Risk Reduction Strategies For Assisted Conception In Women With Polycystic Ovary Syndrome

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I confirm that the work submitted is my own work and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Superovulation in assisted conception can create Ovarian Hyperstimulation Syndrome (OHSS). Morbidity and even mortality that can occur with OHSS should be avoided by using the lowest risk and safest treatment strategy. Women with Polycystic Ovary Syndrome (PCOS) are at high risk of over response due to the ample number of antral follicles capable of responding to stimulation. Diagnosis of PCOS is based on a collection of subjective symptoms, signs and laboratory investigations. Anti-Mullerian Hormone (AMH), produced by the granulosa cells of the antral follicles, is elevated in women with PCOS. In a consecutive series of women presenting to an infertility clinic, the finding of increased AMH in those with PCOS was confirmed. Furthermore, AMH was shown to correlate with anovulation and hyperandrogenism. A single AMH value is interchangeable with any of the Rotterdam diagnostic criteria. Proposed values are 29pmol/L for polycystic ovarian morphology and 45pmol/L for either anovulation or hyperandrogenism using the generation II assay. Metformin has been shown to reduce the risk of OHSS in an agonist IVF cycle. The antagonist cycle is recommended for those at high risk of over-response. In a randomised double-blinded placebo controlled trial on 153 recruited patients; metformin was shown to have no effect on the incidence of OHSS in an antagonist cycle. There was no improvement in clinical pregnancy or live birth rate. The trial highlighted the discrepancy in clinical outcome between a White Caucasian and South Asian population. Avoidance of superovulation is an attractive option offered by *in vitro* maturation (IVM). A pilot study of 30 IVM cycles proved that immature oocytes can mature and fertilise *in vitro* at similar published rates. Unfortunately, no clinical pregnancies were created despite adequate transferred embryo quality. Although no incidence of OHSS, IVM appears to have been superseded by alternative approaches with replicable higher pregnancy rates.

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Abbreviations

AFC	Antral follicle count
AMH	Anti-Müllerian Hormone
AMH GEN II	AMH generation 2 assay
AMHR2	AMH type 2 receptor
AMPK	Adenosine 50 monophosphate-activated protein kinase
ANOVA	One way analysis of variance
AR	Androgen receptor
ART	Assisted reproductive technologies
ASRM	American Society for Reproductive Medicine
ATP	Adenosine triphosphate
AUC	Area under the curve
BFGF	Basic fibroblast growth factor
BMI	Body mass index
BMP	Bone morphogenetic protein
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CC	Clomiphene citrate
cGMP	Cyclic guanosine monophosphate
CI	Confidence interval
COC	Cumulus oocyte complex
CYP450c17	Cytochrome p450c17
CPR	Clinical pregnancy rate
CV	Coefficient of variation
CYP19a1	Cytochrome p19a1 (Aromatase)
DENND1A	DENN domain containing protein 1a
DF	Dominant follicle
DHEA	Dehydroepiandrostenedione
DNA	Deoxyribonucleic acid
DSL	Diagnostic Systems Laboratory
E2	Oestradiol
EC	Egg collection
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EMM	Embryo maintenance medium
ERK	extracellular-signal-regulated kinase
ESET	Elective single embryo transfer
ESHRE	European Society of Human Reproduction and Embryology
ET	Endometrial thickness / Embryo transfer
FAI	Free androgen index
FBC	Full blood count
FBN-3	Fibrillin-3
FDA	Food and drug administration
FET	Frozen embryo transfer
FF	Follicular fluid
FORT	Follicle Output Rate
FSH	Follicle stimulating Hormone
FTO	Fat mass & obesity-associated protein
GDF-9	Growth differentiation factor-9
GDM	Gestational diabetes mellitus
GLUT	Glucose transporter

GnRH	Gonadotrophin releasing hormone
GV	Germinal Vesicle
GWAS	Genome-wide association study
HA	Hyperandrogenism/-aemia
hCG	Human chorionic gonadotrophin
HFEA	Human Fertilisation & Embryology Authority
HIV	Human Immunodeficiency Virus
HOMA	Homeostasis model assessment
HRT	Hormonal replacement therapy
IBMX	Isobutylmethylxanthin
ICSI	Intra-cytoplasmic sperm injection
IGF-1	Insulin-like growth factor-1
IGFBP-1	Insulin-like growth factor binding protein-1
IL	Interleukin
INSR	Insulin receptor gene
IOT	Immunotech
IQR	Inter-quartile range
IR	Insulin resistance
IRS	Insulin receptor substrate
IS	International standard
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
Kg	Kilograms
KGF	Keratinocyte growth factor
KITL	Kit ligand
LBR	Live birth rate
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LCRM	Leeds Centre for Reproductive Medicine
LGI	Leeds General Infirmary
LFTs	Liver function tests
LH	Luteinising Hormone
LOD	Laparoscopic ovarian drilling
LoQ	Limit of quantification
LTHT	Leeds Teaching Hospitals Trust
MAD	*Mother against dpp gene*
MAPK	Mitogen-activated protein kinases
MD	Mean difference
MHRA	Medicines and Healthcare products Regulatory Agency
MI	Metaphase 1
MIS	Müllerian Inhibiting Substance
MII	Metaphase 2
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NGF	Non-growing follicles
NICE	National institute for health and care excellence
NIH	National Institutes of Health
ns	non significant
OA	Oligo-anovulation
OCP	Oral contraceptive pill
OHSS	Ovarian hyperstimulation syndrome
OMM	Oocyte maturation medium
OR	Oocyte retrieval / Odds ratio

OWM	Oocyte washing medium
PCO	Polycystic ovary
PCOM	Polycystic ovary morphology
PCOS	Polycystic ovary syndrome
PDE	Phosphodiesterase
PI3-K	Phosphatidylinositol 3-kinase
PIBF	Progesterone induced blocking factor
PKA	Protein kinase A
POI	Premature ovarian insufficiency
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PVP	Polyvinylpyrrolidone
QA-B	Quinn's advantage blastocyst medium
QA-C	Quinn's advantage cleavage medium
QA-H	Quinn's advantage medium with HEPES
QC	Quality control
RCOG	Royal College Obstetricians & Gynaecologists
RCT	Randomised controlled trial
RD	Risk difference
R&D	Research & Development
RIA	Radioimmunoassay
RR	Relative Risk
rFSH	Recombinant Follicle Stimulating Hormone
RNA	Ribonucleic acid
ROC	Receiver operator characteristic
SAS	Supra-Regional Assay Service
SD	Standard deviation
SEM	Standard error mean
SHBG	Sex hormone binding globulin
SNPs	Single nucleotide polymorphisms
SPOM	simulated physiological oocyte maturation
SRY	Sex-determining region Y protein
StAR	Steroidogenic acute regulatory protein
T2DM	Type 2 Diabetes Mellitus
TCM-199	Tissue culture medium-199
TFTs	Thyroid function tests
TGF-β	Transforming growth factor Beta
TMB	Tetramethylbenzidine
TVUSS	Transvaginal ultrasound scan
U&Es	Urea & electrolytes
UDP	Uridine diphosphate glucose
UK	United Kingdom
US	United States
USS	Ultrasound scan
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
17-OHP	17 Hydroxy-progesterone

Chapter 1
Literature Review

1.1 Introduction

Increasing use of superovulation in assisted conception has amplified the risk posed by ovarian hyperstimulation. For those with Polycystic Ovary Syndrome (PCOS) the risk is heightened due to the large cohort of antral follicles capable of producing an exuberant response to stimulation. Ovarian hyperstimulation syndrome (OHSS) subjects a relatively young and healthy population of women undergoing assisted conception treatment to the significant risk of morbidity and even mortality. This iatrogenic complication of assisted reproduction technology (ART) is unacceptable and requires that effective measures are put in place to limit the risk of morbidity attached to treatment. For women with PCOS, the often subjective diagnosis and lack of rigorous risk stratification measures can hamper safe assisted conception. Anti-Müllerian Hormone (AMH), a product of the ovarian granulosa cells, is elevated in those with PCOS. There has been suggested correlation with anovulation and hyperandrogenaemia. The hormone test therefore has the potential to aid diagnosis and furthermore, quantify risk for women undergoing ART. When superovulation strategies are used, effective adjuvants that limit risk would be ideal. Metformin, an insulin sensitiser, has been used successfully to reduce OHSS in the conventional long treatment protocols centred on the use of gonadotrophin releasing hormone (GnRH) agonists. During GnRH antagonist treatment cycles, as recommended for women with PCOS, limited evidence is available for the merit of the addition of metformin. Finally, given that exposure to supra-physiological doses of exogenous gonadotrophin are fundamental to the development of OHSS; treatment strategies which eliminate this problem are theoretically favourable. *In vitro* maturation (IVM), the process of immature oocyte collection and laboratory maturation, has been used with limited

success. Optimisation of clinical and laboratory methods could strengthen the argument for use of this technique, particularly if suggested high clinical pregnancy rates could be replicated. This literature review will cover the current evidence available for the diagnosis of PCOS, including the use of AMH for this purpose. The review will also address the use of metformin in an antagonist cycle and IVM for women with PCOS.

1.2 Background

Polycystic Ovary Syndrome is the most common endocrine disturbance to affect women of reproductive years. Presentation occurs through adolescence into adulthood and can have far reaching long term health consequences. Although multifactorial in nature, the absolute aetiology of PCOS remains elusive. A complex interplay within the normal ovary allows recruitment of primordial follicles into the growing follicular pool. A coordinated transition allows an antral follicle and oocyte to progress and ultimately reach ovulation. Unfortunately in PCOS, this coordination is often lost. Insulin resistance, rebound hyperinsulinaemia and hyperandrogenism are considered central to the pathophysiology of PCOS (1). These actions contribute to the anovulatory subfertility, menstrual disturbance and stereotypical ultrasound features of a polycystic ovary (2).

In addition to subfertility, the long term health consequences of PCOS include type two diabetes mellitus (T2DM) and endometrial carcinoma (3). A systematic review found an associated 2.5-fold increase in impaired glucose tolerance and a 4-fold increase in T2DM in PCOS (4). Furthermore, unopposed oestrogen secretion stimulates unchecked endometrial proliferation, which left unmanaged can lead to hyperplastic change (5). Finally the quality of life is often considered secondary to the clinical manifestations of PCOS. Yet women with PCOS have a lower reported health-related quality of life, in particular with regard to psychological health. Suicide attempt is up to 7 times higher in women with PCOS and there is an increased incidence of eating disorders (6).

1.2.1 Prevalence

Prevalence of the syndrome is dependent on the diagnostic criteria used. The most common and inclusive classification, the Rotterdam consensus (7), gives a broad estimate of 15%. Other diagnostic systems place more emphasis on hyperandrogenism, resulting in a reduced overall prevalence of the condition (8). Socioeconomic status can affect presentation, with those from a lower socioeconomic class often having higher body mass indices (BMI), increased insulin resistance and a tendency toward an anovulatory phenotype (9). In some societies, the opposite occurs with the rich having a higher BMI (10).

PCOS encompasses >90% of the World Health Organisation (WHO) group 2 anovulatory disorders (11). The normo-gonadotrophic anovulation creates an increased time to pregnancy but not necessarily a reduction in final family size (12). Interestingly, it has been shown that women with PCOS may retain fertility with advancing age when compared with age matched non-PCOS counterparts (13). Over time the impact of hyperandrogenism decreases but metabolic disturbance remains a concern (14).

1.2.2 Insulin resistance

Women with PCOS are more insulin resistant than their non-PCOS counterparts, with 50-70% having demonstrable evidence of insulin resistance (15). A compensatory hyperinsulinaemia attempts to maintain normal glucose metabolism. Unfortunately, it is this response that drives many of the outward phenotypic expressions of PCOS. A possible explanation for insulin resistance is a post-receptor signalling pathway-specific impairment within PCOS (16). This impairment allows metabolic resistance but maintains an intact steroidogenic response to insulin within the ovary. Insulin stimulates cytochrome P450c17 and aromatase activity, increasing thecal cell androgen production (17, 18) and granulosa cell oestradiol conversion (19). Increased ovarian exposure to insulin amplifies Luteinising Hormone (LH) and insulin-like growth factor -1 (IGF-1) action; and this in turn contributes to arrested differentiation of

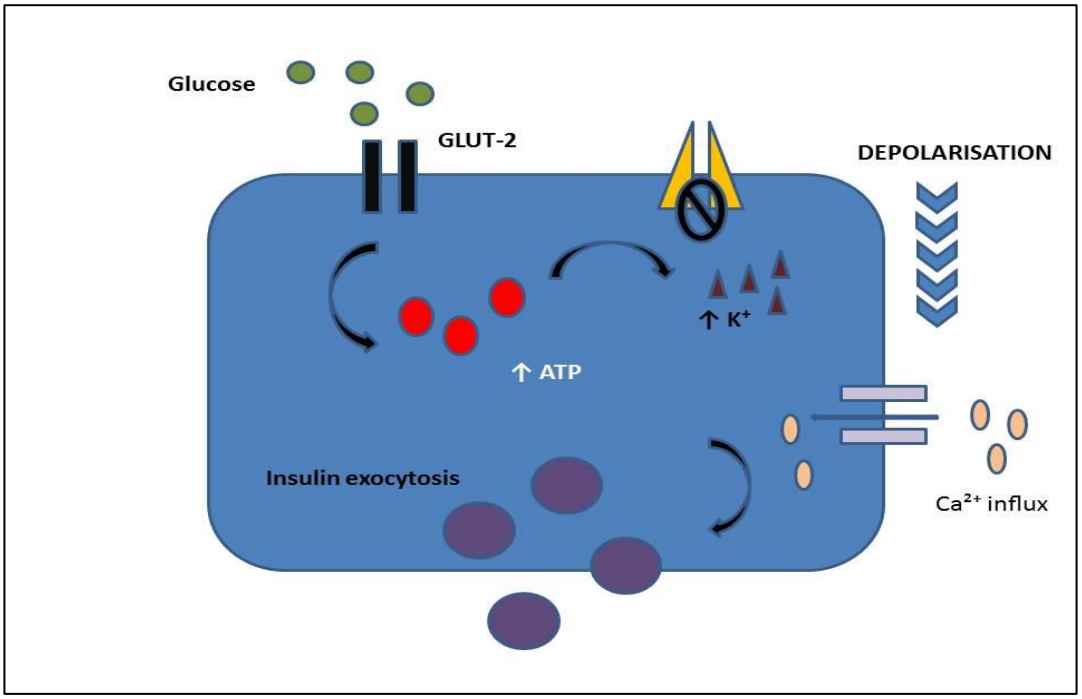
granulosa cells and excessive thecal androgen production (20, 21). Insulin, alongside IGF-1 and androgens, reduces the hepatic production of sex hormone binding globulin (SHBG). This reduction allows a higher proportion of bioactive hormone to remain in the circulation. In addition, there may be increased pulse amplitude and frequency of LH (22) plus a premature follicle sensitisation to the increased circulating LH (23). Obesity amplifies the effect of insulin resistance, through the metabolic products of visceral fat such as free fatty acids or the adipokines including interleukin-1, interleukin-6 and tumour necrosis factor α (24). Obesity is seen in 38-66% of those with PCOS, with clear correlation between symptoms and reproductive outcome (25).

1.2.3 Glucose and insulin interaction

Insulin is produced by the β cells of the islets of Langerhans in the pancreas. Insulin is important in glucose homeostasis and impacts on reproductive and mitotic functions. Circulating levels of insulin rise rapidly in response to elevated blood glucose in a dose dependent manner (26). Insulin is a polypeptide hormone, composed of two polypeptide chains (A-chain and B-chain) linked by two disulphide bonds. Initially insulin is synthesised as a pre-prohormone. On entry to the rough endoplasmic reticulum, a short hydrophobic signal sequence at the N-terminal is cleaved to produce proinsulin. Folding occurs and then transportation to the Golgi complex in micro vesicles. Within the Golgi complex, a protease removes an inactive C-peptide, resulting in active insulin leaving the complex via storage vesicles which associate with zinc (26). A process of exocytosis occurs in response to glucose stimulation of the pancreatic β cells, which requires Potassium (K^+), Adenosine triphosphate (ATP) and Calcium (Ca^{2+}) (27). Glucose enters the β cell via a glucose transporter (GLUT-2), which is concentrated in the microvilli of the canaliculi which lie between the cells. The process of glucose phosphorylation by glucokinase is the rate limiting step of islet glucose use. The main pathways for glucose metabolism include glycolysis (oxidation leading to ATP production), the pentose phosphate pathway (oxidation generates nicotinamide adenine dinucleotide phosphate (NADPH)) and glycogen synthesis via uridine diphosphate (UDP) glucose. During the oxidative metabolism of glucose, intracellular

concentrations of ATP rise, closing K^+ channels. Suppression of the K^+ efflux leads to cell depolarisation. In turn, depolarisation opens a Ca^{2+} channel allowing a rapid Ca^{2+} influx. This activates mechanisms for the vesicle movement and insulin exocytosis (see Figure 1-1) (28, 29). Using a tyrosine kinase receptor for signal transduction, insulin creates a cascade of events including activation of enzymes, translocation of glucose transporters to the plasma membrane and altered transcription processes within the target cell. Rapid glucose uptake into muscle and adipose tissue is initiated by the glucose transporter, GLUT 4. Translocation of this transporter is mediated by phosphatidylinositol 3-kinase (PI3-K). Directed conversion of glucose into glycogen, pyruvate, lactate and fatty acids then occurs. This anabolic property of insulin allows efficient storage of excess nutrients while endogenous substrate mobilisation is supressed. Hepatic production of glucose is suppressed.

Figure 1-1 - Glucose and insulin action



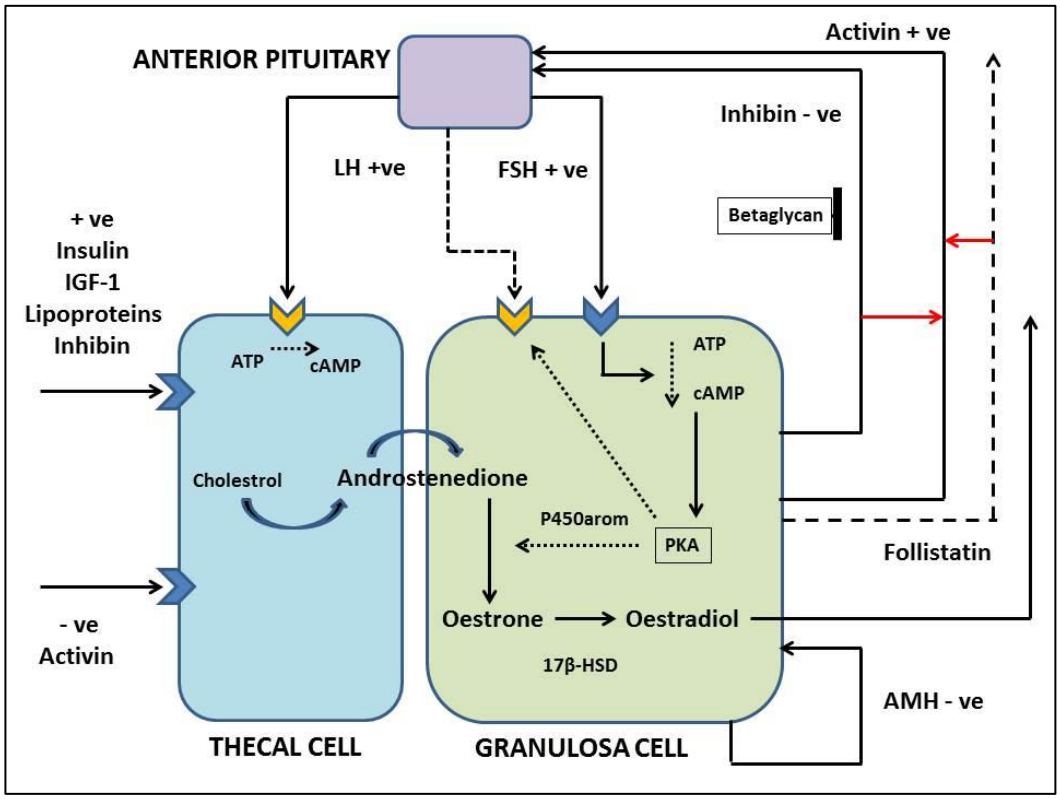
Glucose enters the pancreatic β cell of the Islet of Langerhans via a glucose transporter (GLUT-2). During glucose oxidation, the levels of ATP increase, preventing an efflux of potassium (K^+), which in turn leads to cell depolarisation, allowing rapid influx of calcium (Ca^{2+}). This allows the exocytosis of insulin.

1.2.4 Ovarian steroidogenesis

Ovarian steroidogenesis in the normal ovary requires the coordinated communication between the hypothalamic-pituitary axis and the ovarian thecal and granulosa cells. Figure 1-2 provides a simplified schematic presentation of some of the major pathways, hormones and proteins involved in the steroidogenic process (30). It is recognised that Follicle Stimulating Hormone (FSH) uses a number of signalling pathways to orchestrate its action, including the cyclic adenosine monophosphate (cAMP) dependant protein kinase A (PKA) and PI3K pathways (31, 32). The conversion of ATP to cAMP is driven by stimulation of adenylate cyclase via G protein coupled receptors. The main actions of FSH include stimulation of granulosa cell proliferation, induction of aromatase and aiding LH receptor induction in Graffian follicles prior to ovulation (30). Luteinising hormone on the other hand primarily targets the thecal cells, hence the term '2-cell 2-gonadtrophin theory'. The main action of LH is to stimulate the production of thecal androgens required by the granulosa cell for oestrogen production. Other factors including insulin help to modulate androgen production in the thecal cell and sensitise the granulosa cells to the effects of FSH.

Activin and Inhibin are produced from the granulosa cells and regulate the hypothalamic-pituitary axis. Activin aids granulosa cell proliferation, increases FSH-receptor expression and improves oocyte maturation whilst delaying luteinisation. In contrast, inhibin aids LH-induced production of androgens and increases progesterone production from the granulosa cells (33). Locally produced factors such as AMH limit proliferation of the granulosa cells by reducing the follicle's sensitivity to FSH (34).

Figure 1-2 Pituitary and the two cell two gonadotrophin theory



Gonadotrophins released from the anterior pituitary target the thecal and granulosa cells situated in the ovary. Luteinising hormone (LH; alongside numerous factors including insulin) stimulate the conversion of cholesterol to androstendione, which passes into the granulosa cell. Follicle stimulating hormone (FSH) drives the aromatisation of androgens to oestrone and oestradiol via at least the cAMP driven Protein Kinase A (PKA) pathway and aids the induction of LH receptors on the granulosa cells. Activin and Inhibin produced by the granulosa feedback to the hypothalamic-pituitary axis. Anti- Müllerian Hormone (AMH) exerts an inhibitory control over granulosa cell proliferation. ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, P450arom: cytochrome p450 aromatase, 17β-HSD: 17β-Hydroxysteroid dehydrogenase, -ve: negative, +ve: positive

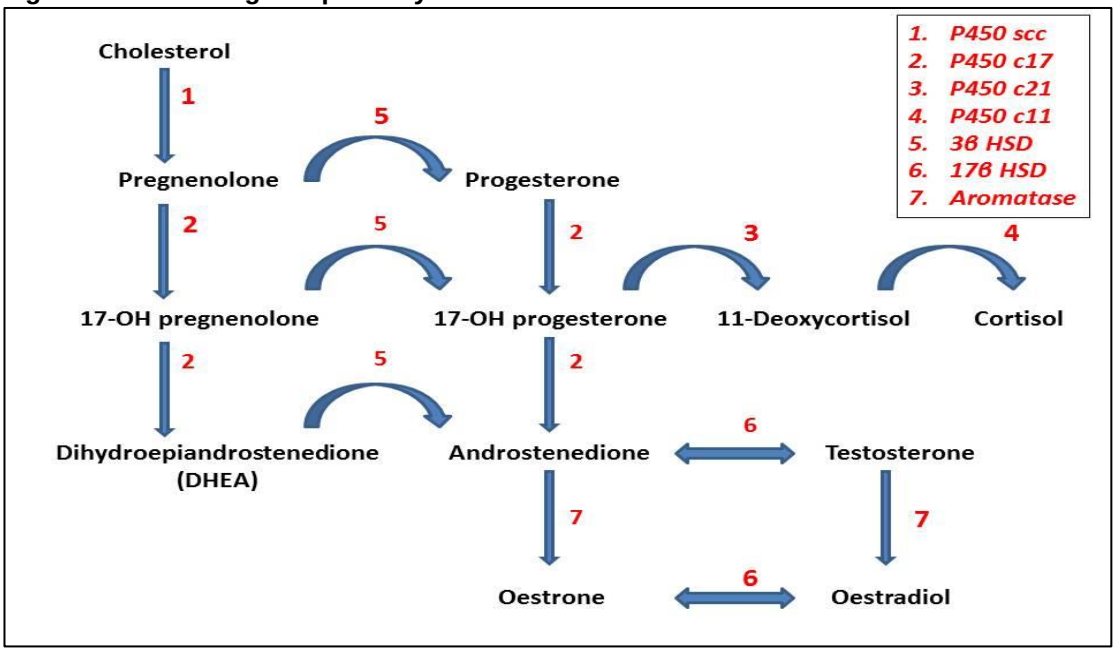
Both the ovary and adrenal gland contribute to hyperandrogenemia (HA) in PCOS. Hyperandrogenemia persists despite either dexamethasone suppression of adrenal steroidogenesis or GnRH agonist ovarian suppression (35). A basal increase in thecal cell androgen production is seen from a polycystic ovary (PCO), irrespective of ovulatory status (36). An exaggerated response to trophic hormones (LH/insulin) also occurs (23, 37, 38). Granulosa cell activity, on the other hand, is determined by ovulatory status. Granulosa cells isolated from anovulatory PCO women have an

increased response to FSH leading to increased oestradiol production. Premature responsiveness to LH also occurs (37). Increased frequency of pulsatile GnRH can selectively increase the LH: FSH secretion. LH stimulates thecal androgen production. Due to the shift in equilibrium and relatively low level of activity of granulosa cell aromatase in pre-antral and early antral follicles, incomplete aromatization of testosterone occurs i.e. more testosterone remains unconverted and therefore increases androgen levels, secondary to the relative deficiency of FSH in the adjacent granulosa cells. This last point contributes to the follicle arrest observed in anovulatory PCOS. Pre-antral/ early antral follicles are responsive to FSH, but the pre-ovulatory follicles are dependant on FSH leading to degeneration of big follicles when there is a relative FSH deficiency. Zhu et al (34, 39) suggest that there is a similar activin receptor expression between normal women and women with PCOS, but increased betaglycan in the latter. Betaglycan is vital for inhibin action and enhanced expression may facilitate antagonism of activin. The authors suggest that compromised FSH-receptor expression may therefore contribute to the accumulation of small follicles. Furthermore, inhibin mediated antagonism of activin and co-factors may lead to increased thecal androgen production.

