expressed per initiated cycle) was 6.5% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, however this was not statistically significant (51.6% vs. 45.1%, p=0.490, Table 4.16). Cumulative LBR (when expressed per initiated cycle) was 4.9% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (50.0% vs. 45.1%, p=0.339, Table 4.16).

Table 4.16: Embryological measures and outcomes stratified by FF VitD status (20 ng/mL cut off).

Variable *VitD Range*	< 20 ng/mL (4.0-19.8)	≥ 20 ng/mL (20.0-72.0)	p-value
Initiated cycles, n	51	62	-
Oocytes			
Total oocytes collected, n	484	668	-
Median oocytes collected per cycle, n	9.0 (6.0)	10.0 (8.0)	0.319
Total MII oocytes collected, n	331	476	-
Median MII oocytes collected per cycle, n	5.0 (6.0)	7.0 (7.0)	0.101
Fertilisation			
Total fertilised (2PN), n	253	365	-
Overall fertilisation rate, n/inseminated [%]	253/331 [76.4]	365/476 [76.7]	0.935
Median fertilised (2PN) per cycle, n	4.0 (3.0)	5.0 (6.3)	0.319
Median fertilisation rate % per cycle, %	75.0 (30.9)	72.5 (33.9)	0.980
Cleavage Embryos			
Total embryos cultured beyond day 2, n	325	530	-
High quality day 3 embryos	157	271	-
Blastocysts			
Total blastocysts formed, n	74	147	-
High quality blastocysts	42	92	-
Blastocysts rate per oocytes collected, n/total [%]	74/484 [15.3]	147/668 [22.0]	**<0.001**
Blastocysts rate per MII collected, n/total [%]	74/331 [22.4]	147/476 [30.9]	**0.008**
Blastocyst rate per 2PN, n/total [%]	74/253 [29.3]	147/365 [40.3]	**0.005**
Mean blastocyst number generated per cycle, n	2.1 ± 2.7	1.8 ± 2.3	0.585
Mean blastocyst development rate, %	15.4 ± 16.2	19.1 ± 18.4	0.264
Cycles developing at least 1 blastocyst, n/total [%]	31/51 [60.8]	45/62 [72.6]	0.184
Transfer & Cryopreservation			
Total embryos transferred, n	59	68	-
Mean embryos transferred per cycle, n	1.2 ± 0.4	1.1 ± 0.3	0.339
Total embryos cryopreserved, n	70	126	-
Mean embryos cryopreserved per cycle, n	1.4 ± 1.7	2.0 ± 2.6	0.128
Median oocyte utilisation rate, %	33.3 (33.9)	39.4 (30.0)	0.479
Median embryo utilisation rate, %	66.7 (66.7)	66.7 (50.0)	0.991

Fresh CPR, n/total [%]	21/51 [41.2]	28/62 [45.2]	0.671
Fresh LBR, n/total [%]	15/51 [29.4]	23/62 [37.1]	0.390
Fresh miscarriage rate, n/total CP [%]	6/21 [28.6]	5/28 [17.9]	0.374
Cumulative CPR (Fresh + Frozen per initiated cycle), [%]	23/51 [45.1]	32/62 [51.6]	0.490
Cumulative LBR (Fresh + Frozen per initiated cycle), n/total [%]	23/51 [45.1]	31/62 [50.0]	0.339

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- MII, metaphase II oocytes; 2PN, two pronuclear zygotes; CPR; clinical pregnancy rate, CP, clinical pregnancy.* Oocyte utilisation rate equals the sum of embryos transferred and frozen, divided by the number of oocytes collected in that cycle. Embryo utilisation rate equals the number of embryos transferred and frozen, divided by the number of 2PN generated in that cycle.

4.2.6 Univariate logistic regression model of patient and cycle characteristics and clinical measures associated with VitD sufficiency

Patient demographics, cycle characteristics, embryological measures and clinical outcomes variables were explored in a univariate logistic regression model to investigate if changes in these variables were associated with an increased chance of a patient having a sufficient FF VitD status (≥ 20 ng/mL) which was assessed as the dependent variable.

For every increasing unit of serum VitD, the likelihood to be in the FF ≥ 20 ng/mL group significantly increased by 9% (OR=1.09, CI=1.05-1.14, p=<0.001, Table 4.17). Similarly, when serum VitD was grouped, patients who were in the serum ≥ 20 ng/mL group were 9.16 times more likely to be in the FF ≥ 20 ng/mL group than those who had a serum VitD level < 20 ng/mL (OR=9.16, CI=3.89-21.58, p=<0.001, Table 4.17). Patients who had a cycle in summer and autumn months were 1.60 times more likely to have a sufficient level of FF VitD compared to patients who has a cycle in winter and spring months, however this was not a significant association (OR=1.60, CI=0.63-4.08, p=0.325, Table 4.17).

Female age at cycle was not associated with patient FF VitD sufficiency (OR=0.96, CI=0.89-1.03, p=0.227, Table 4.17). BMI was not significantly associated with FF VitD sufficiency when expressed as a continuous (OR=0.98, CI=0.91-1.07 p=0.694), or as grouped variable comparing patients within the obese BMI range (≥ 30 kg/m^2) to those in the overweight (25.0-29.9 kg/m^2, healthy (18.5-24.9 kg/m^2) or underweight (< 18.5 kg/m^2) ranges (OR=2.57, p=0.126; OR= 2.23, p=0.147; OR=0.94, p=0.946, respectively, Table 4.17). Caucasian patients were 25% more likely to be FF VitD sufficient, compared to Non-Caucasian patients, however this association was not statistically significant (OR=1.25, CI=0.41-3.83, p=0.696, Table 4.17).

AMH level was not associated with patient VitD sufficiency (OR=1.01, 0.99-1.03, p=0.439, Table 4.17). Patients within the AFC group A (≥ 20 follicles) or group B and C (9-19 follicles) when compared to the AFC groups D and E (< 8 follicles) were not more likely to have a sufficient FF VitD status (OR=1.29, p=0.620; OR=0.60, p=0.281, respectively, Table 4.17).

For every day-3 embryo in culture, the chance of being in the sufficient FF VitD group increased by 10%, however this association was not significant (OR=1.10, CI=0.99-1.23, p=0.065, Table 4.17). Having a sufficient FF VitD status was not significantly associated with developing at least one blastocyst in a cycle (OR=1.71, CI=0.77-3.77, p=0.185, Table 4.17).

Patients who had a successful CP and LB were 18% and 42% (respectively) more likely to be in the sufficient FF VitD group, compared to those who did not achieve a CP or successful LB, but neither of these associations were statistically significant (OR=1.18, CI=0.56-2.49, p=0.671 & OR=1.42, CI=0.64-3.13, p=0.390, Table 4.17).

Table 4.17: Univariate model of variables and their association with FF VitD status (20 ng/mL cut off).

Variable		Likelihood to be FF VitD sufficient (≥ 20 ng/mL) OR (95% CI)	p-value
Season of ET/VitD Test	Winter/Spring	1.00	-
	Summer/Autumn	1.60 (0.63-4.08)	0.325
Serum VitD level, ng/mL		1.09 (1.05-1.14)	<0.001
Serum VitD, grouped	< 20 ng/mL	1.00	-
	≥ 20 ng/mL	9.16 (3.89-21.58)	<0.001
Female age, years		0.96 (0.89-1.03)	0.227
BMI, kg/m^2		0.98 (0.91-1.07)	0.694
BMI Group	≥ 30 kg/m^2	1.00	-
	< 18.5 kg/m^2	0.94 (0.17-5.25)	0.946
	18.5-24.9 kg/m^2	2.23 (0.75-6.57)	0.147
	25.0-29.9 kg/m^2	2.57 (0.77-8.61)	0.126
AMH, pmol/L		1.01 (0.99-1.03)	0.439
AFC Group	D/E (≤ 8 follicles)	1.00	-
	B/C (9-19 follicles)	0.60 (0.24-1.52)	0.281
	A (≥ 20 follicles)	1.29 (0.47-3.59)	0.620
Ethnicity	Non-Caucasian	1.00	-
	Caucasian	1.25 (0.41-3.83)	0.696
Infertility aetiology	Tubal	0.81 (0.22-2.96)	0.746
	Endometriosis	0.81 (0.16-4.22)	0.806
	Unexplained	1.62 (0.76-3.45)	0.214
ART attempt, n		1.05 (0.74-1.48)	0.794
Previous IVF	≥ 3 Events	1.00	-
	0 Events	1.63 (0.34-7.80)	0.544
	1 Event	1.44 (0.27-7.83)	0.670
	2 Events	3.11 (0.41-23.4)	0.270
Stimulation Type	Antagonist	1.00	-
	Agonist/Other	0.87 (0.40-1.91)	0.734
Insemination type	ICSI Only	1.00	-
	IVF or IVF-ICSI Split	1.09 (0.42-2.85)	0.855
rFSH dose, IU	< 200	1.00	-
	≥ 200	0.93 (0.43-2.00)	0.846
Progesterone at trigger, ng/mL		0.92 (0.65-1.29)	0.614
Trigger dose, μg	< 500	1.00	-
	≥ 500	0.67 (0.21-2.15)	0.673
Endometrial thickness, mm		1.25 (0.87-1.80)	0.224
ET day, n		1.07 (0.74-1.56)	0.709
Transferred embryo quality	Low Blastocyst/D3	1.00	-
	High Blastocyst	1.83 (0.77-4.34)	0.168

	Medium Blastocyst	1.13 (0.38-3.30)	0.830
Embryos transferred, n		0.58 (0.19-1.78)	0.576
Oocytes collected per cycle, n		1.04 (0.97-1.12)	0.233
MII oocytes collected per cycle, n		1.06 (0.98-1.16)	0.168
Fertilised (2PN) per cycle, n		1.07 (0.97-1.19)	0.197
Fertilisation rate per insemination, %		0.99 (0.97-1.01)	0.311
D3 embryos in culture per cycle, n		1.10 (0.99-1.23)	0.065
Blastocyst number generated per cycle, n		0.96 (0.83-1.11)	0.582
Blastocyst percentage generated per cycle, %		1.01 (0.99-1.04)	0.262
Proportion of high-quality blastocysts generated per cycle, %		1.00 (0.99-1.00)	0.780
Embryos cryopreserved, n		1.15 (0.96-1.39)	0.137
Oocyte utilisation rate per cycle, %		0.99 (0.98-1.01)	0.916
Embryo utilisation rate per cycle, %		1.00 (0.99-1.01)	0.939
Cycles with at least one Blastocyst	No	1.00	-
	Yes	1.71 (0.77-3.77)	0.185
Cycles with a Clinical Pregnancy	No	1.00	-
	Yes	1.18 (0.56-2.49)	0.671
Cycles with a Live Birth	No	1.00	-
	Yes	1.42 (0.64-3.13)	0.390

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- FF, follicular fluid; ET, embryo transfer; BMI, body mass index; AMH, anti-mullerian hormone; AFC, antral follicle count; ART, assisted reproductive technology; rFSH, recombinant follicle stimulating hormone; ICSI, intracytoplasmic sperm injection; MII, metapahse II oocytes; 2PN, two pronucleate zygotes; D3, day-3.*

Oocyte utilisation rate equals the sum of embryos transferred and frozen, divided by the number of oocytes collected in that cycle. Embryo utilisation rate equals the number of embryos transferred and frozen, divided by the nu mber of 2PN generated in that cycle.

4.2.7 Correlation between serum and FF VitD level

The correlation between serum and FF levels of 25-(OH)D were examined using a bivariate Pearson correlation coefficient analysis. Serum and FF VitD were significantly positively correlated (r=0.798, p=<0.001, Figure 4.3).

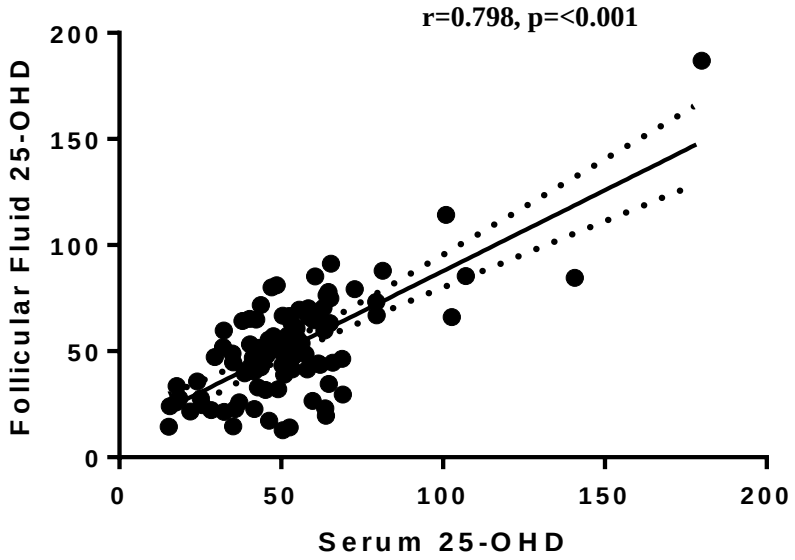


Figure 4.3: Scatterplot representing correlation between serum and FF VitD levels. Serum and FF 25-(OH)D levels were measured on the day of OPU, using the same method to reduce intra- and inter-assay variations. Data is represented as Pearson correlation coefficient (r value). *Abbreviation: 25-(OH)D, 25-hydroxyvitamin D.*

4.2.8 Multivariate model of factors associated with blastocyst development

Based on the individual factors identified in the univariate analysis displayed in Table 4.7 (see section 4.2.8), the final adjusted stepwise, backwards elimination multivariate logistic regression model for blastocyst development consisted of: VitD group (our main study interest), female age at cycle, stimulation type, ART attempt number, rFSH dose, and the number of oocytes collected.

Patients in the ≥ 20 ng/mL group were 2.54 times more likely to develop a blastocyst, compared to those in the < 20 ng/mL group, however this association did not reach statistically significance (OR=2.54, CI=0.88-5.46, p=0.084, Table 4.18). Patients who received an agonist or other ovarian stimulation cycle were 71% less likely to develop at least one blastocyst, compared to patients who received an antagonist stimulation cycle (OR=0.29, CI=0.10-0.81, p=0.019, Table 4.18). For every increasing ART attempt number, the likelihood to develop at least one blastocyst decreased by 57%, this association was statistically significant (OR=0.43, CI=0.26-0.70, p=0.001, Table 4.18). Finally, for every oocyte collected, the chance of developing at least one blastocyst increased by 29% (OR=1.29, CI=1.13-1.48, p=<0.001, Table 4.18).

Table 4.18: Multivariate model of variables and their association with blastocyst development.

Variable		Likelihood for blastocyst development OR (95% CI)	p-value
FF VitD Group, ng/mL	< 20 ng/mL	1.00	-
	≥ 20 ng/mL	2.54 (0.88-5.46)	0.084
ART attempt, n		0.43 (0.26-0.70)	**0.001**
Stimulation type	Antagonist	1.00	-
	Agonist/Other	0.29 (0.10-0.81)	**0.019**
Total oocytes collected, n		1.29 (1.13-1.48)	**<0.001**

OR= Odds ratio, CI= Confidence interval. *Abbreviations- FF, follicular fluid; ART, Assisted reproductive technology. Step 1: All variables were entered into the model. Step 2: Female age was removed from the model. Step 3: rFSH dose was removed and the final model remained.*

4.2.9 Multivariate model of factors associated with clinical pregnancy chance

Based on the individual factors identified in the univariate analysis displayed in Table 4.9 (see section 4.2.10), the final adjusted stepwise, backwards elimination multivariate logistic regression model for CP chance of the following factors were entered in the model: VitD group (our main study interest), female age at cycle, stimulation type, and the transferred embryo quality.

For every increasing year of age at the time of cycle initiation, the likelihood to developing a CP significantly decreased by 10% (OR=0.90, CI=0.83-0.97, p=0.007, Table 4.19). Patients who received a high or medium quality blastocyst were 2.20 and 4.98 times (respectively) more likely to achieve a CP compared to those who received either a low day-3 cleavage stage or poor-quality blastocyst stage embryo, however only the association with medium quality blastocysts was statistically significant (OR=2.20, CI=0.90-5.35, p=0.083 & OR=4.98, CI=1.51-16.50, p=0.008, Table 4.19).

Table 4.19: Multivariate model of variables and their association with clinical pregnancy chance.

Variable		Likelihood for CP OR (95% CI)	p-value
Female age		0.90 (0.83-0.97)	**0.007**
Trans Embryo Quality	Low D3/Poor Blastocyst	1.00	-
	High Blastocyst	2.20 (0.90-5.35)	**0.083**
	Medium Blastocyst	4.98 (1.51-16.50)	**0.008**

OR= Odds ratio, CI= Confidence interval. *Abbreviations- CP, clinical pregnancy; Trans, transferred; D3, day-3. Step 1: All variables were entered into the model. Step 2: Stimulation was removed from the model. Step 3: VitD group was removed, and the final model remained.*

4.2.10 Multivariate model of factors associated with live birth chance

Based on the individual factors identified in the univariate analysis displayed in Table 4.11 (see section 4.2.12), the final adjusted stepwise, backwards elimination multivariate model for LB chance consisted of: VitD group (our main study interest), female age at cycle, stimulation type, and the transferred embryo quality.

For every increasing year of age at the time of cycle initiation, the likelihood to having a successful LB significantly decreased by 17% (OR=0.83, CI=0.75-0.92, p=<0.001, Table 4.20). Patients who received a high or medium quality blastocyst were 2.94 and 4.13 times (respectively) more likely to have a successful LB compared to those who received either a low day-3 cleavage stage or poor-quality blastocyst stage embryo (OR=2.94, CI=1.10-7.89, p=0.032 & OR=4.13, CI=1.23-13.88, p=0.022, Table 4.20).

Table 4.20: Multivariate model of variables and their association with live birth chance.

Variable		Likelihood for LB OR (95% CI)	p-value
Female age		0.83 (0.75-0.92)	**<0.001**
Trans Embryo Quality	Low D3/Poor Blastocyst	1.00	-
	High Blastocyst	2.94 (1.10-7.89)	**0.032**
	Medium Blastocyst	4.13 (1.23-13.88)	**0.022**

OR= Odds ratio, CI= Confidence interval. *Abbreviations- CP, clinical pregnancy; Trans, transferred; D3, day. Step 1: All variables were entered into the model. Step 2: Stimulation was removed from the model. Step 3: VitD group was removed, and the final model remained.*

4.3 Results: Biometrics subset analysis

4.3.0 Introduction

The relationship between VitD deficiency (< 20 ng/mL) and obesity has been well established, and exists irrespective of age, latitude, cut-offs to define vitamin D deficiency and study location (363). Furthermore, obesity is associated with various reproductive issues including anovulation, subfertility, and infertility, as well as increased risk of miscarriage and poor neonatal and maternal pregnancy outcomes (364). Accumulating evidence has found that BMI is inversely related to VitD status and is likely a result of biologically volumetric dilution of VitD and its sequestration in adipose tissue (152, 365, 366).

One study demonstrated that obese women had significantly lower oocyte utilisation rates and fewer embryos cryopreserved (367). Not only does having a high BMI significantly impact the likelihood to conceive, but it also significantly increases the risk of spontaneous abortion (irrelevant to the method of conception), and birth defects (368, 369). Additionally, one investigation found that women who lost ≥ 10% of their baseline weight were significantly more likely to achieve clinical pregnancy, than women who lost < 10% (370). Although data from this area is promising, the lack of homogeneity and standardisation in the definitions of obesity and study protocols has resulted in inconsistencies between reported outcomes (364). Furthermore, since the development and accessibility of bioelectrical impedance analysis, the use and validity of BMI alone is questionable (371-373). One study of body composition in sedentary women reported high body fat and low fat-free mass were significantly associated with infertility (374). Currently there are no studies that investigate the relationship between body composition, VitD sufficiency and clinical outcomes in women undergoing IVF. Based on this, Chapter 4.3 aims to (1) assess how body composition is related to VitD sufficiency (≥ 20 ng/mL). (2) Investigate if body composition is related to the clinical outcomes: blastocyst development, clinical pregnancy, and LB chance, in multivariate logistic regression models.

4.3.1 Patient recruitment

Patient recruitment for the whole cohort was described in detail in section *4.1.1 Patient recruitment.* Of the 287 recruited patients with a fresh ET, 107 had their biometrics measured at the time of OPU and were included in the final subset analysis (total excluded with no biometric measurements, n=180). Based on the IOM guidelines, 39.3% of patients had inadequate serum VitD levels (< 20 ng/mL, n=42), and 60.7% had adequate levels (≥ 20 ng/mL, n=65) (Figure 4.4), while only 2.8% of patients had a serum VitD level of < 10 ng/mL (n=3) and 11.2% of patients had a serum VitD level ≥ 30 ng/mL (n=12, Fig. 4.4).

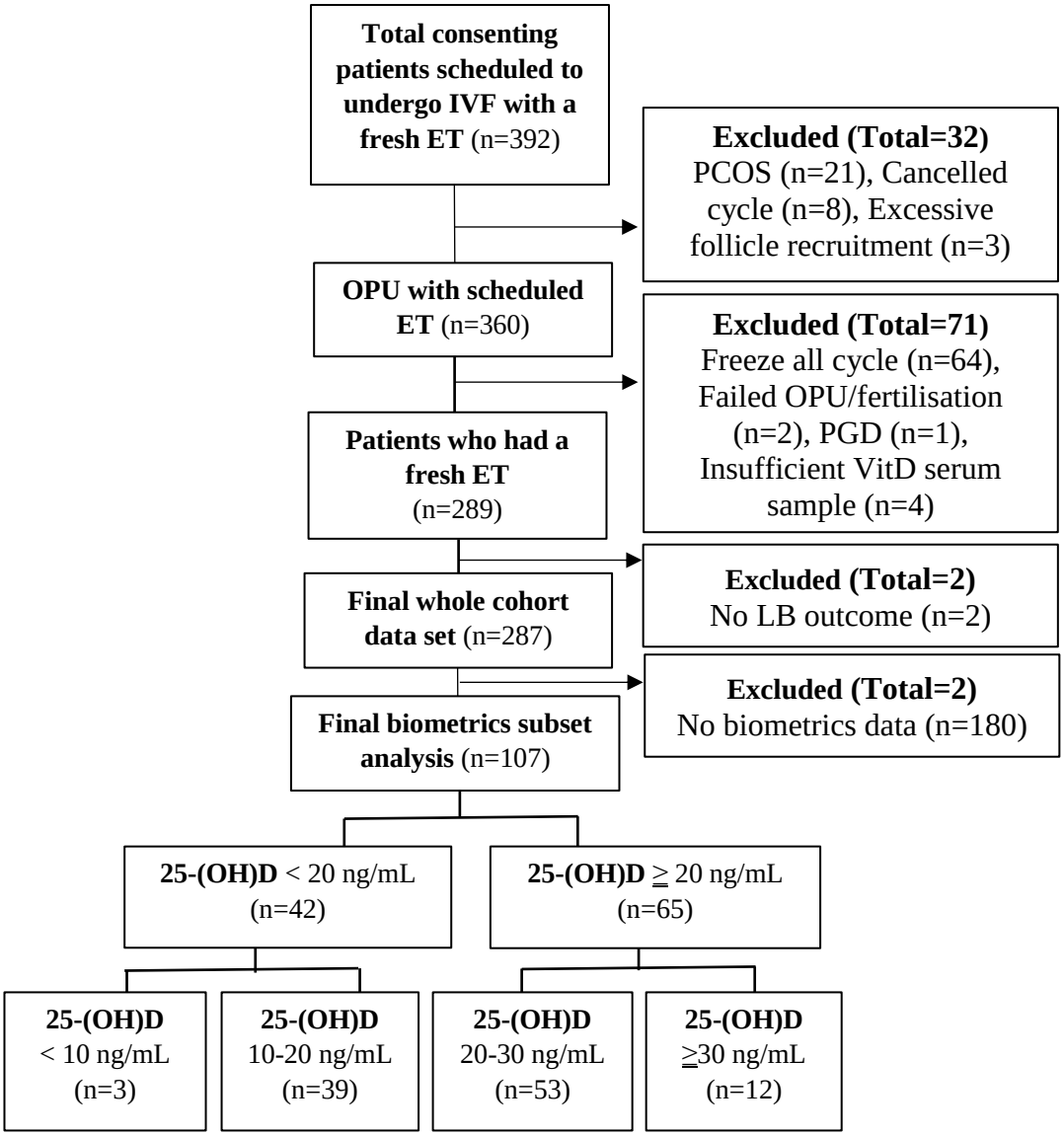


Figure 4.4: Flow diagram detailing the recruitment of consenting patients and final data analysis for biometrics subset. Data for consenting patients was extracted from the PIVET database and cases were removed if the patient was diagnosed with PCOS, there was no fresh ET, the cycle was cancelled, failed, or converted to a freeze all, where there was insufficient sample for VitD measurement, no measurable LB outcome, or no biometrics data at the time of OPU. *Abbreviations- ET, embryo transfer; PCOS, polycystic ovarian syndrome; OPU, oocyte pick up; PGD, pre-implantation genetic diagnosis; 25-(OH)D, 25-hydroxyvitamin D.*

4.3.2 Patient demographics and characteristics

A significantly higher proportion of Caucasian patients were in the ≥ 20 ng/mL VitD group compared to the < 20 ng/mL (93.8% vs. 78.6%, respectively, p=0.018, Table 4.21). There was no increase in the proportion cases where VitD testing or ET was performed during summer/autumn months in the ≥ 20 ng/mL compared to the < 20 ng/mL group (19.0% vs. 9.2%, respectively, p=0.141, Table 4.21). There was no difference in the proportion of patients taking VitD supplementation between the ≥ 20 ng/mL group compared to the < 20 ng/mL group (93.9% vs. 92.9%, respectively, p=0.839, Table 4.21). The ≥ 20 ng/mL group and < 20 ng/mL group were comparable in terms of median female age (p=0.874) and partner age (p=0.373) at the time of cycle, AFC grouping (p=0.860) and previous IVF cycles (p=0.300, Table 4.21).

There were no significant differences in the < 20 ng/mL group compared to the ≥ 20 ng/mL group in terms of infertility aetiologies, including: endometriosis (4.2% vs. 4.2%), tubal defect (11.7% vs. 6.6%), male factor infertility (23.3% vs. 23.4%,) or unexplained infertility (66.7% vs. 76.0%, Table 4.21).

Table 4.21: Patient demographics stratified by VitD status (20 ng/mL cut off).

Variable	< 20 ng/mL	≥ 20 ng/mL	p-value
Initiated cycles, n	42	65	-
Mean Serum VitD level, ng/mL	15.2 ± 3.6	27.4 ± 8.4	**<0.001**
Median Serum VitD level, ng/mL	16.0 (5.7)	25.2 (6.6)	**<0.001**
Serum VitD level range, ng/mL	6.0-19.6	20.2-72.0	**<0.001**
Days between VitD test & ET	4.0	4.2	-
Season of ET/VitD Test, n/total [%]	-	-	0.141
Winter/Spring	34/42 [81.0]	59/65 [90.8]	-
Summer/Autumn	8/42 [19.0]	6/65 [9.2]	-
Female age at cycle, years	34.8 ± 5.0	34.7 ± 4.5	0.874
Partner age at cycle, years	37.5 ± 5.3	38.7 ± 7.7	0.373
Ethnicity, n/total [%]	-	-	**0.018**
Caucasian	33/42 [78.6]	61/65 [93.8]	-
Non-Caucasian	9/42 [21.4]	4/65 [6.2]	-
Consuming VitD supplements, n/total [%]	39/42 [92.9]	61/65 [93.9]	0.839
AFC Group	-	-	0.860
Group A (≥ 20 follicles)	13/42 [31.0]	22/65 [33.8]	-
Group B/C (9-19 follicles)	21/42 [50.0]	29/65 [44.6]	-
Group D/E (≤ 8 follicles)	8/42 [19.0]	14/65 [21.5]	-
Infertility aetiology, n/total [%]	-	-	-
Endometriosis	2/42 [4.8]	5/65 [7.7]	0.549
Tubular defect	5/42 [11.9]	5/65 [7.7]	0.465
Male factor	11/42 [26.2]	17/65 [26.2]	0.997
Unexplained	25/42 [59.5]	41/65 [63.1]	0.712
Previous IVF cycle, n/total [%]	-	-	0.300
First cycle	21/42 [50.0]	39/65 [60.0]	-
Second cycle	13/42 [31.0]	12/65 [18.5]	-
≥ Third cycle	8/42 [19.0]	14/65 [21.5]	-

4.3.3 Patient biometrics

The mean BMI of patients in the ≥ 20 ng/mL group was comparable to the < 20 ng/mL group (24.4 vs. 24.3 kg/m^2, p=0.898, Table 4.22). The mean bone mass was identical between the two groups (2.5 vs. 2.5 kg, p=0.584, Table 4.22). The mean fat mass was 1.6 kg higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, however this was not statistically significant (28.4 vs. 26.8 kg, p=0.211, Table 4.22). The mean muscle mass was significantly higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, with a mean increase of 3.9 kg (36.5 vs. 32.6 kg, p=0.048, Table 4.22). In contrast, the median basal metabolic rate was 56 kJ lower in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, however this was not statistically significant (5648.0 vs. 5704.0 kJ, p=0.783, Table 4.22).

Table 4.22: Patient biometrics stratified by VitD status (20 ng/mL cut off).

Variable *VitD Range*	**< 20 ng/mL (6.0-19.6)**	**≥ 20 ng/mL (20.2-72.0)**	**p-value**
Initiated cycles, n	42	65	-
BMI (kg/m^2)	24.3 ± 4.3	24.4 ± 4.7	0.898
Bone mass (kg)*	2.5 ± 0.4	2.5 ± 0.4	0.584
Fat mass (kg)*	26.8 ± 5.5	28.4 ± 6.7	0.211
Muscle mass (kg)*	32.6 ± 10.8	36.5 ± 9.1	**0.048**
Basal metabolic rate (kJ)*	5704.0 (991.0)	5648.0 (826.0)	0.783

Biometric markers were utilised in a univariate logistic regression model to explore if these variables were individually associated with VitD sufficiency (≥ 20 ng/mL). BMI was significantly associated with VitD sufficiency, with every unit of increasing BMI (kg/m^2), the likelihood of being in the VitD sufficient group decreased by 6% (p=0.017, Table 4.23). For every increasing unit of muscle mass (kg), the likelihood of being in the VitD sufficient group increased by 4%, however this did not reach statistical significance (p=0.051, Table 4.23). Bone mass, fat mass and basal metabolic rate were not statistically significantly associated with VitD sufficiency (p=0.580, p=0.210, p=0.461, respectively, Table 4.23).

Table 4.23: Univariate model of patient biometrics and their association with VitD sufficiency (≥ 20 ng/mL cut off).

Variable	Likelihood to be VitD sufficient (≥ 20 ng/mL) OR (95% CI)	p-value
BMI (kg/m^2)	0.94 (0.90-0.99)	**0.017**
Bone mass (kg)*	0.76 (0.29-2.01)	0.580
Fat mass (kg)*	1.04 (0.98-1.11)	0.210
Muscle mass (kg)*	1.04 (1.00-1.09)	0.051
Basal metabolic rate (kJ)*	1.00 (1.00-1.00)	0.461

OR= Odds ratio, CI= Confidence interval. *Abbreviations- BMI, body mass index.* All parameters displayed here were determined used bioelectrical impedance analysis within a week of ET.

4.3.4 Patient cycle characteristics

The patient cycle characteristics were comparable between the VitD groups in several aspects. In the ≥ 20 ng/mL group, the proportion of patients who received an antagonist cycle was 7.3% higher than in the < 20 ng/mL group, however this was not statistically significant (69.2% vs. 61.9%, p=0.434, Table 4.24). Most patients received insemination via ICSI, and this was not significantly different when stratified by VitD status (81.0% vs. 81.5%, p=0.450, Table 4.24). The median rFSH and trigger dose was not statistically significantly different in the < 20 ng/mL group compared to the ≥ 20 ng/mL group (204.2 vs. 200.0, p=0.849, Table 4.24). Similarly, the median trigger dose was 14.3 µg higher in the < 20 ng/mL group compared to the ≥ 20 ng/mL group (501.2 vs. 486.9, p=0.507, Table 4.24). The mean endometrial thickness was 0.5 mm higher in the < 20 ng/mL group compared to the ≥ 20 ng/mL group, however this was not statistically significant (10.2 vs. 9.7, p=0.491, Table 4.24).

The proportion of patients in the ≥ 20 ng/mL group who received a day-5 ET was 7.1% higher compared to the < 20 ng/mL group, however this was not statistically significant (52.3% vs. 45.2%, p=0.745, Table 4.24). Furthermore, the proportion of patients in the ≥ 20 ng/mL group who received a high-quality blastocyst at ET was 8.3% higher compared to the < 20 ng/mL group (36.9% vs. 28.6%, p=0.518, Table 4.24). Most patients received a single ET, and this was not statistically different when stratified by VitD status, the proportion of the ≥ 20 ng/mL group who received a single ET was only 1.1% higher than in the < 20 ng/mL (89.2% vs. 88.1%, p=0.856, Table 4.24).

Table 4.24: Patient cycle characteristics stratified by VitD status (20 ng/mL cut off).

Variable *VitD Range*	**< 20 ng/mL (6.0-19.6)**	**≥ 20 ng/mL (20.2-72.0)**	**p-value**
Initiated cycles, n	42	65	-
Median Serum VitD level, ng/mL	16.0 (5.7)	25.2 (6.6)	**<0.001**
Stimulation protocol, n/total [%]	-	-	0.434
Antagonist	26/42 [61.9]	45/65 [69.2]	-
Agonist/Other	16/42 [38.1]	20/65 [30.8]	-
Insemination type, n/total [%]	-	-	0.450
ICSI	34/42 [81.0]	53/65 [81.5]	-
IVF	1/42 [2.4]	0/65 [0.0]	-
ICSI/IVF split	7/42 [16.7]	12/65 [18.5]	-
rFSH dosage, IU	204.2 (231.3)	200.0 (175.0)	0.849
Trigger dose, µg	501.2 ± 129.0	486.9 ± 92.4	0.507
Endometrial thickness, mm	10.2 ± 2.2	9.7 ± 2.2	0.491
ET Day, n/total [%]	-	-	0.745
Day 2/3	12/42 [28.6]	15/65 [23.1]	-
Day 4	11/42 [26.2]	16/65 [24.6]	-
Day 5	19/42 [45.2]	34/65 [52.3]	-
Transferred Embryo quality, n/total [%]	-	-	0.518
High quality blastocyst	12/42 [28.6]	24/65 [36.9]	-
Medium quality blastocyst	8/42 [19.0]	8/65 [12.3]	-
Low quality blastocyst/Day 3	22/42 [52.4]	33/65 [50.8]	-
Embryos at transfer, n/total [%]	-	-	0.856
Single ET	37/42 [88.1]	58/65 [89.2]	-
Double ET	5/42 [11.9]	7/65 [10.8]	-

4.3.5 Patient hormonal profile

When stratified by serum VitD status, there was no significant differences in the ≥ 20 ng/mL group compared to the < 20 ng/mL group for any of the hormones assessed (expressed as median values) including: AMH (16.2 vs. 17.4 pmol/L, p=0.783), progesterone at trigger (2.1 vs. 2.0 ng/mL, p=0.719) or OPU (22.5 vs. 16.5 ng/mL, p=0.152), estradiol at OPU (2900.0 vs. 2700.0, p=0.950) or mid-luteal phase (3000.0 vs. 3700.0, p=0.329), prolactin (280.0 vs. 270.0, p=0.495), or luteinising hormone (0.7 vs. 0.6, p=0.517, both at OPU; Table 4.25).

Additionally, there were no significant differences in the ≥ 20 ng/mL group compared to the < 20 ng/mL group for any of the androgen panel hormones assessed (expressed as median values) including: SHBG (84.2 vs. 87.5, p=0.932), total (0.6 vs. 0.6, p=0.975) or free (5.8 vs. 5.0, p=0.670) testosterone, free androgen index (0.7 vs. 0.6, p=0.916) or DHEA (4.1 vs. 5.1, p=0.551; Table 4.25).

Table 4.25: Patient hormonal profile stratified by VitD status (20 ng/mL cut off).

Variable *VitD range*	**< 20 ng/mL (6.0-19.6)**	**≥ 20 ng/mL (20.2-72.0)**	**p value**
Initiated cycles, n	42	65	-
Median Serum VitD level, ng/mL	16.0 (5.7)	25.2 (6.6)	**<0.001**
AMH, pmol/L	17.4 (19.8)	16.2 (22.7)	0.783
Progesterone at trigger, ng/mL	2.0 ± 1.4	2.1 ± 1.2	0.719
OPU			
Estradiol	2700.0 (4400.0)	2900.0 (2275.0)	0.950
Progesterone, ng/mL	16.5 (31.0)	22.5 (23.0)	0.152
Prolactin	270.0 (175.0)	280.0 (215.0)	0.495
Luteinising hormone	0.6 (1.0)	0.7 (1.3)	0.517
Mid-Luteal phase			
Estradiol	3700.0 (4100.0)	3000.0 (2750.0)	0.329
Progesterone, ng/mL	261.0 (160.0)	300.0 (229.0)	0.407
Androgens			
SHBG	87.5 (43.0)	84.2 (64.0)	0.932
Total Testosterone	0.6 (1.0)	0.6 (1.0)	0.975
Free Androgen Index	0.6 (1.0)	0.7 (1.0)	0.916
Free Testosterone	5.0 (6.0)	5.8 (7.0)	0.670
DHEA	5.1 (4.0)	4.1 (4.0)	0.551

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- AMH, anti Mullerian hormone; OPU, oocyte pick-up; SHBG, sex hormone binding globulin (SHBG); DHEA, dehydroepiandrosterone.*

4.3.6 Embryological measures and outcomes

There were no significant differences observed between the two VitD groups (≥ 20 ng/mL vs. < 20 ng/mL) in relation to the median number of total oocytes collected (10.0 vs. 10.0 p=0.690), or number of MII oocytes collected (7.0 vs. 7.0, p=0.675; Table 4.26). Similarly, there was no difference observed between the two VitD groups (≥ 20 ng/mL vs. < 20 ng/mL) for the median number of 2PNs generated per cycle (5.0 vs. 5.5, p=0.972, Table 4.26).

The median fertilisation rate (when expressed as the number of MII oocytes/insemination per cycle) was not significantly increased in the ≥ 20 ng/mL compared to the < 20 ng/mL (75.0% vs. 75.0, p=0.960, Table 4.26). The overall fertilisation rate (when expressed as the sum of total number of 2PNs generated per group/total sum of MII oocytes collected of the whole group) was 1.4% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, however this was not statistically significant (77.0% vs. 75.6%, p=0.648, Table 4.26).

Blastocyst development rate (%) was not significantly different in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (Table 4.26). When expressed per total oocytes collected blastocyst development rate was 2.9% lower in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (17.1% vs. 20.0%, p=0.229). Blastocyst development rate (%) when expressed per MII oocytes collected was 3.8% lower in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (23.6% vs. 27.4%, p=0.232). Lastly, when expressed per 2PNs generated blastocyst development rate was 5.5% lower in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (30.7% vs. 26.2%, p=0.156). In contrast, the percentage of cycles developing at least one blastocyst was 15.9% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, however this did not reach statistical significance (75.4% vs. 59.5%, p=0.083; Table 4.26). The mean number of blastocysts generated per cycle was 0.6 higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, however this was not statistically significant (2.3 vs. 1.7, p=0.759, Table 4.26).

There mean number of embryos transferred per cycle was identical in the < 20 ng/mL group compared to the ≥ 20 ng/mL group (1.1 vs.1.1, p=0.857, Table 4.26). The mean number of cryopreserved embryos per cycle was 0.5 higher in the ≥ 20

ng/mL group compared to the < 20 ng/mL group, however this was not statistically significant (2.0 vs.1.5, p=0.304, Table 4.26). The median oocyte utilisation rate was 3.6% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, although this was not statistically significant (28.6 vs. 25.0, p=0.718, Table 4.26). The median embryo utilisation rate was identical in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (50.0 vs. 50.0, p=0.948, Table 4.26).

Fresh CPR was 5.0% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, however this was not statistically significant (43.1% vs. 38.1%, p=0.609, Table 4.26). Similarly, fresh LBR was 5.9% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, but this was not statistically significant (36.9% vs. 31.0%, p=0.526, Table 4.26). Despite the difference in LBR, there was no statistically significant increase in miscarriage rate in the < 20 ng/mL group compared to the ≥ 20 ng/mL (14.3% vs. 18.8%, p=0.667, Table 4.26).

Cumulative CPR (when expressed per ET) was 7.9% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group, however this was not statistically significant (50.8% vs. 42.9%, p=0.424, Table 4.26). Cumulative LBR (when expressed per ET) was 5.6% higher in the ≥ 20 ng/mL group compared to the < 20 ng/mL group (46.1% vs. 40.5%, p=0.563, Table 4.26).

Table 4.26: Embryological measures and outcomes stratified by VitD status (20 ng/mL cut off).

Variable *VitD Range*	< 20 ng/mL (6.0-19.6)	≥ 20 ng/mL (20.2-72.0)	p-value
Initiated cycles, n	42	65	-
Oocytes			
Total oocytes collected, n	421	702	-
Median oocytes collected per cycle, n	10.0 (6.5)	10.0 (7.5)	0.960
Total MII oocytes collected, n	307	508	-
Median MII oocytes collected per cycle, n	7.0 (7.0)	7.0 (7.0)	0.675
Fertilisation			
Total fertilised (2PN), n	232	391	-
Overall fertilisation rate, n/inseminated [%]	232/307 [75.6]	391/508 [77.0]	0.648
Median fertilised (2PN) per cycle, n	5.5 (4.3)	5.0 (5.0)	0.972
Median fertilisation rate per cycle, %	75.0 (37.3)	75.0 (25.7)	0.960
Cleavage Embryos			
Total embryos cultured beyond day 2, n	296	555	-
High quality day 3 embryos	135	292	-
Blastocysts			
Total blastocysts formed, n	84	120	-
High quality blastocysts	58/84 [69.0]	71/120 [59.2]	0.150
Blastocysts rate per oocytes collected, n/total [%]	84/421 [20.0]	120/702 [17.1]	0.229
Blastocysts rate per MII collected, n/total [%]	84/307 [27.4]	120/508 [23.6]	0.232
Blastocyst rate per 2PN, n/total [%]	84/232 [36.2]	120/391 [30.7]	0.156
Mean blastocyst number generated per cycle, n	1.7 ± 1.8	2.3 ± 2.8	0.759
Mean blastocyst development rate, %	19.5 ± 16.1	21.0 ± 15.0	0.204
Cycles developing at least 1 blastocyst, n/total [%]	25/42 [59.5]	49/65 [75.4]	0.083
Transfer & Cryopreservation			
Total embryos transferred, n	47	72	-
Mean embryos transferred per cycle, n	1.1 ± 0.3	1.1 ± 0.3	0.857
Total embryos cryopreserved, n	64	130	-
Mean embryos cryopreserved per cycle, n	1.5 ± 2.0	2.0 ± 2.5	0.304
Median oocyte utilisation rate, %	25.0 (24.9)	28.6 (23.7)	0.718
Median embryo utilisation rate, %	50.0 (35.0)	50.0 (31.0)	0.948

Fresh CPR, n/total [%]	16/42 [38.1]	28/65 [43.1]	0.609
Fresh LBR, n/total [%]	13/42 [31.0]	24/65 [36.9]	0.526
Fresh miscarriage rate, n/total CP [%]	3/16 [18.8]	4/28 [14.3]	0.697
Cumulative CPR (Fresh + Frozen per initiated cycle), [%]	18/42 [42.9]	33/65 [50.8]	0.424
Cumulative LBR (Fresh + Frozen per initiated cycle), n/total [%]	17/42 [40.5]	30/65 [46.1]	0.563

Mean ± SD; Median (IQR); n/total [%]. *Abbreviations- MII, metapahse II oocytes; 2PN, two pronucleate zygotes; CPR, clincal pregnancy rate; LBR, live birth rate.*

Oocyte utilisation rate equals the sum of embryos transferred and frozen, divided by the number of oocytes collected in that cycle. Embryo utilisation rate equals the number of embryos transferred and frozen, divided by the number of 2PN generated in that cycle.

4.3.7 Multivariate model of factors associated with blastocyst development

The multivariate model presented here was based on the individual factors identified in Table 4.7 and the multivariate model as presented in Table 4.8. Patients in the ≥ 20 ng/mL group were 4.20 times more likely to develop a blastocyst than those in the < 20 ng/mL group (OR=4.20, CI=1.22-14.47, p=0.023, Table 4.27). For every increasing ART attempt, the likelihood of developing a blastocyst decreased by 73% (OR=0.27, CI=0.14-0.55, p=<0.001, Table 4.27). Lastly, for every increasing number of oocytes collected at OPU the likelihood of developing a blastocyst increased by 46% (OR=1.46, CI=1.23-1.73, p=<0.001, Table 4.27).

Table 4.27: Multivariate model of variables and their association with blastocyst development.

Variable		Likelihood for blastocyst development OR (95% CI)	p-value
Serum VitD Group	< 20 ng/mL	1.00	-
	≥ 20 ng/mL	4.20 (1.22-14.47)	**0.023**
ART attempt, n		0.27 (0.14-0.55)	**<0.001**
Total oocytes collected, n		1.46 (1.23-1.73)	**<0.001**

OR= Odds ratio, CI= Confidence interval. *Abbreviations- ART, Assisted reproductive technology. Step1: All variables were entered into the model. Step 2: Female age were removed from the model. Step 3: Stimulation type was removed from the model and the final model remained.*

4.3.8 Multivariate model of factors associated with clinical pregnancy chance

The multivariate model presented here was based on the individual factors identified in Table 4.9 and the multivariate model as presented in Table 4.10. In the adjusted model, for every increasing year of female age at cycle, the likelihood of achieving a CP was reduced by 10% (OR=0.90, CI=0.82-0.99, p=0.030, Table 4.28). Additionally, patients who had either a high or medium quality embryo at ET were 3.40 and 9.52 times (respectively) more likely to achieve a CP than those who received a low-quality blastocyst or day-3 embryo, both were statistically significant associations (OR=3.40, CI=1.35-8.58, p=0.010 & OR=9.52, CI= 2.55-35.53, p=0.001, Table 4.28).

Table 4.28: Multivariate model of variables and their association with clinical pregnancy chance.

Variable		Likelihood for CP OR (95% CI)	p-value
Female age		0.90 (0.82-0.99)	**0.030**
Trans Embryo Quality	Low D3/Poor Blastocyst	1.00	-
	High Blastocyst	3.40 (1.35-8.58)	**0.010**
	Medium Blastocyst	9.52 (2.55-35.53)	**0.001**

OR= Odds ratio, CI= Confidence interval. *Abbreviations- CP, clinical pregnancy; Trans, transferred; D3, day-3. Step 1: All variables were entered into the model. Step 2: Stimulation type was removed from the model. Step 3: VitD group was removed from the model and the final mode remained.*

4.3.9 Multivariate model of factors associated with live birth chance

The multivariate model presented here was based on the individual factors identified in Table 4.11 and the multivariate model as presented in Table 4.12. In the adjusted model, for every increasing year of female age at cycle, the likelihood of achieving a LB was reduced by 16% (OR=0.84, CI=0.76-0.93, p=0.001, Table 4.29). Additionally, patients who had either a high or medium quality embryo at ET were 3.95 and 7.98 times (respectively) more likely to achieve a LB than those who received a low-quality blastocyst or day-3 embryo, both were statistically significant associations (OR=3.95, CI=1.43-10.91, p=0.008 & OR=7.98, CI= 2.16-29.41, p=0.002, Table 4.29).

Table 4.29: Multivariate model of variables and their association with live birth chance.

Variable		Likelihood for LB OR (95% CI)	p-value
Female age		0.84 (0.76-0.93)	**0.001**
Trans Embryo Quality	Low D3/Poor Blastocyst	1.00	-
	High Blastocyst	3.95 (1.43-10.91)	**0.008**
	Medium Blastocyst	7.98 (2.16-29.41)	**0.002**

OR= Odds ratio, CI= Confidence interval. *Abbreviations- LB, live birth; Trans, transferred; D3, day-3. Step 1: All variables were entered into the model. Step 2: Stimulation type was removed from the model. Step 3: VitD group was removed from the model and the final mode remained.*

4.3.10 Results summary

In summary, the current study demonstrated in adjusted analyses that higher serum and FF VitD were significantly related to increased blastocyst development, but not CP or LB chance. Furthermore, serum 25-(OH)D (but not FF) was also independently associated with several embryological measures related to blastocyst development, in an unadjusted analysis. Using a Pearson's correlation indicated serum and FF 25-(OH)D levels were highly positively correlated. Biometric analysis showed that only BMI was significantly associated with VitD status in an unadjusted analysis, but no biometric parameters were associated with any clinical outcomes investigated here. Additionally, self-reported patient ethnicity was also significantly associated with VitD status. In conclusion, female age at cycle, AMH level and transferred embryo quality were the only significant predictors of CP and LB outcome in the present study, while serum 25-(OH)D was associated with FF VitD levels, BMI status, patient ethnicity and blastocyst development.

4.4 Discussion

The present chapter aimed to investigate how VitD sufficiency (in both serum and FF) impacted or was associated with patient characteristics (namely biometrics), embryological measures and clinical outcomes in a heterogenous population of non-PCOS patients undergoing IVF with a fresh ET.

While we found close to half our population was insufficient in serum and FF VitD, studies have reported prevalence of VitD insufficiency in infertile populations between 27-65% (30, 35, 39, 44, 375). This could indicate VitD insufficiency is more prevalent in the sub-fertile population or women of reproductive age (281, 376). When stratified by either serum or FF VitD sufficiency the groups remained well-matched regarding patient demographics and cycle characteristics, except for patient ethnicity which is known to be highly correlated with VitD status (340). VitD is primarily produced in the skin upon exposure to UVB radiation, but a small amount (10%) is also obtained from food sources (5). Patients with darker pigmented skin are at a higher risk of

inefficient synthesis of VitD synthesis in their skin, therefore tend to have a higher prevalence of VitD insufficiency (377). Many other factors are linked to an increased risk of developing VitD insufficiency, including- geographical location, socio-economic status, occupation, body composition, age and gender (103). More specifically, in Australia where around 25% of the population are vitamin D insufficient/deficient, only 5% are taking VitD supplements (101). Australians more likely to take VitD supplementation are women, elderly individuals, those of higher socio-economic status, non-smokers and those who are physically active (64). In contrast, 90% of our cohort were taking VitD supplements. This large discrepancy between the general population and women undergoing IVF is due to the use of pre-conception multi-vitamins, which is recommended to all our patients and which contains various important vitamins and minerals, inclusive of 1,25-(OH)2D3.

Classically VitD is known for its role in calcium and phosphorus metabolism (1). More specifically, VitD has several key roles including intestinal duodenal calcium absorption and renal calcium reabsorption, as well as promoting bone mineralisation (3, 4, 378). The significant impact of VitD insufficiency/deficiency on bone health is prevalent in an aging population, particularly post-menopausal women (379). Despite previous studies demonstrating a relationship between VitD insufficiency and age, we found no such association in our IVF cohort. This discrepancy is likely due to the women in the current study being of reproductive age (< 45 years old).