Insulin action on the normal ovary and PCO is mediated via the insulin receptors located on both thecal and granulosa cells (37, 40). Despite the metabolic insulin resistance in PCOS, insulin driven ovarian steroidogenesis remains intact (41). Insulin increases (see Figure 1-3 for the steroid pathway) aromatase and CYP450c17 action (17) and stimulates oestradiol conversion in the granulosa cells (19, 35). Within the adrenals and gonads, a single CYP450c17 gene catalyses the activity of 17α-hydroxylase and 17,20-lyase activity. The ratio of these enzymes determines the amount of c19 or c21 steroids produced. Increased 17α-hydroxylase activity generates more c21 steroids e.g. cortisol. If both enzymes are active then c19 steroids e.g. sex steroid dehydroepiandrosterone (DHEA) are produced. The c19 steroids androstenedione and DHEA act predominantly as sex-steroid precursors due to a reduced affinity with the androgen receptor. Within the target tissues they are converted to oestrogens or androgens, by aromatase and 17β-hydroxysteroid dehydrogenase respectively. Serine phosphorylation of CYP450c17 increases the ratio

of 17,20-lyase:17αhydroxylase activity and therefore HA. It is proposed that a single kinase may contribute via serine phosphorylation to HA and insulin resistance seen in PCOS (35).

Figure 1-3 Steroidogenic pathway



Conversion of cholesterol to pregnenolone initiates biosynthesis of all steroid hormones. Pregnenolone is converted to DHEA via Δ^5 steroid pathway. Progesterone is converted to androstenedione via Δ^4 steroid pathway. Within the adrenal gland, 17-OH progesterone is either converted to cortisol or the sex hormones. P450 scc: Cytochrome P450 side chain cleavage; 3β HSD: 3β-hydroxysteroid dehydrogenase; 17β HSD: 17β-hydroxysteroid dehydrogenase. P450 c17 includes 17α hydroxylase and 17,20 lyase.

Excess insulin also binds to IGF-1 receptors, enhancing theca cell androgen production in response to LH stimulation (17, 42). In addition, hyperinsulinemia inhibits the hepatic secretion of insulin-like growth factor binding protein-1 (IGFBP-1) leading to increased bio-availability of IGF-1 and 2 (43). These are important regulators of ovarian follicular maturation and further augment ovarian androgen production. A suggested link exists between increased CYP450c17 and reduced phosphorylation of inhibitory Mitogen activated protein kinase - extracellular-signal-regulated kinase 1/2 (MAPK-ERK1/2) in women with PCOS (44, 45). This is in contrast to Munir et al (46) who

suggested that inhibition of MAPK –ERK1/2 has no effect on the 17α-hydroxylase activity of CYP450c17. Increased insulin receptor substrates (IRS) 1 and 2 are seen in PCOS thecal cells and skeletal muscle but not granulosa cells (47). This cell specific alteration in the IRS protein concentration reflects the amplification effect insulin has on thecal androgen biosynthesis. Furthermore, altered Tyrosine auto-phosphorylation of the insulin receptor may be decreased in women with PCOS (48).

1.2.5 Ethnic variation

Throughout the world in different ethnic groups, a wide diversity exists in the prevalence of PCOS and its phenotypic display. For example in the South Asian population the metabolic component is more apparent (49, 50), in both indigenous and immigrant populations (51, 52). This propensity correlates with the exponential rise in type 2 diabetes within this population in recent times (53). A very clear variation in metabolic risk is evident in different Indian populations: Western United States (US) society (17.4%), urban India (13.6%) and rural India (8.4%) (54). Despite having milder features of HA, more pronounced insulin resistance is seen in young South Asian women compared with older obese white women (55). Central obesity appears to be one of the best discriminators for metabolic features of PCOS. Interestingly, SHBG often viewed as a surrogate marker for insulin resistance is lower in South Asian women compared with white women (55). Another interesting comparison uses the often overlooked feature of acanthosis nigricans. When present, it signifies a greater metabolic risk in both South Asian (irrespective of geographical location) and indigenous Thai women (56). Hispanic populations also demonstrate a higher insulin resistance alongside increased obesity (57).Variation in the metabolic component is apparent within white populations. Lower reported levels are seen in Turkish, Czech and south Italian women (2-8%) whereas 43-47% prevalence is seen in American women (58). Different environmental, migration and lifestyle confounders obviously contribute to this variation. Removing geographical factors, African-American and white-American women, have similar clinical manifestations of PCOS with respect to infertility and hyperandrogenaemia (59). Unfortunately, the former group have

increased metabolic demands with increased central obesity, hypertension and mortality.

1.2.6 Genetics

Without doubt, as evidenced by the familial association of PCOS, a genetic component underpins this highly complex and variable condition. The interplay between environment and underlying genetic inheritance is hard to unpick, due to the multiple phenotypic presentations of the syndrome. Kosova and Urbanek (60) highlight the challenges facing scientists whilst trying to identify susceptibility genes. Phenotypic heterogeneity, environmental confounders, small sample size and limitations of the commonly used candidate gene analysis methods are but a few of these challenges. It has been well documented that family clusters of PCOS exist. This extends to all of the main components of PCOS with an increased propensity for HA and T2DM within first-degree relatives of either sex (61, 62). With the advance in genetic studies, the feasibility of genome-wide association studies (GWAS) is promising in the identification of novel genes associated with PCOS (60). The use of candidate gene analysis within PCOS research has highlighted a number of potential susceptibility gene foci such as Fibrillin-3 (FBN-3) (60, 63), insulin and insulin receptor genes (64, 65) and the fat and obesity associated gene (FTO) (66-68). Unfortunately, often due to small sample size and heterogeneous presentation, these studies have failed to be easily replicated. The first publication of GWAS in PCOS originates from China (69). Three distinct regions were identified: chromosome 2p16.3 (containing the Luteinising hormone/choriogonadotrophin receptor gene), chromosome 2p21 (containing the Thyroid adenoma associated gene) and chromosome 9q33.3 (containing the DENN domain-containing protein 1a gene, DENND1A). The latter gene's involvement has had successful replication in European (70, 71) populations allowing plausible recognition as a PCOS susceptibility gene. Single nucleotide polymorphisms (SNPs) in the Thyroid adenoma associated gene have been linked to T2DM, whilst DENND1A encodes a protein involved in Rab35 activated endocytotic trafficking (70).

1.3 Diagnosis of Polycystic Ovary Syndrome

1.3.1 Current diagnostic criteria PCOS

Despite an extensive literature, the diagnostic criteria for PCOS remain a subject of debate within scientific and clinical circles. Stein and Leventhal (72) first described the collection of symptoms (enlarged ovaries, amenorrhoea, infertility and hirsutism) recognised currently as features of PCOS. In 1990, the National Institute of Health (NIH) defined the condition as the presence of chronic anovulation and HA (73). A joint meeting between the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) published the Rotterdam consensus in 2003. To reach diagnosis, 2 out of 3 criteria should be met. These include oligo- or anovulation, clinical and/or biochemical HA and polycystic ovarian morphology (PCOM). Subsequently, it has been proposed that more emphasis should be placed on HA with its presence absolutely necessary for diagnosis (8). Table 1-1 outlines the 3 proposed definitions of PCOS. The most recent meeting of the NIH suggested the continued use of the Rotterdam criteria with focus on the variable phenotypic picture of those with PCOS (74).

Table 1-1 - Diagnostic criteria for polycystic ovary syndrome

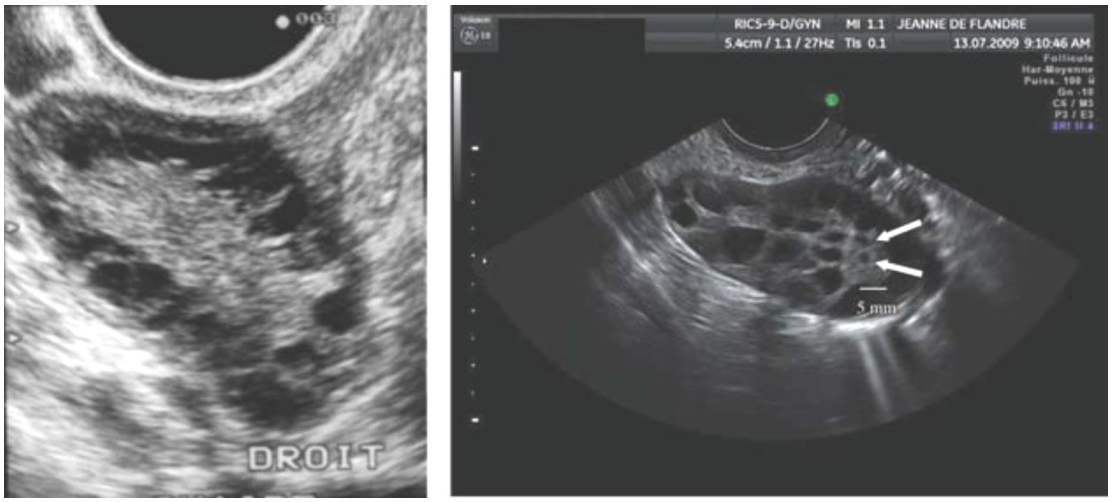
NIH 1990	ESHRE/ASRM Rotterdam 2003	Androgen Excess Society 2006
To include: 1. hyperandrogenism +/or hyperandrogenaemia 2. oligo-/ anovulation 3. exclusion related disorders	To include 2 of the following with exclusion of related disorders: 1. oligo/an-ovulation 2. clinical +/or biochemical hyperandrogenism 3. polycystic ovaries	To include all: 1. hirsutism +/or hyperandrogenaemia 2. oligo/an-ovulation +/or polycystic ovaries 3. exclusion of androgen excess/related disorders

1.3.2 Diagnosis of polycystic ovarian morphology

Ultrasound has provided the ability to define an individual's ovarian dimensions and follicle count, using a relatively acceptable and safe method. The accepted definition for PCOM is at least 1 ovary demonstrating a volume greater than $10cm^3$ or 12 or more follicles 2-9mm (75). Using this definition >75% of women with PCOS will have PCOM (8). The significance of PCOM in an apparently normal women without PCOS, divides opinion. PCOM alone has been reported in >20% of women (76-78). Some believe it is a normal variant of ovarian morphology (79), whilst others consider it a mild phenotype of PCOS (80, 81). Johnstone et al (79) evaluated a Caucasian female population with regular cycles, with and without PCOM (n=257). Although a significant increase was seen in mean testosterone level in the PCOM group, this remained within the upper limit of the normal range. No difference was seen in metabolic parameters including insulin, fasting glucose and lipid profiles. They concluded PCOM alone should not be considered a marker for metabolic dysfunction or cardiovascular risk. This last point is supported by a review by Moran and Teede (82). The review concludes that if PCOS diagnosis is reached using PCOM and either hyperandrogenism or anovulation, the metabolic disturbance is less severe than if both HA and anovulation are present. In contrast, Dewailly et al (83) showed PCOM to be a marker of HA using principal component analysis. They believe that the use of follicle number as a surrogate for HA bridges the gap between the Rotterdam criteria and the Androgen excess groups criteria outlined above. Having PCOM alone has not been shown to have any detrimental effect on overall fertility (84) but gonadotrophin stimulation characteristics are similar to those with PCOS, with respect to oestradiol concentration and number of oocytes retrieved (85). It is accepted that ovarian reserve declines with age. This also applies to the finding of PCOM (79). Use of age-specific ranges for PCOM, have been recommended but not yet quantified (76). The opposite end of the age spectrum, adolescence, also questions the significance of PCOM in healthy volunteers (86). Advances in ultrasound technology and sensitivity, has challenged the validity of the PCOM definition. The ability to accurately measure smaller follicles is evident in Figure 1-4. Over diagnosis of PCOM is plausible, leading to recommendations to

elevate the follicle number used in diagnosis. Dewailly et al (87), found that when comparing a non-PCOM non-PCOS population with a PCOS population, a follicle number of >19 provided the best compromise between sensitivity (87%) and specificity (83%) in identifying PCOM. Excluding those with asymptomatic PCOM may artificially strengthen this level, as it is not a true reflection of the population, particularly if PCOM is regarded as a normal ovarian phenotype. If women with PCOM alone are considered as a separate population, then excluding this ovarian morphology removes a sub-group that imposes bias on the true diagnostic threshold for PCOS. More recent work (88) suggests a threshold of 25 follicles is a better indicator to define PCOM. The operator's ability should not be overlooked as both inter and intra-observer variation are recognised as confounding factors in ultrasonography (89). A mere 51% agreement between 4 experienced observers and 63-74% agreement with their own findings, highlight this inconsistency (90).

Figure 1-4 - Old and new ultrasound technology (87)



The majority of the literature concentrates on follicle number in reference to PCOM. Increased ovarian volume is less common, but remains a recognised diagnostic feature. Johnstone et al (79) suggest that unlike follicle number, the proportion of women with increased ovarian volumes does not trend downward with increasing age. Nor do they find any significant difference in metabolic parameters compared with controls, if this

is the only feature considered. Recommendation to scan when there is ovarian quiescence negates the effect of a dominant follicle on the overall ovarian volume. The findings of increased stromal volume and vascularity may also underpin part of the pathogenesis of both the PCOM and PCOS. Suggested inclusion of 3 dimensional ultrasound may also improve diagnostic ability for PCOM (91, 92).

1.3.3 Diagnosis of hyperandrogenism

The use of clinical and biochemical HA diagnostics as a means to assign PCOS, have been besieged with problems related to optimum diagnostic level, assay design and standardisation of often subjective measurements. Despite published scoring symptoms for hirsutism such as that proposed by Ferriman and Gallwey (93), there are wide discrepancies between patient and physician scores (94) as well as between physician scores alone (95). Acne is also a common finding in women of reproductive years, making its relative importance in PCOS diagnosis questionable (8). Assessment of biochemical HA is no less difficult due to the types of assay and multiple androgen tests available (96). Barth et al (97) summarise the biological and analytical variables that can influence serum testosterone concentrations. The development of liquid chromatography-tandem mass spectrometry (LC-MS/MS) (98) has been shown to surpass the standard radioimmunoassay (RIA) methods used, as it limits laboratory variation (99). Using a variation of this method, the reference level for women without PCOS is <1.8nmol/l (100). Testing the diagnostic performance of testosterone, using receiver operator characteristics, in a consecutive cohort of fertility patients, the area under the curve (AUC) was 0.75. Interestingly, the AUC for the discarded criterion of raised LH: FSH was very similar at 0.72.

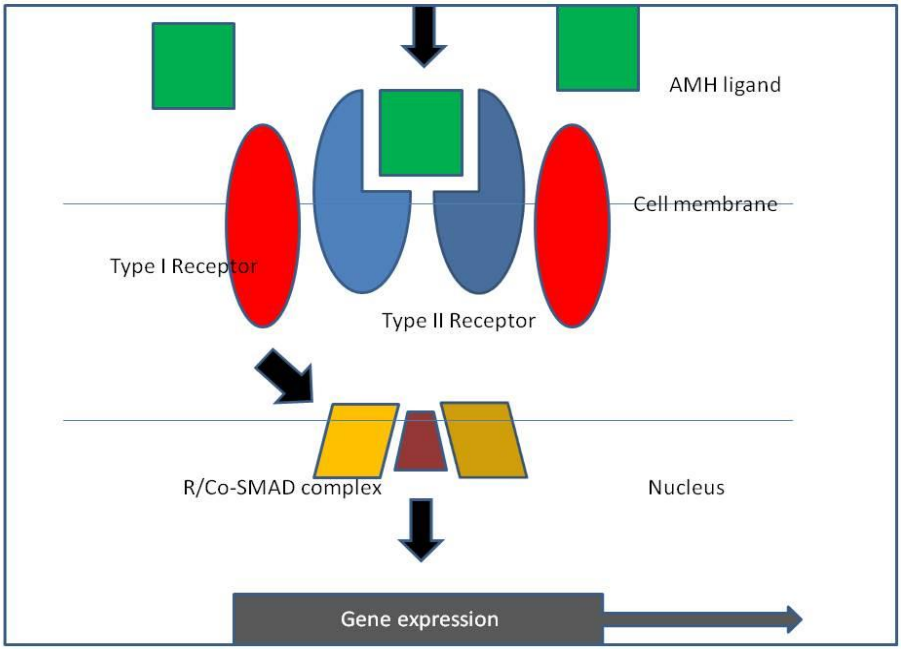
In summary, PCOS diagnosis remains a debated topic. Lack of clarity in definition, variability in laboratory measurements and subjectivity in clinical observations allows the debate to continue. Use of ultrasound allows pictorial evidence of ovarian function, revealing a spectrum of disorder from normal variant to metabolic representation. The importance of PCOM in a ‘normal’ population remains unknown despite much literature. Ultrasound requires expensive user-dependant technology. The best

threshold for diagnosis has been questioned. However other pelvic pathologies can be identified at the time of investigation and ease of assisted reproductive techniques assessed. Historical use of less robust testosterone measurements has left a hyperandrogenism definition vague. More accurate methods such as mass spectrometry may help to alleviate this ambiguity. There remains clear need for further less subjective criteria for the identification of PCOS.

1.4 Anti-Müllerian Hormone Physiology

Anti-Müllerian Hormone is a member of a large family of regulatory proteins labelled as the transforming growth factor beta (TGF-β) superfamily, named after its originally discovered member TGF-β1 (101). These proteins have a wide range of actions including roles in cell growth, differentiation and apoptosis. Despite the range of peptides incorporated under the TGF-β superfamily umbrella, the method of cell signalling is essentially uniform. The ligand e.g. AMH binds to a specific 'type II receptor' dimer found in the cell membrane (Figure 1-5). This receptor is a serine/threonine receptor kinase (102). This then allows recruitment and phosphorylation of a 'type I receptor' dimer to form a heterotetrameric complex with the ligand. Once activated, the type I receptor phosphorylates a collection of intracellular proteins, related to the *Drosphilia* gene *Mothers against dpp* (MAD) and now with more genes identified, dubbed 'Smads'. Binding of co-Smads within an R/Co-Smad complex, transduce the TGF-β signal into the nucleus where they act as transcription factors to allow downstream target gene expression of the ligand (103, 104).

Figure 1-5 The AMH receptor

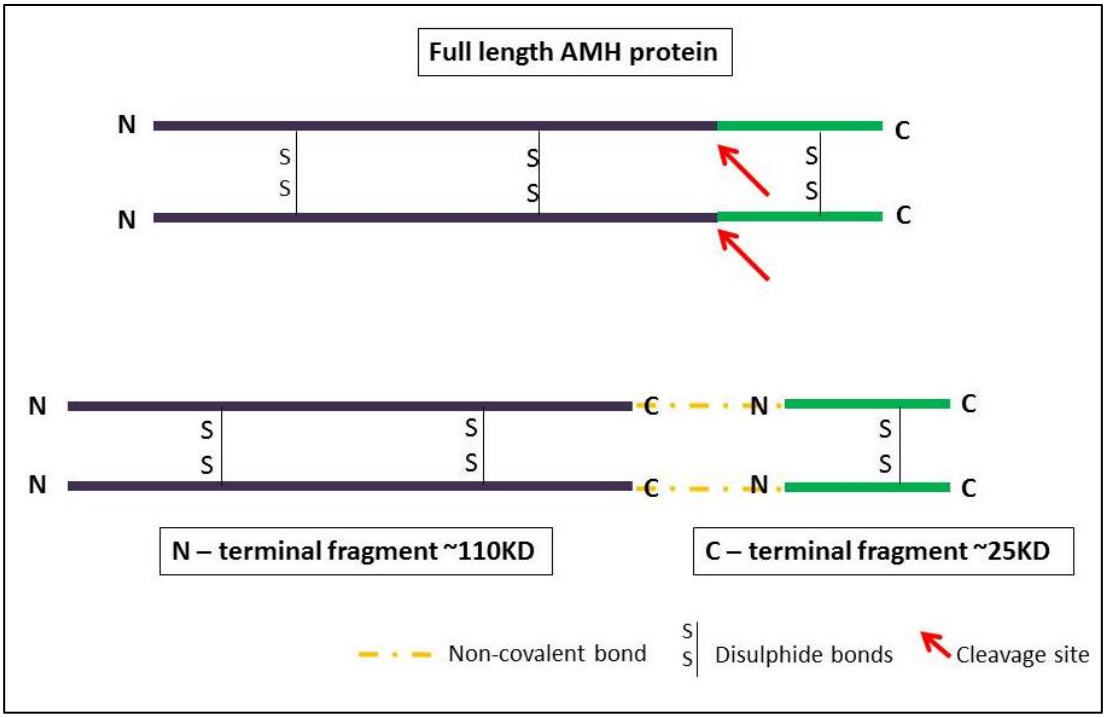


The AMH ligand binds to a type II receptor in the cell membrane, which in turn recruits a type I receptor dimer within the cell. Once recruited the type I receptor binds intracellular proteins, which transduce the signal into the nucleus. This allows downstream expression of the AMH ligand.

In humans, the AMH gene has been located on chromosome 19p13.3 (105), with 5 exons encoding a protein of 560 amino acids. The human AMH II receptor gene is located on chromosome 12 (106). The specific type I receptors remain elusive but potential candidates include bone morphogenetic proteins which activate specific SMADs (107). Prior to secretion AMH undergoes glycosylation and dimerization to form two identical linked 70 kDa monomers. Each of these subunits has a 'mature' C-terminal and a 'pro' N-terminal (see Figure 1-6). Cleavage occurs between these two ends during cytoplasmic transit but remain in contact via a non-covalent bond, resulting in a 140kDa complex in circulation. The biologically active part of the AMH molecule resides in the 'mature' region but requires the N-terminal to maintain this activity (108). This is in contrast to other members of the TGF-β family e.g. TGF-β, who when both parts are associated remains latent (108). Although it has been shown that the C-terminal fragment is active alone, experiments on fetal ovary aromatase action

reveals greater inhibition when both parts of the AMH molecule are in association. This is likely to represent the location of the binding site on the C-terminal.

Figure 1-6 The Anti-Müllerian Hormone protein



AMH protein formed of 2 identical monomers. Plasmin cleavage site highlighted, but fragments remain in association via non-covalent bonds. Both monomers remain in contact via disulphide bonds.