Interestingly in our cohort BMI was the only biometric parameter significantly associated with VitD sufficiency, although on average muscle mass was higher in the sufficient VitD group and in the predictive model was trending towards a significant association with VitD status. The lack of statistical significance is potentially a consequence of the small sample size of the subset analysis. Given the fat soluble and hydrophobic nature of VitD, adipose tissue is the major storage location of VitD (150). Data indicates VitD could be sequestered in adipose tissue and henceforth the bioavailability of VitD is reduced (152). An alternative hypothesis is a volumetric dilution of VitD in individuals with higher body mass could explain the strong link between VitD insufficiency and BMI (366). It is likely that the effect of body composition on VitD is a combination of decreased bioavailability following sequestration in adipose tissue and volumetric dilution leading to lower serum levels. Despite the relationship with VitD insufficiency, biometric measurements were not

associated with any improved embryological measures or clinical outcomes in our cohort.

As expected, serum and FF VitD were highly correlated, a finding that has been reported and well established in several studies to date (38-40, 43, 44). The impact of FF VitD levels on clinical outcome in IVF is inconclusive. In a prospective study Firouzabadi et al., investigated serum and FF levels in 221 IVF patients and found neither serum or FF levels of VitD were related to CPRs which is similar to the current study (50). In another investigation, Anifandis et al., demonstrated FF VitD levels were significantly correlated with the quality of embryos, and high FF VitD levels in combination with decreased FF glucose levels had a detrimental impact on CPR (53). However, this study failed to apply adjusted models to their findings, to account for confounding factors such as embryo quality. In one investigation, higher FF VitD levels were found to be associated with a higher production of mature oocytes and an increased blastocyst formation rate (362). In contrast, Antunes et al., reported lower follicular VitD concentrations predicted a 'better' response to ovarian stimulation protocols, demonstrated by a greater production of larger follicles and higher serum estradiol concentrations (200). A similar study also found a significant positive correlation between FF VitD levels and estradiol and progesterone concentrations (202).We found no such relationship in the present study between serum or FF levels and estradiol concentrations. In a small prospective examination of 80 infertile women undergoing IVF, FF VitD was significantly higher in pregnant women compared to non-pregnant women ($p=0.007$) (34). Once again this was an unadjusted analysis, which does not account for the numerous confounding factors related to IVF outcome success.

The data from our cross-sectional observational study indicated that serum or FF VitD was not related to improved clinical outcomes for CP or LB in women undergoing IVF with a fresh ET, after controlling for various confounders. However, sufficient serum VitD levels were strongly associated with an increased chance of developing more blastocysts per cycle, more high-quality embryos per cycle, and consequently a greater number of blastocysts cryopreserved and a higher oocyte utilisation rate. Blastocysts are generally accepted to have greater implantation potential than cleavage stage embryos (380), and day 5 transfers demonstrated better CPR and LBR in the present study.

One of the potential mechanisms by which VitD may influence reproductive outcomes is through effects on oocyte/embryo development and subsequent quality. In the current study, this aspect was explored in depth as a dependent and independent variable. It was clearly shown in the whole cohort analysis that 17.2% more cases in the sufficient VitD group generated at least one blastocyst (p=0.004). The sufficient VitD group also produced 0.6 more blastocysts per cycle, 0.5 more blastocysts were cryopreserved, and the blastocyst development rate percentage was 8.2% higher than the VitD insufficient group. Stepwise logistic regression analysis adjusting for a range of confounding variables, including- patient ethnicity, BMI, age and season of ET/VitD test, also showed that increasing blastocyst number per cycle (OR=1.32, CI=1.10-1.58, p=0.002), increasing development rate (OR=1.02, CI=1.01-1.04, p=0.001), increasing embryo quality (OR=1.01, CI=1.01-1.02, p=0.040), increasing blastocysts cryopreserved (OR=1.33, CI=1.10-1.60, p=0.004), and increasing oocyte utilisation rates (OR=1.03, CI=1.01-1.05, p=0.004) were all associated with sufficient serum VitD levels (≥ 20 ng/mL). Although we found FF VitD levels were associated with 2.54 times increased chance of blastocyst development in an adjusted analysis, this did not reach statistical significance (OR=2.54, p=0.084). The discrepancy here compared to the strong significant association between serum VitD and blastocyst development, is likely an issue of the smaller subset cohort size (n=113).

Surprisingly, EUR which is calculated as the proportion of embryos transferred and frozen from the total number of 2PN zygotes in each case, was not associated with serum VitD levels (≥ 20 ng/mL, p=0.091), but this could reflect the larger but insignificant number of embryos transferred in the insufficient VitD group (< 20 ng/mL). The potential cellular or biochemical mechanism(s) relating to the effect of serum or FF VitD levels on blastocyst development are currently unknown. However, a recent study has shown that VitD as part of a “Mediterranean” diet impacts embryo morphokinetic makers and possibly blastocyst development by accelerating the fourth cell division event (5 cell to 8/9 cell) and reducing time for these division events (213). It was also associated with an increase in KIDScores on day 3, which indicated enhanced embryo quality. However, the major weakness of this morphokinetic study was that it was underpowered, and the intervention contained other nutritional components such as omega-3 fatty acids and olive oil. Consequently, this adds

significant complexity to the study and prevents the acquisition of firm conclusions, although the findings are interesting and worthy of further exploration.

In the present study, no difference was observed in the number of oocytes and MII oocytes collected and fertilisation rate, and this is in line with several other investigations that failed to demonstrate a clear relationship between these embryological measures and serum VitD (30-33, 35, 38, 39, 44, 48, 51, 375) Conversely, one prospective study showed that fertilisation rates tended to be higher when VitD was ≥ 30 ng/mL, however this study failed to account for transferred embryo quality (32). Another study revealed a similar trend towards greater fertilisation rates, but it did not reach significance (38). However, this was not observed in the current study.

Relatively few studies have investigated the association between serum VitD and embryo quality. The earliest study by Rudick et al., demonstrated that serum VitD was not associated with embryo quality as measured by the amount of fragmentation and number of cells at day 3 cleavage stage (31). Comparable findings were reported recently where the mean number and/or percentage of top quality embryos were not different between VitD groups (30, 48). Interestingly, a prospective study with 335 participants reported that 16% of replete VitD cases generated 3 or more top quality embryos per cycle, where only 6% of VitD insufficient cases generated 3 or more top quality embryos (375). This study also revealed that 16% of replete cases received a blastocyst transfer, where only 6% of insufficient cases received a blastocyst transfer. This could suggest that women with sufficient VitD status are more likely to generate blastocysts, in line with our data in the current study.

The potential association between VitD status and CP and LB outcomes was also examined, and both were higher in the sufficient VitD group, with a crude CP difference of 9.9% and a LB difference of 7.1%. However, these differences were not statistically significant. Furthermore, in stepwise logistic regression analysis with CPR and LBR as binary dependent variables, VitD status (serum or FF) was not included as a significant variable in the final adjusted model. Instead, the most important and significant parameters for both CPR and LBR were female age, ovarian reserve (indicated by AMH level) and transferred embryo quality, all of which have consistently been shown to be central to CP and LB chance in numerous reports from our clinic (326, 381, 382). In a FET cycle study, VitD status was not associated with

CPR and the crude difference was only 5% (383). A recent prospective study also demonstrated that the crude LBR was greater in sufficient VitD groups by approximately 13-14% (p=0.004) (33). However, following adjustment for major confounders such as female age at cycle, BMI, smoking status, ethnicity, FSH level, treatment type, infertility type and duration, the adjusted difference was only 9% and became statistically insignificant (p=0.250) (33). Nonetheless, early retrospective studies suggested that the LBR in VitD replete (≥ 30 ng/mL) IVF patients could be 4-28% higher than those considered insufficient (< 30 ng/mL) (31, 42) while another report indicated a significant 13% difference in LBR for higher VitD status patients (p=0.015) (30). Two recent systematic reviews reported a decreased chance of LB with VitD insufficiency (OR=0.74, CI=0.58-0.90) (384), while VitD sufficiency was associate with an improved LBR (OR=1.33, CI=1.08-1.65) (385). However, this was not demonstrated in the present study, and it remains uncertain whether VitD influences LBR.

The main limitation of the current study was the failure to reach our recruitment target of 320 patients, to reach statistical significance for CP and LB outcomes. Although 392 patients consented to the research, data was only available for 287 eligible individuals, which may play a role in the insignificant trends observed for CP and LB for example. In addition, the reasons why patients were reluctant to participate in an observational trial at a private clinic were not clear, and this was not captured in the trial design or by questionnaire. The target of 320 patients was calculated based on a 15% difference in CPR (30, 383), and the difference observed for CPR and LBR between VitD groups was 9.9% and 7.1%, respectively in favour of the VitD sufficient (≥ 20 ng/mL) group. However, while the cohort was insufficiently powered to detect significant differences in the primary outcomes, it did demonstrate highly significant differences in the secondary outcomes of embryological measures, namely blastocyst development. Even though recruitment fell short of the required number from the CPR power calculation, this study still represents one of the largest clinical trials examining the relationship between serum VitD and IVF outcomes, after the trials by Sufen et al., (n=2577), Chu et al., (n=500), and Paffoni et al., (n=335), and recruited slightly more patients than recent investigations by Drakopoulos et al. (n=283) and van de Vijver (n=280) (33, 48, 375, 383, 386).

Another limitation was that ≥ 90% of patients were taking VitD supplementation, but the specific dose and duration was not recorded. However, we have reduced these potential effects by sampling for VitD status at OPU and within a very close time frame to the outcomes of interest, mainly blastocyst development and CP chance. Previous reports have shown that VitD found in common commercially available pregnancy multivitamins was not effective in mitigating VitD insufficiency at such low doses, and it takes extensive time periods of supplementation to alter VitD status (387). Finally, another related limitation was that the VitD measurement and the outcome of LBs spanned across multiple seasons and therefore it was difficult to establish a direct relationship between VitD measured at OPU and later stage clinical outcomes such as LB or neonatal outcomes. Consequently, future longitudinal studies that follow up with a VitD blood test closer to the expected delivery date would be necessary to further elucidate this relationship.

The major strength of this study was that it was conducted in a large sample size of 287 infertility patients, and it demonstrated a highly significant correlation between serum VitD level and blastocyst generation in an IVF patient population, while showing no association with CP or LB outcomes. This was evident after accounting for a wide range of confounding variables previously demonstrated to impact IVF outcomes such as female age at cycle, BMI, AMH, quality of transferred embryo, ovarian stimulation protocol and patient ethnicity among others (388). This study included FF VitD level, patient biometrics and ethnicity data which has not been examined in many other VitD studies, and never in the depth of the current study (319, 383). Interestingly, ethnicity was shown here to be a significant confounder even in a cohort with a Caucasian majority. It also examined two measures of ovarian reserve (AMH and AFC), which has also been included in very few VitD studies (386). The study was a registered, cross sectional observational prospective clinical trial, which meant that data collection methods and analyses were robust. Furthermore, patients were recruited by researchers blinded to treatment strategies/decisions and independent of treating clinicians to reduce any potential selection bias.

This study is also the third largest study to investigate the relationship between FF VitD and clinical outcomes, following Firouzabadi et al., (n=221) and Antunes et al., (n=197) (50, 200). Another strength is serum and FF VitD samples were quantified

using the same method of detection, stored frozen short term and batch tested to reduce inter-assay variation. Additionally, to the best of our knowledge, the present study is the first to investigate how patient biometrics are related to VitD sufficiency in an IVF patient cohort. Nonetheless, the relationship between serum VitD and blastocyst development was highly significant, and it is entirely possibly that this influence may also moderate CPR and/or LBR, where this relationship has been demonstrated in many other studies (32, 50, 375). However, statistical moderation analysis was not explored in the current study.

Finally, VitD metabolism is complex due to the existence of many VitD metabolites and thousands of VDR binding sites throughout the genome controlling hundreds of genes (25). In current VitD research it is widely accepted that serum 25-(OH)D is the best indication of VitD status (21). Firstly, 25-(OH)D has a half-life of approximately 3 weeks, compared to other metabolites which have a half-life of only a few hours (such as 1,25-(OH)D) (105). Secondly, 25-(OH)D production in the liver is not considerably regulated, whereas metabolites such as 1-25-(OH)D are tightly regulated by calcium and phosphorus metabolism pathways, therefore 25-(OH)D provides an accurate estimate of VitD stores (105). Considering this, 25-(OH)D provides the best indication of VitD status, however future studies implementing multiple VitD metabolites, including the biologically active 1,25-(OH)2D3 may provide more accurate findings.

4.6 Conclusion

In conclusion, our present study provides an in-depth investigation of VitD status in an IVF population of non-PCOS patients. Furthermore, the findings here demonstrate a strong association between VitD sufficiency and an increased chance of blastocyst development, but not CP or LB chance. While in our subset analysis of FF, VitD status did not reach statistical significance, there was a trend towards significance in the adjusted model for blastocyst development. Additionally, VitD remained in the adjusted models for CP and LB, until the final step. This could indicate a potential

relationship between these clinical outcomes and FF VitD status, although a larger sample size is required as the number of factors underlying successful implantation are numerous and therefore require several hundred cases in matched studies. Future prospective studies further elucidating this association and expanding on the current sample size may provide stronger evidence to support a subsequent increase in CP and LB outcomes, as a result of increased blastocyst development.

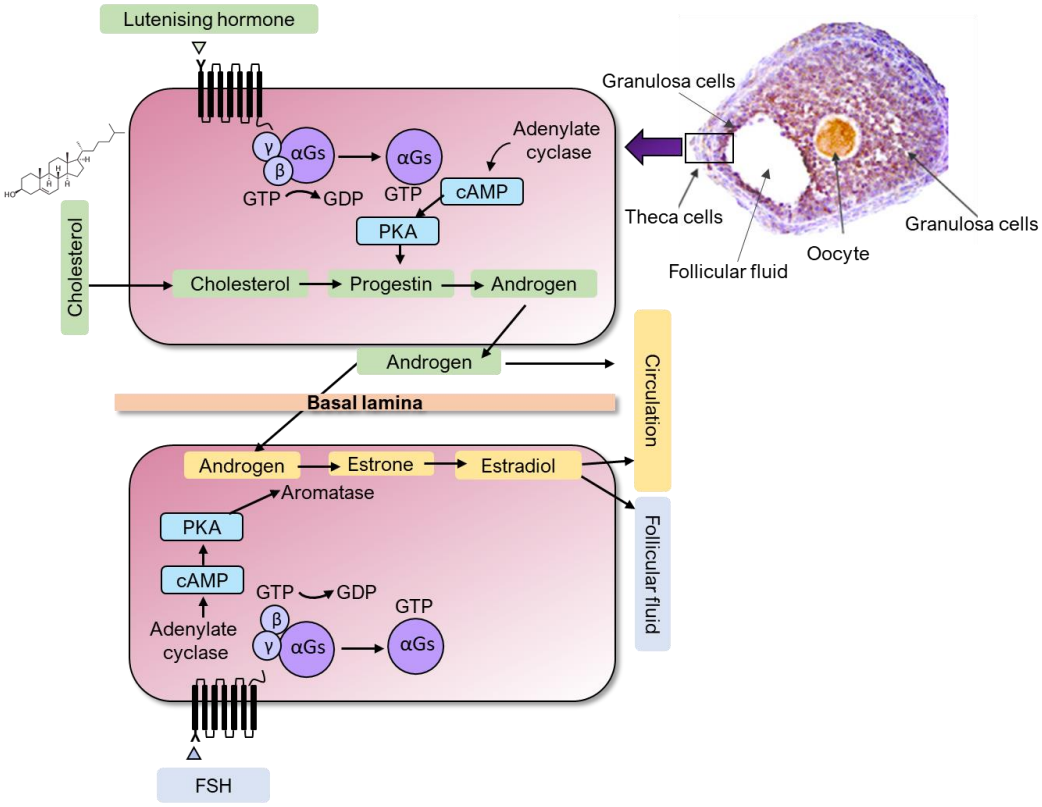
Chapter Five

Characterisation of the human granulosa cell lines: COV434 & KGN

5.0 Introduction

The human ovary is composed of three regions: the outer cortex (containing the follicles), the medulla (consisting of stroma and vasculature) and the hilum (the site of attachment to the mesovarium) (389). Within the cortex are primarily two somatic cell types: theca cells and GCs, which work synergistically in a paracrine manner secreting various growth factors and hormones (223). These ovarian cells are responsible for regulating each other's intracellular function and secretory output, to support folliculogenesis and oocyte maturation (223).

In developing follicles, GCs are described as spherical and/or polyhedral in shape and clustered closely. In atretic follicles, the GCs become flattened and irregularly shaped with an absence of cytoplasmic invaginations, which are projections that connect cumulus cells to the oocyte (390). There are close interactions between theca and GCs that have been extensively studied (391). Research has shown that during folliculogenesis, steroidal and non-steroidal factors produced and secreted by both GC and theca cells influence the proliferation and differentiation of one another (224). There is also a difference in gonadotropin receptor expression between these cell types, such that LH receptors are found exclusively on theca cells, and FSHRs are exclusively found on GCs. LH receptor (LHR) stimulation promotes theca cell androgen production, while FSHR activation influences aromatase expression, which subsequently results in the conversion of theca cell derived-androgens to estrogens (Figure 5.1) (227).



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5218 **Figure 5.1: Granulosa and theca cell interactions.** LH and FSH stimulate adenylate
5219 cyclase via activation of G protein-coupled receptors. The cAMP generated from ATP
5220 activates protein kinase A (PKA) to stimulate expression of steroidogenic enzymes in
5221 theca cells and GCs. In theca cells, this causes the conversion of cholesterol into
5222 progestins and androgens which are secreted and subsequently taken up/utilised by
5223 GCs. Here, androgens are converted to estrogens (such as E1 and E2, previously
5224 described in section 1.4.1, Figure 1.5), the latter of which is secreted via GCs into the
5225 follicular fluid and circulation. *Abbreviations: GDP, guanosine diphosphate; GTP,*
5226 *guanosine triphosphate.*

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Given the invasive nature of collecting ovarian cells, *in vitro* studies are almost exclusively performed on infertile females undergoing IVF procedures where GCs and theca cells can be harvested. For this reason, animal studies were often utilised prior to the development of IVF technologies to allow for the study of ovarian cell structures, interactions, and function. In more recent times, the development of human ovarian cell lines (4) has allowed the molecular study of these cell types in greater depth. COV434 and KGN are two well-studied human granulosa cell lines that both express the functional machinery for steroidogenesis including the cytochrome p450 aromatase (known simply as aromatase) enzyme (254, 392), androgen receptors (393), 17β-HSD (394) and respond to gonadotropin stimulation to secrete estrogens and progestins. These cell lines provide an alternative means to study GCs *in vitro* as primary humans GCs are often restrictive in sample size and accessibility, as well as being exposed to high concentrations of ovarian stimulatory drugs prior to collection.

5.0.1 COV434 cell line

Zhang et al., were the first to establish and characterise the immortalised COV434 cell line in 2000 (260). COV434 cells were generated from a primary human GC tumour derived from a 27-year-old woman who was diagnosed with metastatic GC carcinoma (260). Morphologically, cultured COV434 cells appear granular, and form small, aggregated clusters in cell culture conditions, with some forming intercellular junctions (260). COV434 cells were not observed to have microvilli on the cell surface but contained intracellular lipid droplets and demonstrated a doubling time of 24 hours (260). The addition of recombinant FSH (rFSH, 100 ng/mL) in cell culture medium resulted in the increased expression of the enzyme 17β-HSD and the secretion of E2, and when this stimulation was prolonged, the levels of both continued to significantly increase (260). However, this was not the case when COV434 cells were treated with LH or human chorionic gonadotropin (hCG) in the absence of rFSH (260). Additionally, the production of cyclic adenosine monophosphate (cAMP) was enhanced by rFSH treatment, but not by LH (260). In the same investigation, progesterone (P4) secretion was not altered followings stimulation with rFSH, LH or hCG (260).

5.0.2 KGN cell line

Nishi et al., were the first to establish and characterise the steroidogenic human GC-like tumour cell line (KGN) in 2001 (254). To generate the KGN cell line, enucleated tumour tissue was obtained from a 72-year-old woman who was diagnosed with a granulosa tumour, following re-occurrence of ovarian carcinoma (stage III) (254). Morphologically, KGN cells grow in an adherent monolayer, and appear spindle-shaped in low density seeding, while at high density KGN cells become epithelial cell-like (254). KGN cells have a doubling-time of 46.4 hours, much slower than the COV434 cells which double every 24 hours (254, 260). Aromatase is an important enzyme in the steroidogenic pathway, which converts testosterone to estrogen (395). Aromatase activity was detected in KGN cells by quantifying the amount of [^{3}H]H2O released upon the conversion of [1β-3H] androstenedione to estrone by a modification of a previously published method (396). Aromatase activity increased upon stimulation with FSH, cAMP and human menopausal gonadotropin stimulation, but not hCG (254). Additionally, basal P4 levels were quantifiable and significantly increased upon stimulation with cAMP (254). In recent years, when cultured as spheroids, KGN cells have been shown to express higher basal aromatase expression and E2 secretion when compared to COV434 cells (397).

5.0.3 *In vitro* GC metabolic studies

Evidence has shown that the COV434 cells express the VDR, but not the KGN cell line (232, 398), and previous work from our lab has demonstrated that VitD status influences cell bioenergetics in peripheral blood mononuclear cells (67). However, very little is known regarding the metabolic responses of COV434 and KGN cell lines in basal conditions or under rFSH stimulation, and the bioenergetic response to VitD has never been explored. The way in which these cells utilise cellular energy is key for various molecular processes including steroidogenesis and studies in this area are limited. Of the few studies to investigate metabolic flux in KGN cells, one assessed the mitochondrial bioenergetics following combined peroxisome proliferator-activated receptor gamma (PPARγ) activation and X-linked inhibitor of apoptosis

protein inhibition (399). In this study, basal oxygen consumption rate (OCR), maximal respiration and ATP capacity were approximately 0.40, 0.40 and 0.70 pmol/min/µg/mL (respectively) (399). However, no measurements of glycolytic flux were reported by the authors (399). In another report, researchers assessed mitochondrial bioenergetics in KGN cells following clustered regularly interspaced short palindromic repeats (CRISPR) genetic editing of targeted mutations in tumour suppressor genes (400). In this study, basal OCR, maximal respiration, and ATP capacity were reported as 100, 200 and 70 pmol/min (respectively) (400). Additionally, basal glycolysis and glycolytic capacity, as determined by extracellular acidification rate (ECAR), was approximately 35 and 65 mpH/min in KGN cells (respectively) (400). A PubMed search for the terms "COV434" or "KGN" cell line with "bioenergetics" retrieved 0 and 5 results (accessed on 12/3/21). Importantly, no studies have investigated metabolic bioenergetic profiles in COV434 or KGN cells treated with rFSH and androstenedione.

In contrast, more metabolic studies using primary human GCs exist, and GCs from oocyte donors aged < 35 years have a significantly higher basal OCR compared to older (≥ 38 years) infertile women treated with an antagonist protocol (401).While these GCs were exposed to FSH in follicular fluid, they were not treated with a specific dose of rFSH prior to the bioenergetic assay. These findings could indicate age may significantly impact GC bioenergetics and possibly fertility. The rationale behind why female age and diminished ovarian reserve are associated with infertility and reduced reproductive outcomes is unknown. One explanation could be that at a cellular and molecular level, the normal steroidogenic responses of ovarian cells such as GCs are altered. Alternatively, other cellular metabolic signalling and activity may be reduced or dysfunctional, which could be attributed to the aging or diminished ovarian reserve process. These aspects are not currently known, and very few studies have investigated these cellular responses in GCs.

Since COV434 and KGN cells, are derived from a woman within her reproductive stage of life and a woman from beyond her reproductive window, respectively, these models will allow us to investigate the difference in metabolic machinery between young and old GCs. Furthermore, it is not known how VitD appears to contribute to more positive outcomes in clinical studies. Perhaps VitD can affect the steroidogenic and proliferative response of GCs, mediated through alterations in

metabolism/bioenergetics, and this in manifested in clinical observations. However, before we could determine the influence of VitD on these cell processes, our first aim and that of this chapter was to characterise the COV434 and KGN responses to rFSH and androstenedione stimulation in terms of cell proliferation and viability, steroidogenesis, and metabolism/bioenergetics. In the subsequent chapter, these responses will be examined again but following exposure to VitD.

In addition, no studies have assessed the *in vitro* effect of the human derived rFSH drug Rekovelle™ on human GCs responses. Rekovelle™ is derived from the human cell line PER.C6, by recombinant DNA technology, and the *in vitro* effects on the GC lines COV434 or KGN cell lines has never been reported in the published literature. Therefore, a further aim of this study was to characterise the responses of COV434 and KGN to the human derived rFSH (follitropin delta) drug Rekovelle™.

5.0.4 Chapter Objectives:

To characterise and compare the effect of human derived rFSH (Rekovelle™) and androstenedione (alone and in combination) hormone treatments on COV434 and KGN:

1) cell viability/proliferation, cell cycle and intracellular ROS production.
2) production of the hormones E2 and P4.
3) lipid deposition and markers of lipid metabolic flux and signalling.
4) the glycolytic and mitochondrial bioenergetic profiles.

5.1 Results

5.1.1 COV434 and KGN cell viability following rFSH and androstenedione exposure

Using concentrations reported in the literature, we first evaluated the potential cytotoxicity of androstenedione, rFSH and combination treatments on cell viability to select non-lethal concentrations for cell stimulation. rFSH treatment alone over the 3 concentrations tested (62.5, 125 and 250 ng/mL) for 24 hours did not alter cell viability significantly in either COV434 or KGN cells (Figure 5.2 A). In addition, androstenedione treatment alone over the 3 concentrations tested (1, 5, 10 µM) did not significantly change cell viability for both COV434 and KGN cells (Figure 5.2 B). Furthermore, treatment of both cell lines with androstenedione (1 or 5 µM) in combination with rFSH (62.5 or 125 ng/mL) did not alter cell viability compared to each other or androstenedione or rFSH treatments alone (Figure 5.2 C & D). Consequently, the highest concentrations of rFSH (125 ng/mL) and androstenedione (5 µM) that did not induce cytotoxicity were used in subsequent experiments. This reduced the complexity of having an excessive number of treatment groups in later experiments.

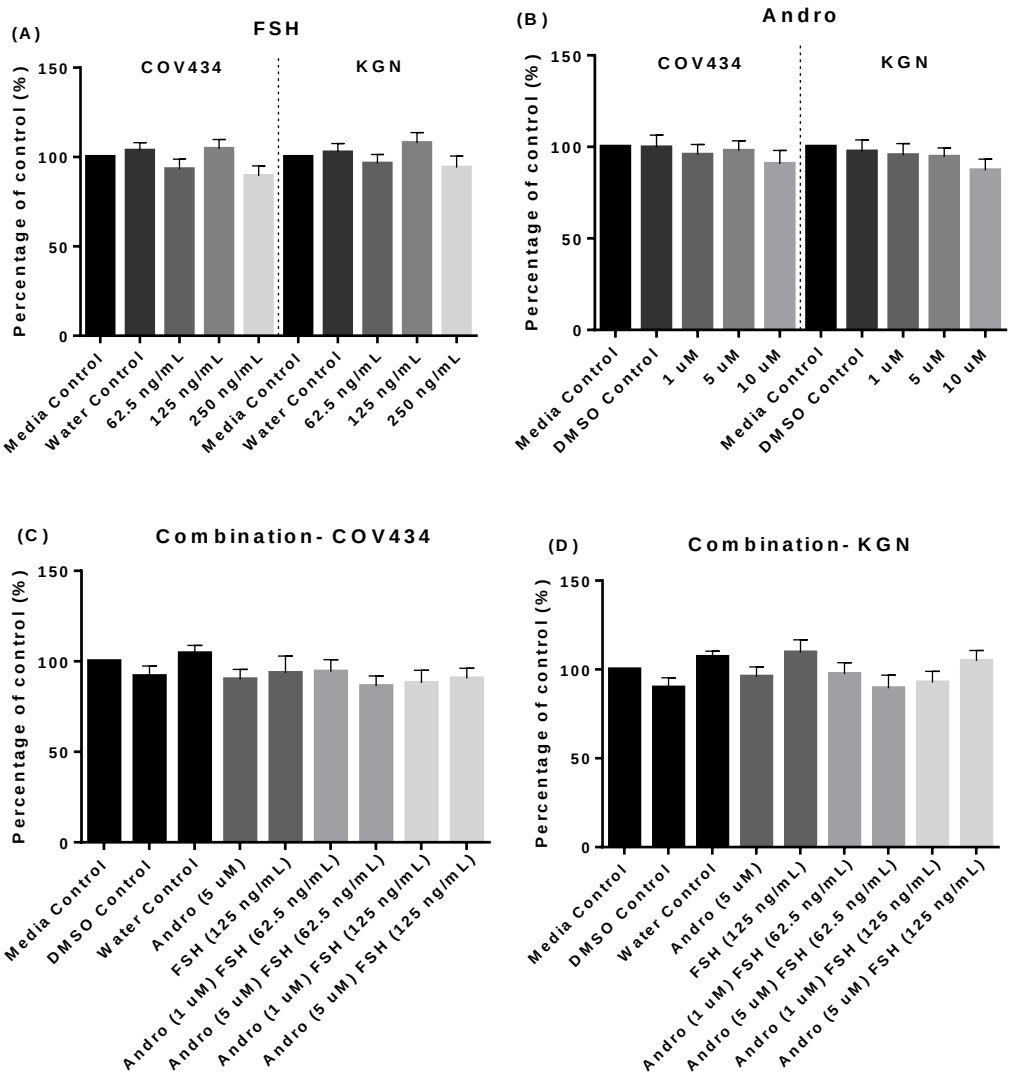


Figure 5.2: **The effect of rFSH & androstenedione treatment on cell viability in COV434 & KGN cell lines.** COV434 and KGN cell lines were exposed to either rFSH (125 ng/mL) or andro (5 µM) alone or in combination for 24-hours and cell viability was assessed using MTT assay. **(A)** rFSH treatment alone **(B)** Andro treatment alone and combination treatment in **(C)** COV434 cells and **(D)** KGN cells. Data is represented as mean ± S.D and each experiment was replicated 3 times with each sample measured in triplicate. *p<0.05, **p<0.01, ***p<0.001. *Abbreviations: DMSO, dimethyl sulfoxide; Andro, androstenedione; rFSH, recombinant follicle stimulating hormone.*

5.1.2 Cell cycle analysis of rFSH and androstenedione treatment

Flow cytometry was used to assess the cell cycle progression in COV434 and KGN cells following rFSH and/or androstenedione stimulation. In COV434 cells, there were no significant changes in cell cycle progression (i.e., G0/G1, S, G2/M phases) following stimulation with androstenedione (5 μM) or rFSH alone (125 ng/mL), or in combination (Figure 5.3). Interestingly, in KGN cells treated with rFSH (125 ng/mL), the G0/G1 phase significantly decreased compared to control (58.4% vs. 63.1%, p=0.046), and this was reflected in non-significant increases in S and G2/M phases. No other parameters of the cell cycle significantly changed in KGN cells (Figure 5.3). Sub G1 populations reflect cells with fragmented DNA, but there were no significant changes observed in either cell line, which indicated no cytotoxicity (data not shown).

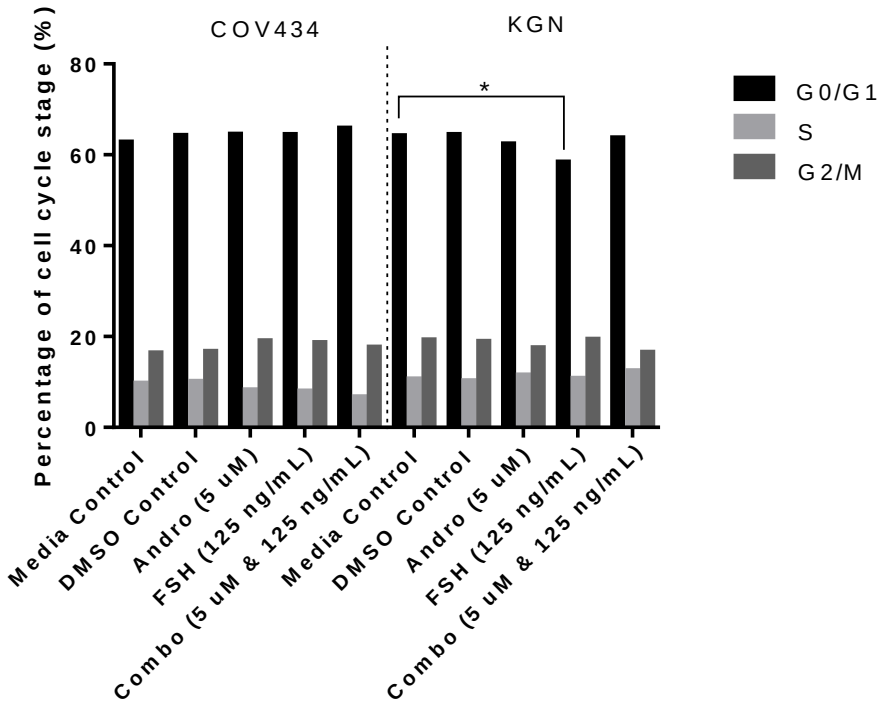


Figure 5.3: **The effect of rFSH & androstenedione treatment on COV434 & KGN cell cycle progression.** COV434 and KGN cell lines were exposed to either rFSH (125 ng/mL) or androstenedione (5 μM), alone or in combination for 24-hours and cell cycle was assessed using flow cytometry. Data is represented as mean ± S.D and each experiment was replicated 3 times with each sample measured in triplicate. *p<0.05, **p<0.01, ***p<0.001. *Abbreviations: DMSO, dimethyl sulfoxide; Andro, androstenedione; rFSH, recombinant follicle stimulating hormone.*

5.1.3 Intracellular ROS analysis of rFSH and androstenedione treatment

Flow cytometry was used to assess general levels of intracellular ROS. In both COV434 and KGN cells treated with androstenedione, ROS slightly increased by 4.5% and 5% compared to solvent control (respectively, Figure 5.4), but was not statistically significant. In contrast, when treated with rFSH, ROS slightly decreased by 5.8% and 3.6% in COV434 and KGN (respectively) compared to media control (respectively, Figure 5.4) but was not statistically significant. Overall, both treatments had a minimum effect on ROS levels. Glucose oxidase (GOX) was used as a positive control for inducing elevated ROS levels. As expected, GOX significantly increased the levels of ROS in both COV434 and KGN cells by 15.6% and 20.8% (respectively, $p<0.001$) compared to media and solvent control (Figure 5.4).

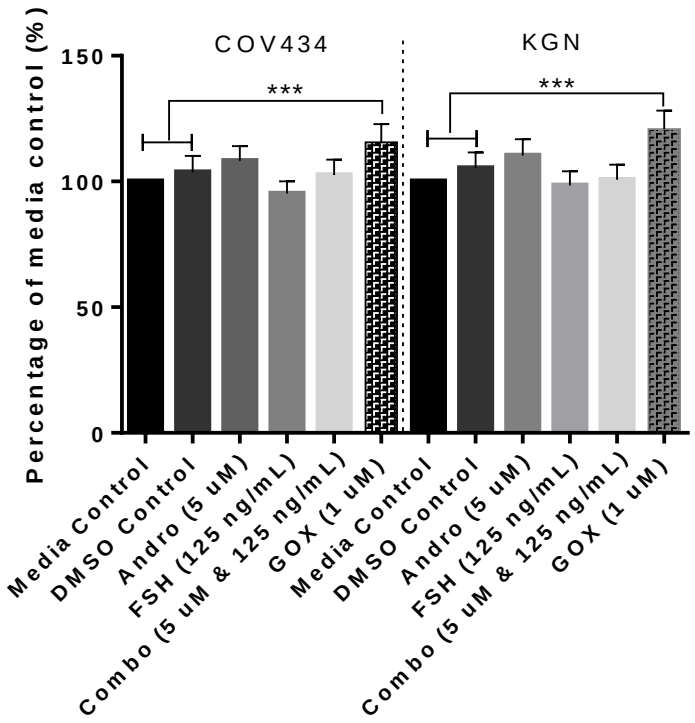


Figure 5.4: **The effect of rFSH & androstenedione treatment on intracellular ROS in COV434 & KGN cell lines.** COV434 and KGN cell lines were exposed to either rFSH (125 ng/mL) or androstenedione (5 µM) alone or in combination, or glucose oxidase (as a positive control) for 24-hours and intracellular ROS was assessed using flow cytometry. Data is represented as mean ± S.D and each experiment was replicated 3 times with each sample measured in triplicate. *p<0.05, **p<0.01, ***p<0.001. *Abbreviations: DMSO, dimethyl sulfoxide; Andro, androstenedione; rFSH, recombinant follicle stimulating hormone; GOX, glucose oxidase.*

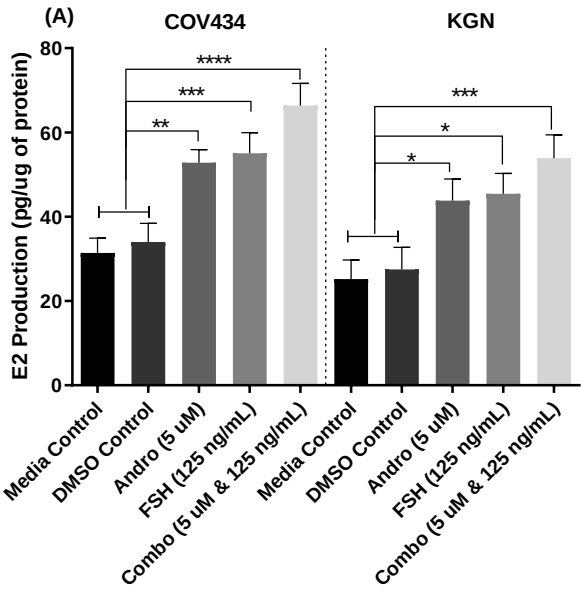
5.1.4 The effect of rFSH and androstenedione stimulation on E2 and P4 hormone secretion from COV434 and KGN cells

To validate the reported responsiveness of both cell lines to rFSH-treatment, they were exposed to androstenedione (5 uM) and rFSH (125 ng/mL) alone and in combination for 24 hours, and the secretory response determined by analysing culture media for E2 and P4 level using ELISA. Exposure to all stimulation regimes significantly increased the release of E2 in both cell types, with the combination generating the highest E2 levels in both cell lines (Figure 5.5 A). For COV434 cells, androstenedione-alone increased E2 from 34.0 pg/µg protein to 52.8 pg/µg protein (increased by 55.3 %), while rFSH-alone promoted a very similar response, increasing E2 from 34.0 pg/µg protein to 55.1 pg/µg protein (increased by 62.1%) (Figure 5.5 A). However, the androstenedione and rFSH combination increased E2 to 66.4 pg/µg protein, which indicated a 95.3 % increase over basal levels (Figure 5.5 A). A similar trend was observed for KGN cells. Here, androstenedione-alone increased E2 release from 27.5 pg/µg protein to 43.8 pg/µg protein (increased by 59.3 %), while rFSH-alone promoted a very similar response, increasing E2 from 27.5 pg/µg protein to 45.4 pg/µg protein (increased by 65.1 %) (Figure 5.5 A). However, the androstenedione and rFSH combination increased E2 to 53.9 pg/µg (increased by 96.0%) from solvent control and was a similar response to COV434 cells above (Figure 5.5).

The P4 secretion response was less pronounced. Exposure of both cell lines to androstenedione alone did not significantly increased P4 secretion beyond control (Figure 5.5 B). rFSH alone increased P4 production slightly but significantly in COV434 cells only, from 26.9 pg/µg protein to 30.1 pg/µg protein (by 11.9%). However, both cell lines increased P4 output in response to androstenedione and FSH combination stimulation. For COV434 cells, the combination increased P4 from 26.9 pg/µg protein to 37.0 pg/µg protein (increased by 37.6 %), while it increased P4 secretion from KGN cells from 34.3 pg/µg protein to 46.2 pg/µg protein (increased by 34.7%, Figure 5.5 B).

Overall, COV434 cells produced higher levels of E2 over all treatment groups compared to KGN cells (Figure 5.4 B), while KGN cells produced higher levels of P4 on average over all treatment groups compared to COV434 cells (Figure 5.5 B).

Additionally, androstenedione and rFSH combination treatment yielded the largest increase in both cell lines for both E2 (Figure 5.5 A) and P4 (Figure 5.5 B) levels, when compared to the individual treatments.



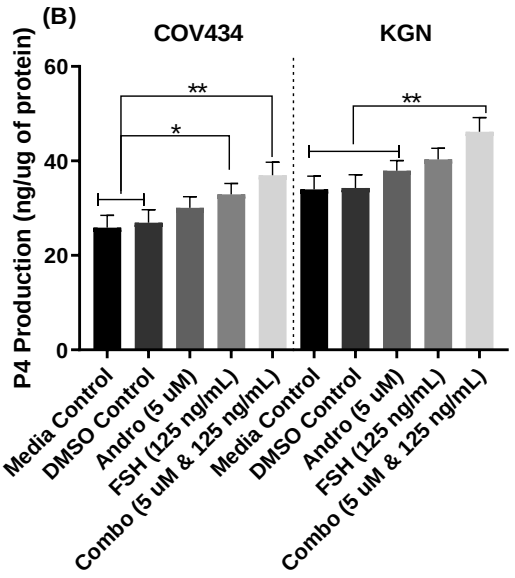
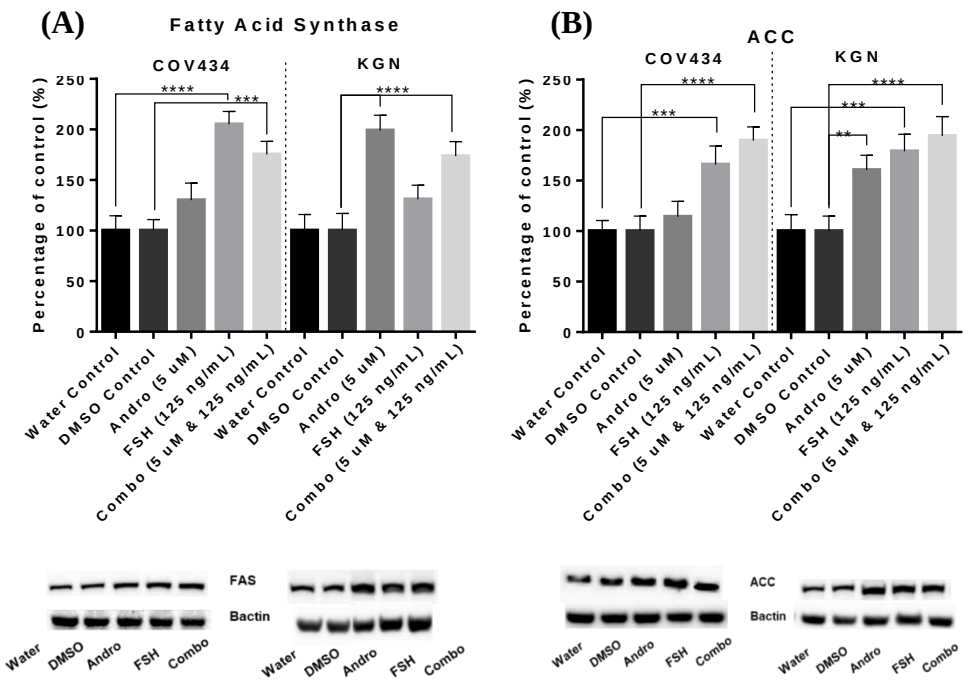


Figure 5.5: **The effect of rFSH & androstenedione treatment on hormone production in COV434 & KGN cell lines.** COV434 and KGN cell lines were exposed to rFSH (125 ng/mL) and androstenedione (5 µM), alone and in combination for 24-hour and the effects on E2/P4 secretion were measured. Secretion of **(A)** E2 and **(B)** P4 in response to stimulation with rFSH, androstenedione or in combination was assessed using relevant ELISA kits. Data is represented as mean ± S.D and each experiment was replicated 3 times with each sample measured in triplicate. *p<0.05, **p<0.001, ***p<0.001. *Abbreviations: DMSO, dimethyl sulfoxide; Andro, androstenedione; rFSH, recombinant follicle stimulating hormone.*

5.1.5 The effect of rFSH and androstenedione treatment on lipid metabolism enzyme expression assessed by Western Blot analysis

The expression of key enzymes involved in lipid metabolism were evaluated in COV434 and KGN cell lines following rFSH and/or androstenedione stimulation for 24 hours. The enzyme expression profiles were different according to the cell line and the response to androstenedione, rFSH, or combination. Stimulation of KGN cells with androstenedione alone increased Fatty acid synthase (FAS) and Acetyl-CoA carboxylase (ACC) expression (by 99.1% and 60.5%, respectively Figure 5.6 A & B), and increased StAR expression in both KGN and COV434 cell lines (by 74.5% and 50.5%, respectively, Figure 5.6 D). However, androstenedione alone did not significantly alter FAS, ACC in COV434 cells, and did not change HMGCR expression in either cell lines. In addition, stimulation of KGN cells with rFSH alone increased ACC and StAR expression (by 79.0% and 69.4%, respectively Figure 5.6 B & D), while rFSH alone increased FAS, ACC, HMGCR and StAR expression in COV434 cell lines (by 105.0%, 65.9%, 63.6% and 63.5%, respectively, Figure 5.6 A-D). rFSH did not significantly alter FAS or HMGCR expression in KGN cells (Figure 5.6 A & C). However, the combination treatment had a broader and stronger effect across the various enzymes and cell lines. It increased KGN and COV434 expression of FAS (by 73.7% and 75.3%, respectively Figure 5.6 A), ACC (by 94.3% and 89.7%, respectively Figure 5.6 B) and StAR (by 112.7% and 113.6%, respectively Figure 5.6 D). The combination also increased HMGCR expression in COV434 cells (by 40.0%, Figure 5.6 C) but did not affect HMGCR expression in KGN cells. The level of increased enzyme expression induced by the combination treatment was largely similar across both cell types.



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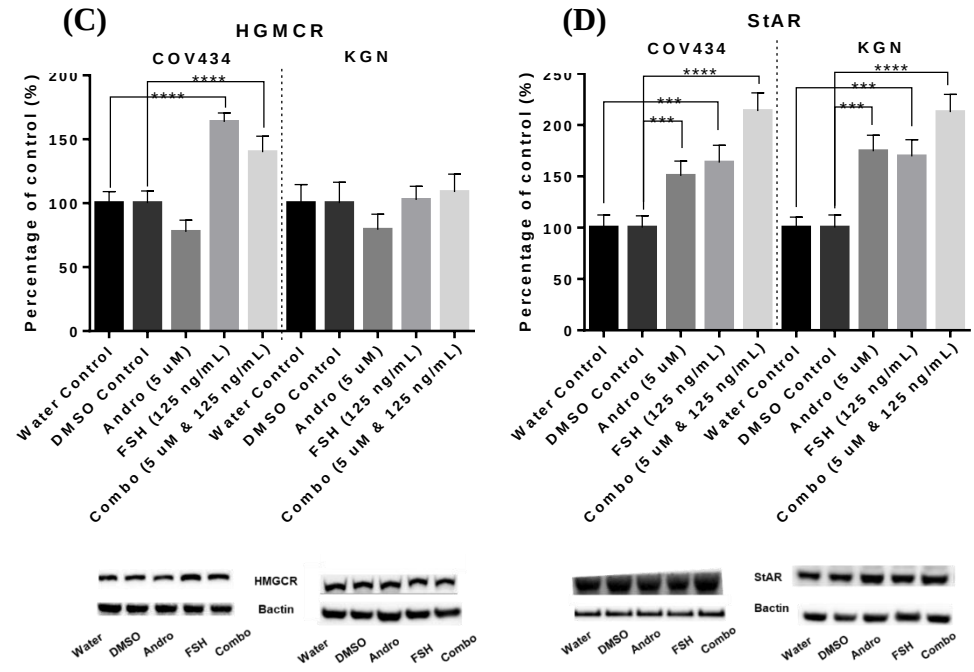

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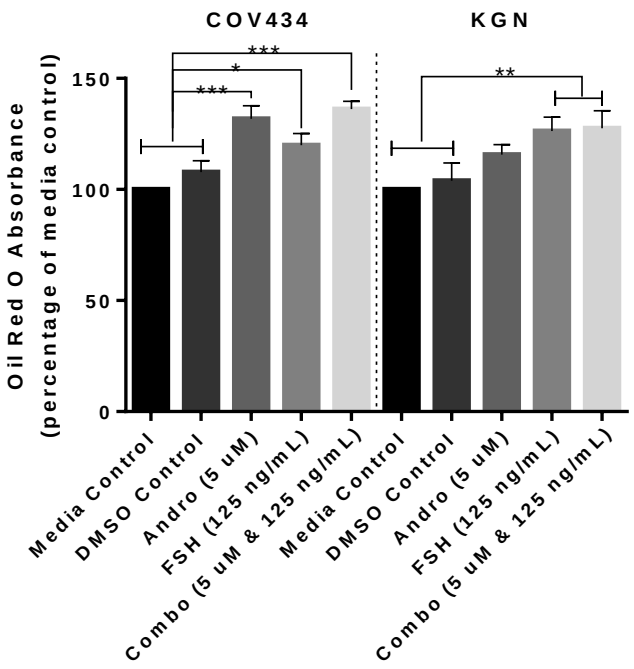
(E) Marker of interest	Stimulation regimes		
	Andro-alone	rFSH-alone	Combo (Andro + rFSH)
FAS	↑ in KGNs	↑ in COV434s	↑ in both cell lines
ACC	↑ in KGNs	↑ in both cell lines	↑ in both cell lines
HMGCR	*No change*	↑ in COV434s	↑ in COV434s
StAR	↑ in both cell lines	↑ in both cell lines	↑ in both cell lines
No significant change in either cell line			
Significant change observed in COV434 cells only			
Significant change observed in KGN cells only			
Significant change observed in both cell lines			

Figure 5.6: The effect of rFSH & androstenedione on lipid metabolising enzyme expression in COV434 & KGN cell lines. COV434 and KGN cell lines were exposed to androstenedione (5 µM) or rFSH (125 ng/mL), alone and in combination for 24-hour and the effects on lipid metabolising enzyme expression determined using Western blot analysis. Proteins evaluated were **(A)** Fatty acid synthase (FAS), **(B)** Acetyl-Carboxylase (ACC), **(C)** HMG-CoA reductase (HMGCR), and **(D)** Steroid acute regulatory (StAR). **(E)** Table to summarise significant results displayed in (A-D). Data is represented as mean ± S.D and each experiment was replicated 3 times*p<0.05, **p<0.01, ***p<0.001. *Abbreviations: DMSO, dimethyl sulfoxide; Andro, androstenedione; rFSH, recombinant follicle stimulating hormone; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; HMGCR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; StAR, steroid acute regulatory protein.*

5.1.6 The effect of rFSH and androstenedione treatment on intracellular lipid deposition

Intracellular lipid deposition was assessed using Oil Red O stain in COV434 and KGN cell lines following rFSH and/or androstenedione stimulation for 24 hours. rFSH and androstenedione, both alone and in combination, significantly increased intracellular lipid deposits in both cell lines (Figure 5.7). Androstenedione significantly increased lipid deposition in COV434 cells relative to media and solvent control (by approximately 13.1%, Figure 5.7 A), but not in KGN cells relative to control (Figure 5.7 A). In contrast, rFSH significantly increased lipid deposition in KGN cells relative to media and solvent control (approximately by 15.7%, Figure 5.7 A). When compared to COV434 cells, the response to rFSH was slightly greater in KGN cells (22.4% vs. 20.0%, Figure 5.7 A). However, the combination treatment showed the greatest response in COV434 cells when compared to the KGN cells (29.2% vs. 23.7%, Figure 5.7 A). A visual representation via bright field microscopy imaging of increased intracellular lipids (quantified in Figure 5.7 A) are displayed in Figure 5.7 B.

(A)



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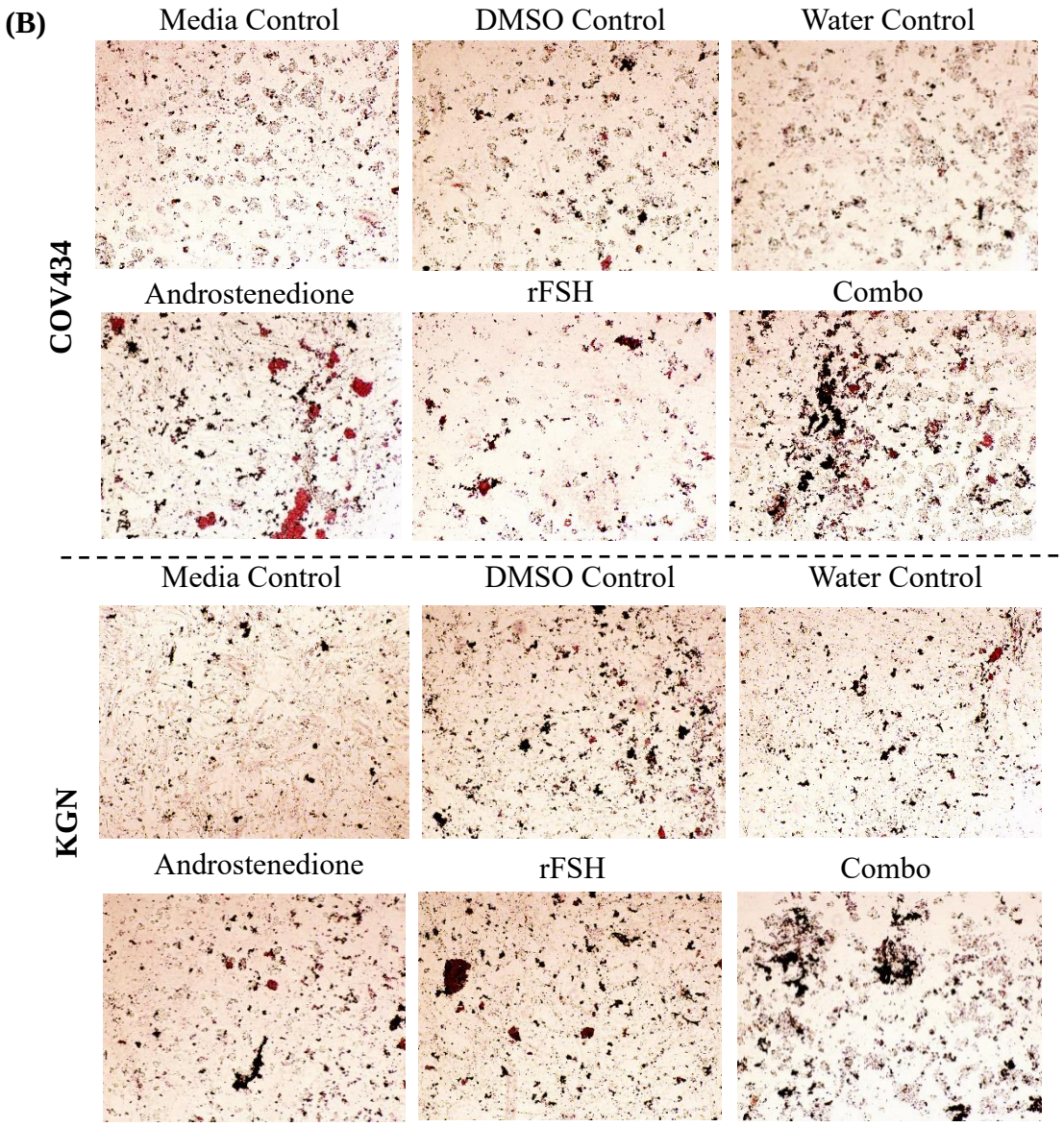
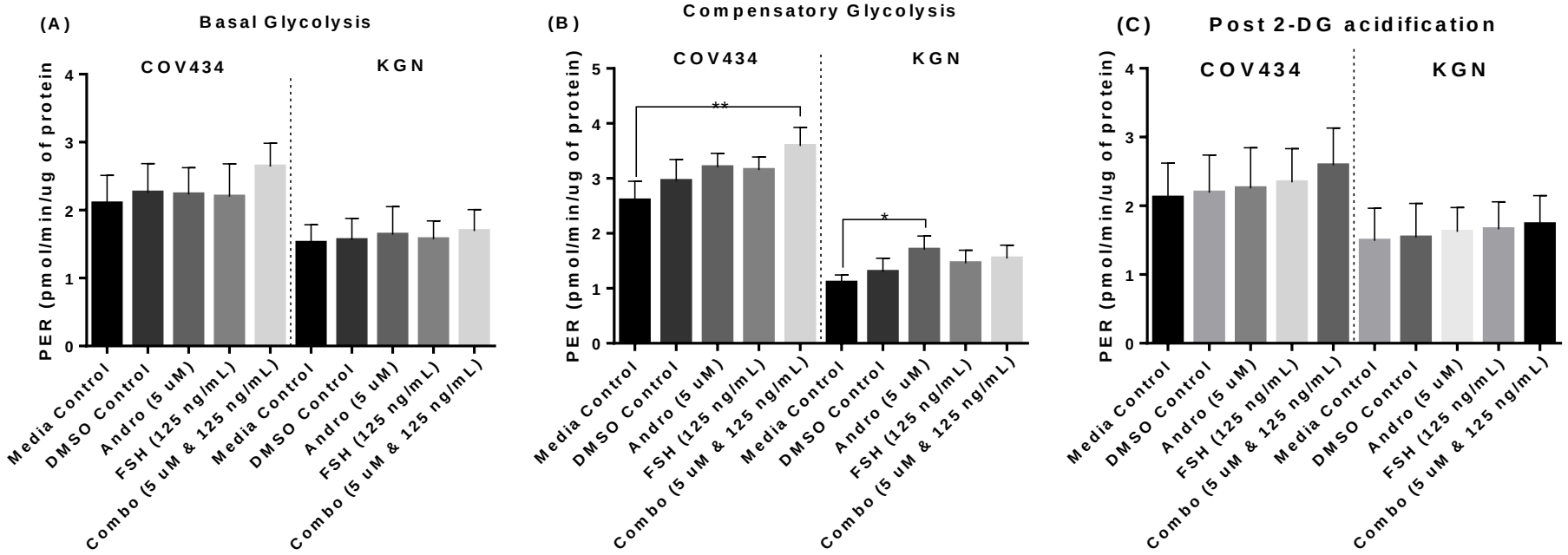


Figure 5.7: The effect of androstenedione & rFSH on lipid deposition in COV434 & KGN cell lines. COV434 and KGN cell lines were exposed to androstenedione (5 µM), rFSH (125 ng/mL), alone and in combination for 24-hour and the effects on lipid deposition determined using Oil Red O stain. Graphs displayed in **(A)** represent absorbance of the eluted Oil Red O stain measured at a wavelength of 518 nm. Bright field microscopy images at 40X magnification displayed in **(B)** are representative from one independent experiment. The independent experiments were replicated 3 times with each sample measured in quadruplicate. Data is represented as mean ± S.D and each experiment was replicated 3 times, *p<0.05, **p<0.01, ***p<0.001. *Abbreviations: DMSO, dimethyl sulfoxide; Andro, androstenedione; rFSH, recombinant follicle stimulating hormone.*

5.1.7 The effect of rFSH and androstenedione treatment on glycolytic metabolic flux

Extracellular metabolic flux analysis was used to evaluate glycolytic bioenergetics in real time through measurements of PER (measurements detailed here defined in Table 2.8) in COV434 and KGN cell lines following rFSH and/or androstenedione stimulation over 24 hours. Exposure of both cell lines to androstenedione and rFSH alone had minimal impact on cell glycolytic bioenergetic responses (Figure 5.8). In KGN cells, androstenedione-alone increased compensatory glycolysis by 23.1% compared to solvent control (Figure 5.8 B). In addition, the combination treatment led to higher PER associated with compensatory glycolysis for COV434 cells (Figure 5.8 B). No changes were observed in either cell line for post-2DG acidification (Figure 5.8 C).


(A)
Basal Glycolysis
COV434
KGN
PER (pmol/min/ug of protein
Media Control
DMSO Control
Andro (5 uM)
FSH (125 ng/mL)
Combo (5 uM & 125 ng/mL)
(B)
Compensatory Glycolysis
**
*
(C)
Post 2-DG acidification

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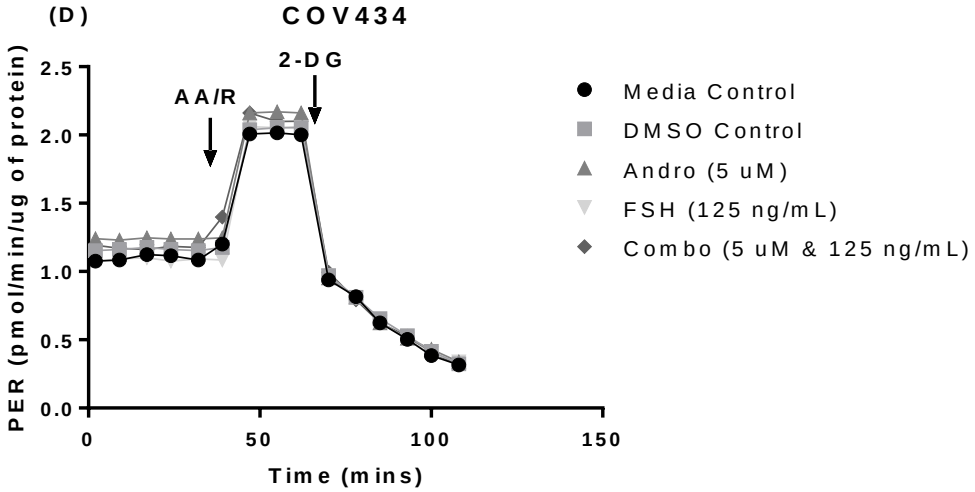

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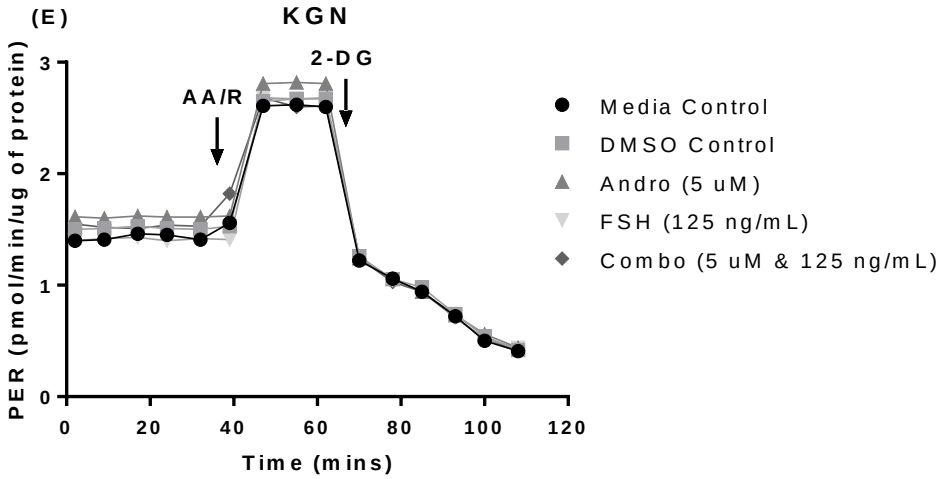

5692

5693 **Figure 5.8**: **The effect of rFSH & androstenedione treatment on glycolytic**
5694 **metabolism of COV434 & KGN cell lines.** Cellular bioenergetics was assessed using
5695 extracellular flux analysis in COV434 and KGN cell lines following 24-hour treatment
5696 with rFSH (125 ng/mL) and androstenedione (5 µM), alone and in combination. PER
5697 was measured to assess **(A)** Basal glycolysis and **(B)** Compensatory Glycolysis.
5698 Representative Seahorse traces are shown in **(D & E)**. Data is represented as mean ±
5699 S.D and each experiment was replicated 3 times with each sample measured in
5700 quadruplicate. *p<0.05, **p<0.01, ***p<0.001. AA/Rotenone were used at an
5701 optimised concentration of 1 µM. 2-DG was used in excess at a concentration of 200
5702 mM. *Abbreviations: DMSO, dimethyl sulfoxide; Andro, androstenedione; rFSH,*
5703 *recombinant follicle stimulating hormone, AA, antimycin A; R, rotenone; 2-DG, 2-*
5704 *deoxyglucose.*

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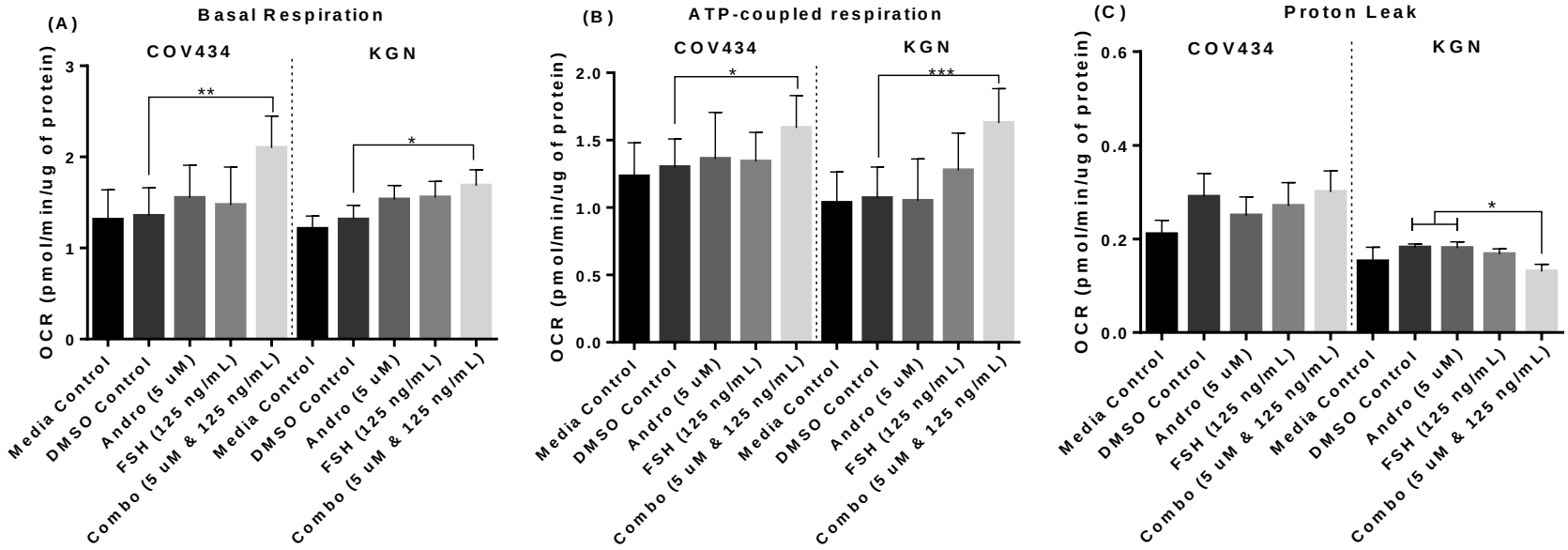
5.1.8 The effect of rFSH and androstenedione stimulation on mitochondrial metabolic flux

Extracellular metabolic flux analysis was used to evaluate mitochondrial bioenergetic parameters as outlined in Table 2.7. Real time measurements of OCR in COV434 and KGN cell lines were recorded following rFSH and/or androstenedione stimulation over 24 hours. Exposure of both cell lines to androstenedione and/or rFSH-alone for 24 hours had minimal impact on cell mitochondrial bioenergetic responses. However, exposure to both agents in combination altered several metabolic parameters. In COV434 cells, androstenedione and rFSH alone did not alter any bioenergetics parameter significantly (Figure 5.9 A-E), but when administered in combination there was a significant increase in basal respiration, ATP-coupled respiration, and maximal respiration (Figure 5.9 A, B, D, respectively). Compared to solvent control these parameters increased by 55.6%, 22.3% and 26.9% (Figure 5.9 A, B & D, respectively). There were minimal changes in bioenergetic parameters for KGN when exposed to androstenedione, although rFSH alone decreased non-mitochondrial respiration in KGNs by 21.9% significantly compared to control (Figure 5.9 E). In KGN cells, basal respiration, ATP-coupled respiration, and maximal respiration were significantly increased in response to combination treatment (Figure 5.9 A, B, & D). Compared to solvent control these parameters increased by 28.2%, 52.3% and 22.7%, respectively (Figure 5.9 A, B & D). The normalised bioenergetics responses demonstrate that all COV434 metabolic rates are higher for OCR per μg of cellular protein in comparison to KGN cells (Figure 5.9 A-E).



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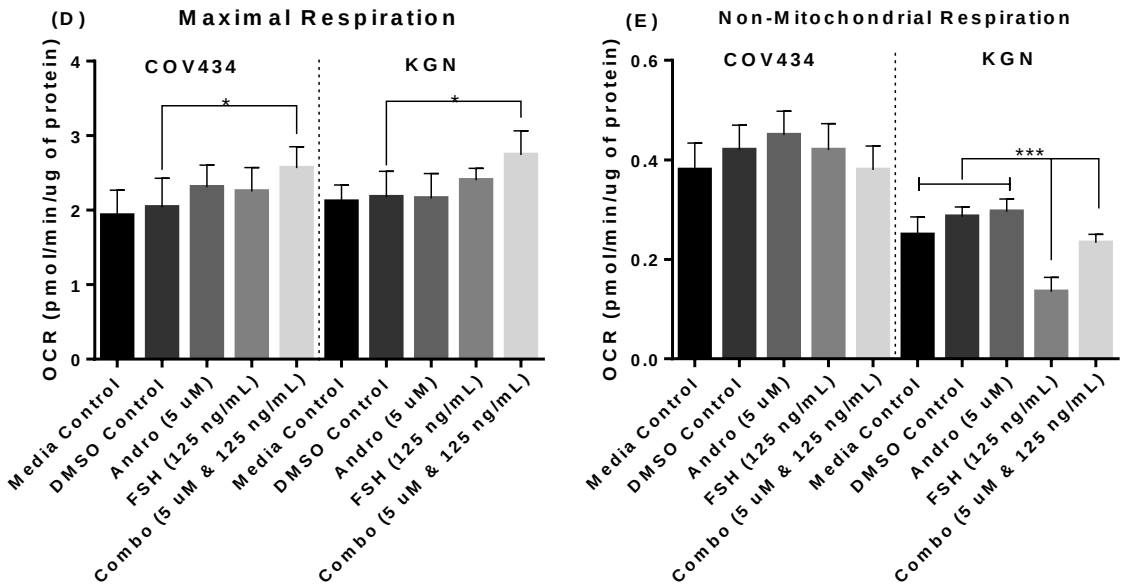
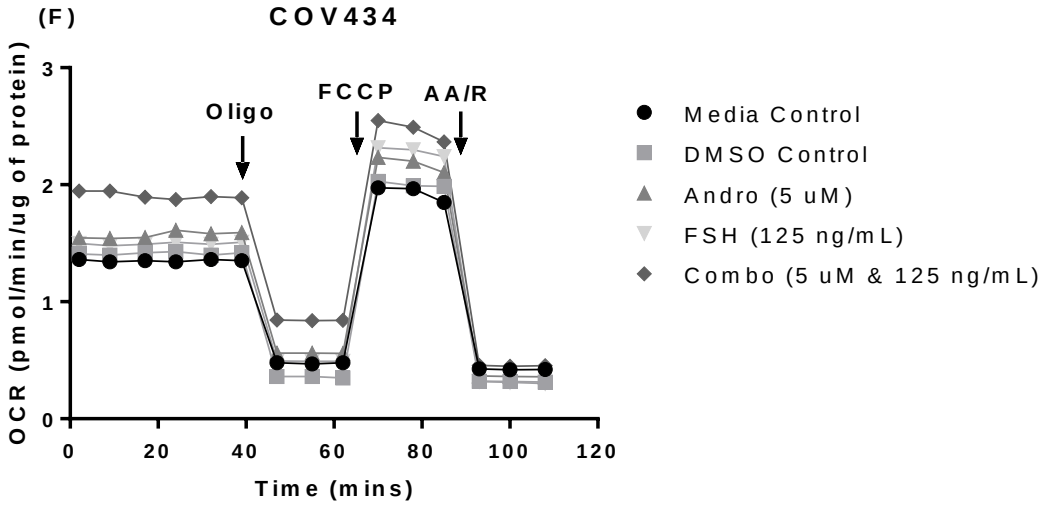

(D)
Maximal Respiration
OCR (pmol/min/ug of protein)
COV434
KGN
*
*
0
1
2
3
4
Media Control
DMSO Control
Andro (5 uM)
FSH (125 ng/mL)
Combo (5 uM & 125 ng/mL)
(E)
Non-Mitochondrial Respiration
OCR (pmol/min/ug of protein)
COV434
KGN
***
0.0
0.2
0.4
0.6
Media Control
DMSO Control
Andro (5 uM)
FSH (125 ng/mL)
Combo (5 uM & 125 ng/mL)

5738



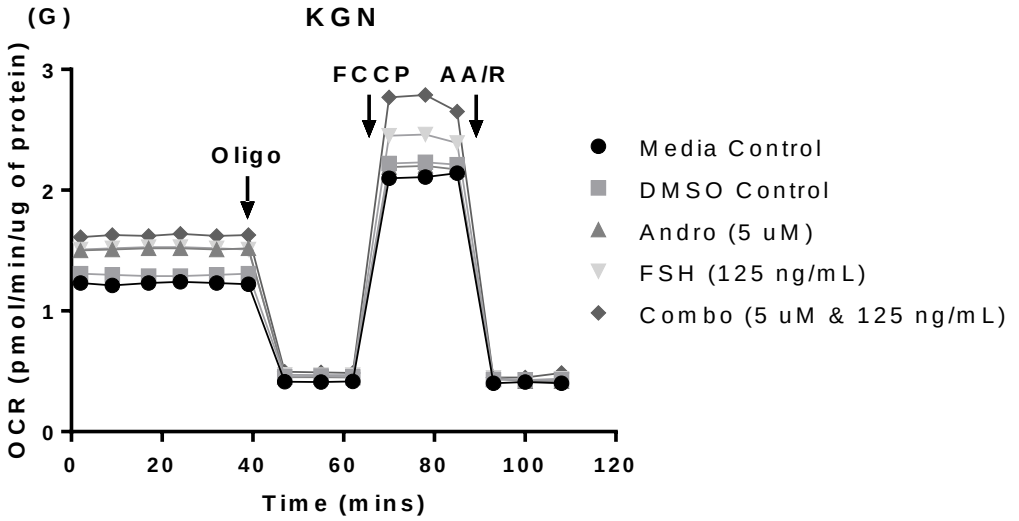
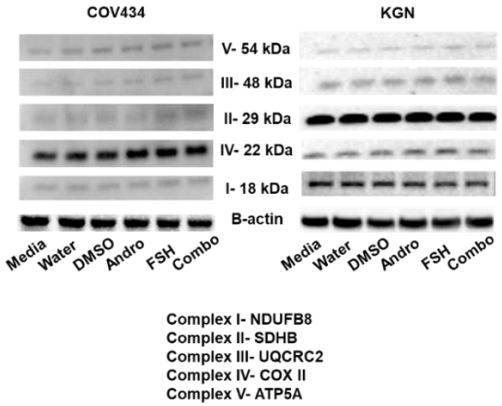


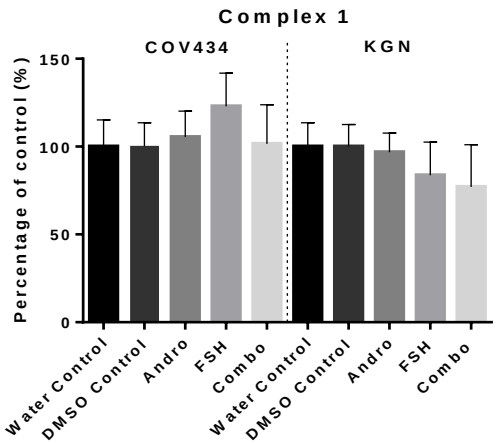
Figure 5.9: **The effect of rFSH & androstenedione treatment on COV434 & KGN mitochondrial bioenergetic parameters.** Mitochondrial bioenergetics were assessed using extracellular flux analysis in COV434 and KGN cell lines following 24-hour treatment with rFSH (125 ng/mL) and androstenedione (5 µM), alone and in combination. OCR was used to determine **(A)** basal respiration, **(B)** ATP-coupled respiration/production, **(C**) proton leak, **(D)** maximal respiration, and **(E)** non-mitochondrial respiration. Representative Seahorse traces are shown in **(F & G).** Data is represented as mean ± S.D and each experiment was replicated 3 times and each sample was measured in quadruplicate. *p<0.05, **p<0.01, ***p<0.001. Oligomycin, FCCP and AA/Rotenone were used at an optimised concentration of 2 µM, 0.75 µM, and 1 µM (each) respectively. *Abbreviations: DMSO, dimethyl sulfoxide; Andro, androstenedione; rFSH, recombinant follicle stimulating hormone; Oligo,*

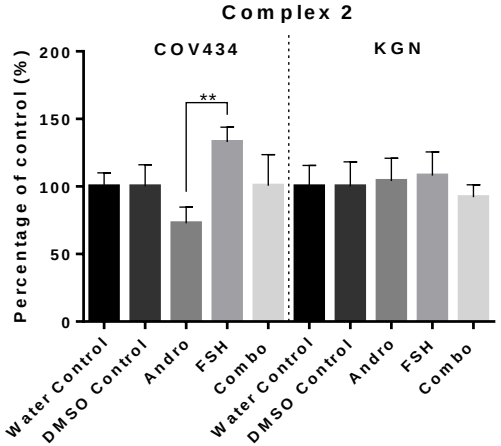
oligomycin; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; AA, antimycin A; R, rotenone.

5.1.9 The effect of rFSH and androstenedione treatment on the protein expression of mitochondrial complexes

COV434 and KGN cells stimulated with androstenedione, rFSH or combination for 24 hours did not significantly alter the protein expression profile of mitochondrial electron transport chain (ETC) complexes I, IV and V in either cell line (Figure 5.10). Conversely, in COV434 cells, rFSH treatment significantly increased the expression of complex II compared to androstenedione treatment ($p=<0.01$; Figure 5.10), while the combination treatment significantly increased the expression of complex III by 81.7% compared to control ($p=0.004$; Figure 5.10). The combination increased complex III expression by 10% in KGN cells, but this was not significant (Figure 5.10).


COV434
KGN
V- 54 kDa
III- 48 kDa
II- 29 kDa
IV- 22 kDa
I- 18 kDa
B-actin
Media
Water
DMSO
Andro
FSH
Combo
Complex I- NDUFB8
Complex II- SDHB
Complex III- UQCRC2
Complex IV- COX II
Complex V- ATP5A


Complex 1
COV434
KGN
Percentage of control (%)
150
100
50
0
Water Control
DMSO Control
Andro
FSH
Combo


Complex 2
COV434
KGN
Percentage of control (%)
200
150
100
50
0
**
Water Control
DMSO Control
Andro
FSH
Combo

5777

5778

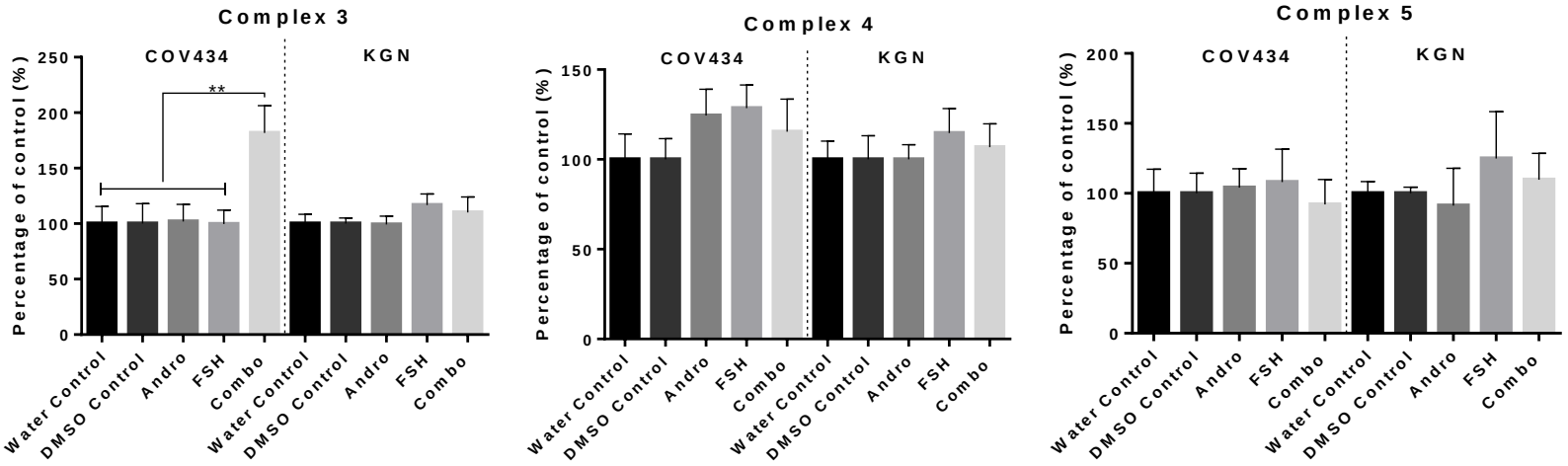


Figure 5.10: **The effect of rFSH & androstenedione treatment on the protein expression of mitochondrial complexes in COV434 & KGN cell lines.** Western blot analysis was used to assess oxidative phosphorylation protein expression in COV434 and KGN cell lines following 24-hour treatment with androstenedione (5 µM) and rFSH (125 ng/mL), alone and in combination. Data is represented as mean ± S.D and each experiment was replicated 3 times. *p<0.05, **p<0.01, ***p<0.001. *Abbreviations: DMSO, dimethyl sulfoxide; Andro, androstenedione; rFSH, recombinant follicle stimulating hormone*

5.2 Discussion

The overall aim for the present study was to understand the effects of rFSH and androstenedione stimulation on the underlying biology of these morphologically different GC lines. Here, we characterised the effect of rFSH and androstenedione on cell viability/proliferation, hormone secretion, lipid metabolic enzyme expression and cellular bioenergetics in the human GC lines COV434 and KGN. Treatment with rFSH and androstenedione for 24 hours did not significantly alter any parameters related to cell viability/proliferation. However, the combination significantly increased the secretion of E2/P4, and lipid deposition in both cell lines. Furthermore, all treatments significantly increased the expression of StAR in both cell lines, a protein that regulates cholesterol transfer into the mitochondrial. ACC expression was also elevated in both cell lines following combination treatments, and this enzyme catalyses the conversion of TCA metabolite acetyl-CoA to malonyl-CoA promoting fatty acid (FA) biosynthesis. Similarly, the combination significantly elevated the expression of FAS in both cell lines, and this multi-enzyme complex catalyses the synthesis of palmitate from acetyl-CoA and malonyl-CoA, in the presence of NADPH. In COV434 cells treated with the combination, there was a significant increase in HGMCR expression, an important enzyme which catalyses the conversion of HMG-CoA to mevalonic acid in the cholesterol synthesis pathway. In both cell lines treated with the combination, several mitochondrial respiration parameters were significantly increased including basal respiration, mitochondrial-linked ATP production, and maximal respiration. For glycolytic metabolism, compensatory glycolysis was significantly increased in KGN cells only. Lastly, for the expression of mitochondrial complexes, only complex III was significantly enhanced in both cell lines.

Overall, it appeared that rFSH and androstenedione activated biosynthetic pathways in both cell lines (FA and cholesterol synthesis), while also increasing cholesterol transfer to the mitochondria and the secretion of sex steroids, E2 and P4. It also appeared that the fuel to meet these energy-demanding processes may originate in altered mitochondrial metabolism, as reflected by an elevation in mitochondrial bioenergetic parameters. All statistically significant results are summarised below in Table 5.1.

Table 5.1: Results summary table of the effect of androstenedione and rFSH stimulation on COV434 and KGN cell lines. All significant findings detailed here were relative to the relevant media or solvent control. *Abbreviations- Andro, androstenedione; rFSH, recombinant follicle stimulating hormone; ROS, reactive oxygen species; E2, estradiol; P4, progesterone; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; HGMCR, 3-hydroxy-3-methylglutaryl-CoA reductase; StAR, steroid acute regulatory protein; 2-DG, 2-deoxyglucose; ATP, adenosine triphosphate; NSC, No significant change (p=≥0.05); Sig, significant change (p=<0.05).*

Table legend	No significant change in either cell line
	Significant change observed in COV434 cells only
	Significant change observed in KGN cells only
	Significant change observed in both cell lines

Experiment	Andro-alone	rFSH-alone	Combination
MTT Cell viability	*NSC*	*NSC*	*NSC*
Cell cycle	*NSC*	Sig. decreased G0/G1 phase in KGN only	*NSC*
Intracellular ROS	*NSC*	*NSC*	*NSC*
E2 secretion	Sig. increased in both cell lines	Sig. increased in both cell lines	Sig. increased in both cell lines
P4 secretion	*NSC*	Sig. increased in COV434 only	Sig. increased in both cell lines
Lipid deposition	Sig. increased in COV434 only	Sig. increased in both cell lines	Sig. increased in both cell lines
	Western Blot analysis of lipid metabolism pathway		
FAS	Sig. increased in KGN only	Sig. increased in COV434 only	Sig. increased in both cell lines
ACC	Sig. increased in KGN only	Sig. increased in both cell lines	Sig. increased in both cell lines

HGMCR	*NSC*	Sig. increased in COV434 only	Sig. increased in COV434 only
StAR	Sig. increased in both cell lines	Sig. increased in both cell lines	Sig. increased in both cell lines
	Bioenergetics (glycolytic)		
Basal glycolysis	*NSC*	*NSC*	*NSC*
Compensatory glycolysis	Sig. increased in KGN only	*NSC*	Sig. increased in COV434 only
Post 2-DG acidification	*NSC*	*NSC*	*NSC*
	Bioenergetics (mitochondrial)		
Basal respiration	*NSC*	*NSC*	Sig. increased in both cell lines
ATP-coupled respiration	*NSC*	*NSC*	Sig. increased in both cell lines
Proton leak	*NSC*	*NSC*	Sig. decreased in KGN only
Maximal respiration	*NSC*	*NSC*	Sig. increased in both cell lines
Non-mito respiration	*NSC*	Sig. decreased in KGN only	Sig. decreased in KGN only
	Western Blot analysis of mitochondrial complexes		
Complex 1	*NSC*	*NSC*	*NSC*
Complex 2	*NSC*	*NSC*	*NSC*
Complex 3	*NSC*	*NSC*	Sig. increased in COV434 only
Complex 4	*NSC*	*NSC*	*NSC*
Complex 5	*NSC*	*NSC*	*NSC*

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There are many registered trials currently underway and there are several clinical studies which have published data relating to the clinical use of the rFSH drug, Rekovelle® (402-412), and one pharmacological report (413). This is the first publicly available *in vitro* study utilising Rekovelle® for the purpose of characterising its effect in COV434 and KGN, and showed that alone and in combination with androstenedione, it induced the secretion of E2 and P4 from both human cell lines. *In vitro* concentrations of rFSH stimulation in the current literature vary from 25 to 200 ng/mL, with the higher doses used in combination with other reagents (such as chemotherapy drugs) to induce apoptosis and oxidative stress (260, 414-416). For this study, an androstenedione and rFSH concentration of 5 µM and 125 ng/mL, respectively, were selected that were not cytotoxic and able to modulate various metabolic and steroidogenic responses. Here, cell proliferation and viability data (MTT assay, cell cycle and ROS) suggested that the concentrations used throughout were not inducing significant alterations to cell growth dynamics and viability, and the concentration range chosen was in line with the drug concentrations outlined in literature (254, 260).

GC function greatly depends on the ovarian folliculogenesis stage, ovulation or the formation of the corpus luteum (417). These changes are tightly regulated by a combination of gonadotropins, steroid hormones, and growth factors (225, 227, 418). In preovulatory follicles, GCs are highly proliferative in nature, while they switch to a non-proliferation phenotype at the terminally differentiated luteal phase (419). Given the proliferative nature of GCs in follicular development, understanding how FSH and androstenedione impact GC cell cycle progression is crucial. Here, stimulation of KGN cells with rFSH significantly decreased the number of cells present in the G0/G1 phase of cell cycle. However, when rFSH stimulation was combined with androstenedione, this effect was diminished. Whether rFSH is directly or indirectly influencing the change in cell cycle of KGN cells remains unclear. Regulatory control of the cell cycle is complex and requires a myriad of factors and molecules, which can be altered by various external signals at several of the cycle stages (420, 421). In the ovary specifically, several key hormones (E2, FSH and LH) play an important role in the progression of follicular growth and regulation of the cell cycle, particularly through their alteration of intracellular cAMP levels (422, 423). High levels of activin (which increases FSH production) have been shown to stimulate GC DNA synthesis

in pre-ovulatory GCs (424). Therefore, it is possible that activin, E2 and FSH regulate cyclin D2, which in turn progresses GCs from the G1 phase to the S phase which reflects an increase in DNA synthesis and as a result, cell proliferation/division. However, activin and cyclin D2 were not measured in the current study, and we did not observe any autocrine proliferative effect from secreted E2 at the 24-hour time-point. Additionally, previous data indicates that androstenedione may modulate cell survival, expression of the estrogen receptor-β and proteins related to apoptosis (425). Cell proliferation, apoptosis and cell cycle are associated processes, whereby when proliferating cells undergo apoptosis their proliferation is inhibited and surviving cells can undergo cell cycle arrest and accumulate in the G0/G1 phase (426, 427). Although, no markers of apoptosis were investigated in this study, there were no changes observed in cell viability or the sub-G1 cell cycle population that indicated no rise in cell death.

Another important cell viability measure investigated in this study was intracellular ROS, which in high levels can lead to oxidative stress resulting in cell death, and in low levels can promote cell proliferation (428). In the current study, ROS levels were not significantly altered by treatment with reproductive hormones, although rFSH treatment did reduce ROS levels below that of control cells in both cell lines. Similarly, animal studies have shown rFSH has protective effects on GCs from oxidative stress by repressing autophagy/mitophagy and apoptosis pathways, as well as reducing ROS levels (429-431). Conversely, ROS accumulation-induced oxidative stress in primary rat GCs and disrupts mitochondrial function, resulting in impaired steroidogenesis via the modulation of steroidogenic responsive genes (432). These genes included cholesterol side-chain cleavage enzyme (CYP11a1), aromatase (CYP19A1), estrogen receptor β1 (ERβ1), and StAR (432). Here, to account for potential toxicity from drug solvents (i.e., DMSO), subtoxic concentrations of the stimulatory drugs (rFSH and androstenedione) were used to reduce any impact on apoptotic pathways or oxidative stress (from drug and solvent) to preserve bioenergetic and steroidogenic functioning.

Whilst the combination treatments did not induce cell proliferation or elevated intracellular ROS in either cell line, they did alter steroidogenic output as shown by increases in E2 and P4 secretion. Interestingly, COV434 cells produced higher levels of E2, while KGN cells produced higher levels of P4, which could reflect the differing

origin of the two cell lines ('younger' GCs vs. 'older' GCs, respectively). When contrasting COV434 and KGN cells E2/P4 secretion in the literature, making comparisons was challenging as there was extensive variation in cell culture conditions, incubation time, hormonal stimulation regimes, methods of measurement and the units reported (summarised in Table 5.2). Additionally, very few studies to date have assessed the endocrine response of COV434 and KGN cell lines within the same study/publication and under the same culture/stimulation conditions (i.e., rFSH/androstenedione combinations).

Table 5.2: Summary of publications reporting E2 and P4 secretion responses in COV434 and KGN cell lines. Incubation refers to the time in which cells were left in culture prior to supernatant collection for assaying. Method refers to the analytical method used to quantify E2 and P4 in cell culture supernatant. Units displayed reflect the units reported by the respective publication. Only literature detailing relevant hormonal stimulation (rFSH or androgen treatments) were included (other types of drugs, stimulators or inhibitors were excluded). Under hormonal stimulation "None" indicates control cells which received no stimulation and maintained in basal culture conditions. Data pertaining to the present study is including as a reference for comparison (highlighted in grey shading). Publications in which raw results were not reported (only percentage change from control) are not included. **"-" =** Not reported/assessed. For percentage increases relative to control, publications in which only control or only hormonal stimulated cell data was reported are excluded in these relevant columns. *Abbreviations: ECLIA, Electro-chemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assays; RIA, radioimmunoassay; E2, estradiol; P4, progesterone; rFSH, recombinant follicle stimulating hormone; Andro, androstenedione; Test, testosterone.*

Publication	Incubation (hours)	Method	Hormonal Stimulation	COV434		% Increase relative to control		KGN		% Increase relative to control	
				E2	P4	E2	P4	E2	P4	E2	P4
Present study	24	ELISA	None	31.4 ng/µg protein	25.9 ng/µg protein	-	-	25.2 ng/µg protein	34.0 ng/µg protein	-	-
			rFSH 125 ng/mL	55.1 ng/µg protein	32.9 ng/µg protein	75.5%	27.0%	45.4 ng/µg protein	40.3 ng/µg protein	19.8%	18.5%
			Andro 5 µM	52.8 ng/µg protein	30.1 ng/µg protein	68.2%	16.2%	43.8 ng/µg protein	37.9 ng/µg protein	27.4%	11.5%
			rFSH + Andro	66.4 ng/µg protein	37.0 ng/µg protein	111.5%	42.9%	53.9 ng/µg protein	46.2 ng/µg protein	113.9%	35.9%
Al-Kawlani (433)	48	ECLIA	None	10 pmol/L	-	-	-	20 pmol/L	-	-	-
Colombe (434)	48	RIA	rFSH 100 ng/mL	-	-	-	-	-	3 ng/10^6 cells	-	-

Gogola (397)	72	ELISA	Test 200 ng/mL	76.2 pg/mL	-	-	-	10 ng/10^6 cells	-	-	-	
			None			-	-	2513 pg/mL	-	-	-	
			rFSH 100 ng/mL	91.3 pg/mL	-	19.8%	-	3202 pg/mL	-	27.4%	-	
Haltia (435)	96	Mass spec	None	-	-	-	-	< 1 nmol/L	-	-	-	
			rFSH 100 ng/mL			-	-	< 1 nmol/L		0.0%	-	
			Test 2 µM			-	-	14 nmol/L		1300.0 %	-	
			rFSH + Test			-	-	52 nmol/L		5100.0 %	-	
Huang (436)	48	EIA	None	-	-	-	-	50 pg/mL/ 10^6 cells	-	-	-	
Lin (437)	24	ELISA	None	-	-	-	-	900 pg/mL	1200 pg/mL	-	-	
Ni (438)	24	RIA	None	-	-	-	-	1500 pg/mL	-	-	-	
Nishi (254)	24	RIA	None	-	-	-	-	<0.01 ng/10^6 cells/mL	0.24 ng/10^6 cells/mL	-	-	
	48					-	-	-	0.26 ng/10^6 cells/mL	-	-	
	72					-	-	170.7 pg/10^6 cells	1 ng/10^6 cells/mL	-	-	

Simon (439)	48	RIA	None	-	-	-	-	0.25 ng/mL	-	-	-
Xie (440)	48	ECLIA	None	-	-	-	-	30 pg/mL	-	-	-
Xu (441)	24	ELISA	None	-	-	-	-	6 pg/mL	-	-	-
			rFSH 100 ng/mL			-	-	11 pg/mL		83.3%	-
Yang (416)	24	ELISA	None	120 pg/mL/ 10^5 cells	65 ng/mL/ 10^5 cells	-	-	-	-	-	-
			rFSH 50 ng/mL	150 pg/mL/ 10^5 cells	100 ng/mL/ 10^5 cells	25.0%	53.8%			-	-
Zhai (442)	48 + 3 hrs stimulation	ECLIA	Test 10^{-7} mol/L	-	-	-	-	1200 pg/mL	-	-	-
Zhang (260)	24	ECLIA	None	600 pmol/L	-	-	-	-	-	-	-
	48			900 pmol/L		-	-			-	-
	72			1700 pmol/L		-	-			-	-
	24			700 pmol/L		16.7%	-			-	-
	48		rFSH 100 ng/mL	1600 pmol/L		77.8%	-			-	-
	72			3500 pmol/L		105.9%	-			-	-

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Gongola et al., is one of two studies that assessed hormone section in both cell lines and in the same publication. They demonstrated endocrine-disrupting chemicals reduced E2 secretion in COV434 and KGN spheroids, although P4 was not assessed (397). E2 secretion for COV434 and KGN cells was lower than the present study, and was 76.2 pg/mL and 2513 pg/mL, respectively in basal conditions, and 91.3 pg/mL and 3202 pg/mL following rFSH (100 ng/mL) stimulation for 72 hours (equivalent to a 19.8% and 27.4% increase, respectively, Table 5.2) (397). There was a large discrepancy between our E2 secretion data and this study, which is possibly due to different culture conditions. Specifically, Gongola et al., grew COV434 and KGN cells in spheroids and collected supernatants after 72 hours, while in the present study cells were grown in a monolayer and supernatants collected after 24 hours. Furthermore, to account for any variations in cell number and to make equitable comparisons between the two cell lines, we normalised our data to cell protein concentration, but Gongola et al. reported E2 release per unit volume. Nonetheless they also demonstrated the responsiveness of both cell lines to rFSH, through increased E2 release. Al-Kawlani et al., also investigated the E2 secretion of COV434 and KGN cells following treatment with the chemotherapy drug doxorubicin but did not stimulate these cells with rFSH or androstenedione (433). These cancer researchers reported unstimulated control COV434 and KGN cells secreted 10 pmol/L and 20 pmol/L (respectively) following 48 hours incubation (433). This was significantly lower than the 31.4 and 25.2 ng/µg protein reported in the current study in unstimulated COV434 and KGN cells following a 24-hour incubation (Table 5.2).

Several other studies have assessed hormonal stimulation in both cell lines individually. Yang et al., investigated the role of micro RNA-431 in rFSH-regulated cell proliferation and hormone secretion of COV434 cells and found pre-treatment of rFSH increased E2 and P4 secretion by 25.0% and 53.8% compared to control cells over a 24 hour incubation period (Table 5.2) (416). Conversely, we found that the E2 response was more sensitive in COV434 cells, with pre-treatment with rFSH for 24 hours increasing E2 and P4 secretion by 75.5% and 27.0%, respectively (Table 5.2). In addition, a report conducted by Xu et al., assessed the molecular regulation of rFSH receptor expression in GCs, by treating KGN cells with rFSH alone and measuring E2 secretion (441). These researchers reported E2 increased by 83.3% following treatment with rFSH (100 ng/mL) for 24 hours (compared to control KGN cells) but did not

stimulate with androstenedione (Table 5.2) (441). In the present study, following treatment with 125 ng/mL rFSH for 24 hours, E2 secretion increased by 65.1% (Table 5.2).

Prior to the present study, Haltia et al., had published the only publication which investigated the effect of both rFSH and androgen stimulation (i.e., testosterone alone and in combination) for 96 hours on KGN cells, and to functionally profile their hormone release response (435). Interestingly, Halti et al., showed rFSH stimulation alone did not alter E2 secretion, while testosterone-alone stimulation increased E2 by 1300% compared to control (435). Additionally, rFSH in combination with testosterone significantly increased E2 secretion a further 3800%, compared to media control alone (435). These increases in E2 secretion in KGN cells following hormonal stimulation are significantly higher than reported in the present study, where androstenedione increased E2 by 73.8% and the combination with rFSH increased E2 by 113.9% in KGN cells (Table 5.2). However, the basal control cell secretion reported here (25.5 ng/µg protein) was significantly higher than those reported by Halti et al., where they were starting at a much lower threshold to begin with (< 1 nmol/L, equivalent to < 3.2 ng/mL; Table 5.2). Some key differences that could explain the discrepancies between all these studies are the differing culture conditions (DMEM/F12 vs. RPMI-1640 media), cell seeding densities, the source/concentration of stimulatory drugs, assay method and the use of data normalisation (or lack thereof). The data in the present study, demonstrating that the effects of rFSH treatment on COV434 cells were mostly in line with the study conducted by Yang et al., (416), where these researchers reported E2 and P4 increased 25.0% and 53.8%, (respectively) compared to 75.5% and 80.2% detailed here (Table 5.2). In KGN cells, our data supports the findings of Xu et al., who used a similar concentration of rFSH and the same treatment time, although their response was 30.6% lower than that reported here (113.9% vs. 83.3% increase, Table 5.2) (441). As the current study appears to be the first to show the effect of androstenedione (alone) or in combination with rFSH on E2 or P4 secretion in these cell lines, it is difficult to make any further direct comparisons with the literature (Table 5.2).

Given there was no observed change in cell proliferation, the altered hormone secretory responses were likely not due to modified biological processes involving increased cell number through enhanced proliferation, but rather alterations in steroidogenesis possibly via molecular regulation of enzyme expression and activity, along with lipid transport mechanisms (i.e., StAR). One of the key functional aspects of GCs is modulation of the steroidogenesis pathway to allow for the hormone secretion in response to gonadotropin stimulation as discussed previously. Firstly, cholesterol is imported into the mitochondria under the action of StAR, where it is then cleaved by the cholesterol side-chain cleavage enzyme (CYP11A1) giving pregnenolone, which can passively diffuse out of the mitochondria (Figure 1.5). At the SER pregnenolone is converted to P4 under the action of 3β-HSD. Next within the SER, P4 is converted to 17-α hydroxyprogesterone and androstenedione both via the enzyme 17-α-hydroxylase. The androgen androstenedione, under the action of aromatase, is converted to estrone which can be synthesised as outlined or indeed be taken up from the extracellular environment (i.e., culture media). Finally, E1 is converted to E2 is via 17β-HSD (Figure 1.5).

Cells can obtain cholesterol as a substrate for further steroidogenesis from two sources (443). Firstly, cells can synthesise cholesterol from the TCA metabolite, acetyl-CoA (443), or secondly, cells can take up cholesterol from the extracellular environment (443). Acetyl-CoA is a crucial metabolite, and is an important molecule for fatty acid synthesis pathway (444), but is also integrated into the cholesterol synthesis pathway. Therefore, a major aim of the present study was to investigate the metabolic flux of lipids as a precursor for E2 and P4 production/secretion via examining the expression of key lipid metabolising enzymes from the fatty acid synthesis and mevalonate pathways. Consequently, we assessed the expression status of the lipid metabolising enzymes: FAS which catalyses the synthesis of long-chain fatty acids (FAs) from acetyl-CoA and malonyl-CoA (445), ACC catalyses the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA (446), Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) catalyses the conversion of HMG-CoA to mevalonic acid, (which limits the rate of cholesterol synthesis a major precursor of sex steroids) (447), and steroid acute regulatory (StAR) protein which mediates the transfer of cholesterol from the outer mitochondrial membrane to the inner

6011 mitochondrial membrane where it is cleaved by the cholesterol-cleavage side chain
6012 enzyme to produce pregnenolone, the first step of steroid synthesis (Figure 5.11) (448).