1.4.1 AMH in males

Although the genetic sex of an individual is predetermined by the inheritance of either an X or a Y chromosome from the paternal line, initial morphological appearance of the embryological gonads is indistinguishable between sexes. Post gonad formation, the crucial event in male sex differentiation is the expression of the *SRY* gene (sex determining region of the Y chromosome), allowing transformation of the gonad supporting cells to differentiate in to Sertoli cells (109). Once testicular differentiation has occurred, testicular hormone secretion promotes masculinisation of the embryo to generate the male phenotype. These hormones include androgens, Insulin-like-3 protein (involved in descent of testes in to the scrotum) and AMH (formerly known as

Müllerian Inhibiting Substance). Expression of AMH occurs shortly after *SRY* gene expression and leads to regression of the Müllerian ducts, which were pre-destined to form the uterus, fallopian tubes and proximal vagina. This degeneration occurs via the direct effect of AMH on the mesenchymal tissue surrounding the ducts. This tissue contains the AMH-receptor type II needed to effect the cranial-caudal regression of the Müllerian system. Defects in the pathway or lack of the type II receptor lead to Persistent Müllerian Duct Syndrome seen in adult males. Normally, the postnatal decline in male AMH concentration runs in parallel with the initiation of spermatogenesis and increasing testicular testosterone production. Over expression in the adult can produce Leydig cell abnormalities and interfere with testosterone production. However, evidence in support of an AMH-induced suppression of *CYP450c17,* the gene necessary for testosterone biosynthesis, exists (110). These findings support the theory that AMH retains an inhibitory autocrine/paracrine role in the adult male steroidogenesis pathway (109). Use of AMH measurement for male infertility assessment is limited when compared with its role in the investigation of the female. The serum concentration of AMH does not seem to be have any predictive capacity on *In vitro* fertilisation (IVF) or intra cytoplasmic sperm injection (ICSI) outcome nor does it reliably differentiate between fertile or infertile males (111). In the case of non-obstructive azoospermic males undergoing testicular sperm extraction, AMH did not predict success (112).

1.4.2 Physiology of AMH

The main physiological effects of AMH have been shown in both animal and human studies. AMH plays an inhibitory role on both the recruitment of primordial follicles and prior to final selection of the dominant follicle. It has also been suggested that AMH acts as the ‘gatekeeper’ of follicular oestrogen production (113). Anti-Müllerian Hormone expression is specific to the granulosa cells of pre-antral and antral follicles. When the follicle reaches the FSH-dependant growth stage, AMH expression drops. A number of studies have shown a steep decline in AMH expression as follicles reach 8mm in diameter (114-116). AMH expression persists in the pre-ovulatory follicle

cumulus cells (117, 118). Atretic follicles lack expression of AMH (119). AMH null-mice show increased oocyte degeneration and follicular atresia (120). Insight into the role of AMH during folliculogenesis has been provided by studies of AMH-knockout mice (121). Although fertile, in the absence of AMH, the mice have a depleted primordial pool and reduced reproductive lifespan due to their lack of inhibitory control from AMH and accelerated recruitment from the primordial pool (121, 122). Histological work on ovarian tissue, confirms the inhibitory effect of AMH on the transition from the primordial to primary follicle (123-125). Carlsson et al (124) evaluated the effect of different AMH concentrations on human ovarian tissue growth *in vitro*. Specimens cultured in the highest concentrations of AMH showed no difference in primordial follicle count compared with the non-cultured sections. Specimens incubated in low concentrations of AMH showed significant initiation of follicular growth and depleted primordial follicle count. Interestingly, AMH exposure also inhibited the stimulatory effect of several other growth factors on primordial follicle recruitment, including basic fibroblast growth factor (bFGF), kit ligand (KITL) and keratinocyte growth factor (KGF) (125). Both the *in vivo* and *in vitro* studies highlight the ability of AMH to reduce the follicle's sensitivity to FSH (34, 126). A further hypothesis involves the ability of raised FSH to down regulate AMH and AMH receptor expression within the granulosa cells, effectively releasing the 'brake' on folliculogenesis (127). Human work confirms a significant inverse relationship between FSH and AMH (128).

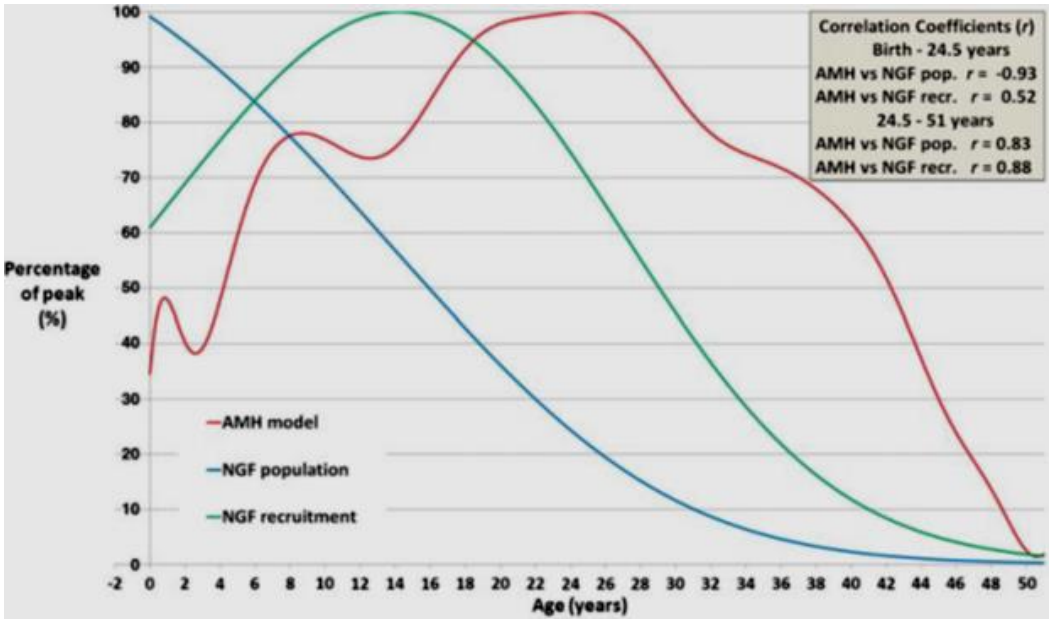
The concept of AMH as the gatekeeper of follicular oestrogen production is supported by a number of studies, summarised by Dewailly et al (113). Anti-Müllerian Hormone ensures little oestrogen is produced from the antral follicles, until the dominant follicle has been selected. At this point, a shift occurs from a low-oestrogen producing state to one of increasing oestrogen production. This differential regulation of AMH by oestrogen and *vice versa*, has been shown through the interaction of the AMH promoter region and the oestrogen beta receptor (129). A number of studies support the inhibitory role of AMH on aromatase action prior to selection of the dominant follicle (130, 131). An intra-follicular inverse relationship exists between AMH, oestrogen concentration and CYP19a1 (aromatase) activity within the granulosa cells

(132, 133). The inverse relationship between aromatase and AMH is also seen in the oocyte cumulus complex of pre-ovulatory follicles in sheep (134).

1.4.3 The effect of age

Dataset analysis has integrated a number of studies, incorporating birth to menopause, with the aim to represent 'normal' female population changes in relation to AMH (135, 136). Analysis of this model describes 4 distinct stages of circulating AMH, with correlation to non-growing follicle (NGF) recruitment. This NGF population (which peaks at 20-22 weeks fetal gestation (137)) should be considered the true reflection of ovarian reserve. There are no available direct methods to assess this number in clinical practice, which lead to indirect methods such as AMH testing. Levels of AMH increase through childhood (stage 1), fluctuate at puberty (stage 2) and have a secondary increase to a peak at 25 years (stage 3). The final stage, over 25yrs of age, shows parallel reduction in both AMH and the NGF population. The dynamics of AMH in relation to the resting pool of follicles in stage 4, allow the reliable use of AMH as an ovarian reserve marker, unlike the 3 earlier stages when the NGF population is still changing (see Figure 1-7).

Figure 1-7 - AMH in relation to NGF population and recruitment. Reproduced from Kelsey et al(135)



1.4.4 Menstrual variation in AMH

An AMH test can be performed at any point within the menstrual cycle because there appears to be little variation in an individual, within or between cycles. This is due to a relatively stable pre-antral to early antral follicle pool and the reduced production of AMH by larger follicles on their approach to ovulation (128, 138). Studies that have tracked AMH profiles in individuals have generally found no significant differences across the reproductive cycle (128, 139). Minor fluctuations do occur although there is little consensus on when or if these peak in each individual cycle (128, 140). Further review suggests that although a potentially significant difference in AMH concentration may occur within the cycle, this is smaller than inter-cycle variability and as such it is not clinically significant (141). Two prospective studies have evaluated and agreed on inter-menstrual cycle variability in AMH concentration (138, 142). The majority of the variability was due to between-subject variation with only 11% of the variability secondary to individual fluctuations. Conversely, Hadlow et al (143) have shown a clinically significant difference in AMH values taken at different stages of the cycle, with a negative trend from follicular to luteal phase. The maximum change was found to be 7.9pmol/L, with over half of the subjects having their ovarian reserve reclassified. It must be noted this study was on a very small sample size.

An interesting hypothesis from Sowers et al (144), depicts two different patterns of AMH dynamics. This is based on AMH level and not the chronological age of the patient. Lower AMH values, showed less variation and correlated with shorter cycle lengths. This was termed the ‘ageing’ ovary, in comparison to the ‘young’ ovary, characterised by higher mean AMH values and significant variations within the menstrual cycle. Others have shown that age also correlates with the ‘young vs. ageing’ ovary patterns (145).

1.4.5 Factors affecting AMH

Contradictory evidence is published for factors that may alter AMH, which is summarised in Table 1-2. Obesity is perhaps the best example. Some studies suggest

no impact of obesity on AMH *per se* (146-148) whilst others suggest a negative correlation with increasing BMI (149, 150). Piouka et al showed a significant inverse relationship with AMH in obesity, irrespective of ovulatory status (151). La Marca et al (152) discussed this finding in a study of a 'normal' population of regular cycling women. A significant decline in AMH was seen with raised BMI (r:-0.1; p=0.03). This was confounded by the stronger relationship that increasing age has with increasing BMI.

Table 1-2 Factors affecting AMH

FACTOR	Negative effect	No effect	Positive effect
BMI	Negative correlation (149-151)	No effect (146-148)	na
Smoking	Negative correlation (153, 154)	No effect (152, 153, 155)	na
Oral contraceptive pill (OCP) / GnRH analogue use	Negative correlation (156-158)	No effect (159, 160)	na
Pregnancy	Negative correlation (161, 162)	No effect (163)	na
Ethnicity	Reduced AMH in African / Hispanic populations (164, 165)	na	na

Smoking, often considered an accelerant of decline in ovarian reserve, is regularly assessed as a covariate in trials. La Marca et al (152) found no difference between smoking and non-smoking groups, although they acknowledge the limitation of not establishing an accurate smoking history of their subjects. These findings corroborate with Dafopoulos et al (155) who also found no difference but had a limited sample size

of 137. Alternatively, from an older population, up to a 44% drop in AMH was seen in active smokers (166) with no relation to dose or duration of smoking. Passive smoking or prenatal exposure to cigarette smoke had no effect on systemic AMH. There is conflicting evidence for the impact of passive smoking on ovarian reserve and time of menopause (167, 168).

In contrast, use of agents to suppress ovulation such as the oral contraceptive pill or GnRH analogues may reduce AMH with time (156, 157). Others report no change in level with 6 months use despite a significant reduction in ovarian volume and follicle count (159, 160). Kallio et al (158) suggested that with continuous use of a combined hormonal contraceptive, regardless of mode of treatment delivery, there was a significant suppression of AMH relative to individual baseline values off hormonal contraceptives (AMH 3.88±3 vs. 1.91±1.5 ng/mL; $p<0.001$). There were similar reductions in FSH, LH, inhibin B and oestradiol (169). These authors suggest that an inhibition of the early FSH-dependent follicular growth associated with continuous hormonal contraceptive use may reduce AMH production through reduction in number of antral follicles releasing AMH. This may even impact on the earlier stages of folliculogenesis. Furthermore, ovarian androgen suppression by such contraceptive use may also reduce promotion of antral follicle growth, therefore impacting on AMH production (169).

It has been reported that serum AMH concentrations fall during pregnancy, which is a situation of prolonged ovarian quiescence (in terms of large antral follicle growth) and suppressed FSH secretion. Postnatal recovery of AMH is then seen (161). A significant reduction is seen in AMH during the second and third trimester. The first trimester correlates with the non-pregnant value taken ~4.5 months after delivery. Obesity also showed an inverse relationship with AMH which correlated at all stages of pregnancy with the early pregnancy BMI (161). In contrast, an earlier study by La Marca et al (163) suggested no change in AMH during pregnancy, although the study design did not track individual values through a pregnancy but employed a cross sectional method of analysis. Both studies (161, 163) conclude that early follicular activity is not abolished in pregnancy due to the presence of detectable AMH throughout. Furthermore, although there are very low levels of FSH and LH in pregnancy, there is enough

available to support early folliculogenesis but not enough for late antral follicle support (170). Nelson et al (161) suggest a plausible evolutionary hypothesis, that reduced AMH and ovarian quiescence help to prevent multiple pregnancies and asynchronous gestations, supported by the rapid recovery of AMH in the early postnatal period.

Variance between ethnicity and AMH is the subject of much debate. A significant proportion of the published work on AMH comes from homogenous cohorts of women with little variation in ethnicity. Disappointingly in some of the largest study cohorts ethnic background is not recorded (171). A study looking at AMH decline in a cohort of 809 American women enrolled in the World Interagency Human Immunodeficiency Virus (HIV) study used multivariate linear regression to establish if there was any difference in AMH decline between white, black or Hispanic women (164). By controlling for confounding factors such as age, BMI, smoking and HIV status, black women were found to have a significantly lower AMH level than white (25.2% lower; p=0.37). Hispanic women also had a lower AMH although this did not reach significance (24.6%; p=0.63).

1.4.6 Other uses for AMH

Ovarian reserve testing has traditionally employed a serum FSH plus dynamic tests such as the GnRH and Clomiphene Citrate stimulation tests (172). With the development of high resolution ultrasound, accurate antral follicle counts (AFC) can be made. A single AMH test may limit observer variability (138), but with the many problems associated with AMH measurements (173), many believe AFC may be as good as AMH as an index of ovarian reserve and so the 2 indices should be used in combination (174). In contrast, recent evidence suggests that AMH is superior to AFC in predicting ovarian response to controlled ovarian stimulation (175). The ability to not only quantify the ovarian reserve, but also reveal the quality of the reserve remains a difficult proposition. Atretic follicles produce less AMH (120) but are still registered within the AFC. In contrast, the larger antral follicles are more likely to respond to gonadotrophins and therefore predict ovarian response (176). Predicting this responsiveness includes a clinical assessment of Follicle Output RaTe (FORT), a

ratio between the pre-ovulatory follicle count and baseline AFC, which shows a negative and independent relationship with AMH (177).

Anti-Müllerian Hormone has been promoted as a test to predict and tailor treatment for superovulation (178). Within ART, its value has been proposed for the prediction of oocyte yield and to a lesser extent live birth rate, allowing individualisation of treatment (179, 180). Adoption of safer strategies for superovulation such as the antagonist cycle can limit the iatrogenic complication of OHSS (181). Improving patient expectations at the lower extreme of reproductive potential may have psychological benefit (182). Age remains the single most important prognostic factor in achieving a successful pregnancy, despite the quantitative merits of AMH as an assessment of ovarian reserve (183, 184). Conflicting evidence questions the relevance of low AMH on untested fecundability (150, 185). Some clinicians have used the detection of very low AMH to deny patients infertility treatment. Although oocyte yield, live birth and treatment success is undoubtedly reduced with reducing AMH, pregnancy can nonetheless be achieved even in those women with a very low serum AMH concentration (186, 187). This treatment limiting strategy therefore seems unjustified and raises ethical questions.

Assessment of the toxic consequences of oncological treatment on the ovary and ovarian tissue using AMH measurement is gaining momentum. Indeed, depending on the chemotherapy regimen used, recovery of ovarian function can be tracked by AMH (188). In both pre and post-pubertal girls treated with chemotherapy, initial detectable levels of AMH showed a progressive decline during the course of treatment. For some the levels recovered to near pre-treatment levels by 12 months following completion of treatment. Unfortunately those treated with alkylating agents show little recovery in comparison with those with non-alkylating agents (189). The alkylating agents are not cell-cycle specific and damage both resting and growing follicles. Furthermore, the damage is rapid, with apoptotic indicators detectable within 12 hours of injection of the alkylating agent, Cyclophosphamide (190). A significant reduction in primordial follicle density has been demonstrated by 48 hours following treatment initiation. Further longitudinal data is required to establish if the AMH recovery patterns reflect future successful fertility (189) For patients with granulosa cell tumours, AMH levels

are significantly raised, suggesting it may have a use as a potential tumour marker (191). At the opposite end of the ovarian spectrum to PCOS, AMH has been shown to be useful in establishing premature ovarian insufficiency (POI) (192-194). In women with Turner's syndrome, a recognised cause of POI, karyotypes with a poor probability of fertility (e.g. 45XO, 45XO/46XY, 46X del(Xq)) had a lower level of serum AMH compared with karyotypes that had a fair probability (e.g. 45XO/46XX, 46X del (Xp)) and of whom went through spontaneous puberty (192). Histological work has shown normal AMH expression in POI pre-antral follicles but a reduced expression in the early antral stages, reflecting a possible defect in antral development (194). Furthermore, serum AMH was detectable in women who had >15 AMH immuno-positive follicles unlike those who had <5 immuno-positive follicles. Serum AMH testing could be used to predict the presence of persistent follicles (194).

Prediction of age of menopause using AMH remains contentious. Complex genetic patterns have emerged controlling the age of menopause, including variants in the AMH receptor gene, which have been implicated in initial follicle recruitment (195, 196). A novel interaction between SNPs on both the AMH gene and AMH receptor gene (AMHR2) has been identified (196). The SNP for the AMH gene alone has been found to be unrelated to age of natural menopause (197), but in unison with the SNP for the AMHR2 there is an apparent negative impact on ovarian ageing, including when controlling for parity, smoking and BMI (196). A proposed mechanism for this involves the SNP for the AMH gene, which influences the expression of mRNAs (198). Furthermore, this mutation can have a protein damaging function (199). It is suggested that AMH can be highly predictive of the onset of menopause when combined with age. Levels become undetectable approximately 5 years prior to the menopause (200) . Broer et al (201) have developed a nomogram based on AMH and age to predict this stage of life. The age ranges are noticeably wide and incorporate the vast majority of years surrounding the average age of menopause. It is therefore questionable whether this provides any clinically useful further information to women contemplating delaying motherhood as suggested in this study. Evidence related to maternal age of menopause suggests AMH decline is more rapid if the mother of the subject entered

into menopause before the age of 45 years (8.6% decline per year vs. 3.2% decline if the maternal mother was over 55 years at time of menopause) (202).

Endometriosis, a condition of abnormal proliferation and apoptotic regulation of endometrial cells distant to the uterine cavity, is implicated as a potential ovarian cause of infertility (203). Surgical treatment for endometriomata can have a significant negative impact on AMH, especially in bilateral cases (204). A novel therapeutic approach to harness the inhibitory role of AMH is under research (205). An intact AMH/AMHR2 pathway has been identified within human endometrial stromal cells. Activation of this pathway *in vitro* reduces cell viability through increased programmed cell death (205). Potential mechanisms for AMH as an adjuvant therapeutic treatment have also been identified in ovarian and cervical cancer lines (206, 207). There is increasing expression of the AMHR2 from benign ovarian tumours to borderline lesions, especially in the non-epithelial tumours (206). Inhibition of ovarian cancer cell growth has been shown via lengthening of the G_1 phase of cancer cells (208). A similar effect has been shown in cervical cancer with induction of the tumour suppressor proteins p16, p130 and p107 (209). In contrast, some evidence suggests that ovarian stromal tumours may be refractory to the anti-proliferative actions of the AMH protein, producing their own bioactive AMH (206). An explanation for this could be defective type 1 receptors blocking AMH signalling (210).

1.5 Anti-Müllerian Hormone As A Diagnostic Test For PCOS

The concept of using AMH as a diagnostic tool for PCOS is not new. Predating the last decade, studies report higher than normal levels of AMH in both serum and follicular fluid in women who meet the different criteria for the diagnosis of PCOS compared with controls. Both Cook et al (211) and Fallat et al (212) demonstrated higher systemic levels of AMH in comparison with normal controls and women with different infertility aetiologies. A polycystic ovary has an increased cohort of antral follicles (213). The ratio of median AMH to AFC has been quoted as a 0.2ng/mL increase per follicle with an increased value of 0.34ng/mL for PCO follicles (214). Nardo et al (214)

demonstrate that although there is a similar increase in serum AMH with AFC between PCOS and non-PCOS women, those with PCOS have a consistently higher AMH level (difference 2.05 ± 0.26 ng/mL). In contrast, Pellat et al (215) show AMH production is increased in anovulatory polycystic ovaries compared with ovulatory polycystic ovaries (215). Indeed, granulosa cells from women with PCOS secrete more AMH *in vitro* than those with normal ovaries (215). This was demonstrated from work with granulosa cells plated at the same density of cell/plate in all 3 types of ovary. The mean AMH production/ granulosa cell (other descriptive statistics such as standard deviation (SD) were not specified) was 0.37ng/mL, 1.56ng/mL and 27.4ng/mL for normal, ovulatory-PCO and anovulatory PCO's respectively. From this study, it should however be noted that the normal ovarian tissue was from an older population of women. It is well recognised that AMH declines with age, but also that those with normal ovaries have a steeper rate of decline in AMH (214). This may have exaggerated the observed difference in granulosa cell AMH production. Not only is AMH over-expressed in the granulosa cells from polycystic ovaries, so too is its type II receptor. This positive correlation includes AMH, AMHR2 and also FSH receptor expression, which could reflect follicle maturation abnormalities (216). Excessive production of AMH may therefore lead to disordered follicular growth and excess androgen production in PCOS (2). The exact cause of elevated AMH in PCO follicles is unknown, but it is plausible that androgens may regulate AMH production as reviewed by Dewailly et al (113). There appears to be an intrinsic defect in PCO thecal cells that leads to overproduction of androgens (36). Androgen and AMH levels appear to have a positive correlation (217). Indeed, animal studies have shown that androgens induce growth of pre-antral follicles (218) which in turn produce more AMH. In contrast, there is evidence that androgen supplementation reduces AMH production (219, 220). Theoretically, this could be due to androgens forcing small-antral follicles to produce less AMH and reduce the overall AMH concentration (220). Prolonged androgen suppression does not appear to have effect on AMH production (221).

Interestingly, morphological comparisons have been made between primordial recruitment in women with polycystic ovaries and AMH-null mice. The AMH-null mice although fertile have a depleted primordial pool and reduced reproductive lifespan

due to lack of inhibitory control from AMH and accelerated follicular recruitment from the primordial pool (121, 122). In contrast, disordered folliculogenesis in PCOS is not purely confined to the antral stage. A hypothesis supported by histological studies purports that primordial follicle recruitment is accelerated leading to an increased primary/pre-antral population (222). Reduced AMH expression determined by immuno-histochemical staining is shown in the primordial follicle and pre-granulosa cells of anovulatory polycystic ovaries in comparison with the normal ovary. Stubbs et al (222) do not comment on the potential confounding factor of obesity in this study. If indeed obesity leads to reduced AMH expression (151), it should be noted that the anovulatory polycystic ovaries in the aforementioned study (222) came from a significantly elevated BMI cohort (34.1kg/m² standard error mean (SEM) 4.5) when compared with either the ovulatory PCO (23.6kg/m² SEM 1.2) or normal ovarian (22.6kg/m² SEM 1.7) groups.

A study to ascertain the cause of secondary amenorrhoea (223) again highlighted AMH use in ovarian reserve assessment. Patients were grouped according to the main causes of secondary amenorrhoea (PCOS, POI and hypogonadotrophic hypogonadism). There was a clear correlation with AMH and the PCOS group. No difference in AMH was seen between the regular cycling control group and the hypogonadotrophic hypogonadal group. Unsurprisingly, AMH was lowest or undetectable within the ovarian failure cohort.

1.5.1 Polycystic ovarian morphology and AMH

There is debate about the use of a single AMH measurement to replace the ultrasound assessment of PCOM. A strong correlation between the AFC and AMH exists (87). Table 1-3 catalogues a variety of cut off values proposed in the literature to diagnose the polycystic ovary and PCOS. It is clearly evident that a consensus has not been reached. Iliodromiti et al (224) conducted a meta-analysis to aggregate data on the diagnostic power of AMH in the diagnosis of PCOS. The extracted data demonstrates specificity and sensitivity for a PCOS diagnosis of 79.4% and 82.8%, respectively, using a cut-off AMH of 4.7ng/mL in symptomatic woman. The Rotterdam criteria were used

to define PCOS in the studies included in the meta-analysis. The majority of diagnostic studies have selected patients from gynaecology or infertility clinics. This has the potential to overestimate the performance characteristics of the test. Studies that have used controls from the general population (225, 226) have found similar AMH cut off values with no associated reduction in sensitivity or specificity, negating any serious effect of selection bias. As highlighted in the meta-analysis detailed earlier (224), there is considerable debate over the equivalence of different AMH measurements quantified by different assays; thus making meaningful comparisons across studies of AMH and PCOM questionable. For example, the significant difference between the Diagnostic Systems Laboratories (DSL) and Immunotech (IOT) / Generation II (Gen II) assay values, hamper the comparison between studies (227). To date relatively few studies have used the Gen II assay (228, 229). In contrast, the work of Dewailly et al (87) has adopted a retrospective design, analysing records from a subset of infertile patients excluding women with unexplained infertility or endometriosis. Interestingly, unlike others this study attempts to categorise women using cluster analysis. They separate the non-PCOS non-PCOM women from the asymptomatic PCOM group. Eliminating the latter removes a potential cohort of women who could be considered to have a silent form of PCOS (230). This 'silent' assumption is not accepted by all who consider PCOM as a normal variant of ovarian morphology (79). On this highly selected population, Dewailly et al (87) use receiver operator characteristic (ROC) curves to establish the best PCOS diagnostic levels for AMH (as measured using the IOT assay), follicle number and ovarian volume of 35pmol/l, 19 and 7ml, respectively. Their data provides an interchangeable value for either follicle number or AMH for PCOS diagnosis. The AUC is 0.973 for AMH, providing strong evidence for its suitability as a diagnostic test for PCOS. More recent work from Dewailly et al (231) follows a similar design comparing PCOS patients, using the stricter NIH criteria, against 'normal' patients without using PCOM within diagnosis. The study population is small, at 59 subjects, with a high proportion of PCOS women. Using ROC curve analysis (AUC 0.97) once again showed a remarkable diagnostic power.

Table 1-3 - AMH values in PCOS diagnosis

Author	Type Study	Number	Category	Type Assay	Result	AMH pmol/L (7.14 x ng/ml)
Casadei et al 2013 (8)	Prospective study	n = 59 Infertility	PCOS/OA or HA/ Asymptomatic	IOT	AMH >33pmol/L for PCOS (+ 13 follicles)	33
Homburg et al 2013 (228)	Prospective study	n = 234 Infertility	PCOS/PCOM/Normal Significant difference between groups	DSL / GEN II	AMH 48pmol/l LH >6IU/L 82.6% PCOS	48
Chao et al 2012 (226)	Prospective Case control	n = 111 Community / Clinic	PCOS/ Premature ovarian failure / healthy volunteers	DSL	3.5ng/ml Sensitivity 74% Specificity 79%	24.99
Eilertson et al 2012 (232)	Cross sectional Case control	n = 262 Preterm / Term Birth	Rotterdam & Androgen XS PCOM/OA/HA	DSL	AMH >20pmol/l Replace PCOM with AMH	20
Woo et al 2012 (225)	Prospective case control	n =140 Gynaecology / advertise	Rotterdam PCOS/non-PCOS/healthy volunteers	IOT	AMH > 7.82ng/ml Sensitivity 75.9% Specificity 86.8%	55.83
Li et al 2012 (233)	Prospective	n = 131 PCOS	PCOS +/- hyperandrogenamia	DSL	AMH 4.23ng/ml for PCOS with HA	30.2
Rosenfield et al 2012 (229)	Prospective cohort-control	n=102	Non-PCOM/Asymp PCOM/PCOS with/without response to androgen testing	GEN II	AMH >6.2ng/mL for PCOS vs non-PCOM; >10.7ng/mL PCOS HA vs Asymp PCOM	44.23 76.4
Dewailly et al 2011 (87)	Prospective – cohort	n = 128	Non-PCOM non-PCOS vs. Hyperandrogenic oligo-anovulation	IOT	>35pmol/L for PCOM (plus AFC >19)	35
Skalba et al 2011 (148)	Prospective	n = 87 (+ 50 control)	Metabolic clinic and relations	IOT	Normal weight/obese PCOS (9.6±3.5 vs 11.2±4.5ng/mL) vs non-pcos (2.5±.8 vs 2.3±0.7ng/mL)	na
Pigny et al 2006 (234)	Prospective	n = 73 (+ control 96)	IVF patients – PCOS/controls	IOT	ROC curve 60pmol/L sensitivity 67% specificity 92%	60
La Marca et al 2004 (235)	Prospective	n = 14 (PCOS) + 15 matched controls	PCOS/Normal	IOT	PCOS vs Normal 5±1.8 vs. 1.3±0.5 ng/ml p<0.5	na

IOT: Immunotech, DSL: diagnostic systems laboratory, GEN II: generation II assay, na: not applicable

The best compromise between sensitivity (95%) and specificity (95%) for PCOM diagnosis was found with a value of 33pmol/L. Both of the French studies (87, 231) propose very similar diagnostic AMH levels to that which is derived from the meta-analysis (using a 7.14 conversion factor from ng/mL to pmol/L, 33.6pmol/L) (224). In marked contrast, a number of studies contest the sensitivity of AMH in predicting PCOS despite good specificity (228, 229, 234). It is of note, that these studies include women with asymptomatic PCOM in their study cohort. Rosenfield et al (229) used age-matched volunteers and PCOS cohorts to delineate between the individual with no evidence of PCOM, the individual with a PCO, atypical PCOS (no hyper-response to 17-hydroxyprogesterone (17-OHP)) and finally typical PCOS (17-OHP hyper-response to GnRH agonist). An AMH (Gen II assay) value of >6.2ng/mL discriminated between a non-PCO and the other 3 groups with a 95% specificity. The sensitivity in discriminating between the non-PCO group from typical PCOS remains high (81%), but drops to as low as 32% if a PCO is found. A ROC analysis indicated that a considerably higher AMH (>10.7ng/mL) was needed to discriminate between the asymptomatic PCO and PCOS with 96% specificity, but sensitivity remained low. Homburg et al (228) support these findings in a similar study design using patients presenting to an infertility clinic. Three groups were identified (controls with no evidence of a PCO/asymptomatic PCOM/PCOS). An AMH >48pmol/l (Gen II assay) had a predictive capacity for diagnosing PCOS with a 98.2% specificity and 60% sensitivity. The inclusion of LH levels of > 6IU/L in the analysis, improved the predictive capacity of AMH for PCOS to 82.6%. This study supports the hypothesis that a finding of asymptomatic PCOM represents a separate cohort of women, midway between those with a normal ovary and PCOS (80).

The capacity of AMH measurement to predict PCOS has been shown to be less reliable in adolescents, who have a lower sensitivity and specificity for diagnosis (236). The reasons for this may be due to the higher incidence of menstrual irregularities (237) and to the multi-follicular appearance of ovaries (238) often seen in this population. Mortensen et al (86) suggest that the finding of a polycystic ovary (based on ovarian volume) in an asymptomatic adolescent is a variant of normal ovarian morphology. They acknowledge that 40% of adolescent girls appeared to have a subclinical ovarian dysfunction linked to PCOS, without any associated HA. This was shown by an elevated

17-OHP response to a GnRH agonist, akin to that seen in PCOS. The difficulties seen in establishing a diagnostic AMH level in adolescent girls is understandable, when considering the different phases of NGF recruitment and AMH as reported by Kelsey et al (136).

1.5.2 LH, insulin resistance, hyperandrogenemia and AMH

A demonstrable significant positive correlation exists between serum concentration of LH and AMH (151). AMH and LH levels are increased in lean PCOS women relative to those who are obese. A positive correlation can be demonstrated between insulin resistance, anovulation and hyperandrogenaemia (87, 239). The factors involved in this link are however complex. The LH-mediated stimulation of androgen secretion from the ovaries is augmented by insulin and insulin-like growth factors, leading to increased androgen production. A significant positive correlation has been shown between the homeostasis model assessment (HOMA), a measure of basal insulin resistance, and AMH level ($r = 0.621$; $p < .05$) (235). This notion is supported by *in vitro* evidence. A study comparing granulosa cell growth and AMH production *in vitro* when cultured independently with either FSH or LH, showed no effect of the gonadotrophins on AMH secretion by cells from the normal human ovary. From the polycystic ovary, AMH production was inhibited by FSH but significantly elevated by LH (215). This correlation may provide an explanation of the failure of anovulation PCOS phenotypes to produce a dominant follicle. The relationship between LH and AMH appears independent of antral follicle number as serum AMH levels are higher in anovulatory women with hyperandrogenaemia without PCOM when compared with ovulatory women with PCOM in combination with raised androgens (73, 240). Increased LH pulse frequency and amplitude (2) and premature sensitisation to LH (241), contribute to follicular arrest (240). Furthermore, there is a reported loss of LH-induced down regulation of the AMH type II receptor within the granulosa cells of anovulatory women with PCOS compared with granulosa cells from women who are normal or ovulatory (129). This combination may potentiate the inhibitory effect of AMH on follicular recruitment.

Studies of treatments aimed at reducing insulin resistance have looked at the effect of treatment on AMH concentration. Protracted (>4 month) use of the insulin sensitising agent metformin is correlated with a reduction in AMH levels, where as a rapid reduction in androstenedione levels is seen within weeks of commencement of metformin treatment. Fleming et al (242) hypothesised that this lag time is due to the time required to recruit a cohort of follicles under a normalised insulin drive. The study in question does not acknowledge the significant reduction in BMI which occurred within the 8 month period or in deed any correlation between weight loss and AMH. Another group confirmed the lack of change in AMH with short term metformin treatment (8 weeks) (243). Further work, showed a differential reduction in AMH concentrations with metformin if hyperinsulinaemia existed in the patients from the outset unlike those women who were normo-insulinaemic (244). An increase in AMH in PCOS with a combination of diet and exercise and orlistat despite reduced testosterone and insulin resistance indices has also been reported (245). Other work by the same group showed a reduction in AMH with diet and weight loss following Sibutramine administration (246).

The use of AMH as a surrogate marker for HA has been proposed (83). Also, AMH has the ability to distinguish between the asymptomatic individual without a polycystic ovary and an individual with PCOS with HA (151). The work of Li et al (233) support this argument by asserting that AMH should only be used for diagnosing PCOS in women with HA due to the relatively improved sensitivity and specificity for this subgroup (HA+ 4.23ng/mL (DSL assay) sensitivity 82% specificity 64%; HA- 3.76ng/mL sensitivity 64% specificity 62%). Notably, the specificity remained poor in this study, unlike other findings which have been discussed previously (228, 229), where a correlation between AMH and androgens has been demonstrable regardless of PCOS status (214). It has been suggested that the term ‘PCO-like abnormalities’ may be more appropriate as the third criterion of the Rotterdam classification (247).

In summary, the diagnostic utility of AMH for identifying PCOS has generated many opinions and has various suggested cut-off values. The wide variation in recommended AMH levels and the debate over its diagnostic capacity, indicate that further work is needed in this field. Validation of AMH reference ranges for local populations and the

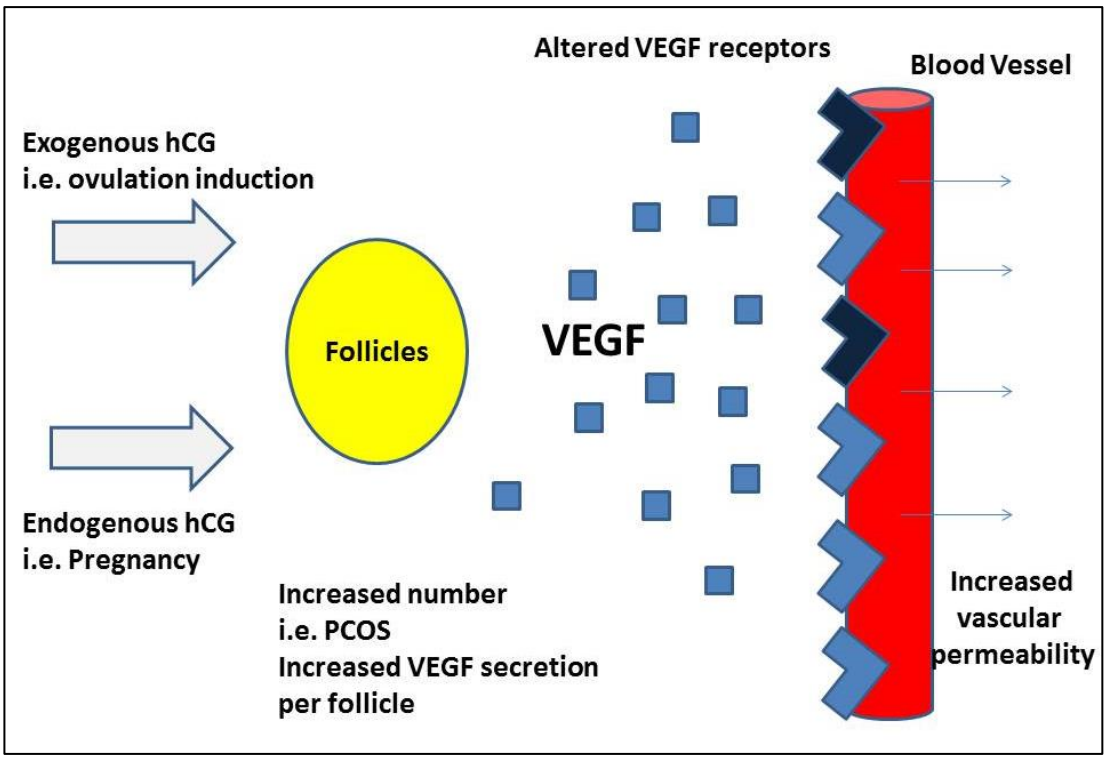
ability to apply these values to the general population are needed. The elimination of specific cohorts of patients in many of the published studies e.g. asymptomatic PCOM, has led to falsely strengthened evidence for the diagnostic use of AMH in PCOS. Given the discrepancy between the different AMH assays, more data must be generated using the Beckman Coulter Generation II assay, to further validate the diagnostic value of AMH before an international standard can be established.

1.6 Ovarian Hyperstimulation Syndrome

The condition of OHSS is a potentially avoidable iatrogenic endpoint of ovulation induction for assisted conception therapy. The condition has a higher incidence in women with PCOS (248). It is most frequently created by exogenous administration of gonadotrophins and is accentuated by pregnancy (249). Vasoactive products released from stimulated ovaries generate increased capillary permeability. Fluid shifts from the vascular compartment into the third space, resulting in intravascular dehydration. Severe manifestations of OHSS include hepato-renal disturbance, thrombosis, adult respiratory distress syndrome and even death. Symptoms of OHSS progress from mild abdominal distension and nausea to oliguria, ascites and haematological disturbance. Hospital admission is needed in severe cases. Careful fluid balance and thromboprophylaxis is imperative. Early OHSS is related to exogenous Human Chorionic Gonadotrophin (hCG). Ovarian hyperstimulation syndrome presenting or continuing after this is often due to endogenous hCG from pregnancy and can follow a protracted course (250, 251). The angiogenic molecule vascular endothelial growth factor (VEGF) is thought to mediate the relationship between hCG and OHSS (see Figure 1-8) (252, 253). This molecule is an endothelial cell mitogen promoting increased vascular permeability and new vessel formation (252, 254). The expression of VEGF has been demonstrated in both granulosa lutein and thecal ovarian cells (255). Furthermore, soluble forms have been found in follicular fluid and ascites in those with OHSS (253, 256). Within the luteal phase, an increase in VEGF occurs, playing a pivotal role in ovarian folliculogenesis (257). There is an increased expression of VEGF in women with PCOS (252). The stereotypical hyper-thecotic stroma seen in a polycystic

ovary (255), with enhanced blood flow (258) and overexpression of VEGF, may contribute to failed diversion of blood between follicles and enhanced androgen steroidogenesis (252). A number of SNPs have been identified in the VEGF gene, that are associated with PCOS (254). Furthermore, insulin has been demonstrated to augment VEGF secretion (259), which is a fundamental feature of PCOS. Unfortunately, although modulation of insulin levels with metformin has been shown to reduce VEGF by some authors (260), this has not been replicated by others (261).

Figure 1-8 Ovarian hyperstimulation and vascular endothelial growth factor.



Exogenous and endogenous hCG stimulate secretion of VEGF which increases vascular permeability. In those with PCOS, an increased concentration of VEGF occurs with altered patterns of VEGF receptor expression and signal transduction.

The action of VEGF is via 2 endothelial tyrosine kinase receptors. The first receptor is expressed in 2 forms, which can either transduce or prevent signal transduction. The combined effect of these differing forms mediates the angiogenic properties of VEGF (262). A reduction in the circulating 'preventing' receptor is seen in those with PCOS

who develop OHSS, who therefore have an increased bioavailability of VEGF (253). It is plausible that women with PCOS who experience OHSS, may have increased serum and follicular concentrations of VEGF secondary to an increased population of granulosa lutein cells and an enhanced secretory ability by the granulosa cells in question (259). A reduction in follicular VEGF has been shown in those with PCOS treated with an antagonist cycle compared with an agonist treatment IVF cycle (263).

The complication of OHSS has not been eliminated in modern assisted conception practice, despite multiple risk reduction strategies. However, maternal death from an IVF cycle or pregnancy is rare. A report from the Netherlands (264), states that 6/100,000 women died from a cause directly related to IVF treatment between 1984-2008. Three of these 6 deaths were related to OHSS, of whom 2 had PCOS. Seventeen deaths were directly related to the IVF pregnancy (42.5/100,000) which was higher than the general maternal mortality in this country. Although the overall incidence of death related to IVF is low, historical reporting of cases of mortality has been sporadic. Significant morbidity may also be under-reported. The risk of both venous and more serious arterial thrombosis is increased in assisted reproductive technologies (ART) and OHSS. Presentation is often unusual such as the upper extremities, making recognition and clinical management hard (265). An estimated 0.08-0.11% of treatment cycles are affected by a venous-thromboembolic event. Although low this represents a 10-fold increase in thromboembolic events in women of reproductive age (266).

The overall incidence of severe OHSS, requiring hospital admission is low (0.5-2%). Clinically significant moderate to severe OHSS affects 1-10% of IVF cycles (248), whilst milder forms can effect 33% of cycles. Women with PCOS or PCOM are at increased risk of OHSS due to the sizeable cohort of antral follicles that are responsive to FSH contained within the polycystic ovary (267, 268). Once the response threshold for the individual is breached, the follicular response may be exuberant, tipping the balance towards OHSS. A study from Oxford (269), reported a clinically significant increase in incidence of severe OHSS in women undergoing their first IVF cycle based on ovarian morphology: normal ovaries (2.7%), PCOM (12.6%) and PCOS (15.4%). Managing and reducing risk related to OHSS, is of paramount importance in what is essentially a

cohort of otherwise 'well-women'. Table 1-4 summarises the main strategies that have been implemented to date and their effectiveness.

Table 1-4 - OHSS reduction strategies

Strategy	Effect
Low dose Stimulation	Use individualised, low dose FSH stimulation (270). Use tests e.g. AMH to tailor treatment (180)
Antagonist Cycle	Competitive binding to GnRH receptors. Significant reduction in OHSS and cycle cancellation when compared to agonist (181).
Metformin	Oral biguanide. Significant reduction in OHSS and potential improved clinical pregnancy rate (260)
Progesterone	Use of Progesterone in luteal phase as alternative to hCG. Significant reduction in OHSS (271).
Cabergoline	Dopamine agonist. Use for secondary prevention of moderate OHSS (OR 0.40 95% CI 0.20-0.77). No difference in clinical pregnancy rate or miscarriage (272).
Human Albumin	Limited evidence for use of albumin or hydroxyethyl starch at point of oocyte retrieval (273, 274)
Coasting	Withholding gonadotrophins whilst maintain pituitary suppression prior to ovulation trigger e.g. hCG – No evidence of benefit (275). Reduction in pregnancy outcome with extended coasting (276)
In Vitro Maturation	Avoidance of gonadotrophins eliminates OHSS risk (277). Reduced clinical pregnancy and live birth rate.
Freeze all cycles	Prevention of late OHSS by prevention of exposure to endogenous hCG (278).
Agonist trigger in Antagonist cycle	Use of GnRH agonist to produce endogenous LH surge (shorter half life when compared to exogenous hCG). Concern regarding inadequate luteal phase support with reduced live birth rate (279).

1.7 The Antagonist IVF Cycle

Prevention of the premature surge of LH and subsequent ovulation is a key element for successful controlled ovarian stimulation. Classically, this was prevented by a GnRH agonist (280). During the course of this protocol, after initial stimulation, down regulation of the GnRH receptors lead to pituitary suppression over a minimum of seven days (281). The drawbacks to this type of therapy include ovarian cyst formation, symptoms of oestrogen deficiency and the increased duration of treatment. Since the

widespread uptake of GnRH agonists for controlled ovarian stimulation, the incidence of OHSS has increased 6-fold (282) due to the synchronisation of follicle development and the high doses of gonadotrophin used to stimulate Graafian follicle development. The agonist approach has increased pregnancy rates through increased oocyte and embryo creation compared to the older regimens using clomiphene citrate rather than necessarily improving embryo quality (283). In contrast to GnRH agonist therapy, treatment with the GnRH antagonist competitively binds to the GnRH receptor, producing its effect within hours of administration (281, 284). The aim, similar to that of the agonist, is to promote synchronous oocyte maturation and prevent follicle maturation arrest by premature luteinisation (281). Early use of the GnRH antagonist was hampered by anaphylactoid reactions. Based on the structure of porcine GnRH, structural modifications led to the development of Nal-Arg and Detrilex. Although these had good anti-ovulatory properties, they also had potent histamine–releasing properties (285). Further pharmaceutical development produced compounds without these unwanted side effects, allowing for the safe use of the antagonist (286).

Early reviews of antagonist cycles were not favourable with regard to clinical pregnancy. The first Cochrane review (287) cited 5 randomised controlled trials (RCTs) comparing the antagonist cycle with the long agonist cycle. There was no difference in rates of OHSS (Relative Risk (RR) 0.51; Odds Ratio (OR) 0.79; 95% Confidence Interval (CI) 0.22-1.18). Significantly fewer clinical pregnancies were seen with the antagonist cycle (OR 0.79; 95% CI 0.63-0.99). Subsequently, 2 reviews disagreed. Al-Inany et al (288) maintained that the antagonist cycle had a lower clinical pregnancy rate. Rates of OHSS were significantly reduced with the antagonist cycle (RR 0.61, 95% CI 0.42 to 0.89 P = 0.01) with less use of techniques such as coasting or cycle cancellation. Kolibianakis et al asserted that the type of GnRH analogue used for pituitary suppression did not affect the pregnancy outcome (289). The most recent Cochrane review (181) concluded that there is no difference in live birth or ongoing pregnancy rate between the analogues used (28 RCTs; OR 0.87, 95% CI 0.77 to 1.00). In the most recent Cochrane review, antagonist protocols have been shown to significantly reduce the risk of OHSS compared with agonist protocols (29 RCTs OR 0.43 95% CI 0.33-0.57) (181). The difference is even more pronounced in women with PCOS, for whom OHSS is more

likely (Risk Difference (RD) -0.10 95% CI -0.14 to -0.07 p < 0.00001). Cycle cancellation due to OHSS, was significantly more likely with an agonist (OR 0.5 CI 0.33-0.76 p=0.001). When the entire population was reviewed the absolute risk reduction was 4% with a corresponding number needed to harm of 25. In lay-man's terms, for every 25 women treated with an agonist 1 more person is likely to develop OHSS (181). The antagonist approach has been acknowledged within National Institute Clinical Excellence (NICE) guidance to have a lower rate of OHSS. The agonist approach is only recommended for those likely to have a low response (290).

Further benefit of using antagonist cycles during assisted conception treatment is that it allows use of a GnRH agonist trigger for final oocyte maturation instead of hCG. The GnRH agonist displaces the antagonist from the pituitary receptor, resulting in a gonadotrophin flare response. The released LH drives oocyte nuclear maturation, although there is a substantial reduction in surge duration (291). Live birth rates have however been disappointing. The 2011 Cochrane review (279) concludes that the agonist trigger should not be employed routinely due to a reduced live birth rate (OR 0.44, 95% CI 0.29 to 0.68; 4 RCTs). The benefit of this alternative trigger is the further reduction in OHSS compared with hCG trigger (OR 0.10, 95% CI 0.01 to 0.82; 5 RCTs). It is well documented that hCG has a longer half-life and supports the formation of numerous corpora lutea compared with LH and so maintains a higher level of oestradiol and thus increased risk of OHSS than LH *per se* (292, 293). In women at high risk of OHSS, the reduction of OHSS risk therefore counterbalances in part the reduced birth rate. Further consideration is needed for optimal luteal phase support, if the agonist trigger is going to be used routinely to treat patients undergoing antagonist cycles.

1.7.1 Antagonist cycle in PCOS

The outcome of IVF in women with PCOS has been reviewed (269, 294). The overall cancellation rate of cycles is increased in PCOS. There is an increased oocyte yield, with lower total fertilisation but similar number of embryos available for transfer. The pregnancy rate remains similar between those with/without PCOS. Although these

findings agree with the majority, some disagree. Engmann et al (295) suggest that women with PCOM without symptoms of PCOS, have a higher clinical pregnancy rate compared to those with normal ovaries.