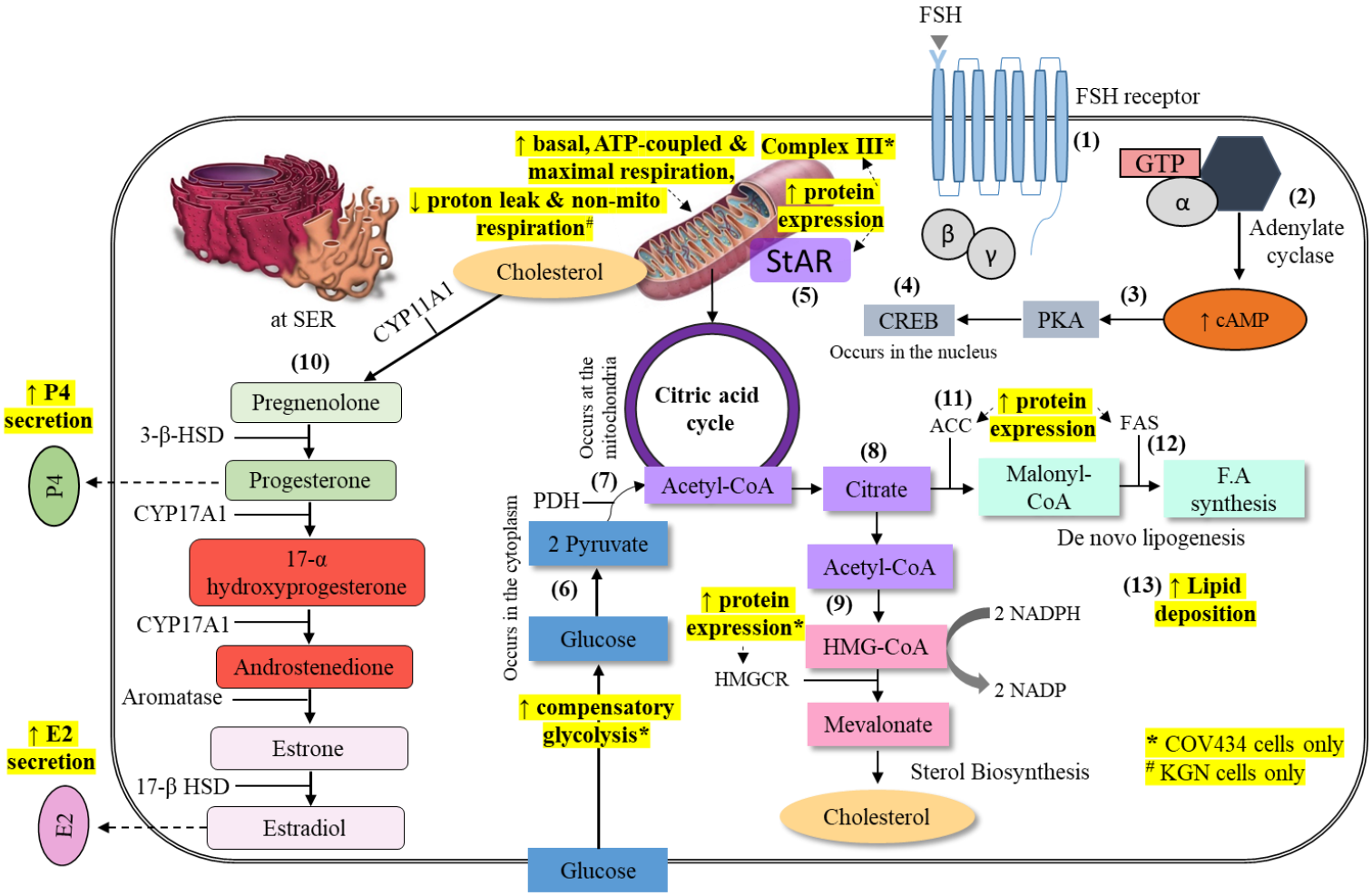


Figure 5.11: GC metabolism in COV434 and KGN cells under stimulated conditions with rFSH and androstenedione. Aspects highlighted in yellow represent results presented within this chapter, with several cellular processes being modulated in COV434 and KGN cells under stimulation with rFSH and androstenedione (arrows in the highlighted text is indicates the direction of change following rFSH/andro stimulation in the current study).

Firstly, upon stimulation, **(1)** FSH binds with the FSH-specific G protein-coupled receptor (FSHR) on the cell membrane (449) (Figure 5.11). This binding causes the splitting of the βγ dimer from the α subunit, which then **(2)** binds to and activates adenylate cyclase (449). Adenylate cyclase converts ATP into the second messenger cAMP (450). **(3)** Increased cytosolic levels of cAMP levels activate the protein kinase (A) pathway, which is how GC exert their steroidogenic effects (451) (Figure 5.11). **(4)** PKA activation acts on cAMP response element-binding protein (CREB) located in the nucleus (451). CREB functions as a transcription factor, binding to the cAMP response element (CRE) of the promoters of its target genes, upon phosphorylation at Ser133 by PKA (452). **(5)** StAR transcription is directly activated by PKA phosphorylation, which not only elevates StAR mRNA, but also acts directly on StAR protein to stimulate its activity (as demonstrated here by increased StAR protein expression, Figure 5.11) (226). StAR, which was increased in both cell lines in response to the rFSH/androstenedione combination, stimulates the initial mitochondrial metabolism of cholesterol to pregnenolone by enhancing cholesterol transfer from the outer membrane to cytochrome P450 11A1 (P450scc) in the inner mitochondrial membrane (226). Given the presence of androstenedione (which is usually supplied by the theca cells), the cells in the current study could have directly utilised the supplied androstenedione to directly produce E2 (228).

Additionally, glucose present in cell culture medium is taken up via the glucose transporters (GLUT) located on the cell membrane (453). **(6)** In the cytoplasm, glucose is converted to pyruvate via glycolysis (Figure 5.11) (454). **(7)** This pyruvate is then converted to acetyl-CoA in the mitochondria via the action of pyruvate dehydrogenase (PDH, Figure 5.11) (454). Acetyl-CoA is a pre-cursor to FA synthesis as well as a substrate for the TCA cycle, although acetyl-CoA cannot leave the mitochondria (444). Acetyl-CoA undergoes condensation with oxaloacetate in the mitochondrial matrix, forming citrate **(8)**, which is then transported to the cytosol (Figure 5.11) (444). From here acetyl-CoA can be utilised in the cholesterol biosynthesis pathway or the lipogenesis pathway (226). For sterol biosynthesis acetyl-CoA undergoes a two-step process and is converted to β-HMG-CoA (226). **(9)** HMG-CoA is then converted to mevalonate via the action of HMGCR, another enzyme whose expression was enhanced in response to rFSH-alone and in combination with androstenedione in COV434 cells (Figure 5.11) (455). This process continues with several additional

steps, resulting in the production of cholesterol which can be utilised in the steroidogenic pathway **(10)** to further produce and secrete steroid hormones, such as E2 and P4, as observed in the current study (Figure 5.11) (226). **(11)** For the lipogenesis pathway, ACC expression was elevated following stimulation with rFSH and androstenedione (Figure 5.11) (446). **(12)** Finally, malonyl-CoA under the action of FAS can be used for FA synthesis by reacting with additional acetyl-CoA from the TCA forming FAs (e.g. palmitate), and then further reacting with glycerol from glycolysis to form **(13)** triglycerides which are deposited and stored in lipid droplets (Figure 5.11) (456).

A vital pathway for sterol synthesis is the HMG-CoA pathway (455). Cytosolic and ER enzymes synthesise cholesterol from acetyl-CoA (447). Active HMGCR and the cholesterol biosynthesis pathway is regulated by sterol response element binding protein 2 (SREB-2), a member of the family of transcription factors known as SREBs (457). If sterol availability is low, the ER protein SREB cleavage-activating protein is transported to the Golgi, where HMGCR synthesis is increased (as observed in the current study), resulting in increased cholesterol synthesis (458). Here, increased secretion of E2/P4 could potentially mean there was depletion in the sterol availability which would explain the elevation in HMGCR expression and suggests enhanced cholesterol biosynthesis. Moreover, in response to low availability of cholesterol, cells can increase the gene expression of proteins that stimulate biosynthesis of cholesterol, such as HMGCR (455). Additionally, rising levels of sterols increase the susceptibility of HMGCR enzyme to undergo ER-associated degradation (457).

Steroidogenic cells can uptake circulating low-density lipoproteins via receptor-mediated endocytosis, directing the cholesterol to endosomes or the de novo synthesis of cholesterol can be derived from acetate in the ER (226). Cholesterol is then transported from the SER to the outer mitochondrial membrane in GCs, where it crosses the inner mitochondrial membrane via the action of StAR (226). Expression of the StAR enzyme, alongside the first enzyme in the steroid biosynthesis pathway (cytochrome P450) which are vital for de novo synthesis of steroids, is limited in human GCs (459). As outlined previously, cAMP/PKA signalling is a crucial for this aspect of steroid biosynthesis and StAR expression (460). In GCs cultured *in vitro,* StAR mRNA expression is significantly upregulated by cAMP/PKA activators, particularly rFSH (461, 462). However, steroidogenic regulation can also be

modulated through signal transduction pathways not involving cAMP, including growth factors, macrophage-derived factors, steroidogenic-inducing protein (SIP), and calcium messenger systems (460). In our study, exposure of both cell lines to rFSH, androstenedione or combination, significantly increased StAR protein expression. Furthermore, StAR activity is primarily regulated at the transcriptional level with data demonstrating the first 250 base pairs of the proximal promoter are critical for basal, FSH, and cAMP analogue-stimulated StAR gene transcription (463). Currently, StAR expression has been detectable in human GC lines in basal cell culture conditions (392). Despite this, very little is known about how rFSH (alone or in combination with androstenedione) effects steroidogenic enzyme expression in COV434 or KGN cell lines. Here we found stimulation of COV434 and KGN cells with a combination of rFSH and androstenedione significantly altered the expression of the lipid/steroid hormone metabolism enzymes StAR, FAS, and ACC (pathways summarised in Figure 5.11). Overall, it appeared that the effects of these hormones on E2/P4 secretion, also resulted in the activation four distinct but connected pathways/processes including fatty acid synthesis, cholesterol biosynthesis, and mitochondrial importation of cholesterol and raised mitochondrial-derived ATP levels. However, the degree by which the processes are activated appear to be depending on the cell type, with the "younger" COV434 cells appearing to be more adaptable and responsive.

The major pathway for FA biosynthesis is via the utilisation of acetyl-CoA and NADPH through the action of fatty acid synthase (FAS) enzymes (444). FAs are the major components of these highly crucial lipids and FAS is the lone lipogenic enzyme in humans able to synthesise these all-important FAs de novo (445). FAS levels are abnormally elevated in certain cancers, including ovarian neoplasms (92), but it is unclear if FAS levels are elevated in GCs carcinomas or cell lines, such as the COV434 and KGN cell lines, and in response to rFSH/androstenedione exposure. In the current study, significant elevation in the expression of both ACC and FAS was observed, indicating enhanced lipogenesis which was confirmed by increased lipid deposition. The increased intracellular lipid deposition demonstrated here in both cell lines could be potentially mobilised and used as a source of energy, or to free up cholesterol to be used as a precursor for steroidogenesis (93). However, whether these increased intracellular lipids are FAs, cholesterol, or a combination of both, remains unclear. In conjunction with the increased secretion of E2/P4, enhancement of distinct/connected

pathways (including cholesterol biosynthesis, enhanced mitochondrial importation of cholesterol, and raised mitochondrial-derived ATP), the activation of the FA synthesis pathway (elevated ACC, FAS, and oil red o staining) could proceed to ensure adequate energy and lipid precursor stores are available in anticipation of further stimulatory conditions. One potential biochemical signalling pivot that may control the integration of these pathways and is upstream of the de novo lipogenesis pathway, is 5' adenosine monophosphate-activated protein kinase (AMPK), which regulates the expression of some crucial enzymes directly (ACC and FAS).

It is evident AMPK is a major upstream regulator of numerous key lipid and steroidogenic pathways discussed previously (Figure 5.12). AMPK signalling is an important energy-sensitive mechanism that maintains the optimum cellular energy level by balancing supply and demand for ATP (93). In circumstances of low energy, AMPK is activated to reduce anabolic processes (such as protein and lipid synthesis), while increasing catabolic process (Figure 5.12) (94). In mammalian cells, there are multiple isoforms of each subunit encoded by separate genes (α1, α2, β1, β2, γ1, γ2, and γ3) (95). AMPK activity depends upon phosphorylation of the Thr172 residue in the activation loop of the α-subunit (96). More recent evidence has suggested that AMPK could also mediate hormonal responsiveness in cells, such as FSH stimulation of GC proliferation via inhibition of AMPK activity (97-99). FSH inhibits AMPK phosphorylation at Thr172 residue reducing AMPK activation (Figure 5.12) (98). Additionally, insulin an anabolic hormone, can inhibit AMPK activation in GCs by phosphorylating two serine residues (serine 485/491) at the α-subunit through an Akt-dependent pathway (Figure 5.12) (98). Furthermore, previous data demonstrated AMPK activation (via phosphorylation of the AMPKα on Thr172) causes phosphorylation of ACC at Ser79, resulting in the inhibition of ACC (464). Unfortunately, the current study only examined total ACC expression and not phosphorylated levels. Given time restraints we were not able to elucidate the complexity of the involvement of AMPK signalling in the findings presented here. However, in line with the literature and with support from bioenergetic data (which demonstrated increased ATP production), it is likely that AMPK is not being activated and is possibly inhibited following stimulation with rFSH and androstenedione in COV434 and KGN cells. Therefore, other enzymes are activated downstream of inhibited AMPK including, HMGCR, ACC and FAS and their expression status

increases to meet the cellular demands (Figure 5.12). This is the first study to show rFSH and androstenedione stimulation in COV434 and KGN cells modulated the expression of these enzymes that are related to lipid metabolic flux.

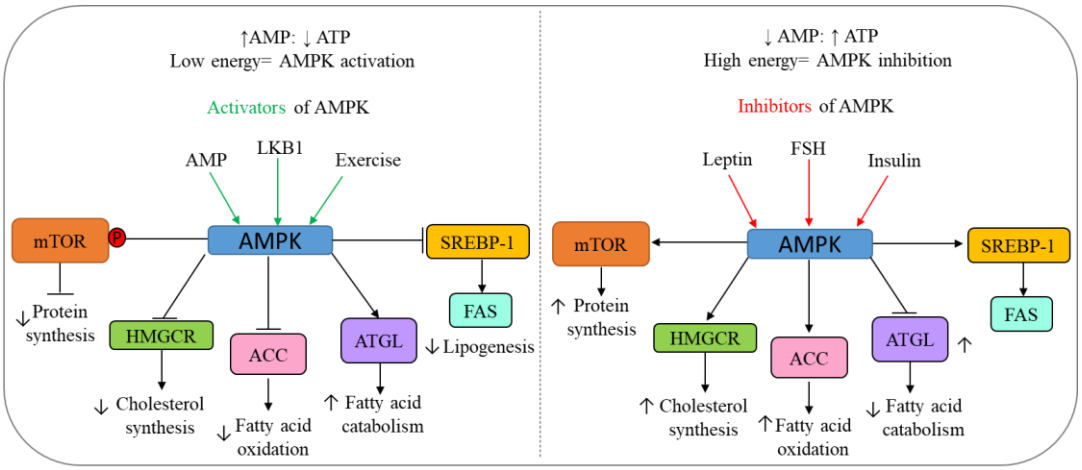


Figure 5.12: AMPK regulation in human GCs. During AMPK activation (left panel) in states of stress or low energy availability, mammalian target of rapamycin (mTOR) is phosphorylated, and protein synthesis is inhibited to redirect metabolism towards increased fatty acid catabolism via adipose triglyceride lipase (ATGL). Conversely, during AMPK inhibition (right panel), hormones (such as FSH) inhibit the activity of AMPK via dephosphorylation of several sites including Thr172 and Ser485/491. As a result, downstream lipid and steroidogenic pathways remain activated, while FA catabolism is inhibited.

Ovarian cells are highly dependent on glycolysis and oxidative phosphorylation for energy provision, depending on the folliculogenesis stage (Figure 5.13) (65). During folliculogenesis, whole follicles increase their glycolytic metabolism as they progress from secondary to preovulatory stages (465-467). Interestingly, paracrine factors secreted from fully matured oocytes are able to promote increased expression of genes related to glycolytic enzymes, when compared to oocytes that are not fully matured (465). However, neither fully developed or maturing oocytes secrete paracrine factors (such as bone morphogenetic proteins) that affect the TCA cycle via the utilisation of TCA cycle intermediates for biosynthesis, therefore these must be supplied via the GCs (465). Oxidative metabolism is highest in primary follicles than at any subsequent stage, indicating that energy needs are greater during a developmental transition (466). The transition rate of quiescent primordial follicles into activated transitional follicles is a critical process in determining the rate at which the ovarian reserve is exhausted (65). Oocytes have a low glycolytic capacity and require GCs to provide them with the products of glycolysis required for development (468). The bioenergetic switching of the follicular stage (oxidative phosphorylation to glycolytic metabolism) are critical determinants of both oocyte quality and ovarian aging (469). In contrast to oocytes, evidence from primary GCs suggests oxidative phosphorylation is not impacted by female age or related to ovarian response (470). Nonetheless, the bi-directional connection between GCs and oocytes is crucial for the delivery of energy substrates, structural components and ions to the maturing oocyte (471).

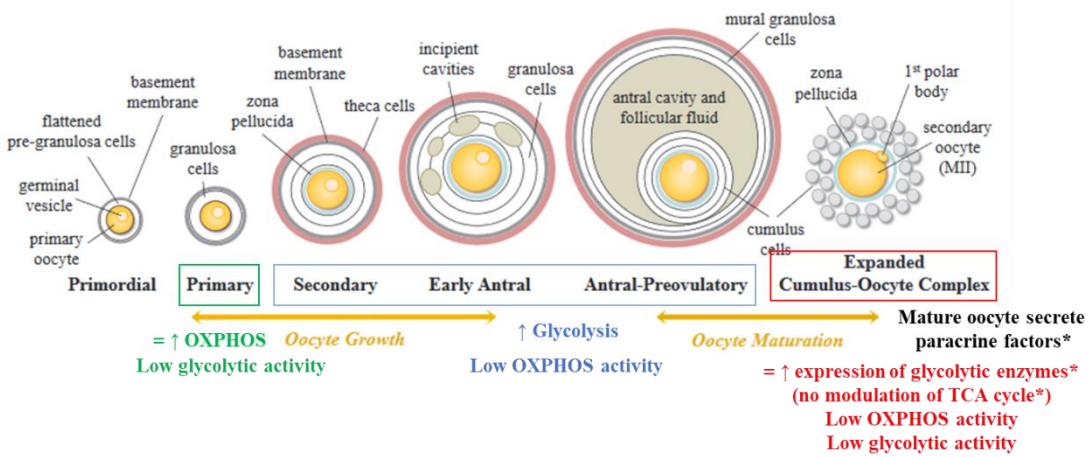


Figure 5.13: Modulation of whole follicle metabolism during folliculogenesis. In primary follicles, oxidative phosphorylation is highest. From the progression of the secondary follicle to the antral-preovulatory follicle, oxidative phosphorylation is downregulated, and glycolytic activity significantly increases to drive this process. Finally, in mature oocytes surrounded by the expanded cumulus complex, metabolism is dampened. To compensate, increased secretion of paracrine factors provided by the GCs drives an increase in metabolism. Additionally, the oocyte can release factors which modulate the expression of glycolytic enzymes within the GCs, increasing their basal glycolysis. Image adapted from Collado-Fernandez et al., (467). *Abbreviations: OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid*.

GCs require key nutrients (such as lipids and amino acids) for biosynthesis pathways, which can also be a source of ATP and macromolecular precursors in some cell types (472). Here, in basal conditions when COV434 and KGN cells were treated with rFSH or androstenedione alone, little change was observed in OCR to suggest a modulation of mitochondrial bioenergetics. However, when treated in combination, several significant bioenergetic changes were observed for both cell lines (Figure 5.11). Firstly, basal OCR significantly increased, which suggests cellular ATP turnover, proton leak or non-mitochondrial respiration has increased. Furthermore, neither proton leak nor non-mitochondrial respiration increased (in fact both decreased in KGN cells while it remained unchanged in COV434 cells), meanwhile ATP-linked respiration was significantly increased. This could all reflect a shortage of available intracellular ATP, or an increased demand for ATP. Given that nutrients are in excess in the culture media and that a variety of biochemical processes are activated that latter is most likely. Specifically, this study showed that presence of rFSH and androstenedione (both of which can individually modulate cell steroidogenesis), significantly increased ATP production in both cell lines as reflected by the oligomycin-inhibited OCR (Figure 5.11). This could indicate a higher demand for initiating and utilisation of ATP for increased cell proliferation or within the steroidogenic pathway and subsequent downstream metabolic pathways, such as FA biosynthesis. Lastly, a significant increase in OCR related to maximal respiration was observed in both cell lines (Figure 5.11). This could indicate one of several possibilities, including increase substrate availability, increased mitochondrial mass and unimpaired ETC integrity. Unfortunately, the reasoning behind the increased maximal respiration demonstrated here in stimulated conditions remains elusive, but it is likely to be the former since nutrients are in excess in the culture media which would increase substrate availability when energy demands are raised. In addition, an increased maximal respiration could be a result of increased glucose uptake or mitochondrial mass, neither of which were investigated here.

Prior to the present study, to the best of our knowledge, only two publications investigated metabolic flux in KGN cells, while it has not been investigated at all in COV434 cells or primary animal/human GCs. Moreover, while our study showed that rFSH at the level chosen did not alter GC proliferation, no previous study has assessed the metabolic flux in COV434 or KGN cells or primary animal/human GCs stimulated

with rFSH and/or androstenedione. In one report, Dong et al., investigated mitochondrial and glycolytic flux using Seahorse XF flux analysis following impairment of tumour suppressor genes in KGN cells. OCR in control KGN cells for basal respiration, maximal respiration and ATP production were approximately 100, 140 and 70 pmol/min, which increased with tumour suppression. The OCR reported in this chapter for basal respiration, maximal respiration, and ATP production of COV434 and KGN cells was between 1.0-3.0 pmol/min/µg protein and cannot be directly compared to previous study. Several key differences exist between these studies. Dong et al., 1) did not use rFSH and/or androstenedione to stimulate KGN cells, so the current findings are novel. 2) They used a 24 well system seeding 50 000 cells/well, while we used a 96 well system with a seeding density of 20,000 cells/well. . 3) Reported raw OCR values only. 4) Used lower concentration of oligomycin (1 µM vs. 2 µM) and FCCP (0.5 µM vs. 0.75 µM) (400).

Dong et al., also assessed glycolysis by quantifying extracellular acidification rate (ECAR). ECAR recorded for basal glycolysis and glycolytic capacity in control KGN cells was 35 and 65 mpH/min (respectively) (400). In our study, glycolysis was measured using the glycolytic rate seahorse assay which is reported as PER rather than ECAR (distinction between these assays detailed in section 2.2.4). The glycolytic function test used in Dong et al. included a 3-injection strategy of glucose, oligomycin and 2-DG (Figure 2.4A), while the glycolytic rate assay in our study was a 2-injection strategy of rotenone and AA in combination, followed by 2-DG. The main advantage of using the newer glycolytic rate assay is the initial injection of rotenone and AA inhibits mitochondrial acidification that was not previously accounted for in the glycolytic test. A crucial limitation of the Dong et al., study was the fact the data pertaining to OCR and ECAR measurements were not normalised (to protein or DNA concentration) to account for potential differences in cell number and glycolytic data did not account for mitochondrial acidification (400).

In another metabolic investigation of KGN cells, Leung et al., examined the effect of peroxisome proliferator-activated receptor-gamma (PPARγ) agonists on KGN cell bioenergetics (399). They reported OCR for basal respiration, ATP production and maximal respiration was approximately 0.4, 0.4 and 0.5 pmol/min/µg of protein, significantly lower than the values reported in the present study (between 1.0-3.0

pmol/min/µg of protein). Several differences are evident here which could explain the discrepancy observed between the Leung et al., study and data presented here. Firstly, Leung et al., used a seeding density of 10,000 cells/well, compared to 20,000 cells/well used in the present study which would explain the higher OCR rate which is positively correlated with cell number (399). Secondly, while Leung et al., used the same concentration of oligomycin (1 µM) as we have used here. However, despite using the same concentration of oligomycin, Leung et al., showed no modulation of OCR following injection with oligomycin. This could suggest either ATP production was not inhibited properly or the membrane was already completely uncoupled (399). The latter has important implications for FCCP uncoupling and they used a higher concentration of the mitochondrial uncoupler FCCP compared to the present study (2 µM vs. 0.75 µM). Considering these two KGN studies, the present investigation is the first to show the effect of rFSH and androstenedione on mitochondrial and glycolytic metabolic flux using the seahorse XF analysis in COV434 and KGN cell lines. It is also the first to directly characterise the metabolic flux in both cell lines together, and the use of normalised raw data to protein levels allowed equitable comparisons between these two morphological and metabolically diverse granulosa cell lines.

While it has previously been well established that COV434 and KGN cells are two distinct and morphologically different cell lines (254, 260), data presented here also indicated for the first time that these are metabolically different cell lines to a small degree. Bioenergetic differences observed between these cell lines both in basal conditions and in treated conditions, could potentially be different due to the origin of these tumour cell lines, or due to the process of establishment of the cell line itself (254, 260). Additionally, 95-97% of adult GC tumours carry a unique somatic mutation in the Foxl2 gene, while KGN cells are heterozygous for the mutation, COV434 cells have a wild-type Foxl2 genotype (473). This supports the current literature that suggests COV434 represents a reproductive age GC cancer (younger), while KGN is considered an adult (post-menopausal/older) GC cancer. Furthermore, in the context of the present study, the age of the donor appears more important than the characterisation of the specific cancer type. Given the decline observed with fertility related to female age, and the biological changes associated with menopause, it is tempting to speculate that ageing process could be a reason why these cell lines

are so metabolically different, or more interestingly, that the metabolic switching contributes at some level to infertility associated ageing.

One of the major issues in assisted reproductive technologies (ART) is advanced maternal aging, which is known to be a major predictor of ART success (54-56). In a comparative study of mitochondria derived from primary mural GCs of 'young' and advanced maternal age IVF patients, younger patients had significantly higher mitochondrial-linked ATP production as measured using a commercially available ATP assay kit (Beyotime, China) (474). Furthermore, the advanced maternal age group had significantly higher abnormal mitochondria and decreased protein expression of the mitochondrial complex V (ATP synthase) (474). Immunoblotting images obtained from lysed whole COV434 and KGN cells displayed in this chapter, suggest some differences in the expression of certain mitochondrial complexes. Stimulation of COV434 and KGN cells with rFSH and androstenedione (alone and in combination) did not significantly alter protein expression of the mitochondrial complexes (I-V). However, COV434 cells appear to highly express complex IV (also known as cytochrome c oxidase), compared to KGN cells in basal culture conditions. Complex IV catalyses the final step in mitochondrial electron transfer chain (ETC), and is regarded as one of the major regulation sites for oxidative phosphorylation (475). This is in line with bioenergetic data presented here which suggests COV434 cells have slightly higher OCR levels associated with increased mitochondrial bioenergetics when compared to KGN cells. In contrast, KGN cells appear to highly express complex II, (also known as succinate dehydrogenase) compared to COV434 cells in basal conditions. Complex II plays a vital role in linking the tricarboxylic acid cycle with the ETC (476). While loss of complex II activity results in increased metabolism of non-essential amino acids, it is unclear if low protein expression of complex II significantly impacts cellular bioenergetics (477). Further investigation of the gene expression of mitochondrial complexes I-V is needed to elucidate any key differences between the COV434 and KGN cell lines.

Furthermore, data presented here demonstrated stimulation with rFSH and androstenedione increased protein expression of mitochondrial complex III in COV434 cells but not in KGN cells. Mitochondrial complex III (ubiquinol-cytochrome c reductase complex subunits) catalyses the reduction of cytochrome c by oxidation of coenzyme Q and the concomitant translocation of 4 protons from the

mitochondrial matrix to the intermembrane space (478), a key step in the oxidative phosphorylation pathway. In mitochondria, complex III is the principal site for ROS generation during the oxidation of complex I substrates (479). Therefore, alterations in complex III could be reflected in intracellular ROS generation. Proton leak and non-mitochondrial derived OCR, which is indicative of mitochondrial complex damage and cytosolic ROS generation (480), did not significantly increase suggesting no increase in mitochondrial-derived ROS. This is in line with our previously discussed general cellular ROS data which showed no significant change in general intracellular ROS levels. Further studies are required to further assess the mitochondrial morphology/mass and specific activity of the mitochondrial complexes in these cell lines.

5.3 Conclusion

Here we aimed to characterise and compare COV434 and KGN cell lines following pre-treatment with rFSH and androstenedione to assess what functional and metabolic changes these hormones would elicit both individually and in combination, and whether the responses varied between the two cell lines. This study was the first in these cell lines to include validation of the drug concentrations used and confirmation that these concentrations resulted in metabolic and signalling changes, without inducing cell stress. We have also characterised the mitochondrial and glycolytic bioenergetic profiles of COV434 and KGN cells demonstrating these cell lines (which were previously known to be morphologically and genetically different) are also metabolically diverse, with COV434 being a more metabolically active cell line compared to KGN cells. Additionally, this study was the first to assess endocrine function (E2 and P4 secretion) of these cell lines within the same study using treatment with both rFSH and androstenedione, alone and in combination. Both cell lines responded significantly to the combination treatment, with COV434 cells producing higher levels of E2 and KGN cells producing higher levels of P4. Moreover, rFSH and androstenedione in combination enhanced several biochemical pathways, as demonstrated by elevated expression of the FAS, ACC, StAR and HMGCR enzymes, which has not been observed before in these cell lines.

A major strength of this bioenergetic and hormone data is both were normalised to cellular protein concentration to ensure consistency across independent experiments, particularly for potential variation in cell density or unpredictable changes in cell growth that may occur, and to also allow a direct comparison of bioenergetic data for two different cell lines. Unfortunately, our data was limited to cell lines and no primary GCs were used to draw comparisons with the COV434 and KGN cell lines. Additionally, our data pertaining to steroidogenic enzyme expression was limited to protein expression and may not fully represent what is happening regarding enzyme kinetics or at the gene expression level. In the future, confirmation and further elucidation of these changes would be vital, as well as assessment of key regulators upstream of the steroidogenic pathway including AMPK, and the direct measurement of metabolite concentrations to determine flux (specific direction of metabolism). Furthermore, the use of lipidomic and metabolomic technologies in the future are critical to assess these findings in greater depth.

Understanding the important role bioenergetics and steroidogenic capacity of these cell lines is key to making informed comparisons between the two, as they represent two metabolically/morphologically different forms of ovarian GC tumours (reproductive age vs. post-menopausal). Furthermore, investigating mitochondrial dysfunction and disorganisation in aging could be vital in further understanding the age-related decline of female infertility and subsequently the improvement of currently available infertility treatments.

Chapter Six

The effect of 1,25-(OH)2D3 exposure on human granulosa cell lines (COV434 & KGN) hormone secretion and metabolism

6.0 Introduction

VitD is an important secosteroid with important roles in skeletal tissues, particularly in calcium and phosphate metabolism (1). However, in more recent years the role of VitD in non-skeletal tissues has become an active area of research (481). In particular, the potential for VitD to regulate molecular pathways associated with numerous chronic metabolic and inflammatory diseases has attracted a great deal of attention (10, 482). More so, VitD metabolites (Table 1.1) and the VDR receptor have been found in numerous non-skeletal tissues throughout the human body, including in both male and female reproductive cells and tissues (29, 58, 81, 232-234, 483). However, the biological relevance and the molecular action of VitD signaling in reproductive cells and tissues remains elusive.

The biologically active form of VitD is known as 1,25-dihydroxycholecalciferol (1,25-(OH)2D3) (Table 1.1) (108). 1,25-(OH)2D3 exerts its pleiotropic effects through a genomic and non-genomic signalling mediated by its VDR (86). In genomic signalling, the VDR is embedded in the nuclear envelope operates as a transcription factor, migrating into the nuclear compartment upon ligand binding, and together with its binding partner RXR, recruits coactivators or corepressors to enhance or repress transcription (86). Furthermore, the VDR is also localised in the inner compartment of mitochondria, where it is hypothesised to have a central role in the regulation of gene sets and in supporting the metabolic requests evoked by nuclear signalling (84). In contrast, the non-genomic activity of VDR occurs at plasma membrane sites or cytoplasm, where VDR modulates several biological processes including calcium reabsorption, calcium flux, insulin secretion, smooth muscle cell migration, and opening of calcium and chloride channels (484). More recently, Consiglio et al., demonstrated a novel VitD signalling pathway in which 1,25-(OH)2D3 can also act as a modulator of mitochondrial activity in human epidermal keratinocytes (267).

Specifically, it was found that when treated with 1,25-(OH)2D3 differentiated keratinocytes had impaired electron transport chain (ETC) transcription and increased lipid deposition (267). Consiglio et al., concluded that in keratinocytes VDR exerts a general role as inhibitor of mitochondrial ETC and acts as facilitator of the diversion of mitochondrial acetyl-CoA towards biosynthetic pathways such as lipid production or biosynthesis of cholesterol (267). Furthermore, silencing of the VDR decreased de novo synthesis of cholesterol and increased respiratory chain activity oxidises metabolic intermediates, preventing their utilisation in biosynthetic pathways (266). However, it is unclear if this method of VitD signalling occurs in other cell types, such as reproductive tissues and cells, such as the GCs.

While it is possible to obtain human granulosa cells (GCs) during *in vitro* fertilisation (IVF) cycles with oocyte aspiration, the numbers of GCs retrieved from this process are limited. Conversely, obtaining human GCs outside the context of IVF is a highly invasive procedure, and therefore most of the work understanding GC biology in this area is conducted in animal studies. Several animal studies have investigated the effect of 1,25-(OH)2D3 on GC proliferation, cell cycle, ROS production, hormone secretion and signalling. Data from goat GCs show that 1,25-(OH)2D3 treatment promotes GC proliferation, through alterations in cell-cycle related genes, such as Cyclin B1 and Cyclin D1 (237, 485). Furthermore, 1,25-(OH)2D3 was also able to significantly decrease intracellular ROS levels through the regulation of antioxidant enzymes (SOD2 and CAT) as shown by flow cytometry (237).

Additionally, 1,25-(OH)2D3 treatment in GCs from various animal models can modulate several key reproductive hormones. In hen GCs treated with 1,25-(OH)2D3, along with an increase in follicular cell proliferation, there was a significant increase in mRNA expression of Anti-Mullerian hormone (AMH) (62). This could suggest 1,25-(OH)2D3 has a role in follicle recruitment through modulation of AMH signalling. In porcine GCs, 1,25-(OH)2D3 was able to modulate both insulin- and follicle-stimulating hormone (FSH)-induced progesterone secretion *in vitro* (64). However, the molecular actions of how this occurs is not fully understood. When goat GCs were treated with 1,25-(OH)2D3, it was demonstrated that estradiol (E2), progesterone (P4) and cAMP levels were all significantly increased (237). Furthermore, when co-incubated with rFSH, these findings were significantly

increased further than when treated with 1,25-(OH)2D3 alone (237). These increases in steroid hormones were accompanied by significant increases in the mRNA expression of VDR, steroid acute regulatory protein (StAR) and 3β-hydroxysteroid dehydrogenase (3β-HSD) (237). This demonstrates 1,25-(OH)2D3 can modulate key reproductive hormones, by increasing key enzymes which promote steroid biosynthesis (StAR) and the conversion of progesterone to androgens (3β-HSD). In contrast, a study of primary GCs of dehydroepiandrosterone-induced PCOS mice, investigated whether 1,25-(OH)2D3 (100 nmol/L) treatment for 24 hours and AMP-activated protein kinase (AMPK) played a role in GC hormone production, and showed that 1,25-(OH)2D3 decreased E2 and P4 secretion (486). The authors also reported 1,25-(OH)2D3 activated AMPK-α and acetyl-CoA carboxylase (ACC), while also reducing the gene expression of steroidogenic enzymes such as StAR and 3β-HSD, leading to reduced hormone output (486). However, it is unclear what the mechanism is behind 1,25-(OH)2D3 activation of AMPK-α or if this effect is observed in non-PCOS GCs.

In cultured primary human GCs obtained from women undergoing IVF, 1,25-(OH)2D3 treatment (100 nmol/L) for 48 hours *in vitro* significantly increased aromatase and 3β-HSD activity in both non-PCOS and PCOS patient GCs (238). Additionally, intracellular ROS was higher in GCs from PCOS patient compared to non-PCOS patients, and treatment with 1,25-(OH)2D3 attenuated this elevated ROS (238). A report from human GCs demonstrated 1,25-(OH)2D3 treatment stimulated P4, E2 and E1 production (63). Furthermore, 1,25-(OH)2D3 and insulin were shown to act synergistically to increase E2 production further when compared to 1,25-(OH)2D3 alone (63). Interestingly, while calciotropic hormones vary minimally across the menstrual cycle, there is some evidence that women with a VitD status < 30 ng/mL have lower mean E2 and free E2 levels compared to women with a VitD status $\geq$ 30 ng/mL (487). This decrease in mean E2 concentration appeared to be most marked during the ovulatory and mid-luteal peaks (487).

While it is evident from numerous animal and human studies that 1,25-(OH)2D3 can modulate both increases in cell proliferation and steroidogenesis in mammalian GCs, the cellular metabolism driving such functional changes has not yet been explored. Understanding the effect of how 1,25-(OH)2D3 effects cellular metabolism could further elucidate key aspects of how VitD is able to drive changes in

steroidogenesis and lipid metabolism, potentially through increased production of cellular energy necessary to drive these pathways. Furthermore, very little is known about whether 1,25-(OH)2D3 attenuates or enhances the effects of rFSH and androstenedione, key hormones which drive human GC functioning. The overall aim of the proceeding chapter was to investigate the impact of 1,25-(OH)2D3 treatment on human GC metabolism.

6.0.1 Chapter Objectives

To characterise and compare the effect of the biologically active form of 1,25-dihydroxycholecalciferol (known throughout as 1,25-(OH)2D3) alone as well as in combination with rFSH and androstenedione hormone treatments on COV434 and KGN:

1) cell viability/proliferation, cell cycle and intracellular ROS production.

2) E2 and P4 hormone production.

3) intracellular lipid deposition.

4) mitochondrial and glycolytic bioenergetic responses/profiles.

6.1 Results

6.1.1 The effect of VitD treatment on cell viability

The potential cytotoxicity of various concentrations of 1,25-(OH)2D3 treatments on cell viability was first evaluated to select non-lethal concentrations for subsequent cell experiments. 1,25-(OH)2D3 treatment over the four concentrations tested (25, 50, 100 & 200 nmol/L) for 24 hours did not alter cell viability significantly in either COV434 or KGN cells (Figure 6.1 A & B). Additionally, 1,25-(OH)2D3 treatment

(200 ng/mL) in combination with of rFSH (125 ng/mL) and androstenedione (5 μM) for 24 hours did not alter cell viability significantly in either COV434 or KGN cells (Figure 6.1 C & D).

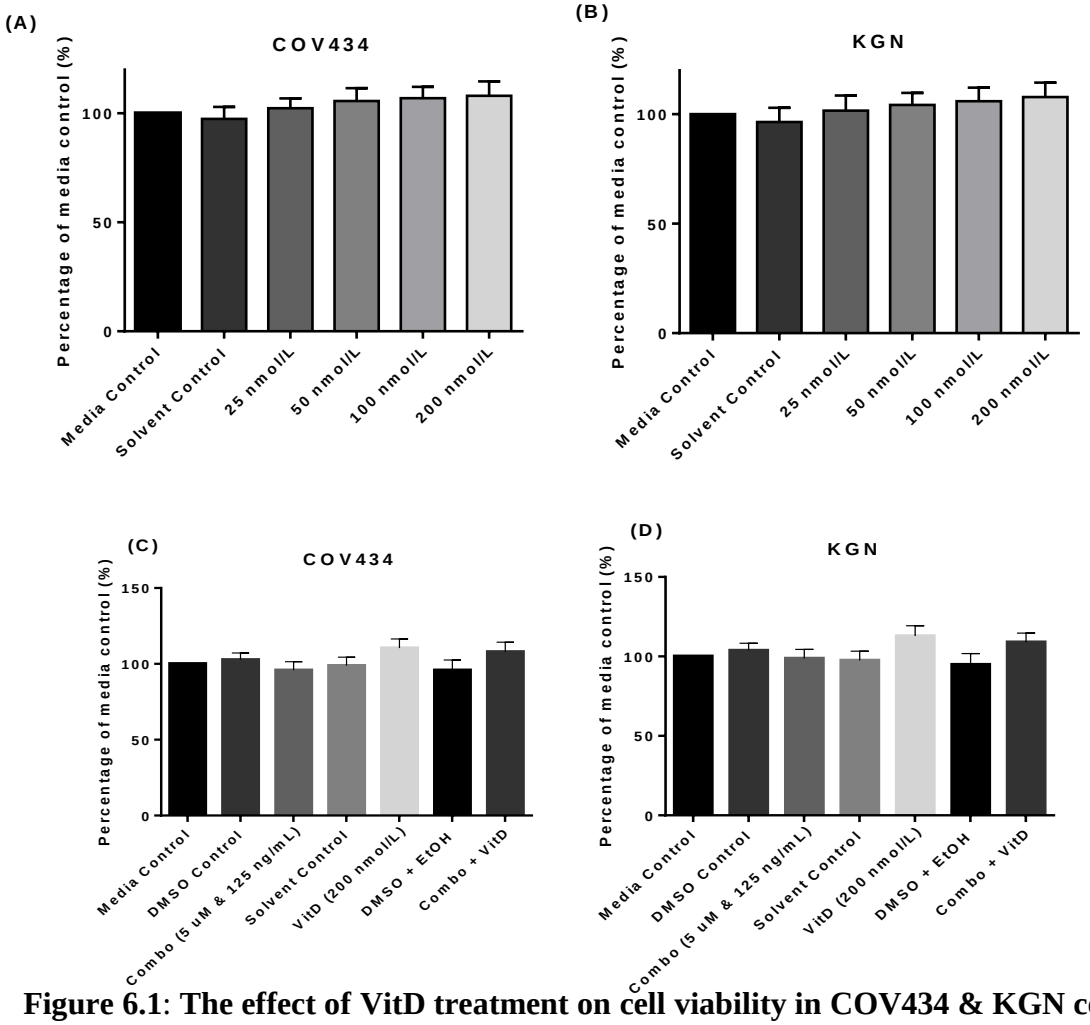
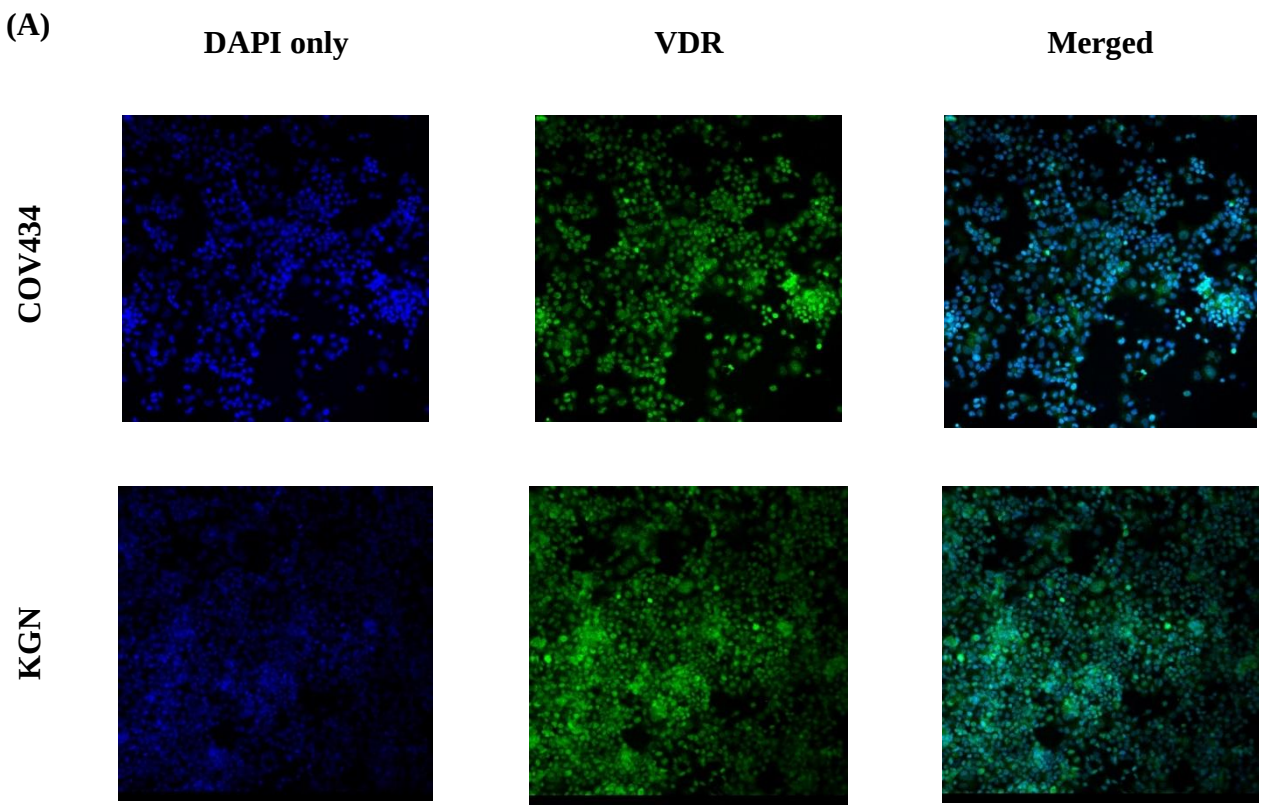


Figure 6.1: **The effect of VitD treatment on cell viability in COV434 & KGN cell lines.** COV434 and KGN cell lines were exposed to various concentrations of VitD alone or in combination with rFSH and androstenedione for 24-hours and cell viability was assessed using MTT assay. **(A)** COV434 cells and **(B)** KGN cells were treated with various concentrations of 1,25-(OH)2D3 (25, 50, 100, 200 nmol/L) for 24 hours. **(C)** COV434 and **(D)** KGN cells were treated with 1,25-(OH)2D3 alone (200 nmol/L) or in combination with combination of rFSH (125 ng/mL) and androstenedione (5 µM) for 24 hours. Solvent control refers to EtOH (0.2%) and is the control for 1,25-(OH)2D3 (200 nmol/L). DMSO control refers to the control for the combination treatment (rFSH + androstenedione). DMSO + EtOH refers to the control for the combination (rFSH + androstenedione) and VitD treatment. Data is represented as mean ± S.D and each experiment was replicated 3 times with each sample measured in triplicate. *p= <0.05, **p= <0.001, ***p= <0.001. *Abbreviations: DMSO, dimethyl sulfoxide; VitD, vitamin D; EtOH, ethanol; Andro, androstenedione, rFSH, recombinant follicle stimulating hormone.*

6.1.2 COV434 and KGN cells express the VDR

Immunofluorescent staining, and Western Blot analysis was used to evaluate if the VitD receptor (VDR) was expressed in COV434 and KGN cells. Immunofluorescence staining demonstrated the VDR was in the nucleus of both COV434 and KGN cells (Figure 6.2 A). This was confirmed via Western Blot analysis in which VDR was expressed in COV434 and KGN cells (Figured 6.2 B). When treated with 1,25-(OH)2D3 (200 nmol/L), VDR expression increased in COV434 and KGN cells by 1.76 and 1.58 (fold change, respectively) compared to control ($p=0.013$ & $p=0.021$, respectively, Figure 6.2 B).


(A)
DAPI only
VDR
Merged
COV434
KGN

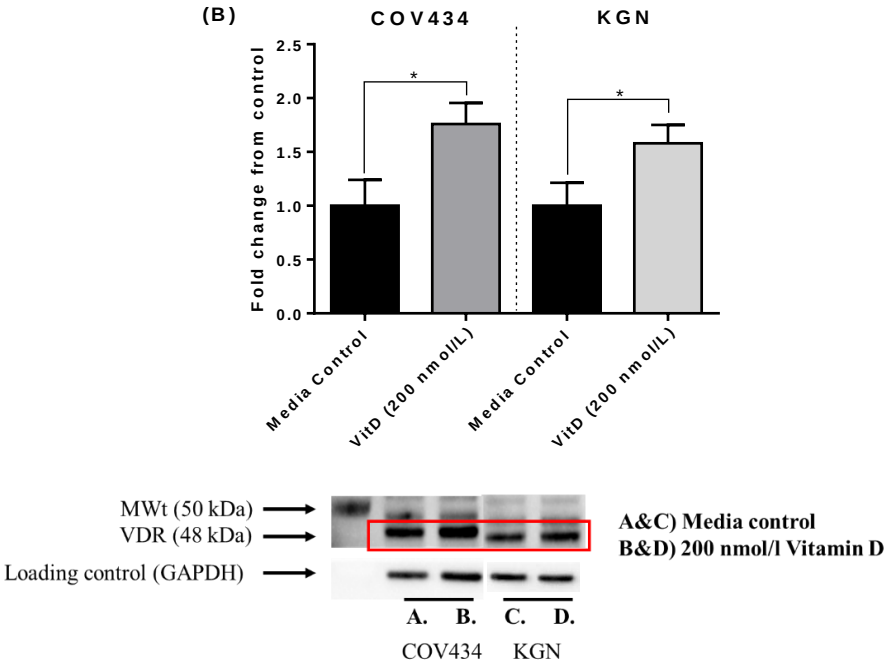


Figure 6.2: **The presence of the VitD receptor in COV434 & KGN cell lines. (A)** COV434 cells and KGN cells were maintained in media for 24 hours and then fixed and stained with the nuclear stain DAPI and the Anti-VDR antibody to assess the presence and location of the VDR. To quantify VDR expression **(B)** COV434 and KGN cells were treated with or without 1,25-(OH)2D3 (200 nmol/L) for 24 hours and whole cells were lysed, and Western Blot analysis was performed. Images in **(A)** are representative of one independent experiment (n=3). Data is represented as mean ± S.D and each experiment was replicated 3 times with each sample measured in triplicate. *p= <0.05, **p= <0.001, ***p= <0.001. *Abbreviations: VitD, vitamin D; VDR, vitamin D receptor; MWt, molecular weight; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.*

6.1.3 The effect of VitD treatment on cell cycle

Flow cytometry was used to assess the phases of cell cycle in COV434 and KGN cells following 1,25-(OH)2D3 treatment alone and pre-hormonal stimulation. There were no significant changes in the COV434 cells, for any phases of the cell cycle (G0/G1, S, G2/M phases) following treatment with hormonal pre-stimulation or 1,25-(OH)2D3alone, or in combination (Figure 6.3). In KGN cells treated with the double solvent (DMSO + EtOH) and combo + 1,25-(OH)2D3, the G0/G1 phase significantly increased compared to media control (64.14% vs. 76.23% & 75.45, respectively, Figure 6.3). However, combo + VitD was not significantly altered relative to its solvent control (DMSO + EtOH). No other parameters of the cell cycle significantly changed in KGN cells (Figure 6.3).