A meta-analysis (296) comparing outcomes between the antagonist and agonist cycle in PCOS agrees with others regarding the risk of OHSS (297). The overall risk of moderate to severe OHSS is reduced with the antagonist cycle using pooled data (RR 0.60 95% CI 0.48-0.76 $p<0.0001$). Due to inconsistent classification of severity of OHSS, without data aggregation there was no difference between the cycles reporting severe OHSS alone. The authors conclude that reduction in severe OHSS following antagonist cycles in women with PCOS should be viewed with caution due to inconclusive results. Like other studies (289, 298, 299) a shorter duration of stimulation and a lower total dose of gonadotrophin was used in the antagonist cycle. There was no difference in oestradiol on day of hCG, number of oocytes retrieved, cycle cancellation, clinical pregnancy rate (RR1.01 95% CI 0.88-1.15) or miscarriage rate (RR 0.79 95% CI 0.49-1.28).

A theory for the higher OHSS rate in the agonist cycle despite similar oocyte yield has been suggested (251). An increased dose of gonadotrophin is required to overcome the pre-treatment blockade of endogenous FSH following pituitary suppression (300). Normally, the ovulatory follicle(s) cohort selection commences at the start of the cycle. GnRH agonist down regulation may interfere with this selection process, reducing atresia of small antral follicles and allowing more midsized follicles to be generated (301). Another interpretation is that following down regulation the low/basal levels of FSH remain sufficient to support the growth of multiple small antral follicles up to the 2mm gonadotrophin dependent stage (302). During controlled ovarian stimulation, large doses of gonadotrophin allow this sizeable cohort to respond on mass creating the outcome of OHSS.

1.8 Insulin Sensitising Agents

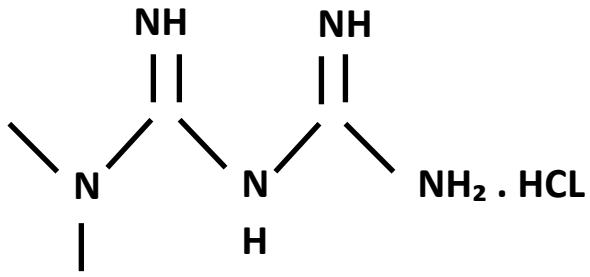
The action of insulin sensitising agents could reasonably be a viable treatment for PCOS. Addressing the ovarian insulin imbalance could break the cycle of hyperinsulinaemia and HA that is pivotal to the pathogenesis of PCOS (303). Debate surrounds the automatic treatment for insulin resistance for those with PCOS (20, 304, 305). A consensus prevails that these agents should only be used when impaired glucose tolerance is seen. Interestingly, metformin use in adolescents with PCOS may attenuate disease progression (306). Some evidence suggests that early use of metformin in high risk women e.g. low birth weight / precocious puberty, may limit onset of hirsutism and androgen excess. It has been suggested that there may be a critical window of development prior to the onset of menarche, that if targeted is more effective than treating post-menarche (307). At present the prescribing of metformin in adolescent girls remains unlicensed (308); however an RCT addressing quality of life in the adolescent found no improvement with metformin when compared to placebo (309).

The vast majority of literature focuses on metformin, discussed below. Other insulin sensitisers have been reviewed. An improvement in ovulation and HA has been shown with Thiazolidinediones (Glitazones). This family of drugs are a selective ligand of the nuclear transcription factor peroxisomes proliferator activated receptor γ. In Type II diabetics, blood sugar levels are lowered, unlike non-diabetics, where only a lowering of insulin is seen. Promising evidence for Troglitazone suggested improvement in all parameters of PCOS (310). Clinical use was aborted due to the significant risk of hepatotoxicity, leading to the agent being withdrawn from the market. Other Glitazones (Rosiglitazone/Pioglitazone) have a reduced hepatic effect but remain category C (animal studies have shown adverse effect on fetus but benefits may outweigh risk of drug use), according to the Food and Drug administration (FDA) due to animal fetal growth restriction (311). The unwanted side effect of weight gain further negates any drug benefit in PCOS women. D-Chiro-Inositol, a buckwheat derivative, also has insulin sensitising properties. Initial work suggested an improvement in ovulation, androgen profile and plasma triglyceride concentrations (312). However, this was not replicated in a large unpublished trial (20).

1.8.1 The pharmacology of metformin

Metformin is a member of the oral biguanide family of oral anti-diabetic drugs (313). The compound is produced from a heating reaction between equal amounts of dimethylamine hydrochloride and 2-cyanoguanidine. Figure 1-9 shows the structure of metformin which has a molecular formula of C4H11N5 • HCL. Under fasting conditions, the bioavailability of metformin is 50-60%. Renal tubular secretion occurs with a plasma elimination half life of ~6.2 hours (314).

Figure 1-9 - Metformin structure



The main modes of action of metformin include:

- Suppression of hepatic glucose production
- Reduced intestinal absorption of glucose
- Increased peripheral glucose uptake and utilisation, improving insulin sensitivity

1.8.2 Metformin safety profile

Metformin readily crosses the placenta in healthy pregnancies and those complicated by gestational diabetes (315). A 10 year retrospective review of the use of metformin and glibenclamide in early pregnancy, show no association with fetal anomaly; unlike the independent association with elevated mean glycated haemoglobin (316). These findings were in agreement with a Canadian meta-analysis (317) that showed no

increase in congenital malformation. Pregnant women with PCOS are at increased risk of pre-eclampsia. Despite early work suggesting an association between metformin and pre-eclampsia (318), further literature showed no ill effect (319). They also showed a protective effect against the development of gestational diabetes (GDM). In women who had had a previous pregnancy complicated by GDM, the metformin group had a further 14% GDM pregnancies compared with 40% of those without metformin (p=0.004). Similar findings have been reported (320). Use of metformin is often limited by patient compliance due to frequent gastrointestinal side effects (321).

1.8.3 The action of metformin on the ovary

The effect of metformin on follicular steroidogenesis seems to be two fold: (i) it has an indirect effect of reduced insulin excess on the ovary; and (ii) it has a direct effect on the ovary itself (322). Clinical data has shown a reduction in the CYP450c17 activity (323). A direct suppression of androstenedione has been found with a preferential suppression of 17,20-lyase over 17αhydroxylase activity (324). See Figure 1-3 for an outline of the steroid pathway within the ovary. De Leo et al also showed an increase in IGFBP1 following metformin treatment, reducing the active availability of IGF- 1 *in vivo* (43). The evidence for androgen reduction is perhaps more robust, than of either insulin concentration or SHBG (242, 325). Teissier et al (326), suggest that the follicular endocrine microenvironment was critically important for oocyte quality. In mouse models, elevated androgens have been reported to generate more meiotically incompetent oocytes (327) particularly in response to higher concentrations of either androstendione or testosterone (328). Mechanisms for this effect included elevated follicular fluid oestradiol levels and chromosome displacement on the metaphase plate. The poor oocyte quality recorded in hyperandrogenic women with PCOS has contributed to this hypothesis (329). In contrast, it has been suggested that pre-treatment supplementation of women undergoing ART treatment with testosterone may improve follicle growth by increasing granulosa cell FSH receptor levels and improving FSH sensitivity. Furthermore, testosterone pre-treatment may induce expression of anti-apoptotic microRNAs which may limit follicular atresia (330). These

opposing views may in part be related to the type of androgen in question (331). The action of metformin may modulate FSH-stimulated aromatase activity within the granulosa cells, so reducing the circulating oestradiol concentration (21). Further work has shown an inhibition of insulin-stimulated aromatase mRNA expression due to silencing of key promoters, via the MEK/ERK pathway (332). These authors have also shown that metformin increases the translocation of the GLUT-4 to the plasma membrane of human granulosa luteal cells, improving glucose uptake (333). Regardless of its direct effect on ovarian cells, the treatment response to metformin is hampered by obesity. Indeed, an inverse relationship exists between BMI and metformin treatment efficacy (334). This may explain the varied results obtained with treatment dependant on trial selection criteria, particularly in the morbidly obese (335).

1.8.4 Clinical use of metformin for the treatment of women with PCOS

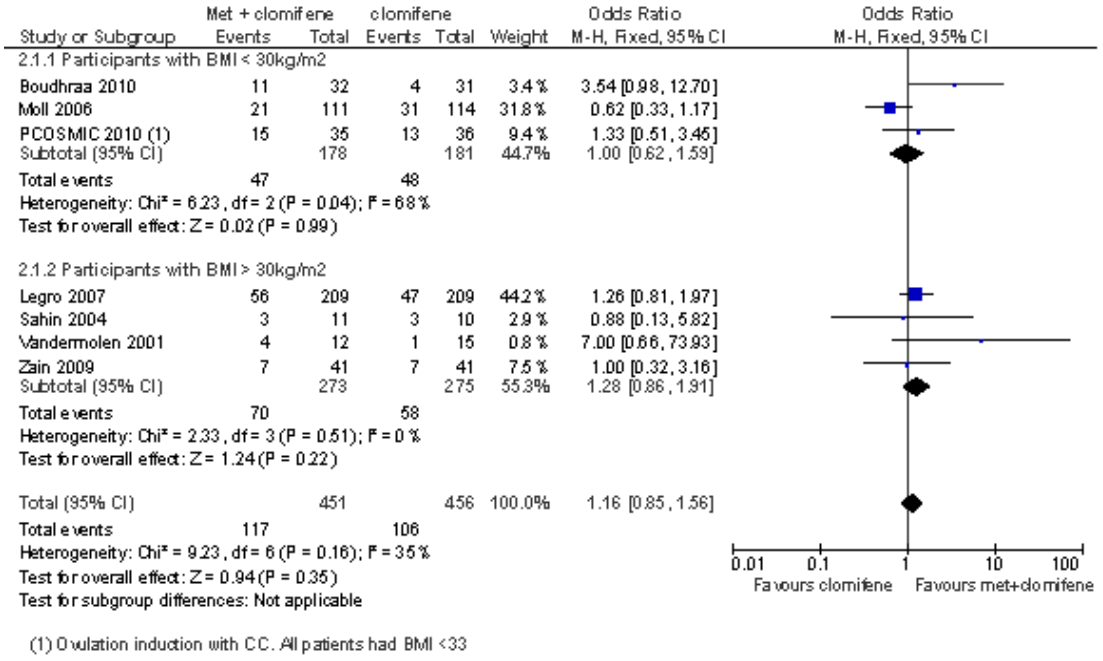
Early studies suggested an improvement in reproductive function and possible benefit to long-term health when insulin sensitisers were used to treat women with PCOS (336). Large prospective, randomized studies have, however, failed to demonstrate a consistent benefit of metformin. Indeed, metformin is not effective at achieving weight loss. Although some biochemical parameters may improve this does not translate into a significant benefit in outcomes, whether for the dermatological manifestations of hyperandrogenism (337) or the enhancement of fertility (338). A prospective randomized, double blind, placebo-controlled multicenter study evaluated the combined effect of metformin and lifestyle modification on obese anovulatory women (BMI > 30 kg/m^2) with PCOS (339) . Individualized patient assessment by a research dietitian set sustainable realistic goals with an average reduction of energy intake of 500 kcal per day. Both the metformin-treated and placebo groups managed to lose similar amounts of weight. An improved menstrual cycle was observed in those who lost weight but did not differ between the two arms of the study, reinforcing the belief that weight reduction holds the key to improved reproductive function (339).

The use of metformin combined with clomiphene citrate (CC) has been evaluated (313). In a Dutch multicenter trial 228 women with PCOS were randomly allocated to either: CC plus metformin or CC plus placebo (340). There was no significant difference in rates of ovulation (metformin 64% vs. without 72%), ongoing pregnancy (40% vs 46%) or spontaneous miscarriage (12% vs 11%). More women discontinued therapy in the metformin group (16% vs 5%) due to gastrointestinal side effects. The largest study, named *The Pregnancy in Polycystic Ovary Syndrome (PPCOS) trial*, sponsored by the NIH in North America enrolled 626 anovulatory women with PCOS (341). Randomization to 3 different treatment arms occurred for a total of 6 cycles or 30 weeks and included: a) metformin 1000 mg twice daily plus placebo, b) CC 50 mg day 3–7 of cycle plus placebo, or c) combined metformin 1000 mg twice daily plus CC 50 mg/day (days 3–7). Live birth rates (LBRs) were 7.2% (15/208), 22.5% (47/209), and 26.8% (56/209), respectively. An increased pregnancy loss rate was seen in the metformin-alone group (40.0% vs 22.6% vs. 25.5%, respectively). The results of this study may not be applicable to all women with PCOS as the average BMI was greater than 35. Dose escalation of metformin did not show significant improvement in features of insulin resistance (342).

The latest Cochrane review on metformin and ovulation induction in women with PCOS included 38 trials with a total of 3495 participants (ranging from 16 to 626 per study) (338). Trial duration ranged from 4 to 48 weeks with a median daily dose of metformin of 1500mg. Pregnancy was reported by most trials, but rarely live birth rates. Although metformin was effective in achieving ovulation in women with PCOS (metformin vs. placebo OR 1.81 (95% CI 1.13 to 2.93) and metformin plus CC vs. CC alone OR 1.74 (95% CI 1.5 to 2.0)), these benefits were not translated into LBRs (7 RCTs, 907 participants; OR 1.16 95% CI 0.85 to 1.56) (Figure 1-10). Live birth rates were significantly better in obese women with PCOS taking clomiphene than those taking metformin alone (2 RCTs, 500 participants; OR 0.30 95% CI 0.17 to 0.52 (341, 343)). For women with CC-resistance, it was suggested that the combined use of CC with metformin would improve the chance of ovulation although the numbers of women studied were relatively small and the data did not translate into an increased live birth rate. A significantly higher incidence of gastrointestinal disturbance was associated

with metformin (5 RCTs, 318 participants; OR 4.27, 95% CI 2.4 to 7.59), but no serious adverse effects were reported. Therefore, the benefit of metformin in women with anovulatory PCOS appears to be limited. Furthermore, the review did not demonstrate any benefit from metformin in improving weight loss, insulin sensitivity or lipid profiles (338).

Figure 1-10 - Metformin combined with clomiphene compared to clomiphene alone (338)



(1) Ovulation induction with CC. All patients had BMI <33

In contrast, a large study from Finland recorded an increased chance of pregnancy from 40.4% to 53.6% (OR 1.61, 95%CI 1.13-2.29) with metformin treatment. A total of 329 women received either metformin (1500mg-2000mg/day) or placebo for 3 months prior to fertility treatment and then during fertility treatment and up to 12 weeks of gestation if a conception occurred (344). Obese women seemed to gain the greatest benefit from metformin. Although there was no reduction in miscarriage rate, live birth rate was significantly increased in those who received metformin (41.9% vs 28.8%, p = 0.014).

1.8.5 The use of metformin in IVF treatment cycles

The insulin sensitizing properties of metformin are deemed a risk prevention strategy for OHSS in women with PCOS but not PCOM alone (345). One of the largest RCTs to compare metformin versus placebo in IVF cycles in women with PCOS, randomised 101 consecutive cycles (260). Metformin at a dose of 850mg twice a day was used for a period of roughly 4 weeks prior to egg collection, within a conventional long GnRH agonist protocol. No differences between placebo and metformin for total FSH dose (1300units vs. 1200units, p=0.94), number of oocytes retrieved (16.2±7 vs. 17.3±7.4) or overall fertilisation rate (52.9% vs 54.9% p=0.641) were recorded. However, a significant increase in clinical pregnancy rate (CPR) beyond 12 weeks (38.5% vs. 16.3%, p<0.05) was seen with a significant reduction in cases of severe OHSS (3.8% vs. 20.4% p= 0.02), even when age, BMI and total FSH dose were taken into account. Work by Kjotrod et al (346) found similar benefits in the pregnancy rate; lean women with PCOS had a higher live birth rate. In a further study by the same group, a total of 150 patients were randomised to receive either 2000mg/day of metformin or placebo for 12 weeks prior to IVF treatment. The live birth rate was significantly higher in the metformin group (48.6 versus 32.0; 95% CI: 1.1 to 32.2; P = 0.0383) (347). A similar reduction in OHSS was seen in a subsequent Cochrane review (OR 0.27 95% CI 0.16-0.47) (348) and meta-analysis (349). Further studies, have shown an increased total dose of stimulation required with added metformin but a lower oestradiol concentration on day of hCG trigger (350).

Historically, the GnRH agonist cycle has been used to treat infertility but the development of the GnRH antagonist has seen significant reduction in OHSS. A single small trial has used metformin in such a cycle. Although underpowered for OHSS reduction, a trend towards reduced incidence is encouraging (351). 40 patients were randomised to either 2 months pre-treatment of 1500mg/day of metformin prior to commencing an IVF cycle or to simply starting IVF. A statistically significant reduction in number of ampoules of FSH used (18±6 vs. 24±8 p<0.05) and oestradiol concentration on the day of hCG (2400±600 vs. 3370±900 p<0.05) were noted. Although no differences in embryo quality or oocyte yield were observed, an improvement in number of mature metaphase II oocytes was recorded. Although numbers were small,

fewer cycles were cancelled after metformin treatment and there was a lower incidence of OHSS.

An underlying factor in the development of OHSS is VEGF (see Figure 1-8). This glycoprotein acts to induce vascular permeability and regulates physiological and pathological angiogenesis, as discussed in Chapter 1.5. Insulin increases expression of VEGF within vascular smooth muscle via the PI3-K pathway and nitrous oxide pathway (352). By reducing insulin secretion, metformin may attenuate VEGF release and therefore reduce OHSS risk (349). A significant reduction in VEGF was shown by Tang et al (260) within the metformin treatment group of their study.

The benefits of metformin therapy may extend into subsequent frozen embryo transfer cycles. A retrospective study highlighted that for those women who had used metformin within fresh assisted conception treatment cycles, in their subsequent frozen cycle they had a significantly improved live birth rate (28.6% vs. 12.3%). This was most significant in women who had a 'freeze all' fresh attempt due to OHSS risk, whereby a 9-fold increase in live birth was seen (353). Although there was no apparent difference in embryo quality, a potential improvement in the hormonal milieu surrounding the follicles provided by metformin may lead to a metabolic improvement in the embryo. Furthermore, there was a trend for improved freeze-thaw survival of embryos in metformin treated fresh cycles. The improved follicular environment could influence the ability of the oocyte to enhance early embryo development and viability prior to zygotic genome activation (353, 354). It is highly unlikely that there would be any residual impact on uterine environment from metformin use in previous cycles on subsequent frozen cycles, especially given the average 8 month duration between cryopreservation and thaw.

1.9 *In Vitro* Maturation Of Oocytes

In vitro fertilisation techniques have many potential drawbacks when they are applied to the treatment of women with PCOS and PCOM. High cost, intense monitoring and medication side effects are only a few. The most serious risk is OHSS, which is related

to the gonadotrophin dose, stimulation length and underlying infertility aetiology. *In vitro* maturation of oocytes has been proposed as a patient friendly alternative to conventional therapies that will eliminate OHSS in women with PCOS undergoing infertility treatment. The potential benefit of IVM in the treatment of PCOS would seem clear, as it avoids the excessive ovarian stimulation required to retrieve multiple mature oocytes during conventional ART cycles. Edwards et al (355) described the successful fertilisation of *in vitro* matured oocytes. Subsequent reports of pregnancy are abundant. Cha (356) achieved a healthy triplet pregnancy in a woman with primary ovary failure. Trounson (357) showed potential for successful pregnancy in women with anovulatory PCOS. Recent literature suggests clinical pregnancy rates of 20-48% can be achieved (358-360). Despite a wealth of literature regarding IVM, high quality evidence in the form of RCTs comparing IVM against a standard IVF protocol in PCOS is sparse. The most recent Cochrane review (361) highlighted that no completed RCTs could be included. The authors highlighted 3 on going registered studies (362-364) addressing this question although recruitment had yet to start or had been suspended in at least 2 of the trials.

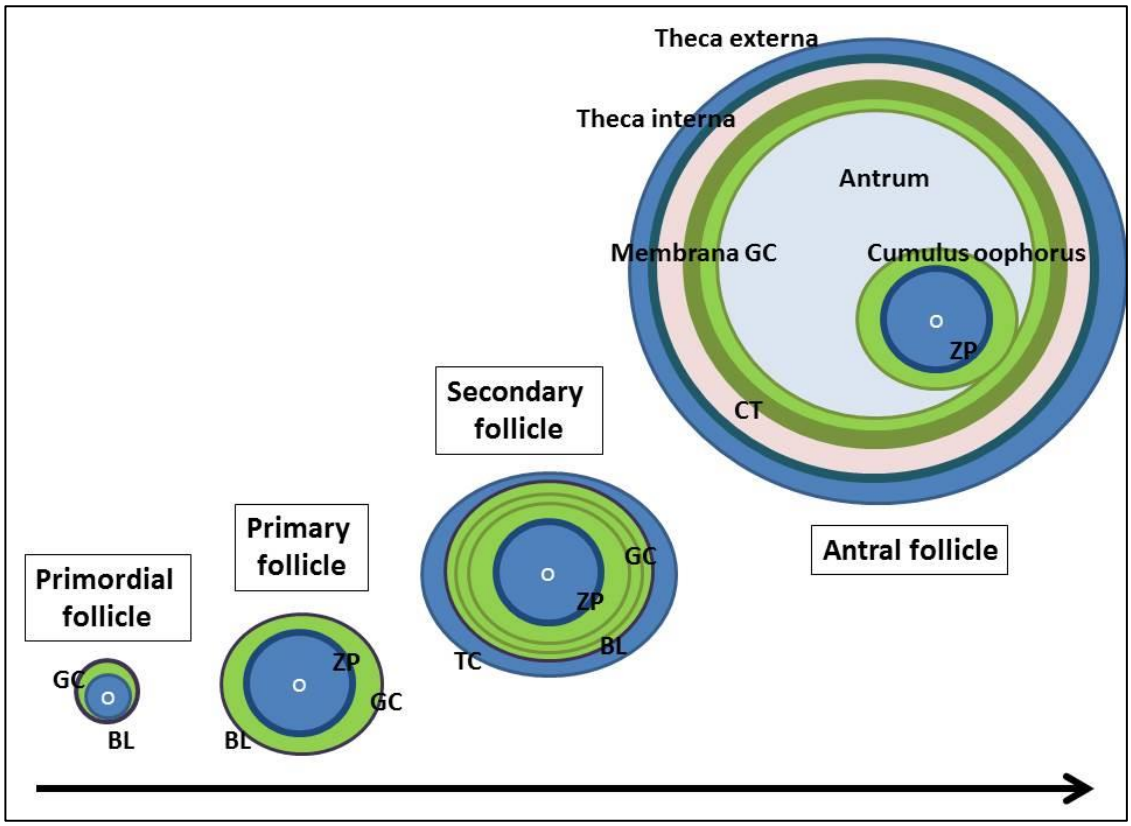
In vitro maturation involves immature egg collection followed by a period of laboratory 'maturation'. This should not be confused with the technique of *in vitro* follicle culture, whereby the follicle and oocyte architecture and interactions are maintained in extended culture in either bi-dimensional or three-dimensional *in vitro* culture systems (365-367). During IVM oocytes are cultured for approximately 24-48hrs only. When meiotic maturation is completed the oocytes can be fertilised, usually adopting ICSI. Oocyte maturation covers the recommencement and completion of the first meiotic division of the presumptive gamete to metaphase II (MII) as is manifest by the extrusion of the first polar body. This progression would normally occur prior to ovulation *in vivo*. Cytoplasmic maturation must accompany meiotic maturation to allow appropriate fertilization and to support early embryo development up until embryonic genome activation (368). Not all germinal vesicle (GV) oocytes progress either through germinal vesicle breakdown to metaphase I (MI) or on to MII. The cellular processes incurred during IVM must include protein synthesis and cumulus

cell-oocyte interaction, that mimic maturation *in vivo* in order to a produce a fertile gamete (368).