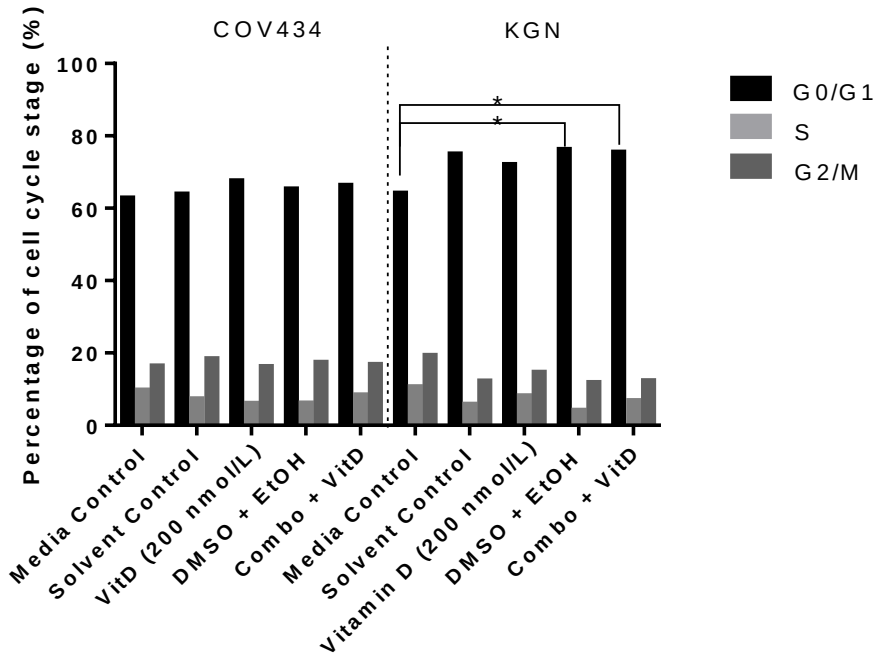


Figure 6.3: **The effect of VitD treatment on cell cycle in COV434 & KGN cell lines.** COV434 and KGN cell lines were exposed to 1,25-(OH)2D3 for 24-hours and cell cycle was assessed using flow cytometry. Data is represented as mean ± S.D and each experiment was replicated 3 times with each sample measured in triplicate. *p= <0.05, **p= <0.001, ***p= <0.001. *Abbreviations: VitD, vitamin D; DMSO, dimethyl sulfoxide; VitD, vitamin D; EtOH, ethanol.*

6.1.4 The effect of VitD treatment on intracellular ROS

Flow cytometry was used to assess general levels of intracellular ROS. In both COV434 and KGN cells treated with various concentrations of 1,25-(OH)2D3 (25, 50, 100 & 200 nmol/L), only the highest concentration of 200 nmol/L significantly reduced intracellular ROS by 11.8% and 10.9% relative to solvent control (103.6% vs. 91.8% 104.6% & 93.7%, Figure 6.4). Glucose oxidase (GOX) was used as a positive control for inducing elevated ROS levels. GOX significantly increased the levels of ROS in both COV434 and KGN cells by 16.9% and 21.2% (respectively) compared to media control (116.9% vs. 121.2%, Figure 6.4).

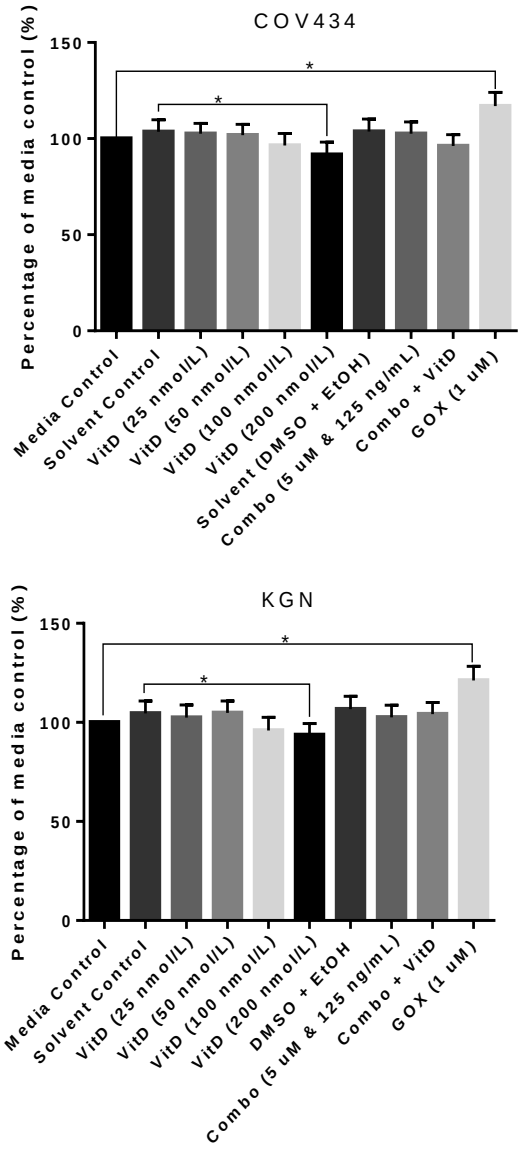
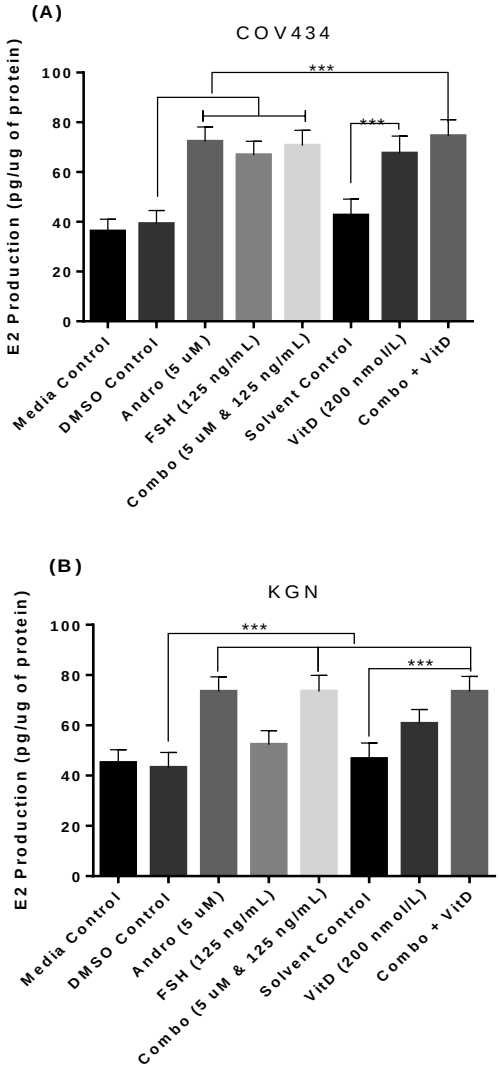


Figure 6.4: **The effect of VitD treatment on intracellular ROS in COV434 & KGN cell lines.** COV434 and KGN cell lines were exposed to either 1,25-(OH)2D3 alone or in combination with rFSH and androstenedione for 24-hours and intracellular ROS was assessed using flow cytometry. Glucose oxidase (GOX) was used as a positive control to induce intracellular ROS. Data is represented as mean ± S.D and each experiment was replicated 3 times with each sample measured in triplicate. *p= <0.05, **p= <0.001, ***p= <0.001. *Abbreviations: VitD, vitamin D; DMSO, dimethyl sulfoxide; EtOH, ethanol; Andro, androstenedione; rFSH, recombinant follicle stimulating hormone; GOX, glucose oxidase.*

6.1.5 The effect of VitD treatment on estradiol production

Both cell lines, were exposed to 1,25-(OH)2D3-alone and in combination, for 24 hours to determine the hormonal secretion response of E2. Exposure to 1,25-(OH)2D3-alone (200 nmol/L) significantly increased the production of E2 in COV434 cells compared to solvent control by 58.2% (67.4 vs. 42.6 pg/ug protein, Figure 6.5 A). In COV434 cells treated with 1,25-(OH)2D3 and the combination treatment (rFSH and androstenedione), E2 secretion increased a further 16.8% (75.0 vs. 42.6 pg/ug protein, Figure 6.5 A). In KGN cells 1,25-(OH)2D3 alone did not significantly increase E2 secretion compared to control (Figure 6.5 B). However, in KGN cells treated with 1,25-(OH)2D3 and the combination treatment (rFSH and androstenedione), E2 secretion significantly increased by 57.3% (73.3 vs. 46.6 pg/ug protein, Figure 6.5 A).



6790

6791 **Figure 6.5**: **The effect of VitD treatment on E2 production in COV434 & KGN**
6792 **cell lines.** COV434 and KGN cell lines were exposed to VitD-alone and in
6793 combination with rFSH and androstenedione, for 24-hour and the effects on E2
6794 production were measured. E2 production in **(A)** COV434 and **(B)** KGN cells was
6795 assessed using relevant ELISA kits. Data is represented as mean ± S.D and each
6796 experiment was replicated 3 times with each sample measured in triplicate. *p= <0.05,
6797 **p= <0.01, ***p= <0.001. *Abbreviations: DMSO, dimethyl sulfoxide; Andro,*
6798 *androstenedione; rFSH, recombinant follicle stimulating hormone; VitD, vitamin D;*
6799 *EtOH, ethanol.*

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6.1.6 The effect of VitD treatment on progesterone production

Both cell lines, were exposed to 1,25-(OH)2D3-alone and in combination, for 24 hours to determine the hormonal secretion response of P4. Exposure to 1,25-(OH)2D3-alone (200 nmol/L) significantly increased the production of P4 in COV434 cells compared to solvent control by 31.4% (72.4 vs. 55.1 pg/ug protein, Figure 6.6 A). In COV434 cells treated with 1,25-(OH)2D3 and the combination treatment (rFSH and androstenedione), P4 secretion increased by 25.6% (69.2 vs. 55.1 pg/ug protein, Figure 6.6 A). In KGN cells 1,25-(OH)2D3-alone significantly increase P4 secretion compared to solvent control by 26.0% (67.8 vs. 53.8 pg/ug protein, Figure 6.5 B). Additionally, in KGN cells treated with 1,25-(OH)2D3 and the combination treatment (rFSH and androstenedione), P4 secretion significantly increased by 28.1% (68.9 vs. 53.8 pg/ug protein, Figure 6.5 B).

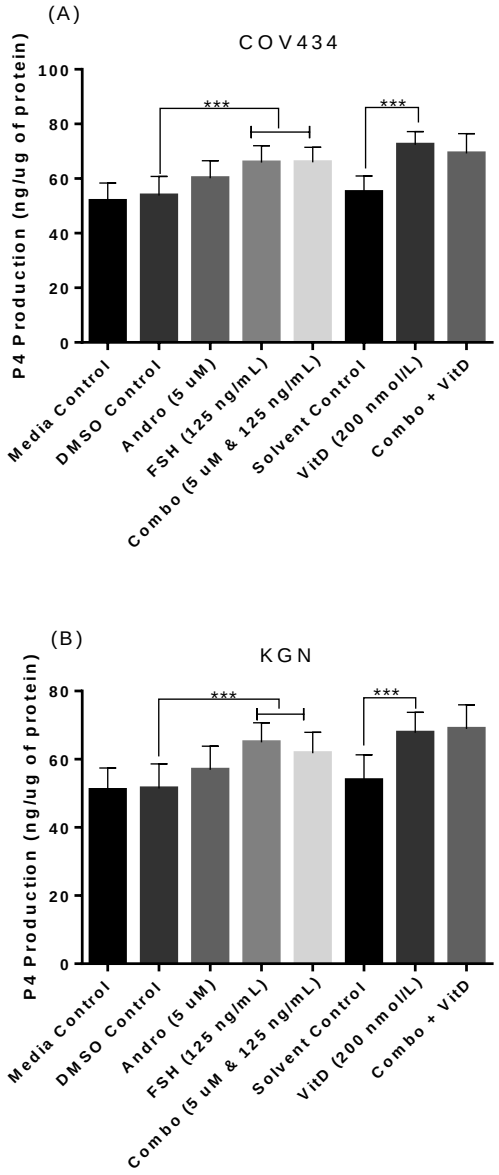
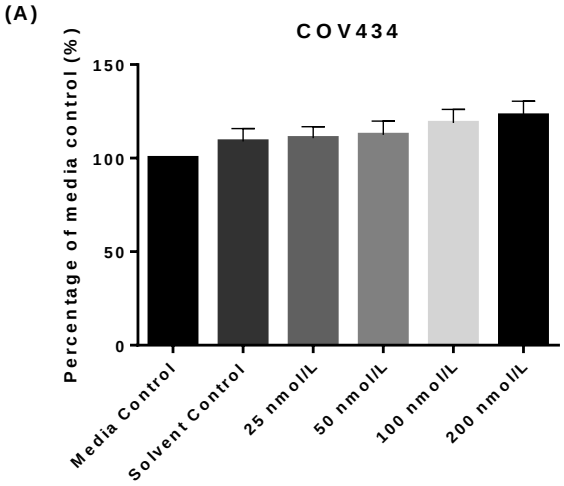
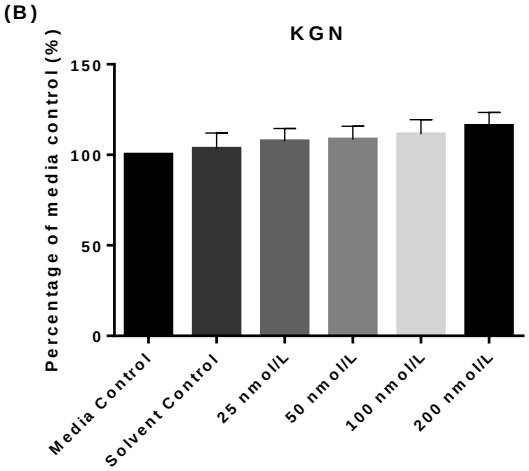


Figure 6.6: **The effect of VitD treatment on P4 production in COV434 & KGN cell lines.** COV434 and KGN cell lines were exposed to 1,25-(OH)2D3-alone and in combination with rFSH (125 ng/mL) and androstenedione (5 µM), for 24-hour and the effects on P4 production was measured. P4 production in **(A)** COV434 and **(B)** KGN cells was assessed using relevant ELISA kits. Data is represented as mean ± S.D and each experiment was replicated 3 times with each sample measured in triplicate. *p= <0.05, **p= <0.01, ***p= <0.001. *Abbreviations: DMSO, dimethyl sulfoxide; Andro, androstenedione; rFSH, recombinant follicle stimulating hormone; VitD, vitamin D; EtOH, ethanol.*

6.1.7 The effect of VitD treatment on lipid deposition

Intracellular lipid deposition was assessed in COV434 and KGN cell lines following 1,25-(OH)2D3 treatment at various concentrations (25, 50, 100 & 200 nmol/L) for 24 hours. In COV434 and KGN cells, 1,25-(OH)2D3 treatment did not significantly increase intracellular lipid deposition at any of the concentrations tested (Figure 6.7).





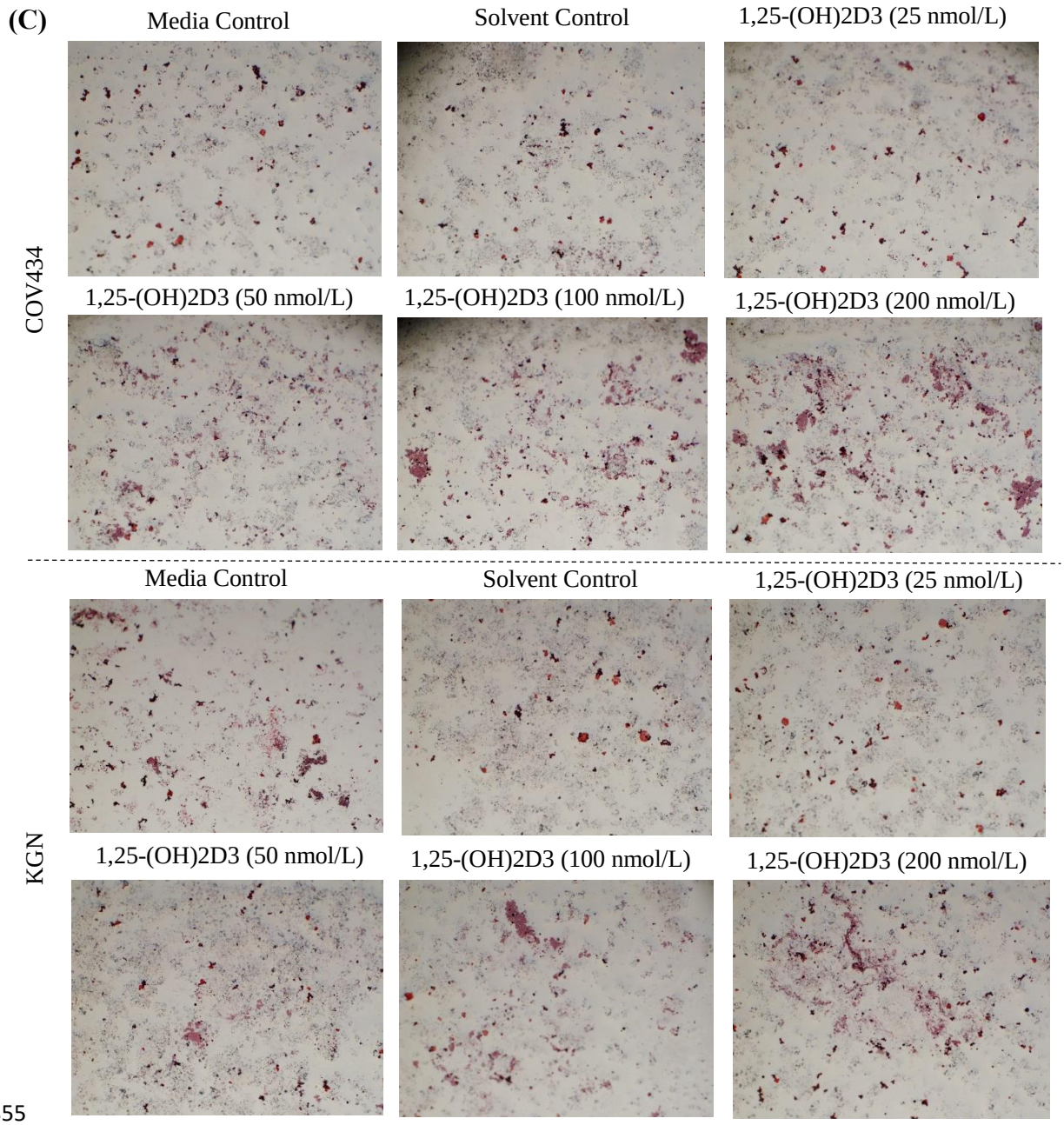
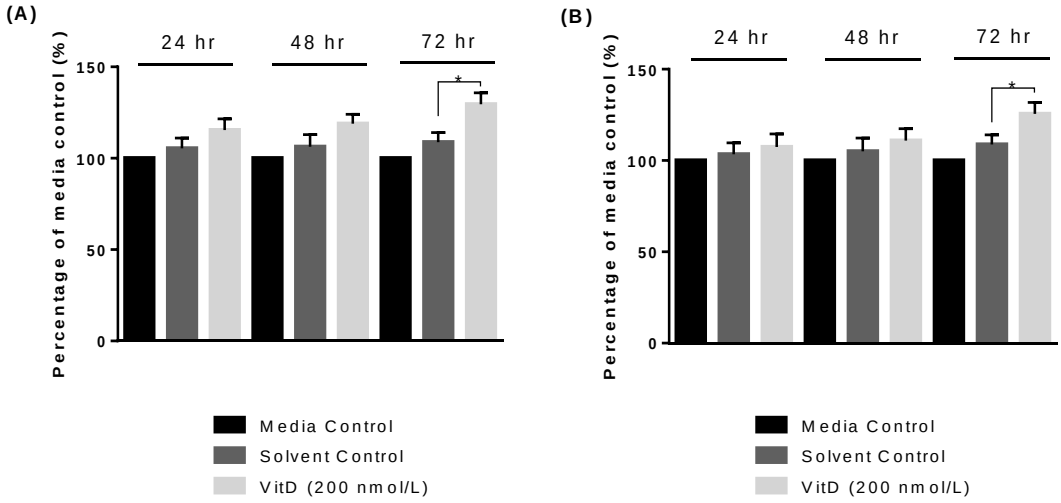
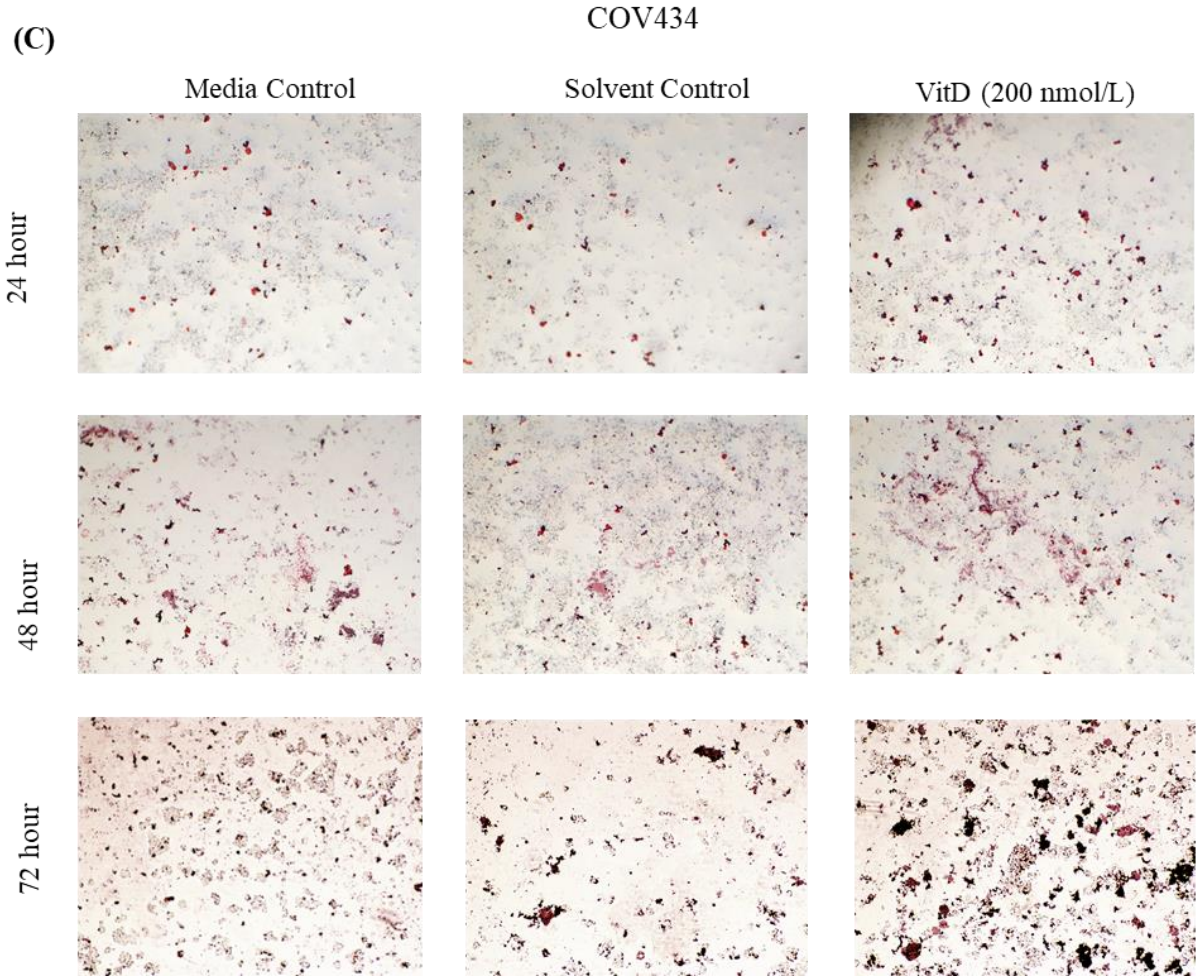


Figure 6.7: The effect of VitD on lipid deposition in COV434 & KGN cell lines. COV434 and KGN cell lines were exposed to various concentrations of 1,25-(OH)2D3 for 24-hour and the effects on lipid deposition were investigated. Gross lipid deposition was assessed using oil red O staining. Graphs for **(A & B)** represent absorbance eluted from the oil red O stain in **(A)** COV434 and **(B)** KGN cells. Bright field microscopy images at 40X magnification displayed in **(C)** are representative from one independent experiment. The independent experiment was replicated 3 times with each sample measured in quadruplicate. Data is represented as mean ± S.D and each experiment was replicated 3 times.

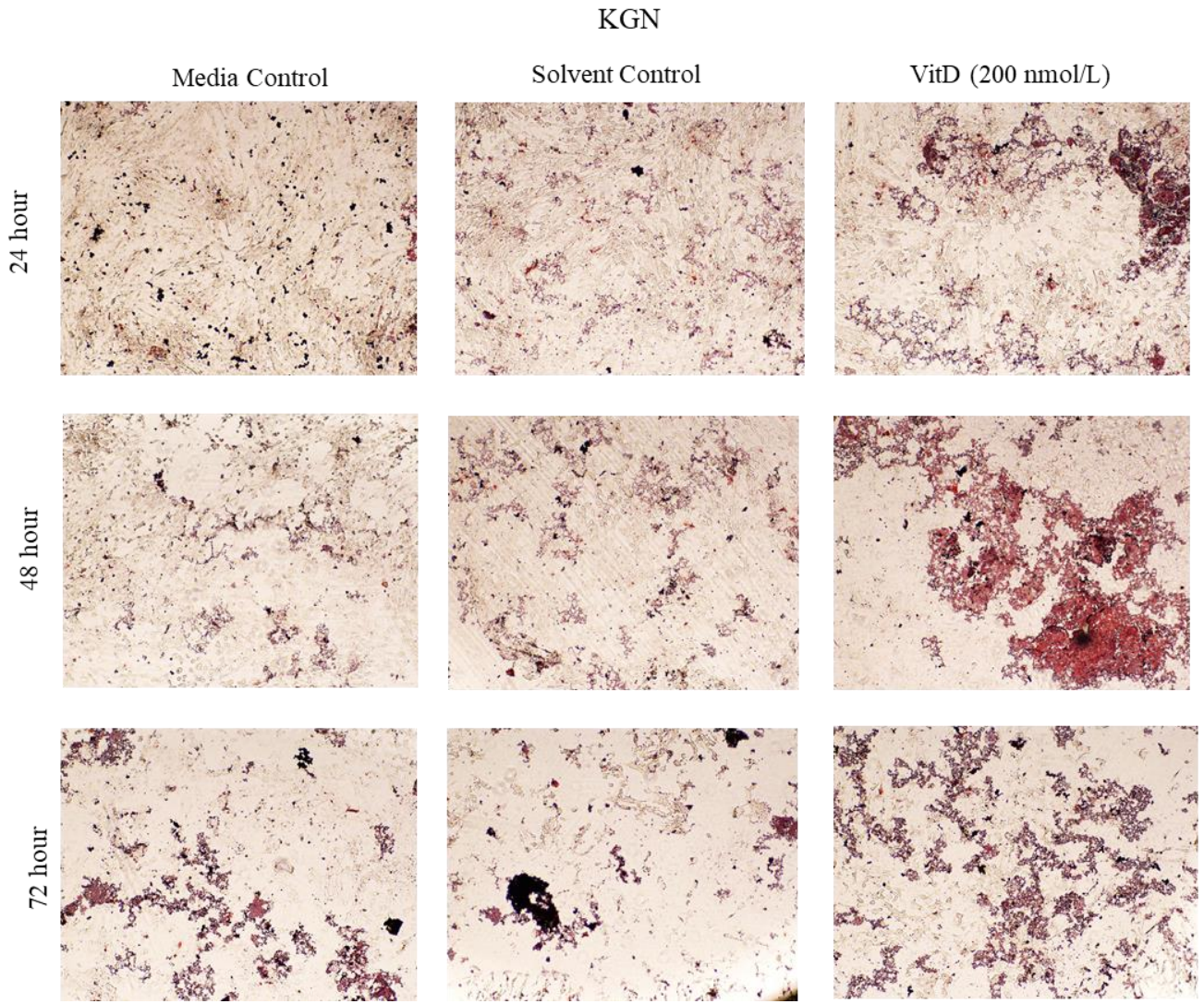
Intracellular lipid deposition was then assessed in COV434 and KGN cell lines following 1,25-(OH)2D3 treatment with 200 nmol/L for 24, 48 and 72 hours. 1,25-(OH)2D3 treatment for 24 or 48 hours did not significantly increase intracellular lipid deposition in either cell line (Figure 6.7). However, 1,25-(OH)2D3 treatment for 72 hours significantly increased lipid deposition in both COV434 and KGN cells by 19.7% and 17.6%, respectively (109.8% vs. 129.5% & 107.9% vs. 125.5%, Figure 6.8 A & B).




(C)
COV434
Media Control
Solvent Control
VitD (200 nmol/L)
24 hour
48 hour
72 hour

6882

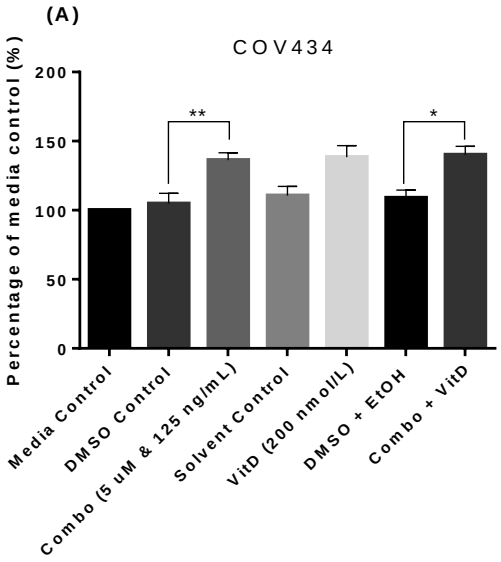
6883

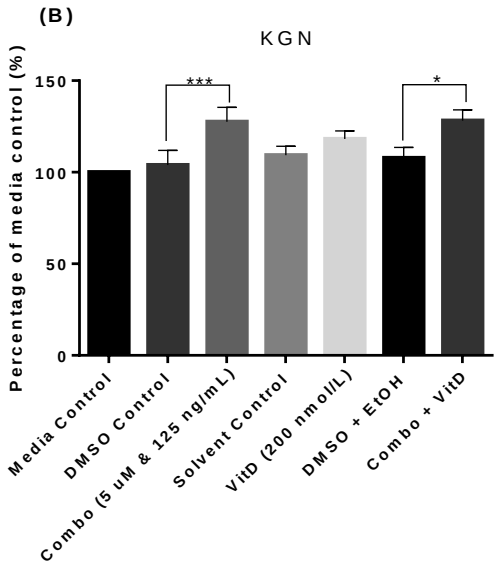

KGN
Media Control
Solvent Control
VitD (200 nmol/L)
24 hour
48 hour
72 hour

6884

Figure 6.8: The time dependent effect of VitD on lipid deposition in COV434 & KGN cell lines. COV434 and KGN cell lines were exposed to 1,25-(OH)2D3 (200 nmol/L) for 24, 48 and 72-hours and the effects on lipid deposition were investigated. Gross lipid deposition was assessed using oil red O staining. Graphs for **(A & B)** represent absorbance eluted from the oil red O stain in **(A)** COV434 and **(B)** KGN cells. Bright field microscopy images at 40X magnification displayed in **(C)** are representative from one independent experiment. The independent experiment was replicated 3 times with each sample measured in quadruplicate. Data is represented as mean ± S.D and each experiment was replicated 3 times, *p= <0.05. *Abbreviations: VitD, vitamin D.*

Intracellular lipid deposition was then assessed in COV434 and KGN cell lines following 1,25-(OH)2D3-alone (200 nmol/L) or in combination with rFSH (125 ng/mL) and androstenedione (5 μM) for 24 hours. 1,25-(OH)2D3-alone did not significantly alter lipid deposition in either cell line (Figure 6.9 A & B). However, when 1,25-(OH)2D3 was used in combination with rFSH and androstenedione, lipid deposition increased significantly in COV434 and KGN cells by 31.0% and 20.3% compared to solvent control (140.0% vs. 109.0% & 128.2% vs. 107.9, respectively, Figure 6.9 A & B).





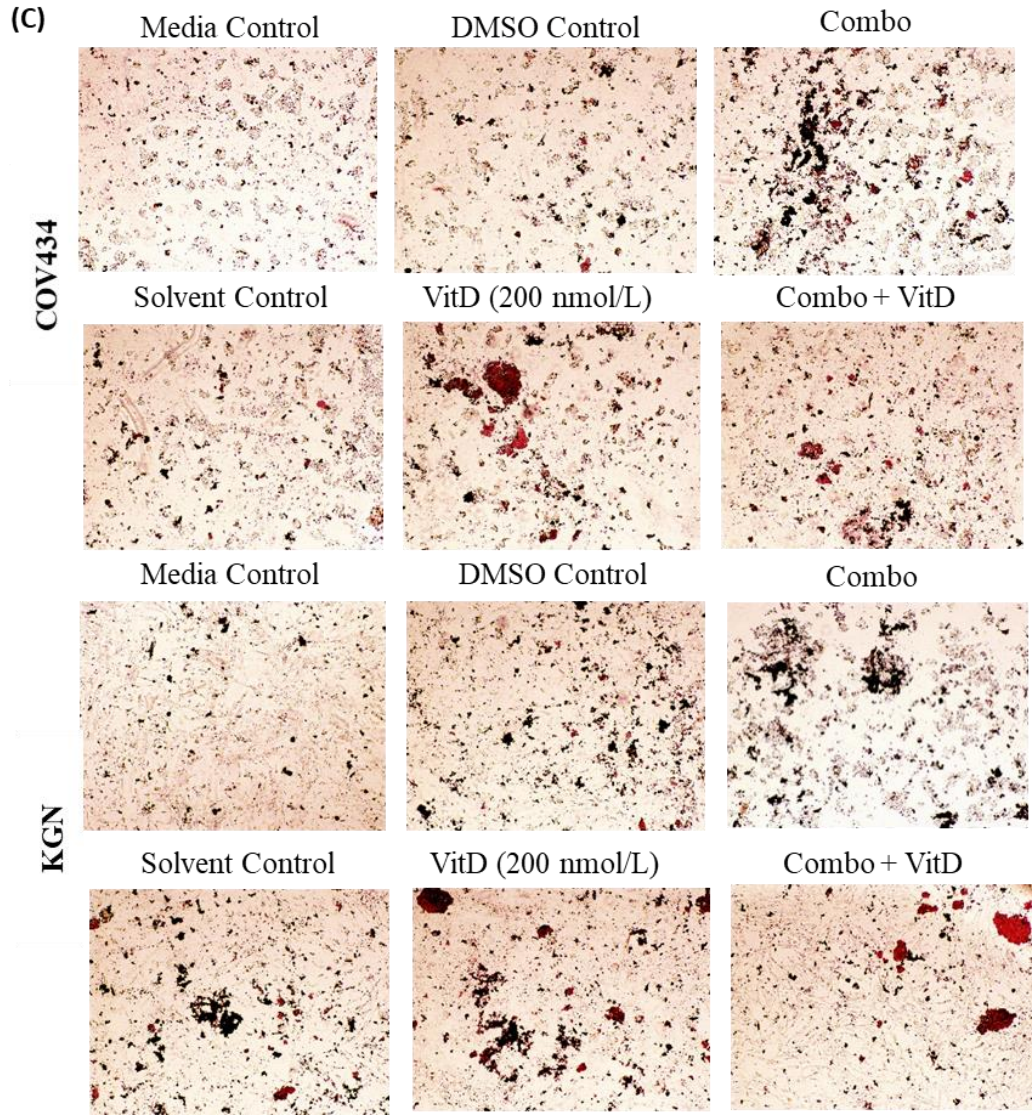
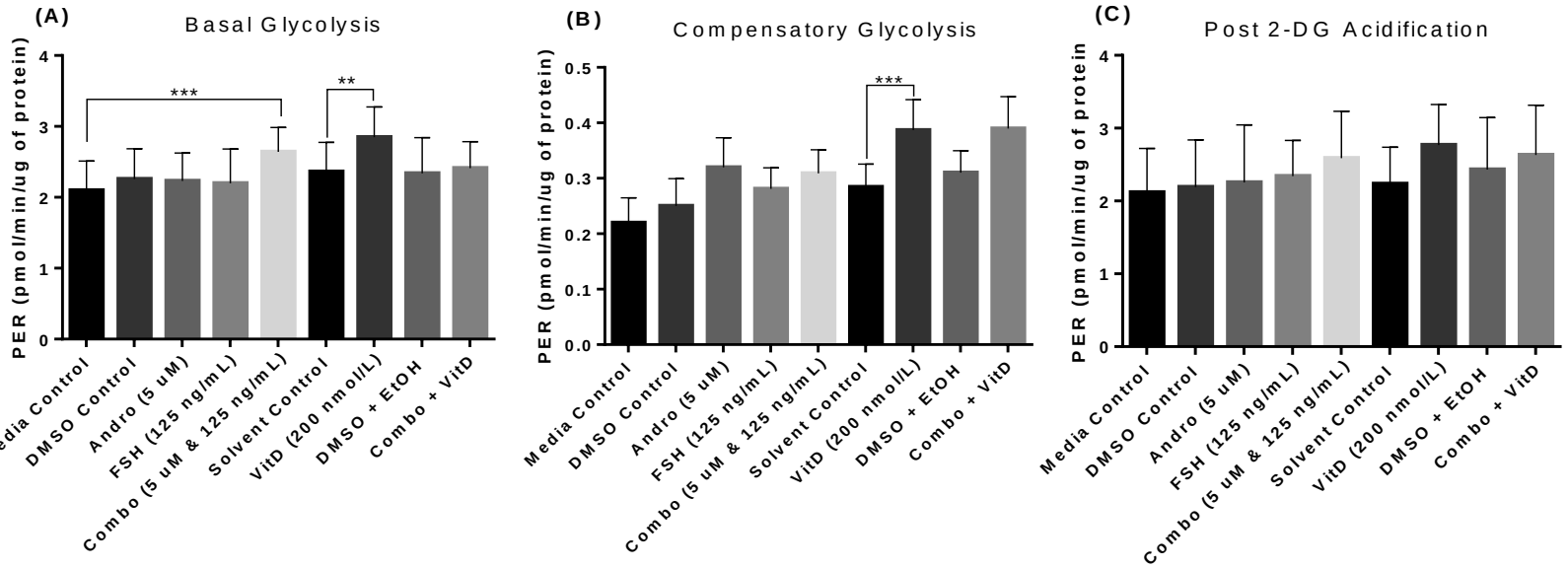


Figure 6.9: The effect of VitD alone and in combination with rFSH and androstenedione on lipid deposition in COV434 & KGN cell lines. COV434 and KGN cell lines were exposed to 1,25-(OH)2D3 (200 nmol/L) alone or in combination with rFSH (125 ng/mL) and androstenedione (5 µM) for 24 hours. Gross lipid deposition was assessed using oil red O staining. Graphs for **(A & B)** represent COV434 and KGN cells (respectively) absorbance eluted from the oil red O stain. Bright field microscopy images at 40X magnification displayed in **(C)** are representative from one independent experiment. The independent experiment was replicated 3 times with each sample measured in quadruplicate. Data is represented as mean ± S.D and each experiment was replicated 3 times, *p= <0.05, **p= <0.01, ***p= <0.001. *Abbreviations: DMSO, dimethyl sulfoxide; VitD, vitamin D; EtOH, ethanol; Andro, androstenedione; rFSH, recombinant follicle stimulating hormone.*

6.1.8 The effect of VitD treatment on glycolytic bioenergetics

Extracellular flux analysis was used to evaluate glycolytic bioenergetics in real time through measurements of PER in COV434 and KGN cell lines following 1,25-(OH)2D3 treatment alone and in combination with rFSH (125 ng/mL) and androstenedione (5 µM) for 24 hours. 1,25-(OH)2D3-alone significantly increased PER associated with basal glycolysis and compensatory glycolysis in COV434 cells by 20.8% and 39.3% (2.85 vs. 2.36 pmol/min/ug of protein & 0.28 vs. 0.39 pmol/min/ug of protein, Figure 6.10 A & B). However, there were no alterations in PER associated with post 2-DG acidification in COV434 cells (Figure 6.10 C). In KGN cells treated with 1,25-(OH)2D3-alone, compensatory glycolysis was significantly increased by 40.7% (0.27 vs. 0.38 pmol/min/ug of protein, Figure 6.10 E). No change was observed in KGN cells treated with 1,25-(OH)2D3-alone for PER associated with basal glycolysis and post 2-DG acidification (Figure 6.10 D & F). The combination of 1,25-(OH)2D3 and rFSH and androstenedione, did not significantly alter PER for any of the glycolytic parameters in either COV434 or KGN cell lines (Figure 6.10 A-F).


(A)
Basal Glycolysis
PER (pmol/min/ug of protein)
0
1
2
3
4
***
**
Media Control
DMSO Control
Andro (5 uM)
FSH (125 ng/mL)
Combo (5 uM & 125 ng/mL)
Solvent Control
VitD (200 nmol/L)
DMSO + EtOH
Combo + VitD
(B)
Compensatory Glycolysis
PER (pmol/min/ug of protein)
0.0
0.1
0.2
0.3
0.4
0.5
***
Media Control
DMSO Control
Andro (5 uM)
FSH (125 ng/mL)
Combo (5 uM & 125 ng/mL)
Solvent Control
VitD (200 nmol/L)
DMSO + EtOH
Combo + VitD
(C)
Post 2-DG Acidification
PER (pmol/min/ug of protein
0
1
2
3
4
Media Control
DMSO Control
Andro (5 uM)
FSH (125 ng/mL)
Combo (5 uM & 125 ng/mL)
Solvent Control
VitD (200 nmol/L)
DMSO + EtOH
Combo + VitD

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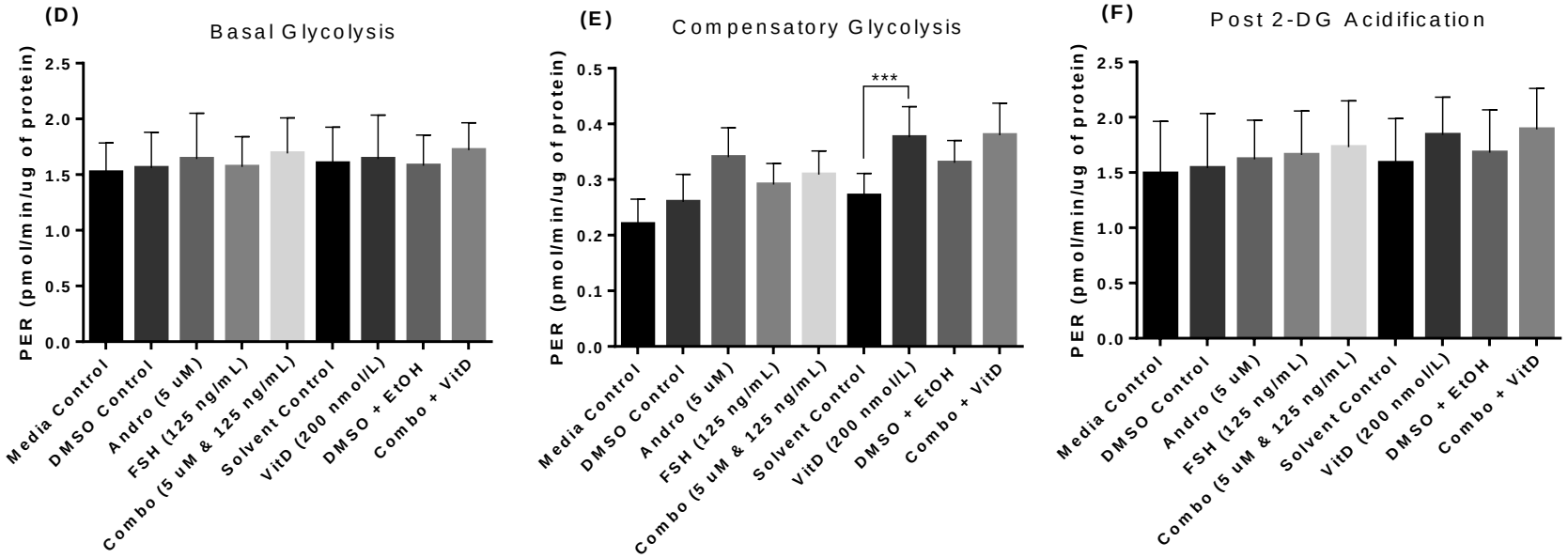

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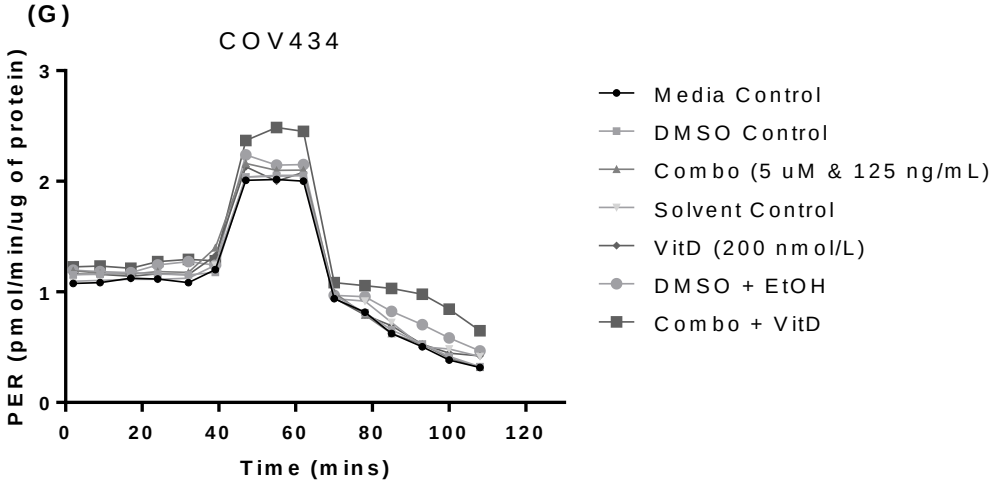

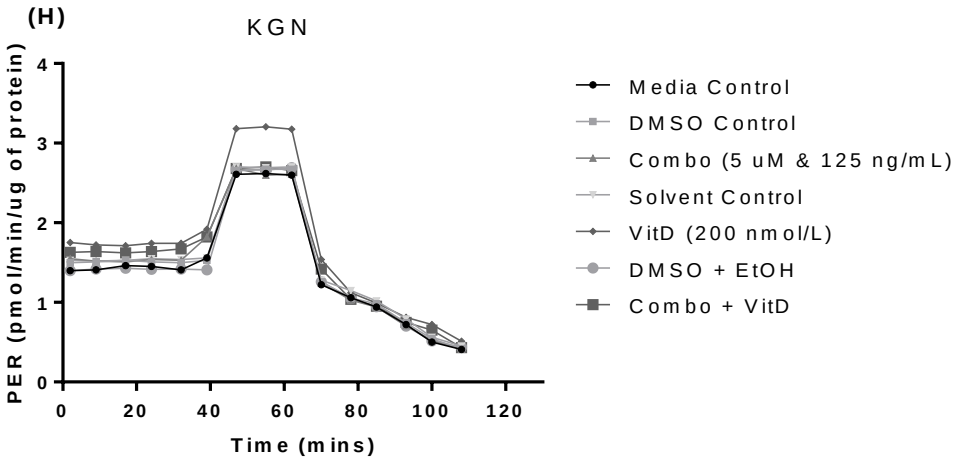

Figure 6.10: **The effect of VitD treatment on glycolytic metabolism of COV434 & KGN cell lines.** Cellular bioenergetics was assessed using extracellular flux analysis in COV434 and KGN cell lines following 24-hour treatment with 1,25-(OH)2D3-alone and in combination with rFSH (125 ng/mL) and androstenedione (5 µM). PER was measured to assess **(A)** Basal glycolysis and **(B)** Compensatory Glycolysis and **(C)** Post 2-DG acidification. Representative Seahorse traces are shown in **(D & E)**. Data is represented as mean ± S.D and each experiment was replicated 3 times with each sample measured in quadruplicate. **p= <0.01, ***p= <0.001. *Abbreviations: DMSO, dimethyl sulfoxide.*

6.1.10 The effect of VitD treatment on mitochondrial bioenergetics

Extracellular flux analysis was used to evaluate mitochondrial bioenergetics in real time through measurements of OCR in COV434 and KGN cell lines following 1,25-(OH)2D3-alone and in combination with rFSH (125 ng/mL) and androstenedione (5 µM). In COV434 cells exposure to 1,25-(OH)2D3-alone for 24 hours had minimal impact on cell mitochondrial bioenergetic responses for basal respiration, ATP production, proton leak and maximal respiration (Figure 6.11 A-D. However, 1,25-(OH)2D3 in combination with rFSH and androstenedione significantly increased basal respiration and ATP production by 33.2% and 40% compared to solvent control (2.73 vs. 2.05 pmol/min/ug of protein & 2.03 vs. 1.45 pmol/min/ug of protein, Figure 6.11). In contrast, 1,25-(OH)2D3-alone significantly increased basal respiration, ATP production and maximal respiration by 41.3%, 36.2% and 28.2% compared to solvent control (1.95 vs. 1.38 pmol/min/ug of protein & 1.63 vs. 1.04 pmol/min/ug of protein & 3.14 vs. 2.45 pmol/min/ug of protein, Figure 6.11 E, F & H). Treatment with 1,25-(OH)2D3-alone or in combination with rFSH and androstenedione did not modulate proton leak in COV434 or KGN cells (Figure 6.11 C & G).

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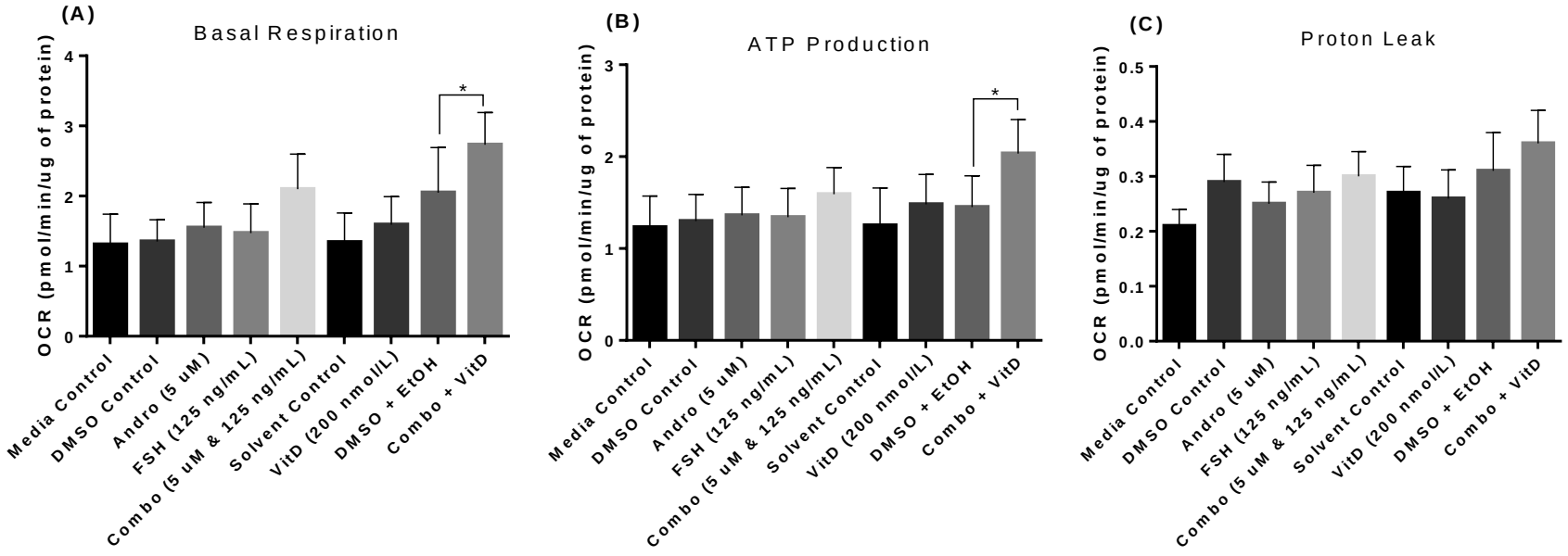

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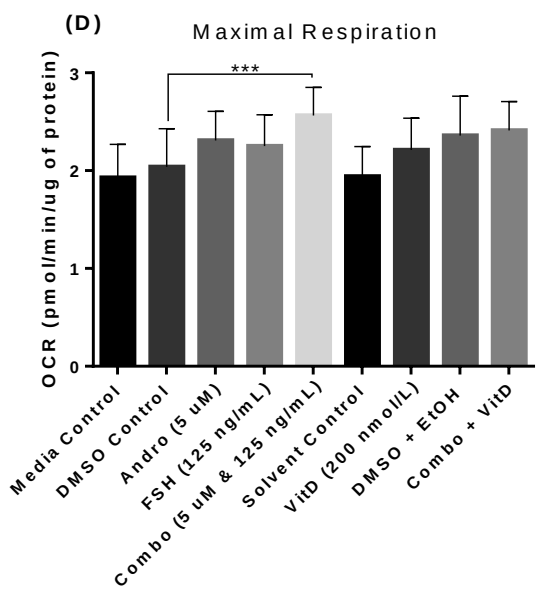

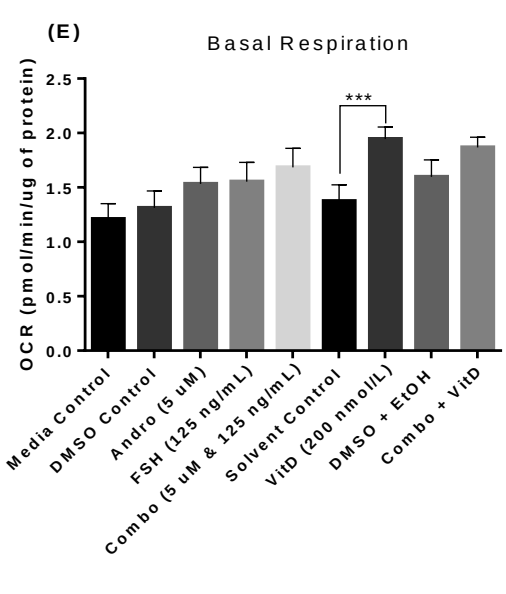

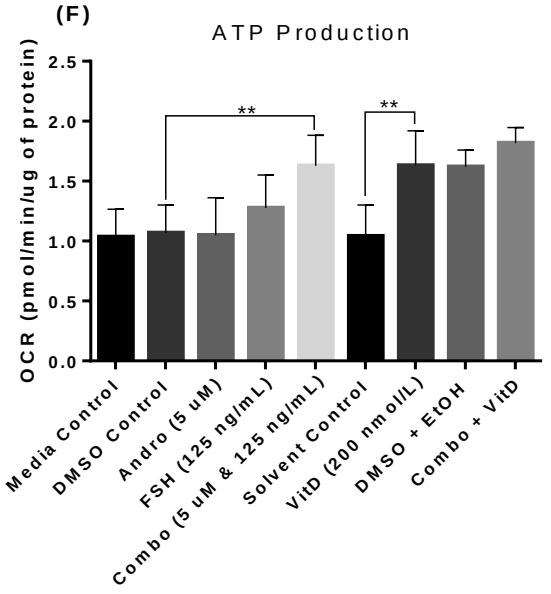

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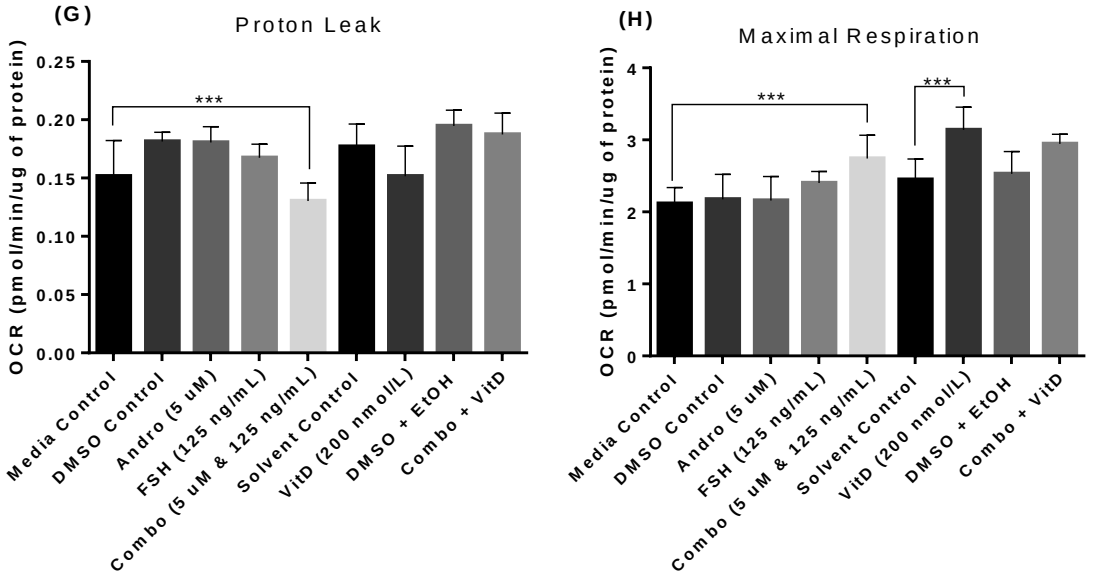

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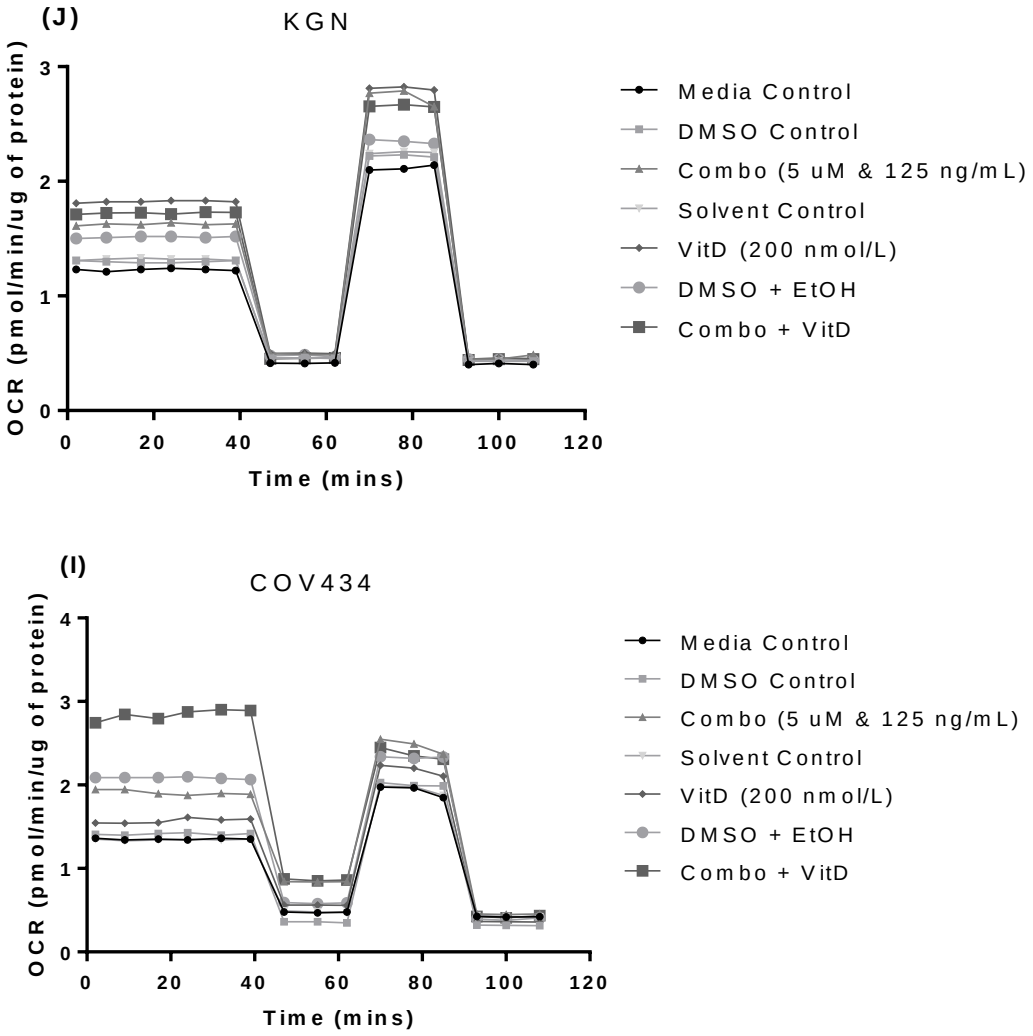

Figure 6.11: **The effect of VitD treatment on mitochondrial bioenergetics of COV434 & KGN cell lines.** Cellular bioenergetics was assessed using extracellular flux analysis in COV434 and KGN cells (respectively) following 24-hour treatment with 1,25-(OH)2D3-alone and in combination with the combination of rFSH (125 ng/mL) and androstenedione (5 µM). OCR was used to determine **(A & E)** Basal respiration **(B & F)** ATP-coupled respiration/production **(C & G**) Proton leak **(D & H)** Maximal respiration. Representative Seahorse traces are shown in **(I & J).** Data is represented as mean ± S.D and each experiment was replicated 3 times and each sample was measured in quadruplicate. *p= <0.05, **p= <0.01, ***p= <0.001. *Abbreviations: DMSO, dimethyl sulfoxide; VitD, vitamin D; EtOH, ethanol.*

6.1.11 The effect of VitD treatment on mitochondrial density

Mitochondrial density was assessed in COV434 and KGN cell lines following treatment with 1,25-(OH)2D3-alone (200 nmol/L) for 24 hours. In COV434 and KGN cells, 1,25-(OH)2D3 treatment did not significantly increase the density of mitochondrial DNA (mtDNA) relative to nuclear DNA (nDNA) (Figure 6.12).

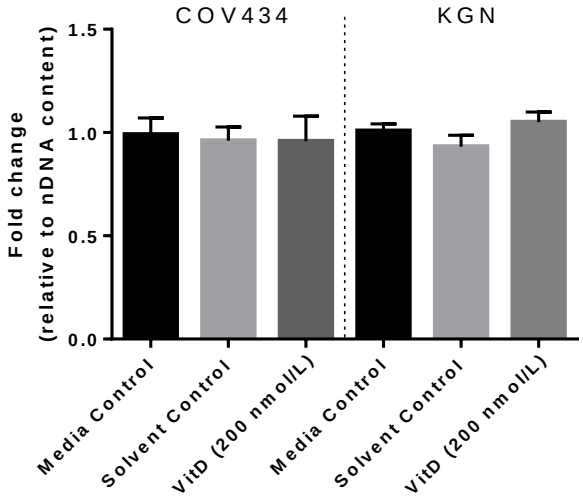

Figure 6.12: **The effect of VitD treatment on mitochondrial density in COV434 & KGN cell lines.** COV434 and KGN cell lines were exposed to 1,25-(OH)2D3-alone for 24-hours and mitochondrial density was assessed using qPCR. Data is represented as mean ± S.D and each experiment was replicated 3 times with each sample measured in triplicate. *Abbreviations: VitD, vitamin D; mtDNA, mitochondrial DNA; nDNA, nuclear DNA.*

6.3 Discussion

In this current study, it was demonstrated that the VDR was present in both COV434 and KGN cell lines. Immunofluorescent staining also showed VDR expression was localised in the nucleus. 1,25-(OH)2D3 exposure for 24 hours (25-200 nmol/L), or when added in combination with rFSH (125 ng/mL) and androstenedione (5 µM), did not alter cell proliferation as determined by the MTT viability assay and cell cycle analysis. VitD-alone (200 nmol/L) significantly reduced intracellular ROS production, although when treated in combination with rFSH (125 ng/mL) and androstenedione (5 µM) for 24 hours this effect was diminished. 1,25-(OH)2D3 exposure for 24 hours at 200 nmol/L, directly promoted E2 and P4 secretion from COV434 and KGN cell lines. In addition, 1,25-(OH)2D3 stimulation with the combination of FSH (125 ng/mL) and androstenedione (5 µM), further enhanced E2 and P4 release compared to solvent control. Treatment with 1,25-(OH)2D3-alone (200 nmol/L) significantly enhanced intracellular lipid deposition at the 72-hour time point, but not at 24 or 48 hours. Additionally, 1,25-(OH)2D3 treatment in combination with FSH (125 ng/mL) and androstenedione (5 µM) for 24 hours, significantly enhanced intracellular lipid deposition. Basal glycolysis was significantly increased in COV434 cells treated with 1,25-(OH)2D3 (200 nmol/L), but not in KGN cells. However, compensatory glycolysis significantly increased in both COV434 and KGN cells following treatment with 1,25-(OH)2D3 (200 nmol/L). There were no other significant changes observed in glycolytic parameters in either cell line or following treatment with VitD (200 nmol/L) in combination with FSH (125 ng/mL) and androstenedione (5 µM). In KGN cells treated with 1,25-(OH)2D3-alone (200 nmol/L) for 24 hours, there was a significant increase in basal respiration, ATP production and maximal respiration. There was no modulation of mitochondrial bioenergetics in COV434 cells under these same treatment conditions. No additional increase was observed in any mitochondrial bioenergetic parameters (basal respiration, ATP production, maximal respiration, or proton leak) when cells were treated with 1,25-(OH)2D3 (200 nmol/L) in combination with FSH (125 ng/mL) and androstenedione (5 µM) for 24 hours. Taken together, this study demonstrated that in both cell lines, the VDR was expressed, 1,25-(OH)2D3 could directly promote E2 & P4 secretion, potentiated rFSH/androstenedione-induced hormone release and increased lipid deposition (but only following 72 hours exposure). 1,25-(OH)2D3-alone and in combination with

rFSH and androstenedione also had cell line specific effects on glycolytic and mitochondrial metabolism, increasing several parameters in both cell lines.

To date, one previous study has investigated the presence of the VDR in COV434 cells, specifically at the mRNA and protein expression levels. The authors performed real-time PCR analysis of COV434 cells and compared this to primary human GCs, where the mRNA expression of the VDR was significantly higher in the latter (232). This was then confirmed via Western Blot analysis, where the VDR was detectable at the protein level (232). Another report detected the VDR protein in KGN cells, although multiple non-specific bands were obtained and therefore the researchers could not determine which was the specific band for VDR (488). To investigate these further, electrophoretic mobility shift assays were performed, which detects DNA binding proteins. The principle being that a nucleic acid with a bound-protein, has less mobility through a gel matrix than free nucleic acid, indicating protein–nucleic acid interactions (489). In this case the interaction of human VDR and RXRα proteins was investigated. Briefly, a constructed human RXRα expression vector (pGEM-3Z/hRXRα) was used. These plasmids and the TNT T7 Quick Coupled Transcription/Translation System (Promega), human VDR and RXRα proteins were synthesised *in vitro*. The oligonucleotide containing VDREs was from the human CYP24 promoter. Double-stranded oligonucleotides were labelled and purified, then combined with the *in vitro* transcribed/translated proteins to form a final reaction mix. These were then loaded onto gels, dried and the DNA-protein complexes were detected (488). Following this, endogenous VDR protein expression was confirmed to be present in KGN cells (488). In the current study, the protein expression of the VDR in basal conditions and following treatment with VitD (200 nmol/L) was confirmed via Western Blot analysis, with specific bands observed in both cell lines. Furthermore, treatment with 1,25-(OH)2D3 significantly increased the protein expression of VDR in both cell lines. The VDR was also detected using immunofluorescent staining, where it was localised within the nucleus (as determined by the merging with a nuclear stained image of the same cells). Previous studies have determined the VDR has several subcellular localisations including within the caveolae of the cell membrane, nuclei and the mitochondria (57). Unfortunately, here whole cell lysates were used and further studies investigating the specific localisation of the VDR within human GCs following subcellular fractionation is crucial to confirm and elucidate these findings.

Previous data from *in vitro* animal studies demonstrate that 1,25-(OH)2D3 treatment promotes GC proliferation through modulation of cell cycle associated genes (237, 485). However, very little is known about how 1,25-(OH)2D3 treatment alters cell proliferation in human GCs or cell lines. In the current study, 1,25-(OH)2D3 treatment for 24 hours did not alter cell proliferation (as determined by MTT assay) or significantly alter any stages of the cell cycle in either cell line, compared to control. Furthermore, in KGN cells 1,25-(OH)2D3 treatment in combination with rFSH and androstenedione significantly increased the G0/G1 phase of cell cycle compared to media control but not when compared to the relevant solvent (DMSO & ethanol) control. In contrast, no significant changes were observed in COV434 cells in any of the treated groups, compared to control. Based on these findings, 1,25-(OH)2D3 alone or in combination with rFSH and androstenedione, did not appear to alter cell proliferation/viability or cell cycle following 24-hour treatment in COV434 or KGN cells.

Other studies have reported VitD eliciting various immunomodulatory, anti-inflammatory, antioxidant, and anti-fibrotic actions (490). Furthermore, 1,25-(OH)2D3 induces the expression of several molecules related to antioxidant defence including glutathione (GSH), GPx, CAT, and SOD, as well as supressing NADPH oxidase expression (237, 491). In a study of non-PCOS and PCOS women undergoing IVF, primary human GCs were collected and treated *in vitro* with 1,25-(OH)2D3 (100 nmol/L) and the effect on ROS markers were analysed (238). SOD (which has powerful anti-inflammatory actions) activity and gene expression were significantly increased in both groups of GCs treated *in vitro* with 1,25-(OH)2D3 for 48 hours compared to control (238). Furthermore, GPx activity (a cytosolic enzyme that catalyses the reduction of hydrogen peroxide to water and oxygen) was significantly reduced in both groups treated with 1,25-(OH)2D3 compared to control (238). In the current study, it was imperative to first establish if 1,25-(OH)2D3 caused elevated intracellular ROS that could negatively impact cell growth, steroidogenesis, and cell bioenergetics and which were the major biochemical outcomes of interest for this chapter. Here, 1,25-(OH)2D3 treatment alone significantly reduced intracellular ROS in both cell lines, whilst in combination with rFSH and androstenedione there was no effect on intracellular ROS. Therefore, 1,25-(OH)2D3 appeared to have a potential

antioxidant effect on COV434 and KGN cells, although further studies are required to confirm any changes in gene expression.

The major role of human GCs is to secrete vital reproductive hormones (such as E2 and P4) to support the developing oocyte and follicle. In one investigation of primary human GCs, 1,25-(OH)2D3 decreased AMH receptor II and rFSH receptor mRNA expression, while promoting 3β-HSD expression and activity leading to increased progesterone secretion (59). In another study of human GCs derived from women undergoing IVF, 1,25-(OH)2D3 treatment alone or in combination with androstenedione and pregnenolone, significantly enhanced E1 and P4 secretion (238). Additionally, treatment with the precursors in combination with 1,25-(OH)2D3 for an additional 24 hours further significantly elevated E1 and P4 production/secretion (238). This is in line with the findings presented in the current study, where 1,25-(OH)2D3 alone or in combination elevated E2 and P4 production/secretion in both cell lines. As expected, the levels of both E1 and P4 detailed in the previously mentioned study were significantly higher than those reported here, given the fact these were primary human GCs exposed to ovarian stimulatory drugs *in vivo* (prior to collection) versus GC lines, as used here. The E1 and P4 secreted by primary human GCs following treatment with 1,25-(OH)2D3 in combination with androstenedione or pregnenolone were approximately 9000 pg/mL and 4000 ng/mL, which translated to a 500% and 3900% increase relative to control (respectively, Table 6.1) (238). However, it is not stated if these control cells were media controls (primary GCs maintained in culture medium) or solvent controls (supplemented with the solvent 1,25-(OH)2D3/androstenedione and pregnenolone were solubilized in). Additionally, the publication using primary human GCs did not use 1,25-(OH)2D3-alone to show a direct effect on hormone secretion or a combination of rFSH and androstenedione with 1,25-(OH)2D3. In contrast, in the current study, 1,25-(OH)2D3-alone was shown to have a direct effect on the secretion of E2 and P4 in both cell lines, these increases ranged from 26.0% to 58.2% (Table 6.1). Furthermore, in the present study, 1,25-(OH)2D3 in combination with rFSH and androstenedione increased the secretion of E2 and P4 further than 1,25-(OH)2D3-alone, except for E2 secretion in COV434 cells which was highest in the 1,25-(OH)2D3-alone treated cells (Table 6.1).

Table 6.1: Comparison of hormone secretion of primary human GC's and the human GC lines, COV434 and KGN. Data from primary GCs derived from non-PCOS women undergoing IVF was obtained from the published study by Masjedi et al., (2020). Data for COV434 and KGN cells refers to results presented in the current study. Control values relate to the relevant solvent controls. *Abbreviations: E1, estrone; P4, progesterone; E2, estradiol; Andro, androstenedione; VitD, vitamin D; rFSH, recombinant follicle stimulating hormone.*

			Primary human GC's		
Hormone measured	**Control**	**Andro + 1,25-(OH)2D3**	**Pregnenolone + 1,25-(OH)2D3**	**% Increase from control Andro + 1,25-(OH)2D3**	**% Increase from control Pregnenolone + 1,25-(OH)2D3**
E1	1500 pg/mL	9000 pg/mL	-	500.0%	-
P4	150 ng/mL	-	4000 pg/mL	-	2566.7%
			COV434 cells		
	Control	**1,25-(OH)2D3-alone**	**rFSH + Andro + 1,25-(OH)2D3**	**% Increase from control 1,25-(OH)2D3-alone**	**% Increase from control rFSH + Andro + 1,25-(OH)2D3**
E2	42.6 pg/µg protein	67.4 pg/µg protein	75.0 pg/µg protein	58.2%	76.1%
P4	55.1 pg/µg protein	72.4 pg/µg protein	67.8 pg/µg protein	31.4%	23.1%
			KGN cells		
E2	46.6 pg/µg protein	61.2 pg/µg protein	73.3 pg/µg protein	31.3%	57.3%
P4	53.8 pg/µg protein	67.8 pg/µg protein	68.9 pg/µg protein	26.0%	28.1%

Besides the main difference in cell type (primary vs. cell line) there are several other key differences in cell culture/treatment conditions which would explain the discrepancies between the values reported in the literature and here. Firstly, the primary human GCs were treated with either androstenedione prior to quantification of E1 or pregnenolone and prior to quantification of P4. Whereas our treatment strategy involved both rFSH and androstenedione in combination for both E2 and P4 measurement. Secondly, these researchers assessed E1 while in the present study E2 (the more potent estrogen) was measured. Thirdly, it is unclear if the human GCs were pooled prior to E1/P4 measurement or an average was reported based on the n=14/20 assessed. Lastly, E2 and P4 secretion detailed here was normalised to protein to account for potential cell number changes, whereas this was not done in the primary human GC study. Based on these differences, it is difficult to draw direct comparisons between these studies. However, the present study was the first to investigate E2/P4 production/secretion in COV434 and KGN cells following treatment with 1,25-(OH)2D3 alone or in combination with rFSH and androstenedione. It was clear that 1,25-(OH)2D3 directly affected hormone secretion and showed a similar enhanced steroidogenic response when used in combination with other key hormones.

The increased steroidogenesis observed here likely reflects an increased bioavailability of lipids, which could be utilised within the steroidogenic pathway. Lipid deposition is a complex process involving lipid transport, uptake, synthesis, and catabolism (492). Currently, very little is known about lipid deposition in human GCs or cell lines, or whether 1,25-(OH)2D3 can modulate intracellular lipid deposition. Previous data demonstrated 1,25-(OH)2D3 treatment (10 nmol/L) for 48 hours significantly increased intracellular lipid deposition by 60.5% in primary human keratinocytes, as measured by Oil Red O staining (267). In the present study, Oil Red O staining demonstrated 1,25-(OH)2D3-alone significantly increased intracellular lipid deposition at 72 hours by 19.7% and 17.6% in COV434 and KGN cells- but not at 24 or 48 hours in either cell line. As expected, this increase was less pronounced than what was reported in human keratinocytes. It has previously been reported that COV434 and KGN cells have minimal intracellular lipid droplets at baseline, when compared to freshly collected primary human GCs (260, 493). When 1,25-(OH)2D3 treatment was used in combination with rFSH and androstenedione for 24 hours, lipid deposition significantly increased, indicating increased lipid metabolic flux in these

cells. Unfortunately, Oil Red O staining does not distinguish between neutral triglycerides and lipids, although it is known to not stain unesterified (free) cholesterol. Consequently, the staining reflects triacylglycerol, esterified cholesterol, and/or wax esters. Therefore, it is unclear what lipid type/s are specifically being stained in the present study. Further investigation using advanced methods, such as lipidomics, may further elucidate the lipid profile in these cells under the stimulatory conditions detailed here. Given the increase in lipid deposition and secretion of E2 and P4, it is likely stored lipids are mobilised to be used as a precursor for the cholesterol pathway (resulting in increased steroid hormone production), or to meet increased cellular energy demands of the cells under these stimulatory conditions.

Many mammalian cells store lipids in their cells to produce metabolic energy, to be utilised in times of insufficient energy sources (494). Cells preserve lipids by converting them into neutral lipids, such as triglycerides and sterol esters (492). The catabolism of lipid droplets into free fatty acids is a crucial cellular pathway that is required to generate ATP, and to provide building blocks for the cell membrane and hormone synthesis (492). Previous work from our lab, which investigated the effect of VitD status on circulating peripheral blood mononuclear cells (PBMC), demonstrated VitD insufficiency adversely influenced bioenergetic parameters of these cells (67). Furthermore, seasonal improvements in VitD were associated with reduced PBMC bioenergetics and whole body energy metabolism (66). However, this was hypothesised to reflect greater inflammation which has an ‘energetic cost; that could account for this reduction in cellular bioenergetics. In contrast, 1,25-(OH)2D3 has been shown to inhibit the ETC in human keratinocytes, while it facilitates the diversion of mitochondrial acetyl-CoA towards biosynthetic pathways. This is more in line with the data presented here, where 1,25-(OH)2D3 was able to directly modulate hormone secretion and lipid deposition, through enhanced bioenergetics.

The VDR has been identified in all key organs of energy metabolism including the pancreas, adipose, liver and skeletal muscle (495). However, in cells of the reproductive system, the precise function of 1,25-(OH)2D3 on cellular bioenergetics is not fully understood yet, although previous animal and *in vitro* cell studies have suggested that 1,25-(OH)2D3 can modulate energy metabolism (68, 496-498). In VDR null mice fed a high-fat diet, lipid accumulation in the liver was reduced due to enhanced fatty acid oxidation and increased expression of uncoupling proteins (UCPs),

UCP-1, UCP-2, which increased energy expenditure (498). Overall, this indicated that intact VitD signalling regulated fat deposition through mitochondrial proton flux. UCP-1 is an important regulator of proton flux and can allow dissipation of the proton gradient across the mitochondrial inner membrane (498). While UCP-2 transports protons and increases the net proton conductance of mitochondria in the presence of specific activators. Molecular studies have shown that the silencing of VDR signalling and impairment of VDR translocation to the mitochondria in cancer cells, promoted elevated mitochondrial respiration and ETC activity through upregulation of cytochrome oxidase enzymes (COX II and IV) (266). In the current study, 1,25-(OH)2D3 enhanced mitochondrial respiration (in KGN cells), which is likely reflect in enhanced steroidogenesis and lipid storage. Stored lipids can then be used as an alternative fuel source by entering the β-oxidation pathway within the mitochondria (Figure 6.13).

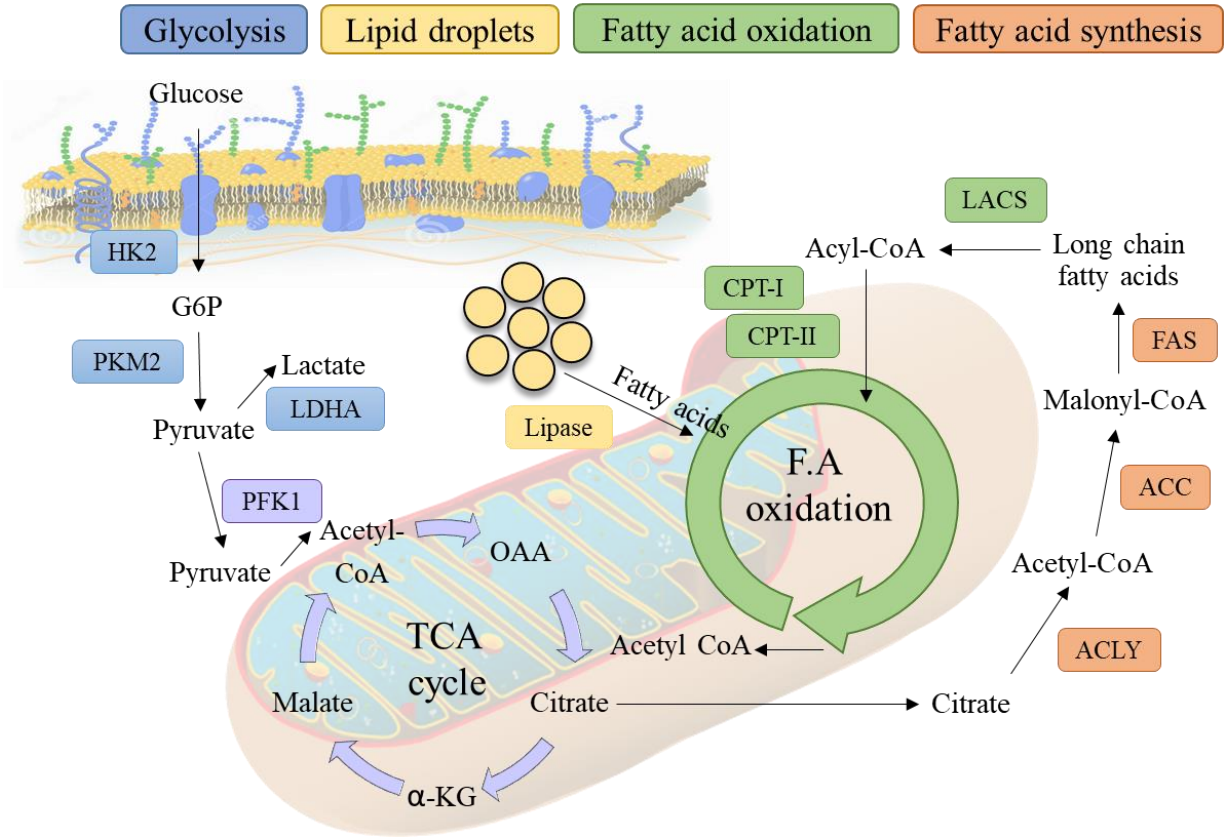

Figure 6.13: Comparison of metabolic pathways of glycolysis and fatty acid oxidation & synthesis. Glucose enters the cell membrane and is converted to pyruvate via glycolysis. Pyruvate is then converted to Acetyl-CoA, which can then enter the TCA cycle. Citrate can exit the TCA cycle and be converted to Acetyl-CoA, which can then be utilised in the fatty acid synthesis pathway. Long chain fatty acids formed by the fatty acid synthesis pathway is converted to Acyl-CoA, which can be shuttled into the mitochondria to enter the fatty acid oxidation (also known as β-oxidation) pathway, creating more Acetyl-CoA.

A recent report investigating human breast epithelial cells demonstrated treatment with 1,25-(OH)2D3 reduced glycolytic and citric acid cycle metabolic flux by decreasing the concentration of key metabolic intermediates, such as acetyl-CoA and oxaloacetate flux from glucose, pyruvate dehydrogenase activity, intracellular succinate levels, and mRNA expression of 3-phosphoinositide-dependent protein kinase-1 (499). Taken together, these *in vitro* cell studies suggest VDR signalling is an important regulator of glycolytic and oxidative metabolism, by altering key aspects of metabolic flux within these pathways. In the context of the present study, no intermediates of these metabolic pathways were assessed, but future studies investigating metabolic flux could further explain results demonstrated here.

Prior to this study the impact of VitD on human GCs bioenergetic parameters (glycolytic or mitochondrial) was never investigated. Here, 1,25-(OH)2D3 treatment for 24 hours significantly increased basal and compensatory glycolysis in COV434 cells, whilst only the latter was significantly increased in KGN cells. Interestingly, the addition of 1,25-(OH)2D3 in combination with rFSH and androstenedione did not enhance any glycolytic bioenergetic parameters further in either cell line, compared to 1,25-(OH)2D3 alone (as observed in lipid deposition). In contrast, previous data suggested 1,25-(OH)2D3 decreased glycolysis by suppression of glycolytic gene expression in various reproductive cell types, such as prostate and breast cells (500, 501). Here, for mitochondrial bioenergetics, 1,25-(OH)2D3-alone significantly increased basal, ATP-linked, and maximal respiration in KGN cells only. Moreover, basal, and ATP-linked respiration were only increased when 1,25-(OH)2D3 was used in combination with rFSH and androstenedione in COV434 cells. The discrepancy between data presented here and previous data could be due to differences in cell types, method of cell line establishment, and/or cell culture and experimental conditions. In contrast, in a human mammary epithelial cell line, OCR was reduced upon treatment with 1,25-(OH)2D3 both in the absence and presence of glucose (502). The authors suggested 1,25-(OH)2D3 inhibits two genes (glutamine synthetase and glutaminase) that affect glutamine entry into the TCA cycle for oxidation and therefore the flux of the cycle and production of reducing equivalents ($NADH/FADH_2$). While it is known that overexpression of glutamate-cysteine ligase protects COV434 cells against oxidative stress (503), it is unknown how glutamine-alone or in combination with 1,25-(OH)2D3 influences the mitochondrial bioenergetics in human GC lines. Additionally,

in an investigation of triple negative breast cancer cells, 1,25-(OH)2D3 was shown to significantly increase OCR in the presence of glutamine, when compared to cells deprived of glutamine (504). The discrepancy between these results and those presented here could potentially be due to cell-specific metabolic variations, how the cell lines were established/their origin ('young' cells vs 'older' cells, Foxl2 mutation (393)) or the cell culture and treatment conditions (use of high glucose DMEM vs. DMEM/F12 media, supplementation with additional constituents such as insulin, 1,25-(OH)2D3 concentration).

Mitochondrial/glycolytic dysfunction at the molecular level is not the only factor that can significantly impair GC functioning. Data from oocyte specific knock-out of mitofusin 1, which is a key regulator of mitochondrial organisation, severely diminishes mitochondrial function resulting in impaired folliculogenesis, oocyte development and subsequently leads to female sterility (505). This suggests mitochondrial dysfunction can result from mitochondrial disorganisation and loss of mitochondrial density, impairing oocyte-granulosa cell interactions that may lead to female infertility. To date, it is unknown if 1,25-(OH)2D3 can alter mitochondrial density in human GCs. The mtDNA copy number of cumulus GCs in IVF patients suggested that at different stages of oocyte maturation, the mtDNA number may undergo self-degradation and replication to meet the energy requirements of the corresponding oocyte and the maturation of the oocyte cytoplasm (506). Evidence from other cell types suggested that with advanced age, mtDNA volume, integrity and functionality decrease due to accumulation of mutations and oxidative damage induced by ROS (507). Additionally, abnormal copy number of mtDNA is related to type, grade and progression of ovarian cancer (508). However, prior to the current study, this had not been investigated in human GCs, and showed that 1,25-(OH)2D3 treatment for 24 hours did not alter mitochondrial density in either cell line. Taken together with increases in cellular bioenergetics, lipid deposition and hormone secretion, treatment with 1,25-(OH)2D3-alone or in combination with rFSH and androstenedione may result in enhanced storage of lipids. These lipids can then be mobilised which can then be used as a precursor for fatty acids via the fatty acid synthesis and oxidation metabolic pathways or steroid hormone production via the cholesterol pathway.

A major strength of this cellular bioenergetic and hormonal secretion data is both were normalised to cellular protein concentration to ensure consistency across independent experiments, particularly for potential variation in cell density or unpredictable changes in cell growth that may occur. Unfortunately, our data was limited to cell lines and no primary GCs were used to draw comparisons with the COV434 and KGN cell lines. Given the fact that these cells are lines derived from cancer cells, it is likely that primary human GCs would respond differently, and this requires further investigation. In the future, confirmation and further explanation of the changes observed here would be vital, as well as assessment of key regulators of the metabolic/steroidogenic pathways including the direct measurement of metabolite concentrations to determine flux (specific direction of metabolism). Additionally, using shorter or longer 1,25-(OH)2D3 treatment times could elucidate the genomic vs. non-genomic effects of VitD in human GCs. The major limitation of the current study was the lack of data to explain the increased functional output of these cells, following 1,25-(OH)2D3 treatment, in particular aspects of the steroidogenesis pathway. Furthermore, the use of lipidomic and metabolomic technologies in the future are critical to assess these findings in greater depth.

6.4 Conclusion

The aim of the current work was to investigate the functional and metabolic changes induced by 1,25-(OH)2D3-alone and in combination with rFSH and androstenedione on the COV434 and KGN cell lines, and whether the responses varied between the two human GC lines. This study was the first to examine (under the above conditions) the E2/P4 hormonal outputs, lipid deposition, cellular bioenergetics, and mitochondrial density of COV434 and KGN cells. The two cell lines responded differently following treatment with 1,25-(OH)2D3-alone and in combination with rFSH and androstenedione, specifically for lipid deposition and cellular bioenergetics. COV434 cells (which represent a ‘young’ human GC line) directly responded to 1,25-(OH)2D3 to increase E2 and P4 secretion, lipid deposition, and glycolysis. Moreover, KGN cells (which represent a ‘older’ human GC line) responded directly to 1,25-(OH)2D3 to increase P4 secretion, lipid deposition, and mitochondrial bioenergetics. Although the

hormone secretion of these cell lines was significantly lower than that of primary human GCs, taken together with the fact these cell lines both express the VDR and respond by enhancing steroidogenesis under the stimulatory conditions detailed here, these cells are a good model to investigate the effect of 1,25-(OH)2D3 on human GC's. The findings discussed here could be translated to a study of primary human GCs to investigate lipid deposition, lipid flux and cellular bioenergetic responses to 1,25-(OH)2D3, to uncover the biochemical role of 1,25-(OH)2D3 in ovarian physiology.

Investigating the important roles of bioenergetics and steroidogenic capacity for these human GC lines is crucial to making informed comparisons between the two, as they represent two metabolically/morphologically different forms of ovarian GC tumours (reproductive age vs. post-menopausal). These findings may have implication for local VitD levels in developing follicles that could influence oocyte development during folliculogenesis. Additionally, 1,25-(OH)2D3 is a relatively inexpensive drug which could potentially be beneficial in assisted reproductive technologies such as *in vitro* maturation, to further enhance the effects of this process. Moreover, understanding mitochondrial dysfunction and disorganisation in aging could be vital in further understanding the age-related decline of female infertility and subsequently the improvement of currently available infertility treatments. Examination of the role of VitD at the follicular and molecular level (in reproductive cells such as GCs) is critical to understand the association between serum VitD levels and IVF outcomes, which remains unclear.

Chapter Seven

Overall Thesis Discussion and Conclusion

7.0 Introduction

The overall aim of the current thesis was to investigate the association between VitD status and clinical IVF outcomes, and the *in vitro* effects of active 1,25-(OH)2D3 on granulosa cell hormone secretion, steroidogenesis, and metabolism. Firstly, a retrospective study (Chapter 3) was implemented to identify gaps in the clinical data collection process, and to inform the prospective cross sectional clinical study (Chapter 4). Secondly, two human GC lines (COV434 and KGN) were characterised after exposure to rFSH and androstenedione to represent *in vivo* stimulated GCs (Chapter 5). Lastly, following characterisation, these same human GC lines were stimulated with 1,25-(OH)2D3 – alone, and in combination with both rFSH and androstenedione to assess if 1,25-(OH)2D3 had direct effects on GCs, or potentiated the response to FSH and androstenedione (Chapter 6).

7.1 Key findings

7.1.1 Chapter Three

This retrospective study found no association between serum VitD status and clinical outcomes of women undergoing IVF including blastocyst development, CP, or LB. Even after controlling as a confounding factor, the inconsistent timing between VitD testing and the start of IVF cycles largely impacted the confidence of these findings. Nonetheless, the main purpose of this clinical study was to determine the feasibility of a prospective study, to identify potential study design issues (such as timing of VitD testing) and to inform study design and statistical analysis of the proceeding prospective clinical study.

7.1.2 Chapter Four

The current prospective cross sectional clinical study found a strong association between VitD sufficiency and an increased chance of blastocyst development, a non-significant rise in CP chance, but no association with LB chance. Moreover, serum VitD and FF 25-(OH)D levels were strongly correlated in this patient cohort. Finally, while serum or FF 25-(OH)D status did not appear to be related to patient biometrics such as fat or muscle mass, there was a strong correlation with VitD sufficiency and BMI status.

7.1.3 Chapter Five

In this validation study of the human GC lines COV434 and KGN cells, rFSH and androstenedione in combination enhanced steroid hormone secretion (E2/P4), and increased enzyme expression in several biochemical pathways, related to cholesterol biosynthesis/transport and fatty acid synthesis. In addition, stimulation with rFSH and androstenedione enhanced lipid deposition, and cellular bioenergetics in these cell lines.

7.1.4 Chapter Six

In this examination of COV434 and KGN cells it was demonstrated that active 1,25-(OH)2D3 directly influenced steroid hormone secretion (E2/P4), and altered intracellular ROS generation, lipid deposition, and cellular bioenergetics. Furthermore, when used in combination with rFSH and androstenedione, these measures were enhanced.

7.2 The link: *in vitro* vs. *in vivo* findings

It was observed that VitD-alone and in combination with androstenedione and rFSH directly increased *in vitro* E2 and P4 hormone secretion, steroidogenesis, and lipid deposition in GC cells, while in the clinical study there was a strong association between serum VitD status and the development of good quality embryos and blastocysts, but no subsequent association with statistically significant CP or LB outcomes. These steroid hormones (E2/P4) support ovarian development and function, which is known to influence downstream oocyte development whereby good quality mature oocytes are more likely to result in a high-quality blastocyst (216). While not shown directly, it is reasonable to speculate that VitD sufficiency *in vivo* and within FF could potentially influence oocyte quality by modulating the secretion of E2 and/or P4 reproductive hormones from GCs which then directly affect follicular growth dynamics and oocyte maturation (509, 510). Indeed, follicular E2 levels regulate preantral follicle development (229), and levels increase as follicles enlarge (509), and this is facilitated by an increased expression of GC aromatase (510). A prospective clinical study by Taheri et al., found E2 levels > 3000 pg/mL on the day of hCG administration was associated with a higher number of good-quality embryos, and improved CPRs and LBRs (14), suggesting E2 levels in IVF may be predictive of successful outcomes. The strong association between VitD sufficiency and blastocyst formation may support this theory, but other evidence has also suggested that follicular components are associated with positive reproductive outcomes.

A lipidomic study published by Montani et al., found that the lipid composition within FF during oocyte collection differs between pregnant and non-pregnant women following IVF (354). Furthermore, pathway analysis within the same study showed enriched functions of these lipids for the process of steroidogenesis (in the pregnant group) and apoptosis (in the non-pregnant group) (354). For example, a range of diacylglycerol kinase (DAGK) and diacylglycerol acyltransferase (DGAT) enzymes were elevated in the pregnant group and these are associated with steroidogenesis and triacylglyceride (fatty acid) metabolism respectively (511, 512). Release of triacylglyceride into follicular fluid is important for oocyte development and elevated levels have been associated with high quality oocytes (354). So perhaps the VitD-induced increased storage of triacylglyceride in lipid droplets of GC observed in the

current study could be critical for future release for follicular fluid in an *in vivo* situation? Interestingly, another key aspect of the pathway analysis by Montani et al., was that the VDR was detected in the interaction network. The VDR had a downstream effect on phospholipase D1 (PD1), which catalyses the hydrolysis of phosphatidylcholine to produce phosphatidic acid and choline (354, 513). This phospholipase influences several biological pathways including membrane trafficking, signal transduction, mitosis, apoptosis, and importantly the creation of cytoplasmic lipid droplets (513). Furthermore, it was suggested that E2 production could be mediated by the VDR which highlights the importance of VitD signalling in ovarian steroidogenesis (354, 514). Taken together, this supports our *in vitro* findings whereby 1,25-(OH)2D3 treatment directly increased lipid deposition, which may be used *in vivo* to create intracellular lipid stores which can then be used as a precursor for steroidogenesis, as discussed in detail in Chapter 5.3 and support the development of oocytes with enhanced developmental competence.

Conversely, another lipidomics examination conducted by Shehadeh et al., found that the accumulation of plasma lipids is lower in the FF of positive-outcome patients (such as triacylglycerols, diacylglycerols, and cholesteryl esters) (40). However, others were enhanced in this same group including total sphingolipids, lysophospholipids, phospholipids, and glycosphingolipids. Interestingly, there was an accumulation of VitD derivatives in the follicular fluid of positive outcome patients including 25-(OH)D and 1,25-(OH)2D3 (40). These findings suggest there is a shift in the positive-outcome FF group downstream of cholesterol metabolism, whereby lipid signalling is altered and perhaps lipid stores are being depleted (in contrast to being stored, as shown in Chapter 6.2), and these are utilised to produce steroid hormones and/or to meet the energy demands of the developing oocyte. The accumulation of VitD derivatives identified here, including 25-(OH)D (which was measured within serum and FF in Chapter 4.2, and shown to be strongly associated) and 1,25-(OH)2D3 (which was used in the *in vitro* studies detailed in Chapter 6.2), supports the notion that adequate VitD within FF is positively associated with clinical outcomes in IVF (40), as previously speculated elsewhere and here within Chapter 4.5. Interestingly, an examination of metabolomics found that the composition of metabolites related to lipid metabolism within FF was also altered relative to female age (515). More specifically in the 'young' patients (average age at cycle was 29.4 years), there was an up-

regulation of arachidonic acid, which affects oocyte development, and lysophosphatidylcholine (which was previously discussed to be downstream of the VDR) participates in the regulation of follicular development and oocyte maturation (515).

While lipids are an important source for many biological processes such as fatty acid biosynthesis, steroidogenesis and cellular bioenergetics (all discussed in detail in Chapter 5.3), excessive accumulation of lipids can cause lipotoxicity (516). However, this was not the case in the present study, as determined by intracellular lipid staining, enhanced cellular bioenergetics and unchanged intracellular ROS (which would be suggestive of lipotoxicity via lipid peroxidation/oxidative stress (516)), as detailed in Chapters 5.3/6.3. A study from Raviv et al., examined lipid content in GCs as well as lipid/hormonal and C-reactive protein (CRP) in the serum of women < 40 years old undergoing IVF. The authors demonstrated the women who achieved CP had lower intracellular lipid droplets in their GCs, lower serum high density lipid elevated E2 levels on day of OPU (in FF and serum) and serum triglycerides, compared to women who did not achieved CP (517). Additionally, women with a higher BMI ($\geq$ 30 kg/m^2) had higher CRP levels, LDL cholesterol, but there was no difference in the lipid content of GCs based on BMI status (517). An alternative hypothesis could be that the lipid stores within GCs are being utilised as a steroidogenesis precursor in the women who conceived, hence decreased lipid content within GCs (518). This theory supports the findings presented here in Chapter 5 and 6, whereby increased lipid deposition was associated with enhanced secretion of E2 and P4, alongside enhanced lipid flux through the fatty acid synthesis, cholesterol biosynthesis and steroidogenic pathways (discussed in detail previously in Chapter 5.3). It is important to note some key limitations of the Raviv et al., report, firstly the sample size of this study was only n=41, and secondly the authors speculate that patients who did not conceive and had higher lipid content, meaning these GCs were undergoing lipotoxicity, yet no measures of lipid peroxidation, oxidative stress or GC dysfunction was investigated to support this suggestion (7).

Prior to this study (Chapter 4) little was known about whether VitD was associated with embryo quality or blastocyst development. It was shown in Chapter 5 that StAR expression was significantly elevated under stimulation with rFSH and androstenedione, while previous work by others has demonstrated VitD and StAR

expression are closely related. In particulate StAR levels are increased in women with PCOS (who are also more likely to be deficient in VitD henceforth were excluded in the present clinical study detailed in Chapter 4) and unexplained infertility. Additionally, StAR expression in these women undergoing IVF was positively correlated with GC E2 content, which supports the current study findings where rFSH and androstenedione stimulation resulted in elevated StAR expression and E2 secretion (Chapter 5.2). Interestingly, transcriptome analysis of StAR gene expression in cumulus cells found StAR expression was upregulated in low-quality oocytes and embryos (519). Additionally, StAR expression was higher in cumulus cells from abnormal 2PNs than from oocyte yielding high-quality embryos, suggesting StAR is involved in embryo development from as early as 2PN formation (519). While this suggests a relationship between StAR expression and oocyte/embryo quality, it is important to note the authors only looked at the mRNA level which does not reflect the protein level/post-translation modification, therefore transport of cholesterol to the mitochondria may not necessarily be impaired in this circumstance (520). On the other hand, the opposite was true for the present study - here it was demonstrated StAR protein expression increased under stimulation with rFSH and androstenedione, but the gene expression of StAR was not investigated. Furthermore, StAR protein expression in GCs of IVF patients has been shown to be altered depending on the infertility aetiology (521).