To understand the intricate system of oocyte maturation it is prudent to understand the lifecycle of the oocyte housed within the follicular structure (see Figure 1-11). The stock of primordial follicles formed between 5 - 9 months gestation, constitutes the ovarian reserve. This supply of oocytes services the entire reproductive lifespan of the individual. Folliculogenesis is the term used to describe primordial follicle recruitment into the pool of growing follicles; this process ends in either ovulation or atresia. In humans, this process can take up to 6-9 months to complete (30). Two distinct phases of folliculogenesis are described. The pre-antral (gonadotrophin-independent) stage of follicle development incorporates the growth and differentiation of the oocyte which accompanies the transition from the primordial to secondary follicle. The antral (gonadotrophin-dependant) stage describes the growth and formation of the antral follicle that is under the control of FSH, LH and multiple growth factors produced from both the oocyte and its surrounding somatic cells (369). The primordial follicle contains a small oocyte (~25µm diameter) surrounded by a single layer of squamous granulosa cells contained within a basal lamina. The histological hallmarks of the activation of primordial growth include the transition from squamous to cuboidal granulosa cells alongside oocyte growth. Evidence suggests that the oocyte itself has significant control over the activation and growth of primordial follicles (370). Two major signalling pathways are involved. These are the restraining phosphatidyl inositol 3-OH-kinase (PI3K)-AKT-Foxo3a (371) and activating pathway mammalian target of rapamycin (mTOR) (372). Furthermore, oocyte and granulosa cell derived proteins are also crucial regulators of primordial follicle survival and recruitment. Oocyte derived proteins include helix-loop-helix transcription factors and homeobox transcription factors that regulate expression of downstream genes including growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15). These proteins promote granulosa cell proliferation (30, 373). Kit ligand produced by the granulosa cells interacts with the c-kit tyrosine kinase receptor on the oocyte which promotes growth and primordial follicle activation (374). Factors separate to the oocyte-granulosa cell follicle unit are also responsible for the transition from primordial follicle

to primary oocyte. These include stromal produced KGF (also known as fibroblast growth factor-7), BMP-4, and BMP-7 (375, 376). In addition, AMH produced from growing antral follicles feeds back locally with the ovary to inhibit primordial recruitment (124).

Figure 1-11 Folliculogenesis from primordial to antral follicle



Transition from primordial follicle (squamous granulosa cells surrounding an oocyte) to primary follicle (zona pellucida present and cuboidal granulosa cells). Increasing layers of granulosa cells with the appearance of theca represent the secondary follicle before antrum formation and the antral follicle. GC: Granulosa cells O: oocyte BL: basal lamina ZP: zona pellucida TC: thecal cells CT: connective tissue

The primary follicle is recognised by 1 or more cuboidal granulosa cells arranged in a single layer around the oocyte. Expression of the FSH receptor commences with FSH being required for progression of follicle growth through the pre-antral stages (302). In concert, the oocyte dramatically increases in size to ~120µm developing the zona pellucida, an extracellular matrix. This growth coincides with the reactivation of the

oocyte genome. The highly transcriptionally active oocyte generates enough protein and mRNA to support immediate growth, maturation and subsequent embryo development (30). During the primary stage, intimate interdigitating intracellular connections are made between oocyte and granulosa cell (377). Within these connections, gap junctions facilitate transport of metabolites and signalling molecules which ultimately allow the resumption of meiosis (378). The secondary follicle is recognised by an increased number of granulosa cells forming multiple layers around the oocyte with the acquisition of a thecal layer (30). The granulosa cell layers change from simple cuboidal to a stratified columnar epithelium arrangement. Oocyte derived GDF-9 and BMP-15 are critical for this transition (379). The thecal layer differentiates through the secondary follicle stage to include a theca externa (smooth muscle cells) and theca interna. Alongside the thecal layer formation, new vessel formation occurs allowing a circulation of nutrients and gonadotrophins (380). By the end of the pre-antral stage, if an oocyte is removed from the follicular environment it has the potential to resume meiosis (381). It is widely recognised that cAMP plays a pivotal role in preventing meiotic resumption (382). High levels of oocyte derived cAMP are maintained by activation of a G –protein coupled receptor. Cyclic guanosine monophosphate (cGMP) transits via the gap junctions from the cumulus cells and inhibits phosphodiesterase 3A (PDE3A). This in turn prevents PDE3A-mediated cleavage of cAMP, maintaining meiotic arrest as shown in mice (383).

The antral follicle is characterised by the formation of a cavity filled with follicular fluid. It provides the medium that surrounds both granulosa cells and oocyte and through which regulatory proteins must pass through (384). The quantity of follicular fluid determines the size of the follicle. A dominant follicle has extensive proliferation of granulosa cells, thecal cells and follicular fluid compared with atretic follicles which rarely grow past the medium follicle size (30). The spatial organisation of the granulosa cells in relation to the oocyte, lead to 3 distinct subtypes: the membrana, peri-antral area and the cumulus oophorus. Receptors for FSH are expressed within all 3 subtypes, but the membrana also express aromatase and LH receptors (30). Oocyte morphogens including GDF-9 and BMP-15 function as gradient signals to enable the differentiation of the granulosa cells. The majority of follicles are destined for atresia. It is believed

that FSH acts as a pro-survival factor for follicles inhibiting apoptosis (385). In a highly orchestrated symphony of gonadotrophins, growth factors and signalling pathways (30), during the follicular phase of each menstrual cycle the dominant follicle responds to the pre-ovulatory surge by resuming meiosis. Following the LH surge, the mural granulosa mediate cAMP production which reduces cGMP production and leads to closure of gap junctions (386). A reduction in cGMP entering the oocyte enables PDE3A to activate and cleave cAMP and therefore allow meiotic resumption. Following ovulation, the luteinised granulosa cells and theca cells undergo remodelling to form the corpus luteum, the cells retain a high capacity to produce progesterone and oestradiol to support an implanted pregnancy (30).

1.9.1 Clinical application of IVM

Women with PCOM or PCOS are prime candidates for IVM as their naturally occurring large cohort of antral follicles provides a large reservoir of follicles for collection, increasing oocyte yield and potential embryo production. Trounson (357) showed a significantly increased oocyte retrieval between a PCO (15.3 oocytes) and non-PCO ovary (2.8 oocytes). No difference in yield was seen in anovulatory or ovulatory PCOS. Indeed, the majority of studies only consider women with polycystic ovaries as candidates for IVM. While women with normal ovarian morphology could receive IVM, the success rates from conventional ART cycles far outweigh that what can be attained through IVM. Jaroudi et al (387) employed IVM to avoid cycle cancellation in those at risk of OHSS following a long protocol IVF cycle. The majority of these women had demonstrable PCOM. Human chorionic gonadotrophin was withheld and oocyte retrieval completed. Of 21 cycles, 20 retrieved oocytes (average 8.1 oocytes per cycle) with a 70.8% maturation rate; of these 17 cycles had on average 2.6 embryos transferred (44 embryos per 17 cycles) which resulted in a clinical pregnancy rate of 9.5%. The other potential use of IVM is in fertility preservation. For some fertility preservation patients the protracted time frames of IVF/ICSI cycles are not appropriate (388) and/or supraphysiological doses of gonadotrophins may aggravate hormone dependant cancers (389, 390). For very young girls or young women who lack a life

partner and for whom IVF/ICSI treatments are not appropriate, ovarian tissue preservation and subsequent in vitro culture followed by IVM or the collection and maturation of oocytes for vitrification may be the better treatment option e.g. Turner's syndrome (391).

Despite the availability of oocytes for retrieval, oocyte quality may be reduced in women with polycystic ovaries. For example, *in vitro* matured oocytes collected from regular cycling women versus anovulatory women, showed a higher maturation and embryo development rate (392). There was no difference in embryo development between unstimulated and super-ovulated regular cycling women (368, 392). However, the initial CPRs were unsatisfactory at 1-2% (356, 357, 393). By changing the gonadotrophin priming and timing strategies, success rates of IVM now range from 16-40%. In 2008, Suikkari et al (394) reviewed the published LBR of 671 IVM cycles from which 108 children were born, and reported a 16.1% birth rate. The Oxford Fertility Unit (358) have compared matched PCOS/PCO women undergoing IVM or IVF. The LBR for these 2 groups of patients is 15.9% and 26.2 % respectively. Other studies have reported exceptionally high clinical pregnancy rates (43.9%) and live births (42.4%) (359). The latter group (359) adopted an FSH/LH only priming strategy, reduced oocyte culture duration (24-26 hours) and single blastocyst transfer compared with the former group (358) who used hCG priming pre oocyte retrieval and extended oocyte maturation up to 48hrs. Both used similar endometrial priming strategies based around oral oestradiol and vaginal progesterone supplementation.

1.9.2 Gonadotrophin priming

Early work (395) improved oocyte retrieval and maturation potential with a truncated course of FSH. An increased follicle number $\geqslant$4mm (11.7$\pm$1.2 vs. 7.6$\pm$1.2 p=0.039), oocyte yield (7.5$\pm$1.2 vs. 5.2±1.3) and meiotic progression to MII were seen following 6 days of FSH pre-treatment (71.1% vs. 43.5% $p < 0.05$). Further clinical work (396) found similar outcomes in women with PCOS. There was no difference in oocyte yield but oocytes had a significantly increased maturation potential when harvested after

gonadotrophin priming (59% 92/156 vs. 44% 36/81 p<0.05). Indeed, implantation only occurred in women who had been primed with FSH before oocyte retrieval (21.6% 8/37). It is plausible that the gonadotrophin priming may influence the endometrial receptivity through increased follicular oestradiol production, although no difference was seen in endometrial thickness between the primed and non-primed group. In support of this idea, a significant increase in serum oestradiol was measured prior to oocyte aspiration.

During ART treatments hCG is routinely used 35-37 hours prior to oocyte harvest to trigger oocyte nuclear maturation. This strategy has also been adopted in IVM. For example, a Canadian RCT (397) randomised 24 IVM cycles to receive either no priming or 10000IU of hCG, 36 hours prior to oocyte retrieval. No difference was seen in oocyte yield but maturation was significantly improved with priming (84.3% vs. 69.1% p <0.05). Speed to maturation was improved at 12 hours (8.8±3.6% vs. 0%) and 24 hours (78.2±7.1% vs. 4.9±2.5% p<0.001). Other studies have shown lower maturation rates (398). An increased dose of hCG did not improve maturation but reduced fertilisation (399). It can however be questioned whether the use of an hCG trigger *in vivo* really represents IVM or whether this strategy more closely reflects a minimal stimulation IVF treatment cycle.

The combined effect of FSH priming *in vivo* and the use of hCG as trigger is debated. Lin et al (400) found the addition of FSH to hCG primed cycles did not improve oocyte maturation, fertilisation or clinical pregnancy rate. Son et al (401) showed no difference in total maturation or fertilisation rate using both methods. At oocyte retrieval, MII oocytes were collected in the hCG primed group (11.4% vs. 0%) with more cumulus expansion. Higher CPRs were seen (39.1% with hCG priming vs. 25% with FSH priming vs. 22.2% with no priming). In mouse studies (402), the benefit of hCG over FSH priming was not confirmed. More recent work by Junk et al (359) has used FSH alone, with a maturation rate of 69.7% and fertilisation of 71.4%. Blastocyst formation was 41.7%, double that of Son's previous work (401).

1.9.3 *In vitro* maturation cycle planning

The optimum timing for oocyte collection during IVM cycles is unclear. Resumption of meiosis in oocytes from larger follicles (9-15mm diameter; 34.5%) is significantly higher than in oocytes from smaller follicles (3-4mm; 8.8%) (403). Larger oocytes display higher maturation rates (86-105µm vs. 106-125µm; 22.2% vs. 60.0%) (404). Endometrial thickness > 5mm and the presence of no dominant follicle >10mm or >14mm at the time of harvest are the most common planning factors. This evidence has led to oocyte collections for IVM being conducted on day 9-16 of the cycle in PCOS women. Apparent discordancy exists with regard to the effect a dominant follicle has on IVM outcome. In 1999, an RCT suggested that the presence of a follicle of > 10mm, led to a significantly reduced blastocyst rate (56.5% vs. 35.7%) (405). In contrast, more recent work suggested higher success rates if a dominant follicle was present. A retrospective review (406), using an endometrium thickness of >6mm to time hCG priming, grouped collections into 3 (dominant follicle <10mm/10-14mm/14mm). No significant difference was seen in oocyte maturation, fertilisation or cleavage rates following IVM but embryos transferred from group 2 had a significantly increased CPR. Further work has also shown comparable embryo quality, between oocytes from <10mm and >10mm follicles (407). This observation is important for those women with anovulatory PCOS who may not produce a follicle greater than 10mm without stimulation.

Endometrial preparation is a major limiting feature of IVM cycles. Supplementary oestradiol and progesterone are routinely used as gonadotrophin priming seems to have little impact on the endometrial thickness (395, 400). A strategy that improves the synchrony between the endometrium and embryo has been a frozen embryo transfer (FET) cycle. Unfortunately IVM outcomes do not seem to improve with FET, with a significantly reduced survival of zygotes and cleavage stage embryos being recorded; most pronounced in women with PCOS (408). The clinical pregnancy rate following freeze-thaw IVM transfers was 6.7% compared to the centres' standard freeze-thaw IVF cycles (24%) and ICSI cycles (20%). More recent work shows comparable survival and cleavage between vitrified oocytes matured *in vitro* or *in vivo* (409). De Vos et al (410) have shown a significant improvement in clinical pregnancy

rate using vitrified and warmed embryos instead of a freshly transferred embryos after IVM (31.8% vs. 9.4% p<0.03). These authors attribute this result to optimised endometrium prior to transfer. Furthermore, it has been suggested that vitrification rather than slow freezing is optimal for more sensitive *in vitro* matured oocytes or embryos. A possible reason for this is less cellular trauma caused by ice crystal formation in slow freezing methods (411). A further detrimental effect of IVM is the potential for extended culture to induce hardening of the zona pellucida (412). Consequently, the majority of centres conducting IVM use ICSI to bypass this phenomenon. A retrospective analysis (412) has compared IVM-IVF vs. IVM-ICSI. Although fertilisation was significantly improved (69.3% vs. 37.7%), implantation (14.8% vs. 24.2%) and clinical pregnancy rate was significantly better with IVF alone; this finding was most distinct in those women with PCOS.

It is clear from the literature, that consensus on the optimum protocol for IVM treatment has yet to be reached. This is discussed in more detail in Chapter 3, including an overview of the published protocols and outcomes for the majority of IVM treatments published worldwide for women with PCOS. The majority of centres use hCG priming with fewer adopting gonadotrophin stimulation. Clinical pregnancy and live birth rates continue to show a wide variation. This could in part be related to the variability in the average number of embryos transferred. The legal boundaries within the United Kingdom limit a maximum of 2 embryos for transfer in the populations concerned, whilst many studies outside the UK transfer more.

1.9.4 Safety aspects of the clinical treatment of PCOS with IVM

IVM is safer for the women with respect to OHSS when compared to IVF. Gremeau et al (358) report no episodes of OHSS in a case-controlled study. 8.2% of IVF cases had moderate–severe OHSS requiring hospital admission. Interestingly, these authors noted that of the 97 IVM cycles, 27% had previously either had OHSS or had cycles abandoned due to OHSS; highlighting the high risk group being treated. Other studies confirm the lack of OHSS with IVM treatment (413).

With any experimental treatment regarding an unborn child, safety is of paramount importance. Data available for the safety of the child following IVM is relatively limited. A Finnish team (414) reported on 46 infants born from IVM, of which 20.9% of the mothers had PCO. Preterm birth rate, birth weight and major malformation rate were all within the national average range. Increased minor developmental delay was seen at 1 year (19 vs. 10%) but equalized by 2 years. A small comparative study showed no developmental or growth delay (415). Canadian data showed no increased risk of abnormality when compared with the risk for conventional IVF/ICSI. Other studies have displayed higher abnormality (416, 417), preterm birth and lower birth weight rates (418) when ART is implemented. Interestingly, a higher birth weight with IVM has been reported which links to commercial animal studies with 'large offspring syndrome' (419, 420). Whether the type of ART treatment or the underlying infertility cause, the risk to mother and child should not be overlooked (421).

1.9.5 Epigenetics and chromosomal abnormality following IVM

Epigenetic disruption and imprinting disorders are of concern following the manipulation and extended culture of gamete and embryo during assisted conception. Angelman and Beckwith-Wiedemann syndromes have been associated with ART and specifically with the use of ICSI (422, 423). This incidence could be due to the maturation process or level of gonadotrophin stimulation. Khoueiry et al (424) observed an increased disruption in stimulated IVF oocytes when compared to natural cycle oocytes. Methylation is part of the epigenetic marking to allow parent-of-origin gene expression. By exposing the oocytes to gonadotrophins, fewer GV and MI oocytes were methylated after 28 hours of *in vitro* culture. A limited number of studies have compared the rate of aneuploidy in different assisted conception techniques. A non-significant increase in aneuploidy rates in IVM embryos was seen when compared with IVF controls (60 vs. 33%) (425). Yakut et al (426) display higher aneuploidy in certain chromosomes (11,13,15,21,22) after IVM in comparison to *in vivo* maturation. Oocytes from PCOS patients (427) confirm mice studies (428), with a significant increase in abnormal spindle and chromosome configurations in *in vitro* matured

oocytes (43.7%; 33.3%) when compared to *in vivo* matured oocytes (13.6%; 9.1%). Contrasting work from Vieira et al (429) who compare non-obese *in vitro* matured oocytes from standard IVF cycles found no significant difference in IVM rates or meiotic abnormalities between women with PCOS and other infertility causes. Microarray analysis of oocytes shows some persistent immature gene expression in IVM-MII oocytes that bears resemblance to GV oocytes. This molecular immaturity may play a part in the lower potential of IVM embryos (430). Gene expression is also altered in the cumulus complexes of IVM oocytes with up-regulation of genes involved in deoxyribonucleic acid (DNA) replication and repair (431).

OHSS remains a significant risk despite practical methods already in place, aimed at reducing the iatrogenic complication. Any further reduction in this risk should be sought. Metformin has the potential to provide a simple adjuvant solution. The drug has an established safety record and is easy to administer. Establishing if it offers further benefit in an antagonist cycle will complete the 'jigsaw' for metformin management and IVF. Similarly, IVM is a possible alternative treatment option for high risk women, limiting the exposure to gonadotrophins which potentiate the effect of OHSS. For this to be a viable treatment, efficacy and optimisation of the IVM protocol needs to be attained. Useful comparisons between standard IVF treatment and IVM would be beneficial in establishing a clinical need for this treatment.

1.10 Aims & Objectives

The overarching aim of the work presented in this thesis was to evaluate the efficacy of risk reduction strategies for the treatment of women with PCOS who are undergoing assisted conception. The evidence presented in this literature review highlights the rapid advancement in the science and technology in assisted conception. It is therefore necessary to use the new information to improve potential treatment. On this basis it is possible to hypothesise that:

"The diagnosis and management of treatment strategies for PCOS should be re-evaluated in light of the advances in understanding of AMH and the development and adaptation of treatment modalities such as metformin and IVM, to improve the outcome and management of risk for women with PCOS who undergo ART treatment".

Three inter-linked aims tested this hypothesis. These were:

Aim 1: ***AMH has clinical utility as a diagnostic test for PCOS and can be used as a marker of severity of the condition*****.**

This hypothesis is addressed in Chapter 2. The aims of this chapter were:

(i) To conduct a diagnostic study for PCOS on a prospective consecutive series of women presenting to the Infertility clinic using the AMH Gen II assay.

(ii) To evaluate the use of AMH as a substitute for the Rotterdam criteria of PCOS as a means to diagnose the phenotypic presentations of PCOS.

(iii) To evaluate the impact of age on the diagnostic utility of AMH measurements in women with PCOS.

(iv) To define the cut-off levels for AMH in the diagnostic pathway of PCOS

Aim 2: ***Metformin treatment can significantly reduce the risk of OHSS in women with PCOS undergoing a GnRH antagonist treatment cycle.***

The aims of Chapter 3 were therefore:

(i) To conduct an appropriately powered RCT to evaluate the use of metformin as an adjunct in an antagonist IVF treatment cycle with the aim of OHSS reduction in women with PCOS.
(ii) To establish if metformin had any impact on ovarian stimulation, embryo development and clinical treatment outcome within an antagonist cycle.

Aim 3: ***IVM is an effective alternative to a standard IVF assisted conception cycle for those with PCOS who wish to avoid OHSS***.

The aims of Chapter 4 were:

(i) To conduct a pilot study to optimise conditions for the use of IVM as a treatment strategy in women with PCOS, as a prelude to conducting a full RCT to evaluate the efficacy of IVM as a strategy to treat women with PCOS or PCOM.
(ii) To conduct a cost analysis of IVM as a treatment for PCOS compared with a conventional antagonist protocol.

Chapter 2
Anti-Müllerian Hormone As A Diagnostic Tool For Polycystic Ovary Syndrome

2.1 Introduction

Anti-Müllerian Hormone has an extensive literature base. Its origin, physiology and history have been discussed in detail within Chapter 1 section 1.3. The focus of this chapter is therefore to assess the diagnostic potential of AMH within the context of treatments for women with PCOS and furthermore to evaluate its ability to reflect the severity of disease in individuals. A wide literature is already available highlighting the increased systemic level of AMH in women with PCOS. Despite this, there is little consensus on the best reference range to use in the diagnosis of PCOS or what variable AMH correlates with the most closely. Whether it is purely a reflection of AMH production from the small antral follicles or also the interplay between underlying ovarian hyperandrogenism and anovulation will be discussed.

Targeted assay development, directed at some or all parts of the AMH molecule, has led to the development of a series of AMH assays. The current AMH assay has seen the commercial acquisition of the two previously available assays DSL and IOT under the ownership of Beckmann Coulter (Beckmann Coulter (UK) Ltd, High Wycombe). Beckman Coulter has subsequently enhanced their AMH assay (Generation II). Historically, a number of epitopes have been used to measure AMH levels (432-434). Through this process, the optimal detection antibodies were ascertained (F2B/7A and F2B/12H) and were subsequently used within 1 of the first commercially produced assays by DSL. This assay targeted both the pro-region and mature region of the AMH molecule. With extended exposure the first antibody will also bind to the mature region of the molecule (see Figure 1-6). This ability negates any effect of proteolysis within the sample. An interesting point is the ability for these antibodies to detect not only human AMH but other mammalian species such as Monkey and Bovine.

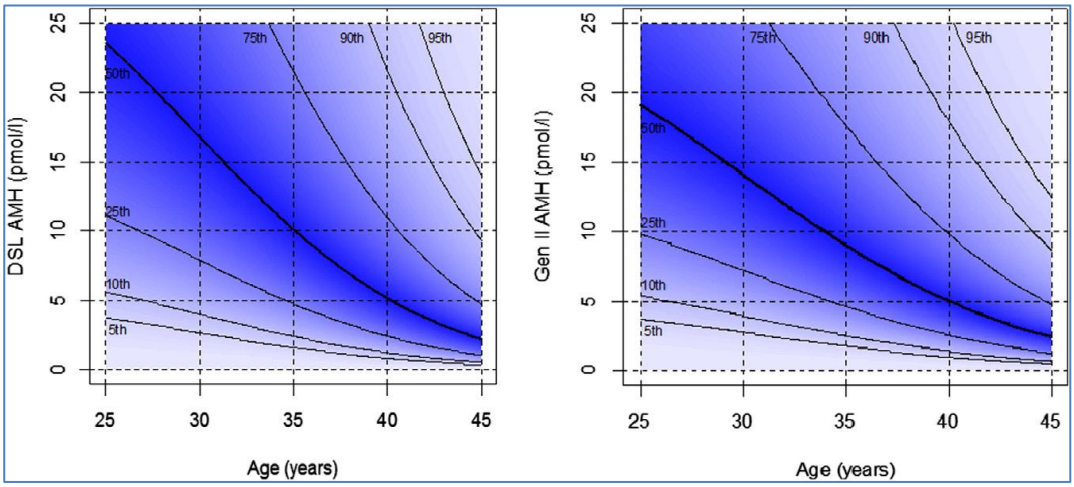
Calibration standards have varied through the history of the AMH assay. The IOT standard has been made in heat-inactivated bovine calf serum, using native AMH and has been adopted into the AMH GEN II assay. With only a single commercial assay available presently (227), impetus to create an international standard has lost momentum, despite the obvious need for such a universally accepted standard. The details of the assay remain proprietary information of Beckman Coulter. With the varying standards, results also vary. Although a significant correlation exists between the DSL and IOT samples, given the primary endpoint measurement, it is important to be aware of the units and normal range for each assay. Traditionally DSL uses pmol/l and IOT uses ng/ml. A simple multiplication conversion would not be appropriate as the different antibodies bind the AMH molecule differently and therefore the two assays do not exhibit a true linear relationship. Furthermore, values with the older DSL assay were initially approximately 40% lower than either the IOT or GEN II assay (435). More recent data has suggested that this difference may no longer exist, as there has been a systematic shift in assay calibration (436). The amalgamation of the antibodies from the DSL assay with the calibration standards of the IOT assay, have produced the only commercial assay now available from Beckman Coulter. Although care is needed when comparing studies retrospectively, from now the comparison of discriminatory cut off levels for normal ranges and discriminators of ovarian function should be easier and more reproducible (227).