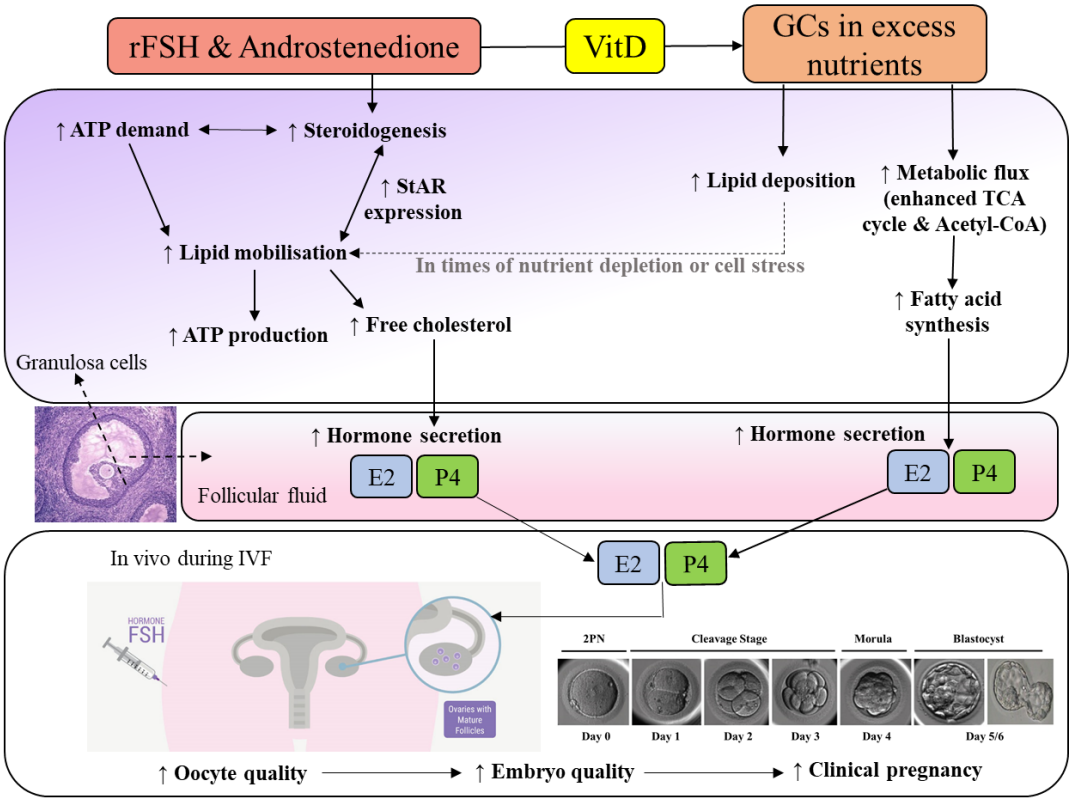

Figure 7.1: Summary of hypothesised *in vitro* and *in vivo* link. VitD increases lipid deposition, and these lipids can be stored and used as a potential energy source in times of need to meet ATP demand. During IVF ovarian stimulation, ATP demand is increased, and stored lipids are mobilised and can be used as precursor for steroidogenesis to produce the hormones E2 and P4, which are released into the circulation and follicular fluid. Enhanced E2 and P4 support oocyte development within the follicle during ovarian stimulation leading to increased oocyte and embryo quality, which is associated with increased CP chance.

7.3 Limitations and strengths

A major strength of the present thesis was the examination of the role of VitD in human fertility both at an *in vivo* level in a clinical setting with IVF patients, as well as on a molecular level using *in vitro* GC lines. Another major strength of the present prospective cross sectional clinical study (Chapter 4) was the fact it consisted of a large sample size and utilised advanced statistical analysis methods. Through this, a strong association was found between VitD sufficiency and an increased blastocyst development which carried over into a trending increase in clinical pregnancy. While the clinical findings presented here are promising, prospective, randomised control trials (RCTs) remain the gold standard in clinical studies, and therefore is a limitation of the present study. Several advantages of a RCTs include providing direct comparisons between groups, minimised bias (selection bias and allocation bias) and confounding factors and enhanced statistical reliability (reducing Type I and II errors). The prospective cohort study presented here provides evidence that a large RCT investigating VitD supplementation during IVF on clinical outcomes, such as blastocyst development and clinical pregnancy, is a worthwhile course of action proceeding this prospective study.

Furthermore, for the chapters which investigated the characterisation (Chapter 5) and impact of VitD (Chapter 6) on COV434 and KGN cell lines, these findings were promising. A major strength of these chapters was the extensive aspects investigated, including bioenergetics, hormone secretion, steroidogenesis, cell viability, lipid flux, and lipid deposition. However, the main limitation is these findings were only demonstrated in human GC lines. Further research is required to elucidate and translate these concepts in primary GCs. GC lines and primary GCs have varied metabolic and functional outputs when compared. Additionally, during the process of oocyte collection in IVF several stimulatory drugs are used which will likely influence the function of the primary GCs collected alongside oocytes. Future studies using primary GCs to confirm proof of concept that VitD levels (within FF) are directly related to steroidogenesis, metabolic functioning, lipid deposition, lipid flux, and clinical outcomes in IVF.

7.4 Significance and future directions

The reported prevalence of VitD insufficiency in infertile populations from previous publications ranges from 27-65% (30, 35, 39, 44, 375). Moreover, close to half the patient cohort in the current retrospective and prospective studies were found to be insufficient in VitD. This could indicate VitD insufficiency is more prevalent in the sub-fertile population or in women of reproductive age (281, 376). Despite numerous improvements in ART processes including within IVF (522), success rates worldwide can still remain low, especially in women of advanced maternal age (523). IVF is not only a financially demanding process, it is also mentally and emotionally demanding on the patients (524). Finding new ways to reduce the burden of multiple cycles for patients undergoing IVF is a crucial aspect of medical research in this field. VitD is freely available via sun exposure and can be natural obtained by the diet as well (5), and is relatively inexpensive in its supplementation form (7). Improving VitD levels in patients to $\geq$ 50 nmol/L (equivalent to $\geq$ 20 ng/mL) could potentially be an important clinical factor for reproductive clinicians and scientists to consider moving forward. Additionally, VitD in serum (which was shown here to reflect FF concentrations of VitD), may potential be used as a biomarker to predict IVF success, in particular for embryological and clinical outcomes, such as oocyte quality, blastocyst development, CPRs, and LBRs.

Prior to the present thesis, the direct effects of rFSH and androstenedione (alone and in combination with 1,25-(OH)2D3) was unknown. Here we have shown these drugs have direct effects of many biological aspects of GCs including hormone secretion, lipid flux of key enzymes, lipid deposition, and cellular bioenergetics. Understanding these molecular functions in human GCs could be crucial for reproductive scientists and clinicians moving forward for *in vitro* maturation processes and embryo culture.

7.5 Conclusion

While recent reports have suggested 25-(OH)D was positively associated with CPRs in women undergoing IVF (25-27), prior to the current prospective study, it was unknown if VitD status was associated with improved blastocyst development in women undergoing IVF. Furthermore, while it was previously known the VDR and VitD metabolising enzymes are present in numerous reproductive cells and tissues (including but not limited to- ovarian, endometrial, and placental (58, 82, 232, 341), prior to this study very little was known about how VitD impacts the steroidogenic and cellular bioenergetic profiles of human GCs. Based on the novel findings presented here, it is plausible to speculate that VitD modulates steroidogenesis of key reproductive hormones and bioenergetic parameters which support ovarian function. Moreover, this may be reflected *in vivo,* where higher levels of VitD in FF (which is strongly correlated with serum VitD, Chapter 4.3) may activate the key biochemical pathways discussed in Chapter 5, resulting in improved blastocyst development (as shown in Chapter 4).

Chapter Eight

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Chapter Nine

Appendices

Appendix I

Appendix I

Trial registered on ANZCTR

Registration number	ACTRN12617001221347
Ethics application status	Approved
Date submitted	7/08/2017
Date registered	21/08/2017
Date last updated	5/03/2020
Date data sharing statement initially provided	5/03/2020
Date results information initially provided	5/03/2020
Type of registration	Retrospectively registered

Titles & IDs

Public title	The Role of Vitamin D in Fertility and In Vitro Fertilisation (IVF) Outcomes
Scientific title	The impact of Vitamin D status on fertility outcomes, reproductive cell metabolism and bioenergetics.
Secondary ID [1]	N/A
Universal Trial Number (UTN)	
Trial acronym	
Linked study record	

Appendix II

PIVET MEDICAL CENTRE
The Miracle Of Life

Curtin University
CURTIN HEALTH INNOVATION RESEARCH INSTITUTE

Ovarian Metabolism Research Study – Female Information & Consent

PROJECT TITLE: The Role of Vitamin D & Related Growth Factors (IGF & PEDF) in Regulating Reproductive Function.

INVESTIGATORS: Dr. Kevin Keane, Prof. Philip Newsholme (*both Curtin University)* & Clinical Prof. John Yovich *(PIVET Medical Centre)*

THE PROJECT

Vitamin D is made in your body from exposure to sunlight, and is an important vitamin for healthy teeth and bones. It is also a nutritional factor and some research indicates that it may control some parts of hormone balance and metabolism. However, it is not clear if vitamin D levels influence IVF outcomes. Our researchers are trying to find out if the level of vitamin D in your blood and ovarian fluid, along with your partner's level, are related to successful IVF outcomes. By studying the level of vitamin D and how it interacts with some of the cells in your body, we may be able to determine how vitamin D affects the level of metabolites and growth factors in your body, which are important for your developing eggs. If we understand these processes, we may be able improve IVF treatments and help more couples succeed in IVF.

AIM

1. We want to collect the fluid & cells from the follicles that you have produced during your egg collection, and to test these for vitamin D, calcium, parathyroid, growth factors IGF and PEDF, insulin, glucose & amino acids. Follicle fluid and the cells floating in it would normally be discarded after your oocyte is removed, as they are not used in the IVF procedure.

2. We would also like to test any **immature eggs** that are deemed not suitable for IVF by your embryologist. In most cases where mature eggs are collected, immature eggs are normally discarded without attempting fertilisation with sperm, as they do not make normal embryos. However, we may be able to use these unfertilised eggs to find out more about their biology **(please initial specific approval for immature eggs below)**. We would not take such eggs if they were your only eggs as PIVET might try to mature them for fertilisation in desperate cases.

3. Finally, we would also like to take a small portion of the blood sample, which the Doctor already collects as part of your assessment, and test this for those nutrients listed in aim 1.

Information that we record about you such as the number/size of your follicles, age and the IVF treatment will be **de-identified** meaning it **will not** have your name on it and your confidentiality will be fully respected.

Risks & Benefits: There are no apparent risks associated with participating in this study, as the samples used are leftovers from your IVF samples. The benefits of this study are that you will get to know your level of vitamin D when it is tested, and if you request it, we can send you a copy of any publication when the study is complete. The work will help us know if vitamin D levels affect IVF outcomes, which can possibly help future IVF patients.

Partner Data: As part of this research we will also need access to your partner's IVF data (age, BMI, treatment etc.), but only if they also complete their own personal consent form similar to this one. If both consent forms are received, both of your data will be used but your confidentiality will be fully respected. If you are about to have surgical egg collection for IVF treatment, we would like you to consider donating this biological material to help us find out more about female fertility. You are not obliged to participate **if you do not wish.** If you choose not to participate, you are assured that it will not affect your normal IVF treatment in any way. If you have any concerns, please feel free to contact us to answer any questions about the project at kevin.keane@curtin.edu.au or (08)92669781.

PIVET MEDICAL CENTRE
The Miracle Of Life

Curtin University
CURTIN HEALTH INNOVATION RESEARCH INSTITUTE

Curtin University Human Research Ethics Committee (HREC) has approved this study (HREC number RD-26-10). Should you wish to discuss the study with someone not directly involved, in particular, any matters concerning the conduct of the study, your rights as a participant, or you wish to make a confidential complaint, you may contact the Ethics Officer on (08) 9266 9223 or the Manager, Research Integrity on (08) 9266 7093 or email hrec@curtin.edu.au.

Thank you and we wish you all the best with your IVF treatment.
The PIVET Research Team

I also confirm that (please initial):

☐ I have been informed of and understand the purposes of the study.

☐ I have been given an opportunity to ask questions.

☐ I understand that my participation is completely voluntary and that I can withdraw at any time and without prejudice. This will not affect my fertility treatment in anyway, and all identifiable data and samples will be disposed of accordingly.

☐ I understand any information which might identify me will not be used in any published materials.

☐ I agree and understand that if the researchers want to test for other measures not listed above, I will be informed.

☐ I agree that the de-identified information (not including your name) from this project can be collated with a similar study from national or international collaborators for the means of publication in esteemed, high quality medical journals.

Are you taking vitamin D supplements? (this will not affect whether or not you are included in the research).....Yes ☐ / No ☐

Please circle your ethnicity.....Caucasian / African / Asian / Other (specify)_____

I allow the use of (please initial your choice)...

Blood...Yes ☐ / No ☐ Follicular Fluid & Cells ... Yes ☐ / No ☐

Immature Eggs...Yes ☐ / No ☐

Signature of participant: _____ Date: _____

Signature of witness: _____ Date: _____

Appendix III

RBMO

ELSEVIER

ARTICLE

Serum Vitamin D status is associated with increased blastocyst development rate in women undergoing IVF

BIOGRAPHY

Kevin Keane is a Research Fellow who obtained his doctorate in cell biology from the Technological University of Dublin in 2011, following a period working in the pharmaceutical industry. His main research interest is reproductive endocrinology, focusing on metabolic factors that regulate reproductive outcomes in IVF patients.

Nikita L. Walz[1,2], Peter M. Hinchliffe[2], Mario J. Soares[3], Satvinder S. Dhaliwal[4,5], Philip Newsholme[1], John L. Yovich[1,2], Kevin N. Keane[1,2,*]

KEY MESSAGE

For every single increase in a blastocyst generated or embryo cryopreserved, the likelihood of VitD sufficiency increased by 32%. However, sufficient VitD level was not related to improved live birth rates. Larger studies should investigate whether the effect on blastocyst development may affect subsequent clinical pregnancy and live birth rates.

ABSTRACT

Research question: To determine the relationship between vitamin D (VitD) status and embryological, clinical pregnancy and live birth outcomes in women undergoing IVF.

Design: Cross-sectional, observational study conducted at a university-affiliated private IVF clinic. A total of 287 women underwent 287 IVF cycles and received a fresh embryo transfer. Patients had their serum 25-hydroxyvitamin D2/D3 (VitD) determined on the day of oocyte retrieval, which was analysed in relation to blastocyst development rate, clinical pregnancy and live birth outcomes.

Results: In stepwise, multivariable logistic regression models, increases in blastocyst development rate, number and quality, along with embryo cryopreservation and utilization rates were associated with women with a sufficient VitD status (≥20 ng/ml). For a single increase in the number of blastocysts generated per cycle or embryos cryopreserved per cycle, the likelihood for the patient to be VitD sufficient was increased by 32% (odds ratio [OR] 1.32, 95% confidence interval [CI] 1.10–1.58, $P = 0.002$ and OR 1.33, 95% CI 1.10–1.60, $P = 0.004$, respectively). Clinical pregnancy (40.7% versus 30.8%, $P = 0.086$) and live birth rates (32.9% versus 25.8%, $P = 0.195$) in the sufficient VitD group versus the insufficient group were not significantly different and VitD sufficiency was not significantly associated with these outcomes.

Conclusion: A strong relationship was observed between blastocyst development and VitD sufficiency. However, there was no association between VitD and clinical pregnancy or live birth outcomes. Further larger studies are needed to investigate whether the observed effect on blastocyst development may have downstream implications on subsequent clinical pregnancy or live birth rates, and on a potential mechanism where sufficient VitD concentrations are linked to improved IVF outcomes.

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KEYWORDS
25-Hydroxyvitamin D
Blastocyst development
IVF

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rbmo.2020.08.014 1472-6483/
Declaration: The authors report no financial or commercial conflicts of interest.

INTRODUCTION

The role of vitamin D (VitD) in calcium and phosphate metabolism is well established (*Hughes et al., 1975*). VitD affects intestinal duodenal calcium absorption (*Cromphaut et al., 2001*) and renal calcium reabsorption (*Bouillon et al., 2003*), as well as promoting bone mineralization by altering chondrocyte and osteoblast differentiation (*Masuyama et al., 2006*). Currently, it is estimated that 1 billion people worldwide have insufficient serum VitD concentrations (*Holick, 2007*). Many studies have reported that dysregulation of VitD homeostasis is associated with various immunological and metabolic pathologies, such as type 1 diabetes mellitus (*Hyppönen et al., 2001*). Consequently, interest in the non-skeletal actions of VitD has increased in recent years (*Bouillon et al., 2008*; *Holick, 2007*). The role and function of VitD in fertility beyond mineral homeostasis is largely unknown (*Keane et al., 2017a*). However, the VitD receptor (VDR) and associated metabolizing enzymes were reported to be distributed across various animal and human reproductive tissues and tracts including the ovary, endometrium, placenta, epididymis, prostate, and in spermatozoa (*Corbett et al., 2006*; *Jensen et al., 2010*; *Thill, 2010*; *Viganò et al., 2006*).

A range of clinical studies have suggested that VitD potentially plays an important role in human fertility. Various prospective cohort studies (*Garbedian et al., 2013*; *Neville et al., 2016*; *Ozkan et al., 2010*; *Paffoni et al., 2014*) and retrospective analyses (*Fabris et al., 2014*; *Franasiak et al., 2015*; *Polyzos et al., 2014*) have examined the associations between VitD concentrations and clinical outcomes in IVF patients including embryological measures, clinical pregnancy and live birth rates. Several studies have reported positive associations between higher serum VitD concentration and clinical pregnancy rate (CPR), embryo quality and live birth rate (LBR) (*Ozkan et al., 2010*; *Paffoni et al., 2014*; *Polyzos et al., 2014*), while others have reported no significant relationship (*Fabris et al., 2014*; *Franasiak et al., 2015*; *Garbedian et al., 2013*; *Neville et al., 2016*; *van de Vijver et al., 2016*). Because previous investigations have been largely inconsistent in terms of outcomes and reference cut-off values for VitD sufficiency, the aim of this study was to contribute to the body of knowledge by examining the correlation between serum VitD status and IVF outcomes within a university-affiliated private IVF facility and in a relatively large study population.

MATERIALS AND METHODS

Study design and patient inclusion/exclusion criteria

This cross-sectional observational study was registered with the Australian and New Zealand Clinical Trial Registry (ACTRN12617001221347). The key inclusion criteria were patients undergoing conventional IVF stimulation and receiving a fresh embryo transfer in the same cycle. The key exclusion criteria were patients with a history of thyroid, renal, liver or metabolic disease including polycystic ovary syndrome (PCOS), and/or those not receiving a fresh embryo transfer in the stimulated cycle. Researchers were blinded to patient treatment and consent was obtained from patients on day 21 of their previous cycle following infertility assessment. A total of 392 patients consented to have serum drawn on the day of oocyte retrieval for 25-hydroxyvitamin D2/3 assessment (total Vitamin D 2/3, both forms referred to as VitD throughout), but only 317 patients received a fresh embryo transfer, a key inclusion criterion. Of the 75 excluded patients, 64 had freeze-all cycles with no fresh embryo transfer, eight patients had cycles cancelled between day 21 of the previous cycle and oocyte retrieval, two were excluded as no oocytes fertilized and one was excluded as embryos underwent preimplantation genetic diagnosis. Of the remaining 317 cycles with fresh embryo transfer, 21 cases were excluded on the basis of PCOS diagnosis, four cases were excluded as not enough serum was available for VitD testing, three antagonist cycles were excluded due to excessive follicular recruitment (≥12 follicles over 12 mm), and another two cycles were excluded as no live birth data were available due to medical termination of pregnancy. The final dataset in this study consisted of 287 eligible patients, who underwent 287 conventional ovarian stimulation cycles, received a fresh embryo transfer between 1 April 2016 and 30 November 2018, and had VitD measured on site. Cases were grouped based on the clinical definitions of VitD insufficiency (<20 ng/ml) and sufficiency (≥20 ng/ml) as per the Institute of Medicine (IOM) and the Endocrine Society clinical practice guidelines (*Holick et al., 2011*; *Rosen et al., 2012*). The flow chart describing the inclusion/exclusion process is shown in FIGURE 1.

Blood collection and analysis, and determination of patient body composition

Non-fasting whole venous blood was collected on site via venepuncture between 07:00 h and 11:00 h on the day of oocyte retrieval. Blood was separated by centrifugation at 250*g* for 10 min at room temperature. Serum was aliquoted, frozen and stored at −20°C (for up to 3 months) and batch tested. Total VitD (VitD2 and VitD3) and progesterone were determined by chemiluminescence immunoassay using an ADVIA Centaur Vitamin D Total Assay kit (Siemens, Bayswater, VIC, Australia). Manufacturer controls were analysed on the day of each batch test and all results were calculated automatically by the ADVIA Centaur XP Immunoassay System (Siemens). For VitD, the assay detection and upper linearity limits were 4.0 and 150.3 ng/ml, respectively, while the intra- and inter-assay coefficients of variation were 4.2 and 11.9%, respectively. This assay has a similar limit of detection and cross-reactivity as compared with the gold standard liquid chromatography-tandem mass spectrometry (LC-MS/MS), and has one of the lowest total error percentages (following LC-MS/MS) compared with other commercially available automated VitD assays (*Ong et al., 2012*). Progesterone was also measured using the ADVIA Centaur and the Centaur Progesterone assay. The assay detection and upper linearity limits were 0.21 and 60.0 ng/ml, respectively, while the intra- and inter-assay coefficients of variation were 4 and 8%, respectively. Patient anti-Müllerian hormone (AMH) was determined using the Elecsys AMH assay on the Roche Cobas e411 module. The assay detection and upper linearity limits were 0.071 and 164.2 pmol/l, respectively, while the intra- and inter-assay coefficients of variation were 1.8 and 4.4%, respectively.

Biometric parameters were obtained at the initial consult and updated every 3 months at visits to the clinic. Height was measured using a stadiometer fixed to a wall. Body composition was determined using a Segmental Body Composition Monitor (Tanita BC-545N; Tanita, Kewdale, WA, Australia) which uses

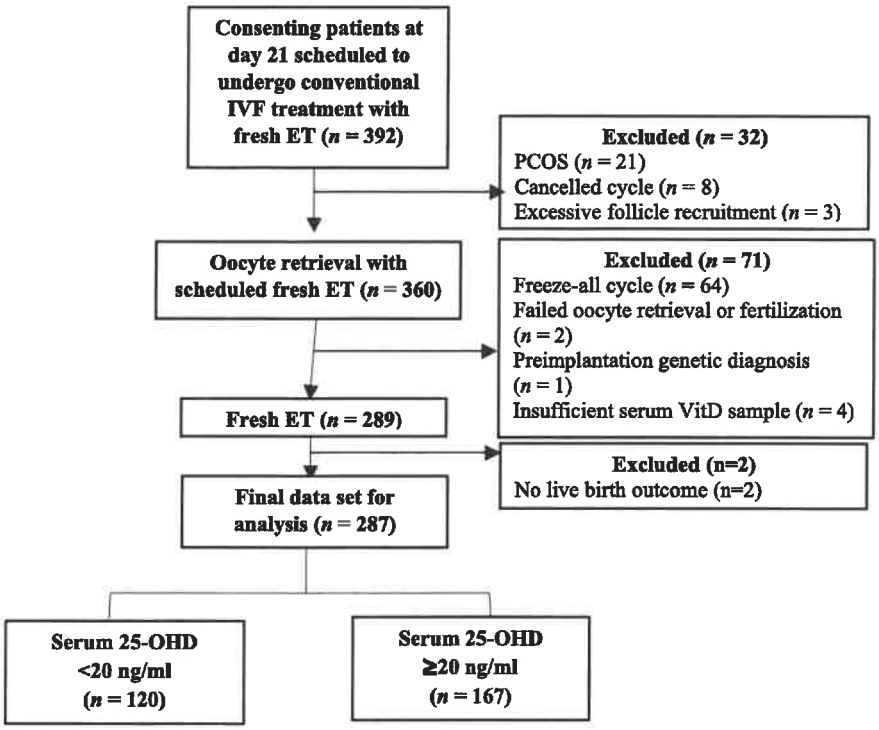

FIGURE 1 Flow chart detailing the recruitment of consenting patients and final data analysis cohort. Data were extracted from the PIVET database and cases were removed if there was no fresh embryo transfer (ET), the cycle was cancelled or failed. 25-OHD = 25-hydroxyvitamin D; PCOS = polycystic ovary syndrome; VitD = Vitamin D.

bioelectrical impedance analysis (BIA) technology. Measurements obtained included weight (kg), body mass index (BMI, kg/m^2), body fat percentage (%), total body water percentage (%), muscle mass (kg), bone mass (kg) and basal metabolic rate (BMR; kJ/day).

IVF cycle procedures

Detailed descriptions of clinical procedures have been published previously (*Keane et al., 2016*), including ovarian stimulation and induction protocols, luteal support, embryo culture and transfer procedures, clinical pregnancy testing and live birth follow-up. Previously published recombinant FSH (rFSH) dosing algorithms were used to select the initial rFSH dose, accounting for various patient factors such as age, BMI, smoking status, basal FSH concentration, antral follicle count (AFC) and AMH values (*Keane et al., 2016*). Selection of the appropriate ovarian stimulation protocol was at the discretion of the clinician and independent of the researchers, but protocol type was examined as a confounder during data analysis. Oocyte maturation was initiated using a single 13,000 IU dose of recombinant human chorionic gonadotrophin (rHCG) (Ovidrel; Merck Serono Australia Pty Ltd, Frenchs Forest, NSW, Australia) for patients with at least two leading follicles (≥18 mm in diameter). For patients with ≤4 follicles, Ovidrel (19,500 IU) was used. Oocyte retrieval was performed 35–37 h post-trigger for all patients. Luteal support for fresh IVF–embryo transfer cycles was based on the number of oocytes recovered at oocyte retrieval, as previously described (*Keane et al., 2016*), and included oestradiol, progesterone pessaries and/or recombinant HCG injections.

Embryo culture, grading and transfer

Following insemination, all two-pronucleate (2PN) zygotes were placed into cleavage-stage medium (Quinn's Advantage™ Sequential Medium, CooperSurgical, Trumbull, CT, USA) to be cultured to day 3. If there were fewer than three high-grade embryos progressing at the 6–8 cell stage at day 3, embryo transfer was considered and blastocyst culture precluded. Cleavage-stage embryos were graded high or low as published previously (*Jacobs et al., 1990*), while blastocysts were graded based on Gardner's classification (*Gardner and Schoolcraft, 1999*) and categorized as high, medium or low quality as previously discussed (*Yovich et al., 2015*). Day 2/3 transfers were included in 27.9% of cases (n = 80). Transfers on day 4 (n = 75) and day 5 (n = 132) made up the majority of cases (72.1%). Single embryo transfer (SET) was used in the majority of cases (86.0%). However, exceptions were made for double embryo transfer (DET) upon written request from patients who had three or more unsuccessful IVF attempts previously, and at the discretion of the treating clinician.

Embryological and clinical outcomes measured

Fertilization rate was calculated as the percentage of 2PN generated from metaphase II (MII) oocytes inseminated for each case and the median for the VitD groups presented. Blastocyst development rate (*ESHRE Special Interest Group of Embryology, Alpha Scientists in Reproductive Medicine, 2017*) for each case was calculated as the percentage of blastocysts generated from the 2PN generated in the cycle. Mean blastocyst development rate was determined for each VitD group and compared. These blastocyst rates were also calculated from the number of oocytes collected and from the MII oocytes collected.

Oocyte and embryo utilization rates were also calculated to determine the

proportion of clinically 'usable' embryos generated per cycle. Oocyte utilization rate was expressed as the total number of embryos (cleavage and blastocysts) transferred and vitrified, as a function of the total number of oocytes retrieved (from a single oocyte retrieval), while embryo utilization rate was the total number of embryos transferred and vitrified, as a function of the total number of 2PN generated. Clinical pregnancy was confirmed via transvaginal sonogram at 7 weeks' gestation following the detection of an intrauterine gestational sac with a fetal heartbeat. After the expected delivery date, live birth outcomes were obtained from the relevant hospital and/or obstetrician.

Statistical analysis

Normally distributed continuous data are represented as mean ± SD, analysed using *t*-tests. Categorical data are represented as number of patients (percentage), analysed via chi-squared contingency tables. Non-normally distributed data are expressed as median (interquartile range, IQR), analysed non-parametrically applying Mann-Whitney *U*-tests. In some cases, for example embryos transferred or cryopreserved, the mean is also presented because the range of values rarely exceeded 3.

Logistic regression models

Because the measured embryological parameters were continuous variables, and the VitD sufficient and insufficient groups were binary variables, univariate and multivariable binary logistic regression models with VitD group as the binary dependent variable were developed. Univariate logistic regression models were used to identify independent variables that were significantly associated with the likelihood of a patient being VitD sufficient (Supplementary Table 1). Each embryological measure was then explored to determine whether increases in the variable were associated with an increased prospect of VitD sufficiency (Supplementary Table 2). Significant independent variables established in this model were then used in stepwise multivariable logistic regression analysis as confounding factors to assess whether the embryological measures remained significant. A similar univariate approach was used for live birth as a binary dependent variable (Supplementary Table 3), which was then adapted to create a final multivariable logistic regression model for live birth.

To reduce multicollinearity, AMH was included instead of AFC in the multivariable models, because the two variables are directly related (this logic was applied to all adjusted models) and in a previous study ovarian reserve was not associated with VitD status (*Drakopoulos et al., 2016*).

Sample size calculation

Previous studies observed a difference of 13% in CPR between the VitD insufficient and sufficient groups (*Polyzos et al., 2014*), and in the present study, an approximate CPR of 40% and 25% for VitD sufficient and insufficient groups, respectively, was estimated. For the sample size calculation, the alpha and beta values were set to 0.05 and 0.2, respectively. A total of 320 participants were required to detect this CPR difference with 80% power. At the end of the study 392 consented to participate, but only 287 were eligible for data analysis.

Ethics approval and trial registration

All recruited patients signed consent forms detailing the research aims and requirements for research purposes. This cross-sectional observational study was approved on 5 January 2016 by the Curtin University Human Research Ethics Committee (HREC), approval number RD 26-10.

RESULTS

Patient recruitment, VitD status and demographics

A total of 287 IVF patients, each with a fresh embryo transfer cycle, were included in the final analysis (FIGURE 1). There was no significant difference between the two groups in regard to the proportion of excluded cases and the reason for exclusion (data not shown). Based on the IOM guidelines, 41.8% of patients had insufficient VitD concentrations (<20 ng/ml, n = 120), and 58.2% had sufficient concentrations (≥20 ng/ml, n = 167) (TABLE 1), while only 3.1% of patients had a serum VitD concentration of <10 ng/ml and 15.3% of patients had a serum VitD concentration >30 ng/ml (data not shown). The range of serum VitD measurements for the whole cohort was 4.0–72.0 ng/ml (TABLE 1).

When stratified by VitD status (<20 ng/ml versus ≥20 ng/ml), respectively), there was no significant difference between the groups in terms of season of embryo transfer/VitD testing and the proportion of patients consuming VitD supplements. Median female and partner age, AMH, AFC, and the proportion of patients with previous IVF treatment and infertility aetiology were also similar and not significantly different (TABLE 1). As expected, a significantly higher proportion of patients within the sufficient VitD group self-reported as of Caucasian descent (96.4% versus 85.8%, P = 0.001, TABLE 1). BMI was not significantly different between the VitD sufficient and insufficient groups (24.9 versus 23.2 kg/m^2, P = 0.088, TABLE 1). From a subset of 107 patients who had their biometrics analysed using BIA, 42 patients (39.3%) were grouped as VitD insufficient, while the remaining 65 patients (60.7%) were VitD sufficient (TABLE 1). The groups were comparable in regard to bone mass, fat mass and BMR. Median muscle mass in the sufficient group was 35.2 kg compared with 29.5 kg in the insufficient group (P = 0.052, TABLE 1).

Patient cycle characteristics

The cycle characteristics were highly comparable between the VitD groups. There was no difference in the proportion of patients receiving antagonist or agonist stimulation, insemination technique (intracytoplasmic sperm injection [ICSI] versus IVF versus ICSI/IVF split), median rFSH dose, trigger dose, progesterone concentration at trigger or endometrial thickness (TABLE 2). There was also no significant difference between the VitD groups in relation to the proportion of cycles with cleavage stage or blastocyst transfer, the quality of embryos transferred (high, medium, low), and the proportion of cases with SET or DET (TABLE 2).

Embryological measures

There were no significant differences observed between the two VitD groups in relation to the number of oocytes collected (median per cycle), number of MII oocytes collected (median per cycle), or median fertilization rate (78.3% versus 77.8%, ≥20 ng/ml versus <20 ng/ml group, respectively, P = 0.835, TABLE 3) or the median fertilized zygotes for each case/cycle (5.0 versus 4.0 2PN, ≥20 ng/ml versus <20 ng/ml group, respectively, P = 0.364, TABLE 3).

The mean blastocyst development rate was 30.0% and 21.8% for those with VitD ≥20 ng/ml and <20 ng/

TABLE 1 PATIENT DEMOGRAPHICS STRATIFIED BY SERUM VITD STATUS

Variable	<20 ng/ml	≥20 ng/ml	*P*-value
Initiated cycles, *n*	120	167	–
Mean serum VitD concentration, ng/ml	15.5 ± 3.4	28.0 ± 8.1	<0.001
Median serum VitD concentration, ng/ml	16.2 (5.1)	25.6 (7.5)	<0.001
Serum VitD concentration range, ng/ml	4.0–19.8	20.0–72.0	–
Season of ET/VitD test	–	–	0.730
Winter/spring	70/120 (58.3)	94/167 (56.3)	–
Summer/autumn	50/120 (41.7)	73/167 (43.7)	–
Female age at cycle, years	35.0 (8.0)	35.0 (8.0)	0.455
Partner age at cycle, years	38.0 (7.0)	38.0 (9.0)	0.918
BMI, kg/m^2	24.9 (8.2)	23.2 (5.9)	0.088
Ethnicity	–	–	0.001
Caucasian	103/120 (85.8)	161/167 (96.4)	–
Non-Caucasian	17/120 (14.2)	6/167 (3.6)	–
Consuming VitD supplements	113/120 (94.2)	160/167 (95.8)	0.524
Bone mass, kg[a]	2.4 (0.4)	2.4 (0.3)	0.739
Fat mass, %[a]	26.4 (9.0)	27.1 (12.9)	0.718
Muscle mass, kg[a]	29.5 (15.4)	35.2 (12.9)	0.052
Basal metabolic rate, kJ[a]	5704.0 (991.0)	5648.0 (826.0)	0.783
AMH, pmol/l	16.7 (19.0)	14.5 (17.2)	0.172
AFC	–	–	0.492
≥20 follicles	40/120 (33.3)	45/167 (26.9)	–
9–19 follicles	55/120 (45.8)	82/167 (49.1)	–
≤8 follicles	25/120 (20.8)	40/167 (24.0)	–
Infertility aetiology	–	–	–
Endometriosis	5/120 (4.2)	7/167 (4.2)	0.992
Tubular defect	14/120 (11.7)	11/167 (6.6)	0.132
Male factor	28/120 (23.3)	39/167 (23.4)	0.997
Unexplained	80/120 (66.7)	127/167 (76.0)	0.080
Previous IVF cycle	–	–	0.797
First cycle	59/120 (49.2)	92/167 (55.0)	–
Second cycle	32/120 (26.7)	40/167 (24.0)	–
≥Third cycle	29/120 (24.2)	35/167 (21.0)	–

Data expressed as mean ± SD, number/total cases (percentage) or median (IQR). Season of VitD testing refers to the season in which venepuncture and serum sample was collected at oocyte retrieval.

AFC = antral follicle count; AMH = anti-Müllerian hormone; BMI = body mass index; ET = embryo transfer; VitD = Vitamin D.

[a] From a subset of 107 patients who had their biometrics analysed (<20 ng/ml *n* = 42 and ≥20 ng/ml *n* = 65).

ml, respectively (P = 0.013, TABLE 3). In addition, the mean blastocyst number generated per cycle was significantly higher in the sufficient VitD group (2.11 versus 1.49, ≥20 ng/ml versus <20 ng/ml group, respectively, P = 0.026, TABLE 3). Several other measures of blastocyst development rate were elevated in the sufficient VitD group, including the percentage of blastocysts developed calculated as a proportion of oocytes and MII collected (TABLE 3). Furthermore, the proportion of cases where at least one blastocyst developed was greater for the sufficient VitD group, occurring in 67.7% of cases, but only 50.8% of cases in the insufficient VitD group developed at least one blastocyst (P = 0.004, TABLE 3).

There were no significant differences in the mean number of embryos transferred, but the mean number of embryos cryopreserved in the sufficient VitD group was significantly higher (1.9 versus 1.4, ≥20 ng/ml versus <20 ng/ml group, respectively, P = 0.030, TABLE 3). However, this was not reflected in a significant difference in median oocyte or embryo utilization rates (TABLE 3).

Finally, there was no significant difference between the VitD groups in CPR or LBR for fresh embryo transfer, or indeed cumulative CPR or LBR when including subsequent cryopreserved embryo transfers (TABLE 3).

Logistic regression model of embryological measures

Each embryological measure was explored to see whether increases in these variables were associated with an increased chance of VitD sufficiency (dependent variable). A series of stepwise logistic regression models were developed for each specific embryological measure, controlling for female age at cycle, season of embryo transfer/VitD test, BMI, ethnicity, AMH, rFSH and trigger dose, progesterone concentration at trigger, stimulation type and IVF attempt number. For univariate analysis of each component refer to Supplementary Table 1. In the multivariable models, only BMI, ethnicity and the embryological measures presented in TABLE 4 remained significant and retained in each regression model (P < 0.05). As expected, Caucasian patients were highly likely to be within the VitD sufficient group (odds ratio [OR] 13.53, 95% confidence interval [CI] 2.63–69.49, P = 0.002), while for each kg/m^2 unit increase in BMI, the patient would be 11% less likely to be VitD sufficient (OR 0.89, 95% CI 0.83–0.95, P = 0.002, TABLE 4). Controlling for these factors, single unit increases in blastocyst number per cycle (OR 1.32, 95% CI 1.10–1.58, P = 0.002), increasing development rate (OR 1.02, 95% CI 1.01–1.04, P = 0.001), increasing percentage of high-quality blastocysts generated (OR 1.01, 95% CI 1.01–1.02, P = 0.040), increasing embryos cryopreserved (OR 1.33, 95% CI 1.10–1.60, P = 0.004) and increasing oocyte utilization rates (OR 1.03, 95% CI 1.01–1.05, P = 0.004) were all associated with VitD concentrations ≥20 ng/ml (TABLE 4). Of note, for each increase in blastocyst generated per cycle, or each increase in cryopreserved embryo per cycle, the chance of VitD sufficiency increased by approximately 32% (TABLE 4). The remaining insignificant embryological measures analysed using this multivariable logistic

TABLE 2 CYCLE CHARACTERISTICS STRATIFIED BY SERUM VITD STATUS

Variable	<20 ng/ml	≥20 ng/ml	P-value
VitD range	**4.0–19.8**	**20.0–72.0**	
Initiated cycles, n	120	167	–
Median serum VitD concentration, ng/ml	16.2 (5.1)	25.6 (7.5)	<0.001
Stimulation protocol	–	–	0.346
Antagonist	77/120 (64.2)	116/167 (69.5)	–
Agonist/other	43/120 (35.8)	51/167 (30.5)	–
Insemination type	–	–	0.258
ICSI	102/120 (85.0)	138/167 (82.6)	–
IVF	3/120 (2.5)	1/167 (0.6)	–
ICSI/IVF split	15/120 (12.5)	28/167 (16.8)	–
rFSH dosage, IU	200.0 (256.3)	200.0 (212.5)	0.962
Progesterone at trigger, ng/ml	2.1 (2.0)	2.0 (1.0)	0.584
Trigger dose, µg	500.0 (0.0)	500.0 (0.0)	0.897
Endometrial thickness, mm	9.4 (2.3)	9.5 (3.0)	0.953
Embryo transfer day	–	–	0.129
Cleavage stage	41/120 (34.2)	39/167 (23.4)	–
Day 4	28/120 (23.3)	47/167 (28.1)	–
Day 5	51/120 (42.5)	81/167 (48.5)	–
Transferred embryo quality	–	–	0.986
High quality	70/120 (58.3)	99/167 (59.3)	–
Medium quality	16/120 (13.3)	22/167 (13.2)	–
Low quality	34/120 (28.3)	46/167 (27.5)	–
Embryos transferred	–	–	0.659
SET	102/120 (85.0)	145/167 (86.8)	–
DET	18/120 (15.0)	22/167 (13.2)	–

Data expressed as number/total cases (percentage) or median (IQR).

DET = double embryo transfer; ET = embryo transfer; ICSI = intracytoplasmic sperm injection; rFSH = recombinant FSH.; SET = single embryo transfer; VitD = Vitamin D.

regression approach are presented in Supplementary Table 2.

Logistic regression model of live birth outcomes

Because live birth success is a binary outcome, it was selected as the binary dependent variable in logistic regression analysis. Each independent variable was examined for the univariate association with successful live birth set as a dependent variable (Supplementary Table 3). The main factors significantly associated with live birth were female age at cycle ($P < 0.001$), AMH ($P < 0.001$), AFC ($P = 0.004$), rFSH dose ($P < 0.001$), ART attempt number ($P = 0.019$), stimulation type ($P = 0.003$) and transferred embryo quality (high versus low $P = 0.008$, medium versus low $P = 0.011$). A stepwise multivariable logistic regression model was developed using these variables along with VitD-associated variables including VitD sufficiency group, season of embryo transfer/VitD test, BMI and ethnicity. Embryo transfer day was not significant in univariate analysis, but was included to control for embryo transfer stage (i.e. cleavage versus blastocyst). The final regression model demonstrated that none of the VitD-associated variables were significantly associated with live birth chance (TABLE 5). Only female age ($P < 0.001$), AMH ($P = 0.034$) and transferred embryo quality (high versus low $P = 0.048$, medium versus low $P = 0.012$) were significantly associated with live birth outcomes and retained in the stepwise multivariable model (TABLE 5).

DISCUSSION

Several studies have indicated that the prevalence of VitD insufficiency in infertile populations can range from 27% to 65% (*Fabris et al., 2014*; *Garbedian et al., 2013*; *Ozkan et al., 2010*; *Paffoni et al., 2014*; *Polyzos et al., 2014*). This suggests that perhaps VitD insufficiency is more prevalent in the subfertile population or in women of reproductive age (*Al-Jaroudi et al., 2015*; *Pal et al., 2016*). Approximately 40% of participants in the current study were VitD insufficient. However, both VitD groups were well matched in terms of patient demographics and cycle characteristics. VitD status was not associated with infertility history or biometric parameters such as fat mass or metabolic rate, as published previously by this group (*Calton et al., 2016*). Conversely, VitD sufficiency was not associated with increased muscle mass in the current study. As expected, VitD was strongly associated with ethnicity as significantly more Caucasian patients were VitD sufficient. While no significant difference in median BMI was observed in the demographic data, increasing BMI was significantly associated with a lower likelihood of VitD sufficiency in multivariable analysis. This meant that both ethnicity and BMI were key factors to control for in subsequent logistic regression analysis.

The data from this cross-sectional study indicate that serum VitD is not related to improved clinical pregnancy or live birth outcomes in women undergoing IVF with a fresh embryo transfer, after controlling for various confounders. However, sufficient VitD concentrations were strongly associated with an increased chance of developing more blastocysts per cycle, a higher percentage of high-quality blastocysts and consequently an overall greater number of embryos cryopreserved, reflected by a better oocyte utilization rate. Blastocysts are generally accepted to have greater implantation potential (*Yovich et al., 2015*). However, there was no difference in day of transfer between the VitD groups and neither factor was associated with improved clinical pregnancy or live birth rates.

One of the potential mechanisms by which VitD may influence reproductive outcomes is through effects on oocyte/embryo development and subsequent quality. Here, this aspect was explored in depth as a dependent and independent variable. It was clearly shown that 16.9% more cases in the sufficient VitD group generated at least one blastocyst

TABLE 3 EMBRYOLOGICAL MEASURES AND CYCLE OUTCOMES STRATIFIED BY SERUM VITD STATUS

Variable	<20 ng/ml	≥20 ng/ml	*P*-value
VitD range	**4.0–19.8**	**20.0–72.0**	
Initiated cycles, *n*	120	167	–
Oocytes			
Total oocytes collected, *n*	1155	1685	–
Median oocytes collected per cycle, *n*	10.0 (7.0)	9.0 (8.0)	0.375
Total MII oocytes collected, *n*	791	1225	–
Median MII oocytes collected per cycle, *n*	6.0 (5.0)	7.0 (6.0)	0.334
Fertilization			
Total fertilized (2PN), *n*	607	968	–
Median fertilized (2PN) per cycle, *n*	4.0 (4.0)	5.0 (5.0)	0.364
Median fertilization rate % per cycle, %	77.8 (38.1)	78.3 (25.9)	0.835
Cleavage embryos			
Total embryos cultured beyond day 2, *n*	557	936	–
High-quality day 3 embryos	348	634	
Blastocysts			
Total blastocysts formed, *n*	170	348	–
High-quality blastocysts	98	221	
Blastocyst rate per oocytes collected, *n*/total (%)	170/1155 (14.7)	348/1685 (20.7)	<0.001
Blastocyst rate per MII collected, *n*/total (%)	170/791 (21.5)	348/1225 (28.4)	<0.001
Blastocyst rate per 2PN, *n*/total (%)	170/607 (28.0)	348/968 (36.0)	0.009
Mean blastocyst number generated per cycle, *n*	1.49 ± 2.07	2.11 ± 2.50	0.026
[A]Mean blastocyst development rate, %	21.8 ± 26.5	30.0 ± 28.3	0.013
Cycles developing at least 1 blastocyst, *n*/total [%]	61/120 (50.8)	113/167 (67.7)	0.004
Transfer and cryopreservation			
Total embryos transferred, *n*	138	189	–
Mean embryos transferred per cycle, *n*	1.2 ± 0.4	1.1 ± 0.3	0.661
Total embryos cryopreserved, *n*	164	320	–
Mean embryos cryopreserved per cycle, *n*	1.4 ± 1.8	1.9 ± 2.3	0.030
[B]Median oocyte utilization rate, %	25.0 (23.9)	29.4 (25.5)	0.314
[B]Median embryo utilization rate, %	50.0 (42.0)	56.0 (54.0)	0.314
Pregnancy, miscarriage and live births			
Fresh clinical pregnancy rate, *n*/total (%)	37/120 (30.8)	68/167 (40.7)	0.086
Fresh live birth rate, *n*/total (%)	31/120 (25.8)	55/167 (32.9)	0.195
Fresh miscarriage rate, *n*/total clinical pregnancy (%)	6/37 (16.2)	13/68 (19.1)	0.712
Cumulative CPR (fresh + vitrified per ET) (%)	65/209 (31.1)	105/295 (35.6)	0.293
Cumulative LBR (fresh + vitrified per ET), *n*/total (%)	46/209 (22.0)	75/295 (25.4)	0.377

Data expressed as mean ± SD; median (IQR); *n*/total (%).

[A] Group mean % derived from the individual total number of blastocysts developed in a cycle as a function of the total number of normally fertilized oocytes (i.e. 2PN) in that cycle.

[B] Group median utilization rates derived from the individual calculated utilization rate for each case. Oocyte utilization rate equals the sum of embryos transferred and vitrified, divided by the number of oocytes collected in that cycle. Embryo utilization rate equals the number of embryos transferred and vitrified, divided by the number of 2PN generated in that cycle.

2PN = two-pronucleate zygotes; CPR = clinical pregnancy rate; LBR = live birth rate; MII = metaphase II oocytes; VitD = Vitamin D.

(P = 0.004). The sufficient VitD group also produced 0.6 more blastocysts per cycle, 0.5 more embryos were cryopreserved and the blastocyst development rate percentage was 8.2% higher than the VitD insufficient group. Stepwise logistic regression analysis, adjusting for a range of confounding variables including patient ethnicity, BMI, age and season of embryo transfer/VitD test, also showed that increasing blastocyst number per cycle, increasing blastocyst development rate, increasing percentage of high-quality blastocysts generated, increasing number of embryos cryopreserved, and increasing oocyte utilization rates were all associated with VitD concentrations ≥20 ng/ml. Surprisingly, embryo utilization rate, which is calculated as the proportion of embryos transferred and vitrified from the total number of 2PN zygotes in each case, was not associated with VitD concentrations ≥20 ng/ml (P = 0.091). The potential cellular or biochemical mechanism(s) relating to the effect of VitD on blastocyst development are unknown. However, a recent study has shown that VitD as part of a 'Mediterranean' diet impacts embryo morphokinetic markers and possibly blastocyst development by accelerating the fourth cell division event (5 cell to 8/9 cell) and reducing time for these division events (*Kermack et al., 2019*). It was also associated with an increase in known implantation data scores on day 3, which indicated enhanced quality. However, the major weakness of this morphokinetic study was that it was underpowered and the intervention contained other nutritional components such as omega-3 fatty acids and olive oil. Consequently, this adds significant complexity to the study and prevents the acquisition of firm conclusions, although the findings are interesting and worthy of further exploration.

No difference was observed in the present study in the number of oocytes and MII oocytes collected and fertilization rate, and this is in line with several other investigations that failed to demonstrate a clear relationship between these embryological measures and serum VitD (*Abadia et al., 2016*; *Chu et al., 2019*; *Fabris et al., 2014*; *Garbedian et al., 2013*; *Mitra et al., 2018*; *Neville et al., 2016*; *Ozkan et al., 2010*; *Paffoni et al., 2014*; *Polyzos et al., 2014*; *Rudick et al., 2012*; *Sufen et al., 2019*). However, while the study by *Abadia et al. (2016)* showed no significant difference in the number of MII oocytes according to 25-hydroxyvitamin D concentrations, a positive relationship with fertilization rates was demonstrated.

Relatively few studies have investigated the association between serum VitD

TABLE 4 FINAL MULTIVARIABLE LOGISTIC REGRESSION MODEL FOR EMBRYOLOGICAL MEASURES SIGNIFICANTLY ASSOCIATED WITH VITD SUFFICIENCY

Variable	Likelihood to be VitD sufficient (≥20 ng/ml) (OR, 95% CI)	*P*-value
Increasing BMI, kg/m^2	0.89 (0.83–0.95)	0.002
Ethnicity		
Non-Caucasian	1.00	–
Caucasian	13.53 (2.63–69.49)	0.002
[A]Increasing blastocyst number per cycle, *n*	1.32 (1.10–1.58)	0.002
[A]Increasing blastocyst development rate (per 2PN), %	1.02 (1.01–1.04)	0.001
[A]Increasing percentage of high-quality blastocysts generated, %	1.01 (1.01–1.02)	0.040
[A]Increasing embryos cryopreserved, *n*	1.33 (1.10–1.60)	0.004
[A]Increasing oocyte utilization rate per cycle, %	1.03 (1.01–1.05)	0.004

Associations are presented as odds ratio (OR) and 95% confidence interval (CI).

[A] In a series of stepwise logistic regression models controlling for female age at cycle, season of ET/VitD test, BMI, ethnicity, AMH, rFSH and trigger dose, progesterone concentration at trigger, stimulation type and IVF attempt number along with each specific embryological measure, only BMI, ethnicity and the embryological measures presented above remained significant and retained in each model ($P < 0.05$).

AMH = anti-Müllerian hormone; 2PN = two-pronucleate zygotes; BMI = body mass index; ET = embryo transfer; rFSH = recombinant FSH; VitD = Vitamin D.

and embryo quality. The earliest study by *Rudick et al. (2012)* demonstrated that serum VitD was not associated with embryo quality as measured by the amount of fragmentation and number of cells at the day 3 cleavage stage. Comparable findings were reported recently where the mean number and/or percentage of top-quality embryos were no different between VitD groups (*Polyzos et al., 2014*; *Sufen et al., 2019*). Interestingly, a prospective study with 335 participants reported that 16% of replete VitD cases generated three or more top-quality embryos per cycle, where only 6% of VitD insufficient cases generated three or more top-quality embryos (*Paffoni et al., 2014*). This study also revealed that 16% of replete cases received a blastocyst transfer, where only 6% of insufficient cases received a blastocyst transfer. This could suggest that women with sufficient VitD status are more likely to generate blastocysts, which was demonstrated in the current study.