Many Assisted Conception Clinics may not offer in house testing for AMH due to cost and the need for additional equipment and so they send specimens by post to external laboratories for analysis. Preparation, handling and storage of the samples affect the viability of the sample and the validity of the result. For example, a potential 31% increase in AMH has been seen with storage of whole blood at 20°C over a 3.5 day period (437) where as there is a more modest variability if it is stored at 4°C. These findings are supported by others (438). Negligible change occurs if serum separation is carried out within 3 hours of sample collection regardless of the subsequent temperature storage. These findings lead the authors to suggest an ‘enzymic breakdown phenomenon’ occurs if there is direct contact between blood cells and serum. External quality assurance testing has demonstrated wide variability between

laboratories even though data reproducibility is good within a laboratory. This may be of relevance clinically when comparing results from different laboratories (439). Trust in the assay has also been negatively affected by technical updates from Beckman-Coulter, suggesting complement interference and dilution may have an effect on the sample value.

The ability to predict a women's potential fertility at any given age has led to the generation of a number of statistical models to assess this parameter (171, 440, 441). These studies have had varying cohort sizes and backgrounds. Some of the largest studies have originated from Glasgow, UK (171, 442). The data suggests a non-linear quadratic equation provides the best model for correlating age and AMH decline. All of the models used within these studies tended to underestimate the natural log of AMH at the older age of the reproductive spectrum. Overall quadratic regression was best within the 25-45 years age bracket. Initially, the older DSL assay was used to generate the regression models (171), but more recently a similar nomogram has been produced for the GEN II (Figure 2-1). Good inter-assay agreement with the newer GEN II assay has been established concurrently by members of the same team (436). However, the only variable to be assessed within this nomogram was age and no consideration was given to other fertility based pathologies such as the presence of polycystic ovaries. The transatlantic external validation of this initial nomogram has provided evidence for its generalised predictive capacity (442). Despite a clearly different cohort of women, still from an infertile population, the quadratic relationship represents the best fit.

Figure 2-1 Comparison of AMH nomograms for age using DSL and GEN II assay (436)



Nelson et al (442) have challenged the scientific accuracy of other nomograms generated at a similar time to their own work due to their reduced sample size and AMH assay amalgamation (440, 443). Seifer et al (444) provide an alternative statistical hypothesis through a large dataset (17,120) showing a stepwise reduction in AMH with age. A mean reduction of 0.2ng/ml/year up to age 35 was seen, with further reduction to 0.1ng/mL/year thereafter. This study includes all women even at extremes of reproductive age, whom as a group were excluded by Nelson (171). These inclusion/exclusion criteria may reflect the differences in relationship seen between AMH and age. All studies acknowledge that sub-groups of patients have not been investigated. A nomogram specific for ethnicity and presence of polycystic ovaries would be a useful research tool.

The unification of different assays to form the single Generation II (Beckmann Coulter) assay has provided a stepping stone towards an international standard needed in this field. The inherent difficulties in combining data from different assays should be laid to rest. Few publications to date (only 2 (228, 229)) have used the Gen II assay in PCOS diagnosis. The present study therefore aims to provide further statistically relevant information to aid the interpretation and dissemination of evidence in support of the use of AMH measurements in the diagnosis of PCOS. Neither of the aforementioned studies (228, 229), used a clearly defined consecutive series. A prospective consecutive series including all patients fulfilling the selection criteria provides the ideal diagnostic

accuracy study (445). For example, the entire group of patients that the test would routinely be carried out on. In the case of AMH testing this may be either in the secondary or tertiary clinical setting. This is to ensure that the outcome of such a study has good clinical applicability. Instigating restrictions in eligibility or not including all women within a given time period may subject the study findings to bias. Two examples of such bias include limited challenge bias (446) and bias created from diagnostic case control (445, 447). The former type relates, for example, to exclusion of more difficult cases. In doing this, there may be expected to be a lower number of false positive or false negative results enhancing the specificity and sensitivity of the study outcome. Studies which select the populations to study using 2 separate selection processes (sampling of those with the target condition and healthy controls) can overestimate the diagnostic performance. A well designed consecutive series therefore provides a superior study design for diagnostic studies and is needed for the validation of the AMH Gen II assay in relation to PCOS.

The aims of this chapter were:

(i) To conduct a diagnostic study for PCOS on a prospective, consecutive series of women presenting to the Infertility clinic using the AMH Gen II assay.

(ii) To evaluate the use of AMH as a substitute for the Rotterdam criteria of PCOS.

(iii) To evaluate the utility of AMH in diagnosing the phenotypic presentations of PCOS.

(iv) To evaluate the impact of age on the diagnostic utility of AMH in PCOS.

(v) To provide appropriate cut-off levels for AMH in the diagnostic pathway of PCOS.

2.2 Materials And Methods

2.2.1 Eligibility criteria and baseline investigations

Formal ethical approval (ethics reference 12/LO/0679) for this project was granted from Leeds West Ethics committee. All patients attending an appointment at Leeds Centre for Reproductive Medicine (LCRM) in either outpatient or treatment clinics were eligible. The only exclusion criteria, was active current hormonal treatment. An information sheet regarding the trial was supplied with verbal discussion during the appointment with the doctor/designated research nurse. If the patient wished to take part written consent was completed, allowing a sample of blood to be taken and stored, alongside routine blood tests. Consent was gained to access the patients' record to allow a concise history to be recorded.

Consecutive patients within the LCRM registrar outpatient clinics were asked to take part. Patients were also included from the 'LCRM treatment' clinic. These patients had completed baseline investigations and were due to commence ART treatments. These treatments included ovulation induction or *in vitro* fertilisation with or without ICSI. Patients were only recruited at the starting point of their treatment cycle prior to hormone administration. These patients were assigned a 'Treatment' code to separate them from the 'Outpatient' group. The number of patients who declined or were not eligible for recruitment was not recorded.

The case notes were reviewed to compile a relevant history for each patient. This included: age, BMI (kg/m^2), ethnic background, duration of infertility, cause of infertility, menstrual cycle, patient reported symptoms of acne or hirsutism, ultrasound ovarian volumes, ultrasound antral follicle count and the requested blood tests as discussed next. Although symptoms of hyperandrogenism were not formally quantified, a patient's subjective perception of either acne or hirsutism was recorded.

The routine clinical serum investigations (see Table 2-1) were completed with the addition of AMH. If possible, phlebotomy investigations were completed between days

1-3 of the menstrual cycle. These included FSH / LH / Oestradiol / Testosterone / Androstenedione / SHBG. Thyroid function tests (TFTs) and prolactin levels were evaluated to rule out other endocrine pathology that may affect the menstrual cycle. Blood samples were taken by the routine phlebotomy services and samples processed by blood sciences staff. In most instances, samples were separated and serum analysed within 12-18 hours of collection (although this was not formally evaluated). Samples for oestradiol, testosterone, androstenedione and SHBG were stored frozen (-20°C) and analysed in the Supra-Regional Assay Service (SAS) laboratory at Leeds (http://www.sas-centre.org/centres/hormones/leeds). A further aliquot of serum was stored for AMH analysis. If an early menstrual phase test was not possible, for example due to patient geographical location, systemic AMH concentration was measured randomly in the cycle (141) and the correctly timed hormonal profile within the last 6 months was recorded. Leeds Centre for Reproductive Medicine is a tertiary centre for reproductive treatment with a treatment radius of several hundred miles. To allow sample storage without delay or to avoid difficulties with transport, samples were taken at the clinic appointment and stored at -20°C; the stage of the menstrual cycle was noted.

A baseline trans-vaginal ultrasound was completed as routine either at LCRM or Leeds General Infirmary (LGI). The scan was usually done at random through the women's menstrual cycle due to availability of scanning appointments within the hospital. Machines used included a Philips HD15 and Philips HD11XE with a C8-4V trans-vaginal probe (Philips healthcare, the Netherlands). Baseline scans were completed by trained ultrasonographers or radiologists. The day of the menstrual cycle was noted. Ovarian volume was measured using 2 perpendicular planes of direction through the ovary. The volume was calculated using the formula $V = 0.526 \times \text{length} \times \text{height} \times \text{width}$. The total number of follicles 2-9mm was recorded, alongside any dominant follicles or cystic structures that could affect the ovarian volume. If the number of follicles exceeded 12 and/or had a volume >10cm^3 on either ovary, a label of PCOM was given.

Table 2-1 Serum blood tests

Serum test	Method	Normal values	International standard (IS)	Quality control mean values with Between batch CV
Gonadotrophins	Siemens Advia Centaur & Siemens reagents (Siemens Healthcare, Camberley, UK)	Female follicular and luteal phases: FSH 1-8 iu/L LH 1-10 iu/L	FSH: WHO 2nd IS94/632 LH: WHO 3rd IS75/537	7.9 cv 6.5% / 45.5 cv 2.9% 3.4 cv 4.6% / 69.6 cv 5.5%
Oestradiol	In house radioimmunoassay (RIA) after ether extraction	Female Premenopausal 200-2000 pmol/L	In house calibrations	248 cv 6.8%
Testosterone (100, 448)	Isotope-dilution liquid chromatography – tandem mass spectrometry (LC-MS-MS) Leeds SAS laboratory	Female adult <1.8 nmol/L	In house calibrations	0.8 cv 5.3% / 5.3 cv 3.5%
Androstenedione (100)	Isotope-dilution LC-MS-MS (Leeds SAS laboratory)	1.4 - 7.4 nmol/L	In house calibrations	3.5 cv 6.5% / 8.7 cv 6.8%
SHBG	Siemens Immulite 2000 & Siemens reagents	Female adult 18-114 nmol/L	WHO 95/560	5 cv 5.7% / 73 cv 8.8%
TFTs (Thyroid Stimulating Hormone (TSH), Thyroxine (T4))	Siemens Advia Centaur & Siemens reagents	TSH 0.2-6.0 mU/L Free T4 10-25.0 pmol/L	WHO 3rd IRP81/565	0.7 cv 3.0% / 26.4 cv 4.4% 11.9 cv 5.7% / 45.7 cv 3.7%
Prolactin	Siemens Advia Centaur & Siemens reagents	<600mU/L	WHO 3rd IS 75/537	153 cv 2.6% / 907 cv 3.3%

A diagnosis of PCOS was given if 2 out of 3 criteria were met, as outlined within the Rotterdam consensus for PCOS (449). Criteria include: oligo-/anovulation, clinical and/or biochemical features of HA and PCOM. Anovulation was defined as the presence of amenorrhoea or oligomenorrhoea (OA; cycle length greater than 35 days).

As previously stated no formal method to assess clinical hyperandrogenism was undertaken and records relied on the patients' perception to provide this information. Biochemical HA was assessed by measuring serum testosterone by tandem mass spectrometry. A value greater than >1.8nmol/L defined laboratory HA.

2.2.2 AMH analysis

One additional serum gel separator tube was taken from the patient. Samples were transported as whole blood from Seacroft Hospital to the central laboratories at either St James' University Hospital or the LGI. They were centrifuged on arrival at the central laboratory within a maximum 9 hours from sampling. The separated serum was stored in aliquots ready for batch analysis at -20˚C. Storage time was less than 18 months. Although some samples had been processed prior to June 2013, due to a field safety notice (FSN 20434-3) issued by Beckmann Coulter, all samples were subsequently reanalysed using the amended protocol. This led to an additional step whereby the sample was mixed with AMH Gen II assay buffer. This was instigated due to complement interference in fresh or freshly frozen undiluted samples producing results up to 70% lower than expected.

Anti-Müllerian Hormone generation II enzyme linked immunosorbent assay (ELISA) was obtained from Beckman Coulter (High Wycombe, Buckinghamshire, UK). This assay is an enzymatically amplified two-site immunoassay. The method was not calibrated against any international standard. Liquid calibrators provided by Beckmann Coulter were traceable to their working standards. The 7 AMH Gen II calibrators were labelled 'A-G'; with 'A' containing 0ng/mL of AMH and 'B-G' containing defined concentrations of bovine AMH in bovine serum with sodium azide. These were prepared into 100ul aliquots and frozen. Once thawed ready for use, the aliquot was stable for 7 days stored 2 to 8°^{c}. One set of quality controls (QC) were provided, containing a low and high concentration of bovine AMH in bovine serum with sodium azide. These were run at the beginning and end of each run, with the same storage and stability as the calibrators. Internal quality control checked that results were within 2 SD limits calculated from previous runs.

Figure 2-2 AMH stepwise assay protocol

- As sample thaws, warm kit room temperature. Mix reagents & samples by inversion. Samples spun at 3000rpm for 10 minutes room temperature.
- Blank plate plan completed and stored for reference, showing position of calibrators, QC and patient samples with appropriate lot numbers and dates.
- Prepare 1 part calibrator, control or test sample with 5 parts AMH Gen II assay buffer (protein based buffered bovine serum albumin with <0.05% Proclin300 and sodium azide, green coloured) e.g. 20uL of sample with 100uL of buffer. Mix thoroughly. No dilution factor required.
- Within 1 hour of preparation, using Eppendorf dispenser (Eppendorf UK Limited, Stevenage, UK) with a 2.5ml barrel dispensing 2 x 50uL (limits risk of reagents splashing into surrounding wells), 120uL of premixed sample pipetted into appropriate wells of Anti-AMH Gen II antibody coated micro-titration microplate strips (flat bottomed polystyrene wells with immobilised ant-AMH IgG).
- Plate covered and incubated on Heidolph Vibramax 100 plate mixer (Heidolph UK, Saffron Walden, UK), set at 600-800rpm for 1 hour at room temperature.
- During first incubation, set up Thermo Wellwash 4 MK 2 plate washer (Thermo Fisher Scientific Inc., Waltham, USA). Waste container emptied and wash container rinsed with distilled water. Sufficient wash concentrate I (buffered saline with non-ionic detergent) diluted 10-fold with distilled water and placed in wash container. Plate washer set for 5 x 400µL washes with ~30 seconds soak between washes.
- Following first incubation plate aligned on washer table and wash cycle run. Wells dry end of wash cycle.
- 100µL of Antibody-Biotin Conjugate (protein based buffer with <0.05% Proclin300 and sodium azide, green coloured) added to each well before incubating, shake for 1 hour and wash as before.
- 100µL of Streptavidin-Enzyme conjugate (streptavidin/horse radish peroxidase in a protein based buffer with <10% methanol) added to each well and incubated, shake 30 minutes and wash as before.
- 100µL of TMB Chromogen solution (tetramethyl benzidene in citrate buffer with hydrogen peroxide) added to each well avoiding direct exposure to sunlight. White paper placed on mixer table beneath plate and mixed for 8-12 minutes. After 8 minutes, top calibrator well checked for definite blue colour. Extremes of ambient temperature can affect incubation time (too long gives erroneous absorbance value for top standard). Visual monitoring for colour development optimised incubation time.
- Do not wash plate before final addition of 100µL Stopping Solution A (0.2M sulphuric acid), whereby colour changes from blue to yellow.
- Plate loaded on to Bio-Rad Benchmark Plus microplate reader (Bio-Rad Laboratories LTD, Hertfordshire, UK) within 30 minutes, connected to Bio-Rad microplate manager version 5.2. Degree of substrate turnover determined by dual wavelength absorbance measurement at 450nm and 600-630nm (directly proportional AMH concentration in individual sample).
- Software automatically displays raw absorbance, absorbance with blank subtracted, a curve, results and plate map. Curve plot cubic as found to give best fit and normally ~straight line.
- Prior to authorisation of results, curve checked for reasonable fit (close to a straight line at least up to middle standard), duplicate % CV's checked for discrepancies and QC should be within 2 SD of mean. Limit of detection / functional sensitivity is 1.5pmol/l (449). The manufacturer kit states Limit of Quantitation (LoQ) is 1.1pmol/L.

All calibrators, QC and patient samples were run in duplicate. Up to 96 samples could be run at once including 37 'unknowns' (patient samples) run in duplicate. All analyses were performed by Ms Nicola Calder in the Research and Development (R&D) department of Blood Sciences, Pathology, Leeds Teaching Hospitals Trust (LTHT). The quality control data included: QC 1 mean 20.6pmol/L, inter assay CV 8.2%; QC 2 mean 66.2pmol/L, inter assay CV 12.3%. The stepwise process of the AMH assay is outlined in Figure 2-2.

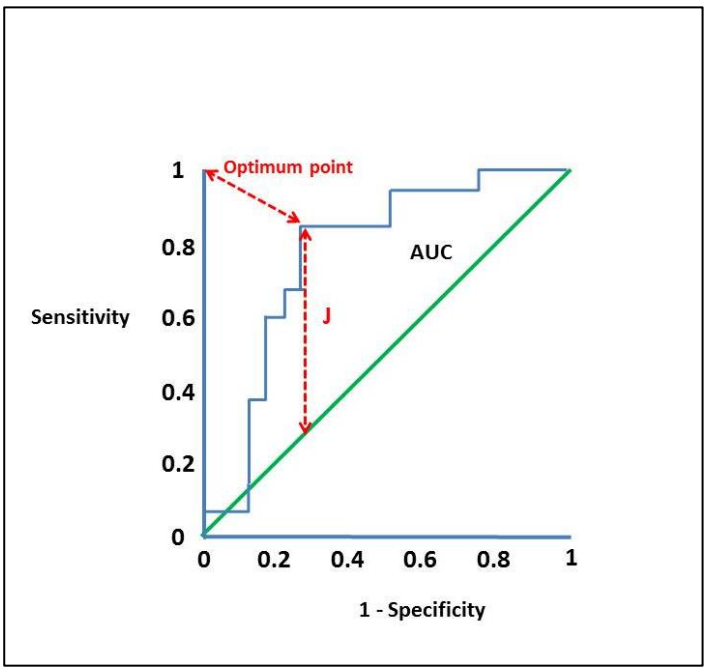
2.2.3 Statistical analysis

Data was processed using IBM SPSS 21 statistics package (IBM Corporation, New York, USA). Descriptive statistics included mean, SD, median and interquartile range (IQR) and geometric mean plus 95% CI. Continuous data was tested for normality using the Kolmogorov-Smirnov test. If data were positively skewed, a log transformation was used where appropriate to allow parametric tests to be completed (Testosterone, Androstenedione, Prolactin and AMH). Data were then back transformed and presented as the geometric mean with 95% confidence intervals. Data that were normally distributed were analysed by Student's t-test and one-way Analysis of variance (ANOVA). Parametric data are presented as mean and SD (age). Data that were not normally distributed including following log transformation, was analysed using Mann-Whitney and Kruskal-Wallis tests. This data is presented as median and the IQR (BMI, FSH, LH and SHBG); contingency tables were employed where necessary with Chi-squared statistic and Fisher's Exact test. Although the p value of <0.05 is often adopted as the cut off significance level in medical research, for this chapter $p<0.01$ was considered significant. This was to limit error artificially created by performing multiple significance tests, thereby reducing the type 1 error rate (450). With AMH as the dependant variable, multiple regression was used to establish if the individual Rotterdam criteria for PCOS were independent predictors of AMH. All statistical results were presented in a comparable manner with other published literature to allow easier interpretation and comparison.

A ROC curve (451) was used to assess the diagnostic value of AMH as a means to diagnose PCOS (sensitivity plotted against 1-specificity). The performance of AMH was quantified by calculating the AUC with a 95% confidence interval. An ideal test would have had an AUC of 1 whereas a value of 0.5 shows no diagnostic ability greater than chance. In simple form, an optimal test will have a curve that runs close to the top left hand corner of the graph. Youden's index is often used in conjunction with ROC curves. The Youden's index (J) is a function of sensitivity and specificity, frequently used to evaluate the overall effectiveness of a diagnostic test (452). It is advantageous over AUC by providing a single threshold diagnostic value which maximises the sensitivity and specificity of the test. The values range from 0 to 1. One implies a perfect test (with no false negatives or positives) and close to 0 implies a test of limited value. The J value is the maximum vertical distance on the ROC curve from the diagonal line of chance, with the intersection on the curve providing the optimal cut off value of the test (see Figure 2-3). The J value is defined over all the cut points (c) as:

$$J = \text{maximum } [\text{sensitivity}(c) + \text{specificity}(c) - 1\}$$

Figure 2-3 Example of a receiver operator characteristic curve, depicting the maximal vertical position from the diagonal line (J) with the area under the curve (AUC) including all the area beneath the blue stepped line.

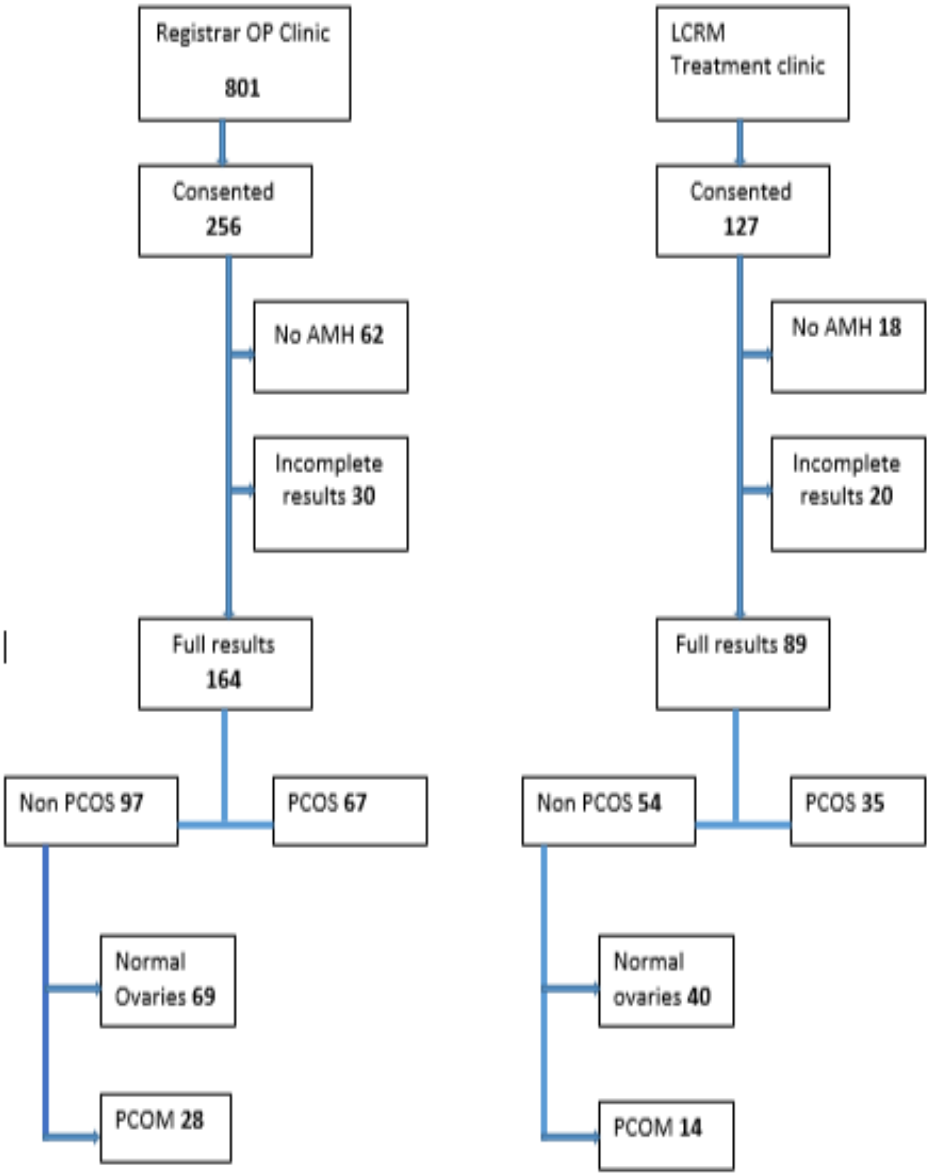


With the suggested AMH values generated from the study, a series of substitutions were then carried out on the consecutive group of women. The substitutions involved all of the individual criteria of PCOS (Rotterdam) and reclassified the population of women with PCOS. Repeated substitutions were then carried out with a higher value of AMH if a significant deviation (over-classification) in the numbers occurred. Fisher's exact test was used to assess if there were significant differences in proportions of women diagnosed with PCOS using AMH *in lieu* of the standard Rotterdam criteria.

2.3 Results

Between June 2012 and May 2013, 383 patients consented to have their AMH blood test taken for research alongside their routine investigations. Figure 2-4 outlines how the patients were recruited and the number of complete results obtained. There were 253 complete (all diagnostic results available) sets of results, grouped as a consecutive series from the outpatient clinic setting and also from the treatment clinic. All diagnostic performance testing used the consecutively recruited outpatient group to ensure results obtained from the study were representative of the population studied. Descriptive results used the entire dataset. Disappointingly, the remaining 130 patients did not have either an AMH test taken and stored or diagnostic tests completed. These patients were therefore excluded from the study analysis. It was also apparent that despite requesting specific information on the baseline ultrasound (total antral follicle count), a variety of reporting styles occurred. The majority of ultra-sonographers reported the entire antral follicle count, but there was a common occurrence for a report to state multiple/12 or more/ polycystic appearance of ovaries. For the purpose of this study, these cases have been labelled as having 24 follicles if both ovaries were involved. This may have underestimated the total number of follicles present and makes comparison with the biological markers less accurate.