The potential association between VitD status and clinical pregnancy and live birth outcomes was also examined, and both rates appeared to be higher in the sufficient VitD group, with a crude clinical pregnancy difference of 9.9% and a live birth difference of 7.1%; however, these differences were not statistically significant. Furthermore, in stepwise logistic regression analysis with CPR (data not shown) and LBR as dependent variables, VitD status was not included as a significant variable. Instead, the most important and significant parameters for both CPR and LBR were female age, ovarian reserve (AMH) and transferred

TABLE 5 FINAL MULTIVARIABLE LOGISTIC REGRESSION MODEL OF SIGNIFICANTLY ASSOCIATED FACTORS WITH LIVE BIRTH CHANCE

Variable		Likelihood for a live birth (OR, 95% CI)	*P*-value
Serum VitD group, ng/ml	<20 ng/ml	1.00	–
	≥20 ng/ml	1.38 (0.75–2.53)	0.298
Season of ET/VitD test	Winter/spring	1.00	–
	Summer/autumn	0.80 (0.44–1.45)	0.461
Female age, years		0.86 (0.81–0.92)	<0.001
BMI, kg/m^2		1.01 (0.95–1.07)	0.853
AMH, pmol/l		1.02 (1.01–1.03)	0.034
Ethnicity	Non-Caucasian	1.00	–
	Caucasian	1.90 (0.55–6.49)	0.308
Embryo transfer day, *n*		1.18 (0.87–1.60)	0.285
Transferred embryo quality	Low	1.00	–
	High	2.07 (1.01–4.24)	0.048
	Med	3.25 (1.30–8.17)	0.012

Associations are presented as odds ratio (OR) and 95% confidence interval (CI).

In a single stepwise logistic regression model including VitD group, female age at cycle, season of ET/VitD test, BMI, AMH, ethnicity, rFSH, stimulation type, IVF attempt number, day of embryo transfer, and transferred embryo quality, only age, AMH and transferred embryo quality remained significant and were retained in the model ($P < 0.05$).

AMH = anti-Müllerian hormone; BMI = body mass index; ET = embryo transfer; rFSH = recombinant FSH; VitD = Vitamin D.

embryo quality, all of which have consistently been shown to be central to clinical pregnancy and live birth chance in numerous reports from this clinic (*Keane et al., 2017b*, *2018*, *2019*). In a study of FET cycles, VitD status was not associated with CPR and the crude difference was only 5% (*van de Vijver et al., 2016*). A recent prospective study also showed that the crude LBR was greater in higher VitD groups by approximately 13–14% ($P = 0.004$) (*Chu et al., 2019*). However, following adjustment for major confounders such as age, BMI, smoking status, ethnicity, FSH, treatment type, infertility type and duration, the adjusted difference was only 9% and insignificant ($P = 0.250$) (*Chu et al., 2019*). Nonetheless, early retrospective studies suggested that the LBR in VitD replete IVF patients could be 4–28% higher than those considered insufficient (<30 ng/ml) (*Rudick et al., 2012*, *2014*), while another report indicated a significant 13% difference in LBR for higher VitD status patients ($P = 0.015$) (*Polyzos et al., 2014*). Two recent systematic reviews reported a decreased chance of live birth with VitD insufficiency (OR 0.74, 95% CI 0.58–0.90) (*Zhao et al., 2018*), while VitD sufficiency was associated with an improved LBR (OR 1.33, 95% CI 1.08–1.65) (*Chu et al., 2017*). However, this was not demonstrated in the present study, and it remains uncertain whether VitD influences LBR.

The major strength of this study was that it was conducted in a large sample size of 287 infertility patients, and it demonstrated a highly significant association between serum VitD concentration and blastocyst generation in an IVF patient population, while showing no association with clinical pregnancy or live birth outcomes. This was evident after accounting for a wide range of confounding variables previously demonstrated to impact IVF outcomes such as age, BMI, AMH, quality of transferred embryo, cycle stimulation protocol and ethnicity, among others (*Loendersloot et al., 2010*). In particular, this study included biometric and ethnicity data, which were not examined in many other VitD studies (*Drakopoulos et al., 2016*; *van de Vijver et al., 2016*). Interestingly, ethnicity was shown here to be a significant confounder even in a cohort with a Caucasian majority. It also examined two measures of ovarian reserve (AMH and AFC), which has also been included in very few VitD studies (*Drakopoulos et al., 2016*). The study was a registered, cross-sectional observational trial, which meant that data collection methods and analyses were robust. Furthermore, patients were recruited by researchers blinded to treatment strategies/decisions and independent of treating clinicians.

The main limitation of the current study was the failure to reach the recruitment target of 320 patients. Although 392 patients consented to the research, data were only available for 287 eligible individuals, which may play a role in the lack of significant differences observed for clinical pregnancy and live birth, for example. In addition, the reasons why patients were reluctant to participate in an observational trial at a private clinic were not clear, and this was not captured in the trial design or by questionnaire. The target of 320 patients was calculated based on a 15% difference in CPR (*Polyzos et al., 2014*; *van de Vijver et al., 2016*), and the difference observed for CPR and LBR between VitD groups was 9.9% and 7.1%, respectively, in favour of the VitD sufficient group. However, while the cohort was insufficiently powered to detect significant differences in the primary outcomes, it did demonstrate highly significant differences in the secondary outcomes of embryological measures. Even though recruitment fell short of the required number from the CPR power calculation, this study still represents one of the largest trials examining the relationship between serum VitD and IVF outcomes, after the trials by *Sufen et al. (2019)* ($n = 2577$), *Chu et al. (2019)* ($n = 500$) and *Paffoni et al. (2014)* ($n = 335$), and recruited slightly more patients than recent investigations by *Drakopoulos et al. (2016)* ($n = 283$) and *van de Vijver (2016)* ($n = 280$). Nonetheless, the relationship between serum VitD and blastocyst development was highly significant and it is entirely possible that this influence may also moderate CPR and/or LBR, where this relationship has been demonstrated in many other studies (*Abadia et al., 2016*; *Firouzabadi et al., 2014*; *Paffoni et al., 2014*). However, statistical moderation analysis was not explored in the current study.

Another limitation was that >95% of patients were taking VitD supplementation, but the specific dose and duration were not recorded. However, these potential effects were reduced by sampling for VitD status at oocyte retrieval and within a very close timeframe to the outcomes of interest, mainly blastocyst development and clinical pregnancy chance. Previous reports have shown that VitD found in common commercially available pregnancy multivitamins was not effective in mitigating VitD insufficiency at such low doses, and it takes extensive periods of supplementation to alter VitD status (*Pittaway et al., 2013*). Finally, another related limitation was that the VitD measurement and the outcome of live births spanned across multiple seasons and therefore it was difficult to establish a direct relationship between VitD measured at oocyte retrieval and later stage clinical outcomes such as live birth or neonatal outcomes. Consequently, future studies that follow up with a VitD blood test closer to the expected delivery date would be necessary to further elucidate this relationship.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2020.08.014.

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Appendix IV

Author declarations

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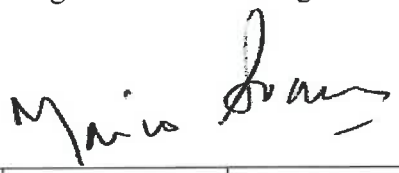
Walz, N.L., Hinchcliffe, P.M., Soares, M.J., Dhaliwal, S.S., Newsholme, P., Yovich, J.L., Keane, K.N. Serum Vitamin D status is associated with increased blastocyst development rate in women undergoing IVF. *Reproductive Biomedicine Online*. 2020, 41, 6: 1101-1111.

Relevant Thesis Chapter: Chapter 4.

Attribution Statement:

Author	Conception & Design	Acquisition of Data and Method	Data Manipulation	Analysis and Statistical Method	Interpretation and Discussion	Final Approval	Total % Contribution
Nikita Walz	30	50	20	30	35	25	35
Co-author acknowledgement: I acknowledge that these represent my contribution to the above research publication. Signed: 							
Peter Hinchcliffe	0	0	40	0	0	0	5
Co-author acknowledgement: I acknowledge that these represent my contribution to the above research publication. Signed:							
Mario Soares	0	0	0	15	5	0	5

Co-author acknowledgement: I acknowledge that these represent my contribution to the above research publication. Signed: 							
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Relevant Thesis Chapter: Chapter 4.

Attribution Statement:

Author	Conception & Design	Acquisition of Data and Method	Data Manipulation	Analysis and Statistical Method	Interpretation and Discussion	Final Approval	Total % Contribution
Nikita Walz	30	50	20	30	35	25	35
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Peter Hinchcliffe	0	0	40	0	0	0	5
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Mario Soares	0	0	0	15	5	0	5
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Satvinder Dhaliwal	0	0	0	20	0	0	10
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Philip Newsholme	0	0	0	0	20	25	5
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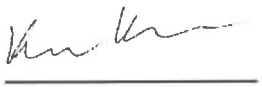
Co-author acknowledgement: I acknowledge that these represent my contribution to the above research publication. Signed: 							

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Relevant Thesis Chapter: Chapter 4.

Attribution Statement: PROF JOHN L YOVICH P:1

Author	Conception & Design	Acquisition of Data and Method	Data Manipulation	Analysis and Statistical Method	Interpretation and Discussion	Final Approval	Total % Contribution
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John Yovich	30	20	20	20	20	25	20
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Kevin Keane	40	30	20	20	20	25	20
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Kevin Keane	40	30	20	20	20	25	20
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Appendix V

Appendix V

Serum Vitamin D status is associated with increased blastocyst development rate in women undergoing IVF

Author: Nikita L. Walz,Peter M. Hinchliffe,Mario J. Soares,Satvinder S. Dhaliwal,Philip Newsholme,John L. Yovich,Kevin N. Keane

Publication: Reproductive Biomedicine Online

Publisher: Elsevier

Date: December 2020

Appendix VI

Molecular and Cellular Endocrinology

Bioenergetic and metabolic characterisation of the COV434 and KGN human ovarian granulosa cell lines

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Keywords:	Granulosa Cells; Metabolism; Bioenergetics; COV434; KGN
Corresponding Author:	Kevin Noel Keane, PhD; BScHons Curtin University Bentley Campus: Curtin University Perth, Western Australia AUSTRALIA
First Author:	Nikita L. Walz, BSc
Order of Authors:	Nikita L. Walz, BSc Philip Newsholme, BSc; DPhill John L. Yovich, PhD; MBBS Kevin Noel Keane, PhD; BScHons
Manuscript Region of Origin:	AUSTRALIA
Abstract:	COV434 and KGN human ovarian granulosa cell (GC) lines are commonly used as an alternative to studying primary GCs. Although these cell lines have been well characterised regarding hormone production and apoptotic signalling, very little is known about the bioenergetic profiles which is vital to driving key functional aspects of GCs. In the present study, we characterised the bioenergetic profiles, hormone production and lipid metabolism of COV434 and KGN cells in basal conditions and stimulated with FSH and androstenedione. COV434 cells produced higher levels of E2 (% vs. %), while KGN cells produced higher levels of P4 (% vs. %), when treated with the combination treatment. COV434 cells were overall more bioenergetically active for both mitochondrial and glycolytic pathways. Both cell lines had significantly increased protein expression of key lipid metabolism pathways, as well as intracellular lipid depositions. In conclusion, COV434 and KGN cells exhibit varied bioenergetic profiles which significantly alter their functionality.
Suggested Reviewers:	John Aitken, PhD University of Newcastle john.aitken@newcastle.edu.au Expert in reproductive cell biology and metabolism
	Yanli Zhang, PhD Nanjing Agricultural University zhangyanli@njau.edu.cn Published on effects of vitamin d on cell prolferation and steroidogenesis in goat granulosa cells Yao, Zhang, Guo, El-Samahy, Wang, Wan, Han, Liu, Wang, Yanli Zhang, Vitamin D receptor expression and potential role of vitamin D on cell proliferation and steroidogenesis in goat ovarian granulosa cells, Theriogenology, Volume 102, 2017,
	Zaher Merhi Albert Einstein College of Medicine, Bronyx zom00@hotmail.com published previously on Vitamin D alters genes involved in follicular development and steroidogenesis in human cumulus granulosa cells doi: 10.1210/jc.2013-4161.

Curtin University

Profs CM Klinge, R Laybutt & CA Stratakis,

Editors-in-Chief

Molecular and Cellular Endocrinology

Re: Bioenergetic and metabolic characterisation of the COV434 and KGN human ovarian granulosa cell lines

Dear Professors Klinge, Laybutt and Stratakis,

I am delighted to submit our cell based study to your esteemed journal, Molecular and Cellular Endocrinology. This study was part of a PhD project which began in 2017 and investigated the in vitro effects of FSH and androstenedione stimulation on hormone secretion, lipid metabolising enzyme expression and bioenergetic response in two human granulosa cell lines. This represents one of the few studies in terms to examine cell metabolic and bioenergetic response following stimulation with gonadotropins.

Overall, our findings show that clinical pregnancy and live birth rates were not significantly associated with Vitamin D status. However, the chance of developing at least one blastocyst was increased in Vitamin D replete patients (≥ 50 nM). As far as we are aware, this is one of the few studies to investigate the relationship between blastocyst development and Vitamin D status in an IVF setting. We feel that this work will be of interest to your readership, and we hope that it can be assessed by your reviewers.

If you need any further details, please do not hesitate to contact me.

Sincerely,

Kevin Keane
Research Fellow (Lecturer),
Curtin School of Medicine,
Curtin Health Innovation Research Institute,
Curtin University.
Tel | +61 8 9266 9781
Fax | +61 8 9266 2342
Email | kevin.keane@curtin.edu.au
Web | http://curtin.edu.au

Title: Bioenergetic and metabolic characterisation of the human ovarian granulosa cell lines: COV434 and KGN.

Authors: Nikita L. Walz[a], Philip Newsholme[a], John L. Yovich[b] & Kevin N. Keane[a]*

a. School of Pharmacy & Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Bentley, Western Australia, Australia.

b. PIVET Medical Centre, Leederville, Western Australia, Australia.

*Corresponding Author

Keywords: Granulosa Cells; Metabolism; Bioenergetics; COV434; KGN

Abstract

COV434 and KGN human ovarian granulosa cell (GC) lines are commonly used as an alternative to studying primary GCs. Although these cell lines have been well characterised regarding hormone production and apoptotic signalling, very little is known about the bioenergetic profiles which is vital to driving key functional aspects of GCs. In the present study, we characterised the bioenergetic profiles, hormone production and lipid metabolism of COV434 and KGN cells in basal conditions and stimulated with FSH and androstenedione. COV434 cells produced higher levels of E2 (% vs. %), while KGN cells produced higher levels of P4 (% vs. %), when treated with the combination treatment. COV434 cells were overall more bioenergetically active for both mitochondrial and glycolytic pathways. Both cell lines had significantly increased protein expression of key lipid metabolism pathways, as well as intracellular lipid depositions. In conclusion, COV434 and KGN cells exhibit varied bioenergetic profiles which significantly alter their functionality.

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1.0 Introduction

The human ovary is composed of three regions: the outer cortex (containing the follicles), the medulla (consisting of stroma and vasculature) and the hilum (the site of attachment to the mesovarium) (1). Within the cortex are primarily two somatic cell types: theca cells and granulosa cells (GCs), which work in a paracrine manner (2). In developing follicles, GCs are described as spherical and/or polyhedral in shape and clustered closely. In atretic follicles, the GCs become flattened and irregularly shaped with an absence of cytoplasmic invagination's (3). There are close interactions between theca and GCs that have been extensively studied (4). Research has shown that during folliculogenesis, steroidal and non-steroidal factors produced and secreted by both GC and theca cells influence the proliferation and differentiation of one another (5). There is also a differential in gonadotropin receptor expression between these cell types, such that luteinising hormone (LH) receptors are found exclusively on theca cells, and follicle stimulating hormone (FSH) receptors are exclusively found on GCs. LH receptor (LHR) stimulation promotes theca cell androgen production, while FSH receptor (FSHR) activation influences aromatase expression, which subsequently results in the conversion of theca cell derived-androgens to estrogen (6).

Given the invasive nature of collecting ovarian cells, *in vitro* studies are almost exclusively performed on infertile females undergoing IVF procedures where GCs and theca cells can be harvested. For this reason, animal studies were often utilised prior to the development of IVF technologies to allow for the study of ovarian cell structures, interactions and function. In more recent times, the development of human ovarian cell lines (4) has allowed the molecular study of these cell types in a greater depth. COV434 and KGN are two well studied human granulosa cell lines that both express the functional machinery for steroidogenesis including the aromatase enzyme (7, 8), androgen receptors (9) 17β-hydroxysteroid dehydrogenase (17β-HSD) (10) and respond to gonadotropin stimulation to secrete estrogen and progesterone. These cell lines provide an alternative means to study GCs *in vitro* as primary humans GCs are often restrictive in sample size and accessibility. However, despite the development of immortalised ovarian cell lines, the focus tends to lie in the study of hormone production and cell viability, while little is known about the metabolic and bioenergetic properties of such cell lines which drive numerous key cellular processes including steroidogenesis and cell proliferation. The aim of this study was to

Commented [KK3]: For introduction. Talk about the two cell model (which you have to an extent), and the role of GCs. What metabolic change must these cells go through to produce steroid hormones. What does the literature say about energy demand when producing hormones What happens to then after the LH surge.

For these two specific cell lines, mention their outputs in response to FSH, levels, if they have been compaired in same paper before. ,

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characterise the metabolic activity of COV434 and KGN and relate this their steroidogenic responses to FSH and androstenedione stimulation.

2.0 Materials and Methods

2.1 Cell culture and reagents

The human cell lines COV434 and KGN were purchased from Sigma and Riken BRC cell bank, respectively. Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM)/Nutrient Mixture F12 Ham (DMEM/F12) containing 2 mM glutamine and supplemented with 10% fetal bovine serum (FBS), 100 u/mL penicillin and 0.1 mg/mL streptomycin (Life Technologies, USA). Cells were maintained in T75 flasks in a humidified incubator with 5% CO_2 at 37°C prior to subculturing.

For hormonal stimulation, both COV434 and KGN cell lines, were exposed for 24 hours to androstenedione (an androgen steroid hormone and intermediate in the biosynthesis of estrone/estradiol and of testosterone from dehydroepiandrosterone), FSH in the form of Rekovelle® (a recombinant FSH produced in human cells) or both androstenedione and Rekovelle® (referred to as FSH from here) in combination. Responses were compared to cells maintained in basal conditions with DMEM/F12 media (media control). Androstenedione (4-Androstene-3,17-dione) was obtained from Merck (Australia) and solubilised in 100% DMSO to create a 100 mM stock. Rekovelle® (72 microgram/2.16 mL pens) was obtained from Ferring Pharmaceuticals (Australia). Both agents were stored in 5 and 10 µl aliquots (respectively) at -20°C prior to experimental use or -80°C for long term storage in order to avoid multiple freeze-thaw cycles.

2.2 MTT cell viability and proliferation

MTT assay was used to measure cell viability and proliferation in COV434 and KGN cells. Cells were seeded on day 1 at 20,000 cells/well in a 96 well plastic culture plate and left overnight in a 37 °C incubator with 5% CO2 to adhere. On day 2 cell supernatant was removed and fresh DMEM/F12 media only or supplemented with androstenedione, FSH or combination. On day 3, MTT reagent was added to the cell supernatant at a final concentration of 0.5 mg/ml. The cell culture plate was left to incubate for 4 hours in a humidified incubator with 5% CO2 at 37°C. Following incubation, cell supernatant was removed and 100 µl of DMSO was added to each well to solubilise the formazan crystals

and the absorbance was read at 550 nm using an EnSpire multimode plate reader (Perkin Elmer, USA).

2.3 Metabolic flux analysis

The seahorse extracellular flux analyser (Agilent Technologies) was used to determine metabolic flux in COV434 and KGN. More specifically, we assessed parameters related to mitochondrial oxygen consumption rate (OCR) and glycolytic proton efflux rate (PER). OCR measurements informed numerous parameters as follows.Cells were seeded at an optimised density of 20,000 cells/well in an XFe96 cell culture plate and left overnight to adhere. OCR and PER were assessed using the Agilent Seahorse XF Cell Mito Stress Test and Glycolytic Rate Assay kits (Seahorse Bioscience, USA). The principle of these assays and injection strategies were performed as previously published (11, 12). For OCR measurements, the optimised injections consisted of base media, 2 µM oligomycin, 0.75 µM FCCP, and 1 µM of each antimycin A and rotenone in combination. For PER measurements, the injection strategy consisted of 1 µM of each Antimycin A and Rotenone in combination, followed by 200 mM 2-DG.

2.4 Western Blot analysis of protein expression

Cells were seeded on day 1 at 500,000 cells/well in a 6 well plastic culture plate and left overnight in a 37 °C incubator with 5% CO_2 to adhere. On day 2 cell supernatant was removed and fresh DMEM/F12 media only or supplemented with FSH and androstenedione, only and in combination. On day 3, cell supernatant was removed, and cells were washed three times with PBS. For cell lysis, 150 µl of ice cold Radioimmunoprecipitation assay buffer (RIPA buffer; Astral Scientific, Australia) with 1 x phosphatase and protease inhibitors (Cell Signalling Technology, USA) were added per well. Protein concentrations were quantified using the Pierce BCA Protein assay kit (Thermofisher, USA) and absorbance was measured at 562 nm using an EnSpire multimode plate reader (Perkin Elmer). Following protein determination, samples were diluted appropriately, centrifuged at 14,000x(g) and NuPAGE® LDS buffer (6 µL/sample) and dithiothreitol reducing agent (2.4 µL/sample) was added. Samples were heated on a heat block at 70 °C for 10 minutes, centrifuged at 14,000x(g) and stored at –20 °C. Proteins (10-15 ug/lane) were separated using SDS-page and then transferred onto nitrocellulose membranes. Antigen detection of membranes was investigated by blocking membranes in 3% bovine serum albumin (BSA) or 5% milk in Tween 20 tris-buffered saline (TBS-T) for

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So for this section start with "Cells were seeded on day 1 at 500,000 cells/well in a 6 well plate and stimulated as above. After washing with PBS, 150 µl of ice cold Radioimmunoprecipitation assay buffer (RIPA buffer; Astra Scientific, Australia) with 1 x phosphatase and protease inhibitors (Cell Signalling Technology, USA) were added per well".

1-hour at room temperature. Primary antibodies were incubated overnight in 1% BSA or 0.05% milk at 4 °C, followed by washing three times on a rocking station (5 mins each wash) with TBS-T. Secondary antibodies were incubated at room temperature in 1% BSA for 30 mins followed by washing three times (5 mins each wash) with TBS-T.

The primary antibodies used (and their respective dilutions) in this study were as follows: Anti-β-actin (1:1000; Cell Signalling Technology, USA), Anti-HMGCR (1:1000; Abcam, Australia), Anti-StAR (1:500; Abcam, Australia), Anti-Acetyl-CoA Carboxylase (1:1000; Cell Signalling Technology, USA), and Anti-Fatty acid synthase (1:1000; Cell Signalling Technology, USA). The secondary antibodies used in this study were anti-rabbit IgG and anti-mouse IgG (1:2000; Dako, USA). To detect bands, 500 µL of Clarity™ Enhanced chemiluminescent substrate (ECL; Bio-Rad, USA) was added per membrane, developed for 5 mins and chemiluminescence was assessed using the Chemidoc™ XRS+ system (Bio-Rad, USA).

2.5 Hormone production

Estradiol (E2) and progesterone (P4) production was detected in cell culture supernatant following stimulation with androstenedione, FSH or combination. Cells were seeded on day 1 at 100,000 cells/well (for a 24-hr treatment) in a 24 well culture plate and left overnight to adhere. On day 2 cell supernatant was removed and fresh media containing either fresh DMEM/F12 media only or supplemented with FSH and androstenedione, only and in combination.

Commented [R19]: As above in terms of stimulation protocol..

2.5.1 Estradiol production

Estradiol (E2) production was detected in cell culture supernatant using a human 17 β-estradiol ELISA kit, catalogue number ab108667 (Abcam, USA). Assaying was performed based on manufacturer's instructions. Briefly, 25 µL of standard, control and culture media sample were pipetted into their respective pre-coated wells. Then 200 µL of 17 β-estradiol-HRP conjugate was added to each well (minus the blank wells). Wells were covered in foil and left to incubate with slow agitation on a plate shaker for 2 hours at 37°C. Following incubation, well contents were aspirated, and wells were washed 3 times with 300 µL/well of washing solution. Next, 100 µL TMB substrate solution was added into all wells and incubated for 30 mins in the dark at RT. Finally, 100 µL stop solution was added into all

wells, and the absorbance was read at 450 nm using an EnSpire multimode plate reader (Perkin Elmer, USA).

2.5.2 Progesterone production

Progesterone (P4) production was detected in cell culture supernatant using a human P4 ELISA kit, catalogue number KA0299 (Abnova, USA). Assaying was performed based on manufacturer's instructions. Briefly, 100 µL of standard diluent (cell culture media) was pipetted into the no substrate blank (NSB) and the substrate blank wells, while 100 µL of sample was added to the appropriate wells. Next, 50 µL of Assay Buffer was added into the NSB wells, while sample wells received 50 µL of blue conjugate and 50 µL of yellow conjugate/well. Wells were covered in foil and left to incubate with slow agitation on a plate shaker for 2 hours at RT. Following incubation, well contents were aspirated, and wells were washed 3 times with 400 µL/well of washing solution. After the final wash was aspirated, 5 µL of blue conjugate was added to the total activity wells. Next, 200 µL pNpp substrate solution was added into all wells and incubated for 45 mins in the dark at RT with no agitation. Finally, 50 µL stop solution was added into all wells, and the absorbance was read at 405 nm using an EnSpire multimode plate reader (Perkin Elmer, USA).

2.6 Lipid deposition

Oil Red O is a fat-soluble diazo dye which stains neutral triglycerides and lipids. Oil Red O staining was used to investigate lipid deposition in the human granulosa cell lines COV434 and KGN. Cells were seeded on day 1 at 20,000 cells/well (for a 24-hr treatment) or 10,000 cells/well (for a 48- or 72-hr treatment) in a 96 well culture plate and left overnight to adhere. On day 2 cell supernatant was removed and fresh media containing either fresh DMEM/F12 media only or supplemented with FSH and androstenedione, only and in combination.

A working solution of Oil Red O stain was prepared by adding 30 mL of 0.5% Oil Red O in 100% isopropanol (Sigma, Australia) with 20 mL ddH2O. After exposure to the various treatments, the cells were washed once with PBS and fixed with 4% paraformaldehyde for 10 minutes. Fixed cells were then washed three times with PBS and stained with the Oil Red O working solution for 30 minutes. Cells were then washed with PBS once, observed immediately for the stained intracellular lipid droplets, and photographed using bright field inverted Nikon microscope. Quantification of the stained area was performed by eluting the stain by incubation with 50 µL of 100% isopropanol/well for 1 hour at room temperature

Commented [KK10]: So cholesterol is a neutral lipid and would say all other sex hormones are too including androstenedione. So oil red o could pick this up and possibi vit D. However, your resuts show that FSH/andro push the level past just that for andro only. Just be prepared for this arrangement from reviewers and especially thesis examiners. You might need to know a bit more about this.

Could you do a quick experiment? Coat some cover slips with BSA, or hyaluronic acid, or Poly D lysine, add some androstane or not for 24 hours and stain. Do the androstane-treated stain more? we can talk about this further.

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For Fig 4, is it work dropping the "water control" and add 48/72 hours. You may have to have as its own figure. Have a think

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on a plate shaker. Absorbance of the eluted solution was measured at 510 nm using an EnSpire multimode plate reader (Perkin Elmer, USA).

2.7 Statistical analysis

All statistical calculations were performed using SPSS statistic version 25 (IBM Corporation, USA). Data is represented as mean ± standard deviation (SD) and independent experiments were replicated a minimum of 3 times. The statistical differences were analysed using one-way ANOVA with multiple comparisons and Tukey post-hoc tests. The statistical significance was defined as $p<0.05$. Graphs were generated using GraphPad Prism version 8 software.

3.0 Results

3.1 Cell viability and Estradiol/Progesterone Secretion

We first evaluated the toxicity of androstenedione, FSH and combination treatments on cell viability in order to select non-lethal concentrations for cell stimulation. Androstenedione treatment only over the 3 concentrations tested (1, 5, 10 μM) did not significantly change cell viability for both COV434 and KGN cells (Figure 1A).in addition, FSH treatment only over the 3 concentrations tested (62.5, 125 and 250 ng/mL) for 24 hours did not alter cell viability significantly in either COV434 or KGN cells (Figure 1B). Furthermore,

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treatment of both cell lines with androstenedione (1 or 5 uM) in combination with FSH (62.5 or 125 ng/mL) did not alter cell viability compared to each other or androstenedione or FSH treatments only (Figure 1C & 1D).

Both cell lines, were exposed to androstenedione and FSH (only and in combination) for 24 hours in order to determine the hormonal secretion response of E2 and P4. Exposure to androstenedione, FSH only and in combination significantly increased the production of E2 in both cells, with the combination having the highest levels in both cell lines (Figure 1E). For COV434 cells, Androstendione increased E2 from 32 pg/ug protein to 55 pg/ug protein (increase by XXX %), while FSH promoted a very similar response, increasing E2 from XX pg/ug protein to XX pg/ug protein (increase by XXX %) (Figure 1E). However, the andro-FSH combination increased E2 to XX pg/ug, which indicated an XX % increased from Andro and FSH only (Figure 1E). A similar trend was observed for KGN cells. Here, Androstendione increased E2 from 24 pg/ug protein to 42 pg/ug protein (increase by XXX %), while FSH promoted a very similar response, increasing E2 from XX pg/ug protein to

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XX pg/ug protein (increase by XXX %) (Figure 1E). However, the andro-FSH combination increased E2 to XX pg/ug, which indicated an XX % increased from Andro and FSH only (Figure 1E). The P4 was less significant. Exposure of both cell lines to Androstenedione only did not significantly increased P4 secretion beyond control (Figure 1F). FSH only increased P4 production slightly but significantly in COV434 cells only, from 28 pg/ug protein to 30 pg/ug protein (by XXx%). However, both cells lines increased P4 output in response to Androstendione and FSH combination stimulation. For COV434 cells, the combination increased P4 from 24 pg/ug protein to 35 pg/ug protein (increase by XXX %), while it increased P4 secretion from KGN cells from from 30 pg/ug protein to 46 pg/ug protein (increase by XXX %) (Figure 1F). Overall, COV434 cells produced higher levels of E2 over all treatment groups compared to KGN cells (Figure 1E), while KGN cells produced higher levels of P4 on average over all treatment groups compared to COV434 cells (Figure 1F).

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3.2 Mitochondrial bioenergetic profile of COV434 & KGN cells

Extracellular flux analysis was used to evaluate mitochondrial and glycolytic bioenergetics in real time through measurements of OCR and PER (respectively), in COV434 and KGN cell lines following hormonally stimulated conditions over 24 hours.

Exposure of both cell lines to Androstendione or FSH only for 24 hours had minimal impact on cell bioenergtic responses. However, exposure to both agents in combination altered several parameters. In COV44 cells, Androstendione and FSH only did not alter any bioenergetics parameter significantly (Figure 2 A-G), but when administered in combination there was a significant increase in basal respiration, ATP-coupled respiration, maximal respiration and compensatory glycolysis (Figure 2 A, B, D & G, respectively). Compared to solvent control these parameters increased by XX, XX, XX and XX% respectively. Similarly, there was minimal changes in bioenergetic parameters for KGN when exposed to Androstenedione and FSH only for 24 hours with the exception of non-miotchondrial respiration, proton leak and compensatory glycolysis, but like COV434, basal respiration, ATP-coupled respiration and maximal respiration were significantly increased in response to combination treatment (Figure 2 A, B, & D, respectively). Compared to solvent control these parameters increased by XX, XX and XX% respectively. Androstenedione only increased compensatory glycolysis by XXX% (Figure 2G), while

FSH only decreased proton leak and non-mitochondrial respiration in KGNs by XXX% and XXX% respectively (Figure 2C & 2E). The normalised bioenergetics responses show that COV434 metabolic rates are higher for both OCR and PER in comparison to KGN cells, (Figure 2H & 2I). In addition, that the combination treatment lead to higher OCR measurements for both cells lines (Figure 2H), but only PER for COV434 (Figure 2I).

3.3 Oxidative phosphorylation protein expression

COV434 and KGN cells pre-conditioned with androstenedione, FSH or combination for 24 hours did not significantly alter the protein expression of mitochondrial complexes I, IV and V in either cell line (Figure 3A). In COV434 cells, FSH treatment significantly increased the expression of complex II compared to androstenedione treatment (p=0.007; Figure 3A), while the combination treatment significantly increased the expression of complex III by 81.7% compared to control (p=0.004; Figure 3A). The combination increased complex III expression by 10% but this was not significant.

3.4 Lipid metabolising enzyme expression & lipid deposition

The expression of key enzymes involved in lipid metabolism were evaluated along with levels of intracellular lipid deposition in COV434 and KGN cell lines following homonal stimulation for 24 hours.

3.4.1 Lipid metabolism protein expression

The enzyme expression profiles were different according to the cell line and the response to androstenedione, FSH, or combination. Stimulation of KGN cells with androstenedione only increased FAS and ACC expression (by XXX% and XXX%, respectively Figure 4A & 4B), and increased StAR expression in both KGN and COV434 cell lines (by XXX and XX%, respectively, Figure 4D). It did not significantly alter HMGCR expression in either cell lines significantly. In addition, stimulation of KGN cells with FSH only increased ACC and StAR expression (by XXX% and XXX%, respectively Figure 4B & 4D), while FSH only increased FAS, ACC, HMGCR and StAR expression in COV434 cell lines (by XX%, XX%, XX% and XX%, respectively, Figure 4A-D). FSH did not significantly alter FAS or HMGCR expression in KGN cells (Figure 4A & 4C). However, the combination treatment had a broader and stronger effect across the various enzymes and cell lines. It increased KGN and COV434 expression of FAS (by XXX% and XXX%, respectively

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Figure 4A), ACC (by XXX% and XXX%, respectively Figure 4B) and StAR (by XXX% and XXX%, respectively Figure 4D). The combination also increase HMGCR expression in COV434 cells (by XX%, Figure 4C) but did not affect HMGCR expression in KGN cells. The level of increased enzyme expression induced by the combination treatment was largely similar across both cell types.

3.4.2 Lipid deposition

In COV434 and KGN cells, FSH and androstenedione, both only and in combination, significantly increased intracellular lipid deposits (Figure 4E). Androstenedione had a greater increase in lipid deposition in COV434 cells (24.8%) relative to control, when compared to KGN cells (11.7%). In contrast, FSH had a slightly greater increase in KGN cells (22.4%) relative to control, when compared to COV434 cells (20.0%). The combination treatment however, showed the greatest response in COV434 cells (29.2%) when compared to the KGN cells (23.7%). A visual representation via bright field microscopy imaging of increased intracellular lipids quantified in Figure 4E are displayed in Figure 4F.

4.0 Discussion

This study characterised the effect of FSH and androstenedione stimulation on E2 and P4 hormone secretion, lipid metabolism enzyme expression and cellular bioenergetics in the morphologically different human GC lines COV434 and KGN. Stimulation with FSH and androstenedione in combination (FSH-Andro), significantly increased E2 and P4 secretion from both cell lines. While FSH-only (FO) and androstenedione-only (AO) treatment also significantly increased E2 secretion in both cell lines, only FO promoted P4 secretion in COV434 cells. The KGN P4 output was not altered in response to FO or AO treatment. Interestingly, all treatments in both cell lines significantly enhanced the expression of steroid acute regulatory protein (StAR), a protein that regulates cholesterol transfer into the mitochondria (13), and the FSH-Andro combination led to the highest StAR expression level. It also led to maximum expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in both cell lines, which catalyse the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA and the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA, respectively (14, 15). The flux towards lipid biosynthesis and storage was also reflected by increased cytosolic lipid deposition in both cell lines following the FSH-Andro combination. In addition, hydroxy-3-methylglutaryl-CoA reductase (HMGCR) which is a rate limiting enzyme for cholesterol synthesis (16), was also elevated in COV434 following FO and FSH-Andro combination stimulation, but this enzyme was not altered in KGN cells following any treatment. Finally, exposure to FSH-Andro led to an increase in several mitochondria bioenergetic parameters in both cell lines (basal respiration, mitochondrial-linked ATP production, and maximal respiration), while also increasing mitochondrial complex III expression. Conversely, glycolytic metabolism remained largely unaffected. Overall, it appeared that the effects of FSH and androstenedione on E2/P4 secretion also resulted in simultaneous activation of four distinct but connected biosynthetic and energy balancing pathways, including cholesterol biosynthesis, fatty acid synthesis, mitochondrial cholesterol transport and raised mitochondrial-derived ATP levels. However, the degree by which these processes are activated appeared to be dependent on the type of hormone stimulation (FO, AO or FSH-Andro), but also the cell type, with the "younger" COV434 cells demonstrating more adaptation and responsiveness, while also displaying a slightly different metabolic responses.

In vitro studies examining the influence of FSH on GCs generally use concentrations from 25 to 200 ng/mL (17-20). For this study, 125 ng/mL was selected as it was in line with the literature (8, 18), was not cytotoxic and was capable of modulating cellular responses, particularly E2 and P4 secretion. A number of studies have shown that both COV434 and KGN cells respond to FSH stimulation by secreting E2 and P4 (20-24). However, when contrasting COV434 and KGN cells E2/P4 secretion responses from previously published studies, difficulty arises as there is extensive variation in cell culture conditions, incubation time, hormonal stimulation regimes, methods of measurement and the units reported. Furthermore, very few studies have compared the endocrine response of COV434 and KGN cell lines within the same study/publication and under the same culture/stimulation conditions (i.e. rFSH/androstenedione combinations). However, Gongola et al. demonstrated endocrine-disrupting chemicals reduced E2 secretion in COV434 and KGN spheroids, although P4 was not assessed (21). In addition, the secretion of E2 from COV434 and KGN cells was lower than the present study, and were 76.2 pg/mL and 2513 pg/mL, respectively in basal conditions, and 91.3 pg/mL and 3202 pg/mL following FSH (100 ng/mL) stimulation for 72 hours (equivalent to a 19.8% and 27.4% increase, respectively, Table 5.3) (21). The discrepancy between these data is that COV434 and KGN cells were cultured in a monolayer in the current study, as opposed to spheroids, and supernatants were collected for analysis after 24 hours exposure rather than 72 hours. In another study, Al-Kawlani et al., investigated E2 release from COV434 and KGN cells following treatment with the chemotherapy drug doxorubicin. However, they did not stimulate the cells with FSH or androstenedione (22). They reported that unstimulated COV434 and KGN control cells secreted 10 pmol/L and 20 pmol/L (respectively) following 48 hours incubation (22). This is again significantly lower than the 31.4 and 25.2 ng/µg protein reported in the current study in unstimulated COV434 and KGN cells following a 24-hour incubation.

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Several other studies have assessed hormonal secretion in both cell lines individually. Yang et al., investigated the role of micro RNA-431 in FSH-regulated COV434 proliferation and hormone secretion and found that exposure to FSH (24 hours) increased E2 and P4 secretion by 25.0% and 53.8%, respectively (20). Conversely, the current study showed that the FSH-induced E2 output was increased to a greater extent than the P4 release in COV434 cell (75.5% v 27.0%, respectively). For KGN cells, FSH-stimulation (100 ng/mL) for 24 hours was reported to increase E2 secretion by 83.3% (23), while in the present study FSH at 125 ng/mL for 24 hours increased E2 release by 65.1 %. Prior to the present study, Haltia et al., published the

only publication which investigated the combined effect of simultaneous FSH and androgen stimulation (i.e. FSH & testosterone for 96 hours) on KGN cells, and profiled the hormone release (24). Interestingly, this study showed that FSH stimulation only did not alter E2 secretion, but testosterone stimulation increased E2 release by 1300% compared to control (24). Additionally, FSH in combination with testosterone significantly increased E2 secretion a further 3800%, compared to media control only (24). These percentage increases in KGN E2 secretion following stimulation are significantly higher than reported in the present study, where androstenedione increased E2 by 73.8% and the combination with FSH increased E2 by 113.9% in KGN cells (Table 5.3). However, the basal control cell secretion in Halti et al., was significantly lower to begin with (< 1 nmol/L) than the current study, where the basal, unstimulated E2 level was 25.5 ng/µg protein. The present E2 data is supported by Xu et al., who used a similar concentration of rFSH and the same treatment time, although their response was 30.6% lower than that reported here (113.9% vs. 83.3% increase) (23). The COV434 data in the present study were mostly in line with the study conducted by Yang et al., (20), where these researchers reported E2 and P4 increased 25.0% and 53.8%, (respectively) compared to 75.5% and 80.2% detailed here, respectively. As the current study appears to be the first to show the effect of androstenedione (only) or in combination with FSH on E2 or P4 secretion in these cell lines, it is difficult to make any further direct comparisons with the literature. In addition, there are other key differences between these studies including differing culture conditions (DMEM/F12 vs. RPMI-1640 media), cell seeding densities, the source/concentration of stimulatory drugs, assay method and the use of data normalisation (or lack thereof). Nonetheless, these previous studies validated the robust E2/P4 secretion response from COV434 and KGNs to FSH stimulation.

Since FSH and/or androstenedione did not change cell viability or cell cycle progression, the increased E2 and P4 secretion is likely to be due to alterations in steroidogenesis, possibly via molecular regulation of enzyme expression and activity, along with lipid transport mechanisms (i.e., StAR), rather than increased cell number/proliferation. One of the first steps in the steroidogenic process is cholesterol import (a major precursor for sex steroid synthesis) from the outer membrane to the inner mitochondrial membrane by StAR (13, 25), which was increased in both cell lines following rFSH/androstenedione exposure. FSH activates adenylate cyclase leading to increased cAMP levels and this rise triggers the protein kinase A (PKA) pathway (26-28). StAR transcription is directly stimulated by PKA phosphorylation leading to enhanced cholesterol-mitochondrial transport (25). Cholesterol is cleaved by the cholesterol

side-chain cleavage enzyme (CYP11A1) giving pregnenolone, which can then be converted to various progestins, androgens and estrogens. In this study, cells were also exposed to androstenedione directly, and under the action of aromatase it can be converted to estrone and ultimately estradiol (29). Therefore, the increased E2 and P4 output observed, was possibly the result of increased cholesterol transport, and/or direct conversion of supplied androstenedione to E2.

Interestingly, GCs can obtain cholesterol as a substrate for steroidogenesis from two sources (30). Firstly, cells can take up cholesterol from the extracellular environment or secondly cells can synthesise cholesterol from the TCA metabolite, acetyl-CoA (30). This is a crucial metabolite, and is an important molecule for the cholesterol synthesis pathway, but is also integrated into the fatty acid synthesis pathway (31). Consequently, a major aim of the present study was to explore changes in the mevalonate and the fatty acid synthesis pathways. The expression status of three key lipid metabolising enzymes was assessed including ACC and FAS involved in fatty acid biosynthesis, and Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) which catalyses the conversion of HMG-CoA to mevalonic acid, (which limits the rate of cholesterol synthesis) (16).

The biosynthesis of fatty acids occurs in nutrient-rich conditions, and is a facilitated by increased acetyl-CoA and NADPH production formed through increased metabolic activity and enhanced glycolysis/TCA cycle flux. ACC converts acetyl-CoA to malonyl-CoA (14), while FAS catalyses the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA (15). Therefore, the increased production of these metabolites from enhanced ACC and FAS activity, leads to the formation of fatty acids (e.g. palmitate). Further reaction with glycerol from glycolysis forms triglycerides which are deposited and stored in lipid droplets (32). In this study there was a significant elevation in the expression of both ACC and FAS, indicating enhanced lipogenesis, which was confirmed by increased oil red o staining of cytoplasmic lipids. Other studies in adipocytes have also shown that FSH can promote lipid biosynthesis and lipid droplet formation, and rising, basal FSH levels may be related to age-associated obesity (33, 34). However for GCs, the biological purpose of FSH-induced intracellular lipid storage following E2/P4 release, would ensure that these lipids are available as a source of energy to maintain steroidogenic processes, and that a store of cholesterol is secured as a precursor for further steroidogenesis and hormone secretion.

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A vital pathway for sterol synthesis is the HMG-CoA pathway, where cytosolic and ER enzymes synthesise cholesterol from acetyl-CoA (16, 35). A key node in this pathway is HMGCR which catalyses the conversion of HMG-CoA to mevalonic acid and allows the metabolic flux towards cholesterol synthesis. HMGCR expression was also elevated in this study but only in COV434 cells. Cholesterol biosynthesis is regulated by sterol response element binding protein 2 (SREB-2), a member of the family of transcription factors known as SREBs (36). If sterol availability is low, the ER protein SREB cleavage-activating protein is transported to the Golgi, where HMGCR synthesis is enhanced, resulting in increased cholesterol synthesis (37). Since it is not clear how FSH may directly regulate HMGCR expression (38), it is more likely that a depletion in the sterol availability from enhanced E2 and P4 secretion led to the elevation of HMGCR expression. While this was only observed for COV434 cells, and it is not clear why this response was not observed in KGN cell, it still may indicate that the treatments used enhanced cholesterol biosynthesis.

For GCs to be able to maintain a secretory response, the management of cellular energy generation and utilisation needs to be carefully coordinated. GCs require nutrients (glucose, lipids and amino acids) which serve as a source of ATP, but also as precursors for biosynthesis of macromolecules (39). Metabolic flux was measured in the current study to determine if the observed changes in steroidogenesis and lipid biosynthesis where concomitantly met with a corresponding alteration in mitochondrial and/or glycolytic metabolism. Only two other studies have measured metabolic flux in KGN cells (40, 41), but it has not been reported previously in COV434 cells. Interestingly, no previous study has assessed the metabolic flux in KGN or COV434 cells following stimulation with rFSH and/or androstenedione. When COV434 and KGN cells were treated with rFSH- or androstenedione-only, there was a slight increase in mitochondrial bioenergetics (OCR), but no significant change in glycolytic bioenergetics (PER). However, when treated in combination, basal respiration, ATP-linked respiration and maximal respiration were increased for both cell lines, which was more prominent for COV434 cells. Basal respiration is made up of the sum of ATP-linked respiration and proton leak (non-ATP respiration). However, the elevation in basal respiration was solely due to the enhancement of ATP-linked respiration, as proton leak was unchanged and decreased in COV434 and KGN cells, respectively. Furthermore, a rise in ATP-linked respiration is connected to either a shortage of available intracellular ATP or an increased demand for ATP, while an increase in maximal respiration is associated with a boost in substrate availability, enhanced mitochondrial mass or unimpaired ETC integrity. Given that FSH-Ando activated a variety of biochemical processes, it was likely that ATP demand increased leading to higher

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ATP-linked respiration in the mitochondria, while maximal respiration was elevated because of an increased substrate uptake and availability due to these enhanced energy demands, as nutrients were in excess in these culture conditions. The increase in these mitochondrial parameters was also possibly facilitated by enhanced electron transport chain complex expression, which was also measured.

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Clearly, the data demonstrated that the treatments activated four distinct but interconnected biosynthetic and energy balancing pathways, including cholesterol biosynthesis, fatty acid synthesis, mitochondrial cholesterol transport and raised mitochondrial-derived ATP levels. One potential biochemical signalling pivot that was not examined here, but may control the integration of these pathways, is 5' adenosine monophosphate-activated protein kinase (AMPK), which directly regulates the activity of ACC and FAS (42). AMPK is an important energy-sensitive node that maintains the optimum energy levels by balancing supply and demand for ATP (43). In low energy situations, activated AMPK reduces anabolic processes (such as protein and lipid synthesis), and increasing catabolic process, while inhibited AMPK allows the reverse (44). FSH inhibits AMPK phosphorylation at Thr172 residue reducing AMPK activation (45), and recent evidence suggested that it could mediate hormonal responsiveness in GC cells (45-47). Taken together, AMPK has the potential to be intimately involved in the biological responses observed in this study.

Overall, KGN cells exposed to FSH and/or androstenedione slightly increased OCR, but there was no significant change in PER. This was largely similar for COV434 cells exposed to FSH- or androstenedione-only, but the combination vastly increased both OCR and PER in COV434 cells. The current investigation is the first to demonstrate that both cell lines were metabolically similar in basal conditions, but metabolically distinct when exposed to FSH-Andro, which may be due to their origin with COV434 coming from a metastatic GC tumour of a 27-year old woman (18), while KGN cells were derived from a 73- year old woman with stage 3 GC carcinoma (8). Stimulation with FSH-Andro appeared to activate lipogenesis and cholesterol synthesis while also increasing ATP demand, which has not been shown previously in these cell lines. Finally, this is the first in vitro demonstration of Rekovelle®-induced secretion of E2/P4 from KGN and COV434 cells (48, 49).

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N.W. & K.K. were the primary investigators of this study and they were responsible for the study concept/design, data extraction and management, statistical analysis and preparation of the manuscript. P.N. aided interpretation of results and edited the final manuscript. K.K. was the primary supervisor of the study.

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Conflicts of interest

The authors declare no conflict of interest.

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Appendix VII

Author declarations

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Walz, N.L., Newsholme, P., Yovich, J.L., Keane, K.N. Bioenergetic and metabolic characterisation of the COV434 and KGN human ovarian granulosa cell lines. *Molecular and Cellular Endocrinology*. 2021. (In submission).

Relevant Thesis Chapter: Chapter 5. Attribution Statement: PROF JOHN L YOVICH

Author	Conception & Design	Acquisition of Data and Method	Data Manipulation	Analysis and Statistical Method	Interpretation and Discussion	Final Approval	Total % Contribution
Nikita Walz	25	50	50	50	25	25	40
Co-author acknowledgement: I acknowledge that these represent my contribution to the above research publication. Signed: 							
Philip Newsholme	25	15	25	20	25	25	20
Co-author acknowledgement: I acknowledge that these represent my contribution to the above research publication. Signed:							
John Yovich	25	15	0	0	25	25	15
Co-author acknowledgement: I acknowledge that these represent my contribution to the above research publication. Signed:							
Kevin Keane	25	20	25	30	25	25	25
Co-author acknowledgement: I acknowledge that these represent my contribution to the above research publication. Signed:							

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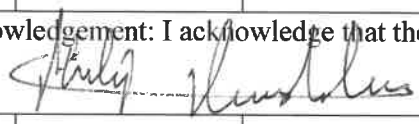
Philip Newsholme	25	15	25	20	25	25	20
Co-author acknowledgement: I acknowledge that these represent my contribution to the above research publication. Signed:  10/5/2021							
John Yovich	25	15	0	0	25	25	15
Co-author acknowledgement: I acknowledge that these represent my contribution to the above research publication. Signed:							
Kevin Keane	25	20	25	30	25	25	25
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Kevin Keane	25	20	25	30	25	25	25
Co-author acknowledgement: I acknowledge that these represent my contribution to the above research publication.							

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