Figure 2-4 Patient recruitment pathway (consecutive series is from the registrar OP clinic)



2.3.1 Patient demographics categorised by PCOS status

Patients meeting the Rotterdam diagnostic criteria for PCOS comprised 40% of the study population. Table 2-2 tabulates the differences in both AMH and baseline variables for women with PCOS, PCOM and normal ovarian morphology. Women presenting with PCOS were significantly younger ($p<0.01$) than those with normal ovaries with a lower mean FSH. LH, Androstenedione and Testosterone were significantly raised ($p<0.01$) in the PCOS population. Women in the intermediate group

with isolated PCOM were significantly younger than those with normal ovaries but similar in age to those with PCOS. Those with PCOM also had a significantly increased ($p<0.01$) androstendione, testosterone and AMH compared with those with normal ovaries. Only 5 instances of deranged TFTs were found which were evenly spaced through the groups. Figure 2-5 depicts the increase in median AMH through the 3 categories, with PCOM being situated part way between normal ovaries and PCOS.

Figure 2-5 AMH concentration categorised by polycystic ovary morphology (outliers: °>1.5xIQR *>3xIQR)

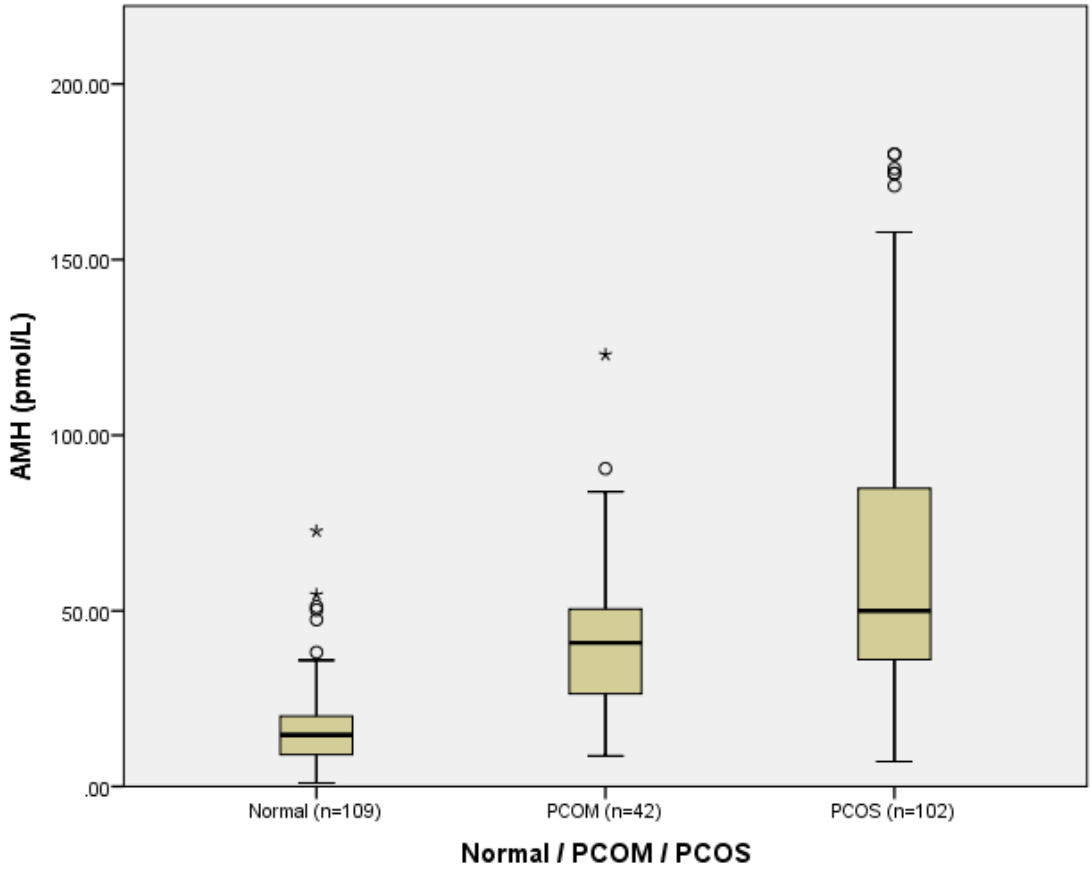


Table 2-2 Baseline demographics by PCOS status

	A Normal *(n=109)*	B PCOM *(n=42)*	C PCOS *(n=102)*	A vs. B	A vs. C	B vs. C	Overall significance value (p)
Age (Years)	33.2 ± 4.3	30.9 ± 4.5	29.6 ± 4.8	2.3, 0.7-3.9, 0.02[3]	3.6 (2.4-4.8), <0.01[3]	1.3 (-0.4-3.0), 0.36[3]	<0.01[1]
BMI (kg/m²)	25 (22.4-27.5)	23.8 (21.0-27.7)	26 (22.0-29.0)	1.2, 0.14[4]	-1.0, 0.20[4]	-2.2, 0.03[4]	0.07[2]
FSH (iu/L)	7.6 (6.2-9.3)	6.3 (5.2-7.7)	6.2 (5.2-7.6)	1.3, 0.001[4]	1.4, <0.01[4]	0.1, 0.94[4]	<0.01[2]
LH (iu/L)	4.9 (3.4-6.3)	5.1 (3.5-6.9)	6.7 (4.4-10.2)	-0.2, 0.49[4]	-1.8, <0.01[4]	-1.6, <0.01[4]	<0.01[2]
SHBG (nmol/L)	56 (39.3-79.0)	62 (48.8-79.5)	47 (29.3-68.5)	-6.0, 0.60[4]	9.0, 0.01[4]	15.0, 0.02[4]	<0.01[2]
Testosterone (nmol/L)*	0.8 (0.8-0.9)	1.0 (0.9-1.1)	1.4 (1.3-1.5)	0.8 (0.7-1.0), 0.02[5]	0.6 (0.5-0.7), <0.01[5]	0.7(0.6-0.9), <0.01[5]	<0.01[1]
Androstenedione (nmol/L)*	3.8 (3.5-4.0)	5.0 (4.3-5.8)	5.8 (5.2-6.4)	0.8 (0.7-0.9), <0.01[5]	0.7 (0.6-0.7), <0.01[5]	0.9 (0.7-1.0), 0.11[5]	<0.01[1]
Prolactin (mU/L)*	206.6 (188.3-226.7)	182.8 (157.0-212.9)	197.5 (186.6-216.0)	1.1 (1.0-1.4), 0.18[5]	1.6 (0.9-1.2), 0.49[5]	0.9 (0.8-1.1), 0.38[5]	0.38[1]
AMH (pmol/L)*	13.2 (11.5-15.4)	37.8 (32.1-44.7)	53.2 (46.2-61.3)	0.4 (0.3-0.4), <0.01[5]	0.3 (0.2-0.3), <0.01[5]	0.7 (0.6-0.9), 0.01[5]	<0.01[1]

Mean ± SD, Median (IQR), *Back transformed Geometric mean (95% CI)
[1]ANOVA [2]Kruskal-wallis test [3]T test (mean difference (95% CI difference), significance level) [4]Mann-Whitney U test (median difference, significance level) [5]T test (ratio geometric mean (95% CI difference), significance level)

2.3.2 Factors affecting AMH

There was no significant association (p>0.05) between AMH and BMI. The AMH value did not change by BMI category within normal, PCOM or PCOS women (Figure 2-6). The lack of association was confirmed by linear regression, using AMH as the dependant variable (0.46, -0.7 – 1.6 95% CI, p=0.43). There were clear correlations between the individual criteria of PCOS (Rotterdam criteria) and serum AMH concentration (Figure 2-7). All of these associations were significant (p<0.01). The AMH concentration increased with follicle number and ovarian volume, with the highest values attributable to what would be classified as PCOM (24 plus follicles). With both evidence of hyperandrogenism (Figure 2-7) and increasing cycle length (Figure 2-7D) a significant (*p* <0.01) increase in AMH concentration was seen. Table 2-3 used multiple regression to show the independent variables used to diagnose PCOS were related to AMH level. Testosterone, PCOM and cycles >3 months remain significant predictors of AMH when controlling for BMI. This outcome remains correct whether the log transformed or the raw data were used. Although there were correlations between the predictor variables, none of these correlated highly.

Table 2-3 Multiple regression: Dependant variable log (AMH)

Model		Unstandardized coefficient		
		B (slope)	**95% CI for B**	**Significance level**
1	**(constant)**	1.13	1.08 – 1.19	<0.01
	PCOM	0.56	0.48 – 0.63	<0.01
2	**(constant)**	1.16	1.10 – 1.21	<0.01
	PCOM	0.44	0.36 – 0.51	<0.01
	Log $_{10}$ Testosterone	0.54	0.37 – 0.70	<0.01
	Normal vs. >3 month cycles	0.12	0.00 – 0.23	0.04
	Normal vs. <3 month cycles	0.07	-0.02 – 0.15	0.14

Note R^2 = 0.46 for model 1; ΔR^2 = 0.10 for step 2 (p<0.001)

Dependant variable: log (AMH) pmol/L

Figure 2-6 Anti-Mullerian hormone and body mass index. A: Anti-Mullerian hormone by PCOS status categorised by body mass index; B: Anti-Mullerian hormone and body mass index correlation

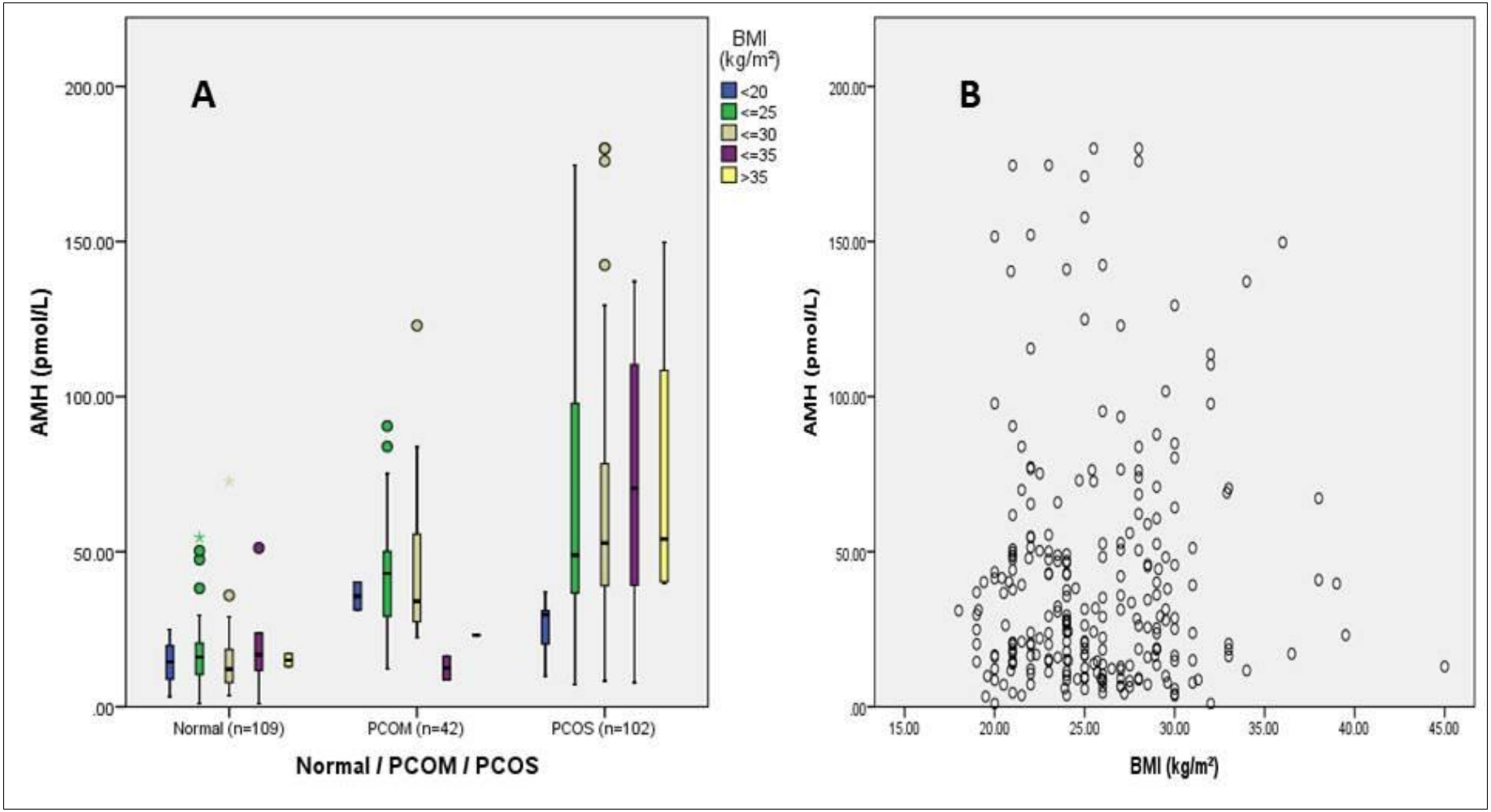
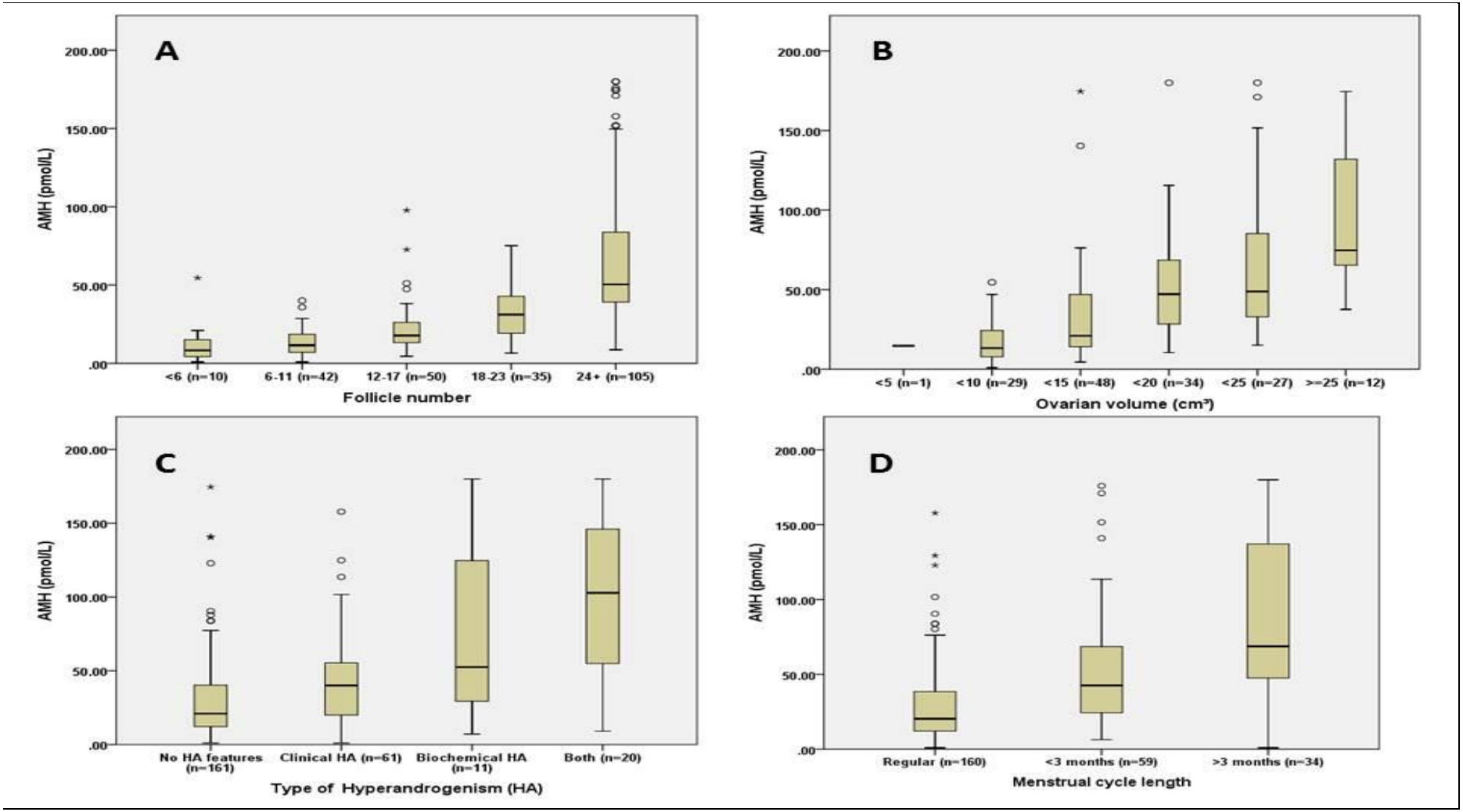
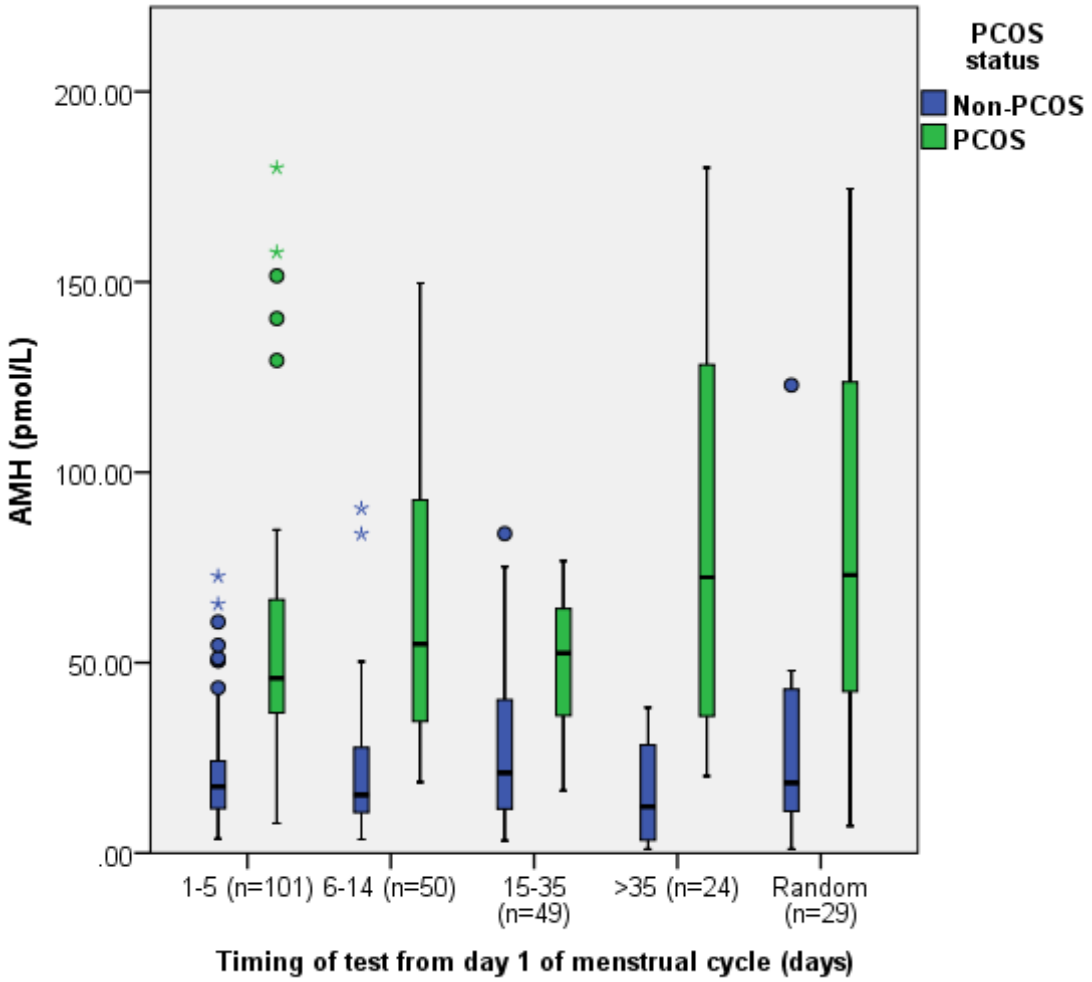


Figure 2-7 AMH serum concentration and individual criteria of PCOS (Rotterdam) A: Total follicle number B: Combined ovarian volume C: Features of hyperandrogenism D: Menstrual cycle length (outliers: °>1.5xIQR *>3xIQR)



Within this study population, the timing of the AMH test within the menstrual cycle was recorded (Figure 2-8). If it was taken within a 35 day cycle, there was no difference in AMH value when grouped by early follicular, late follicular and secretory phases. If taken after 35 days since the last menstrual period then there was a significantly higher AMH (0.54, 0.37-0.78, p=0.001). Of the study population, 29 patients were excluded from this calculation as the menstrual timing was not recorded.

Figure 2-8 AMH concentration by timing of test in menstrual cycle between women with and without PCOS (outliers: °>1.5xIQR *>3xIQR)



Ethnicity (Figure 2-9) and parity (Figure 2-10) were the final 2 parameters reviewed. Separating the population according to ethnicity, generally into Black, White or South Asian revealed no significant difference in systemic AMH concentrations (*p*=0.14). When the two largest populations (White and South Asian) were compared, although there was a difference in geometric mean (26.9 (23.7–30.5 95% CI) pmol/L vs. 35.0 (25.3-48.6 95% CI) pmol/L) of serum AMH, this was not significant (0.8, 0.6-1.1, p=0.13). Likewise with parity, there was no difference in serum AMH concentration whether a woman had had a successful pregnancy or not (1.2, 0.3-1.8, *p*=0.23).

Figure 2-9 Anti-Müllerian Hormone concentration comparison between the White and South Asian population separated by Polycystic Ovary Syndrome (outliers: °>1.5xIQR *>3xIQR)

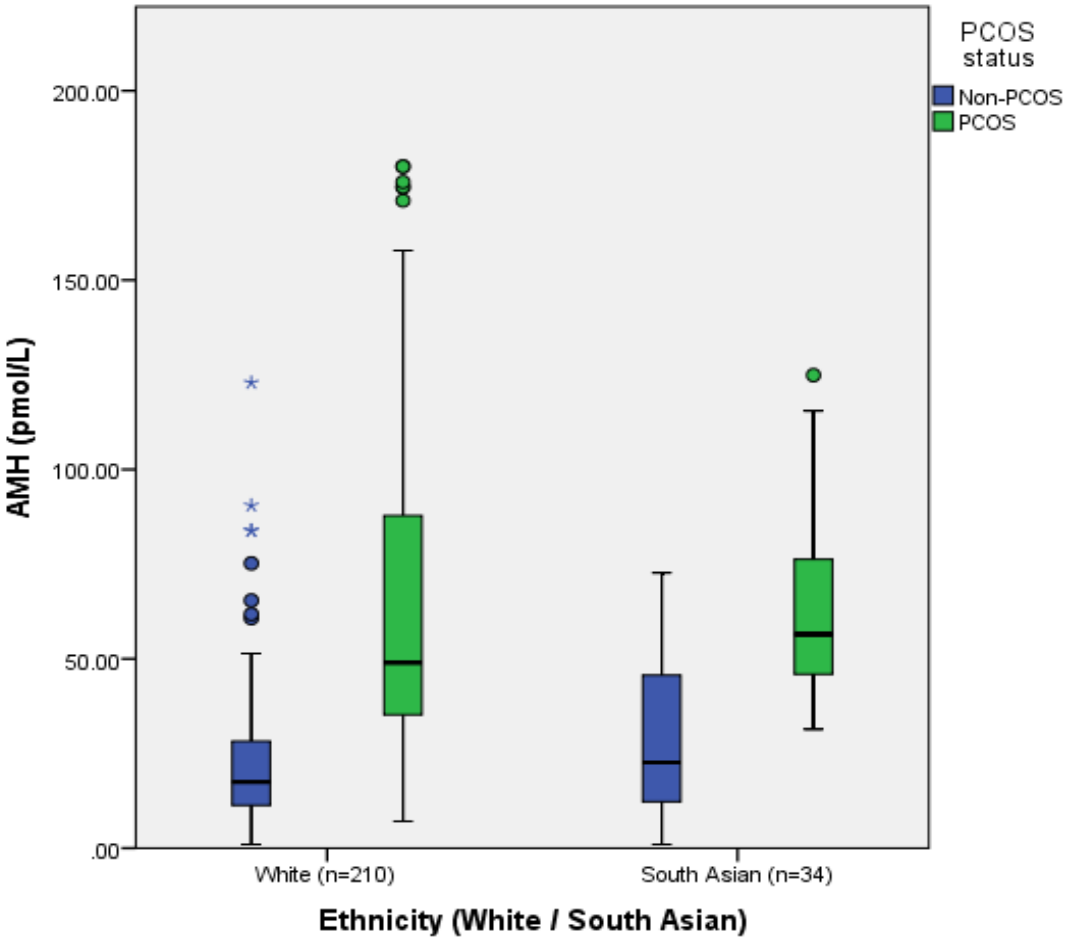
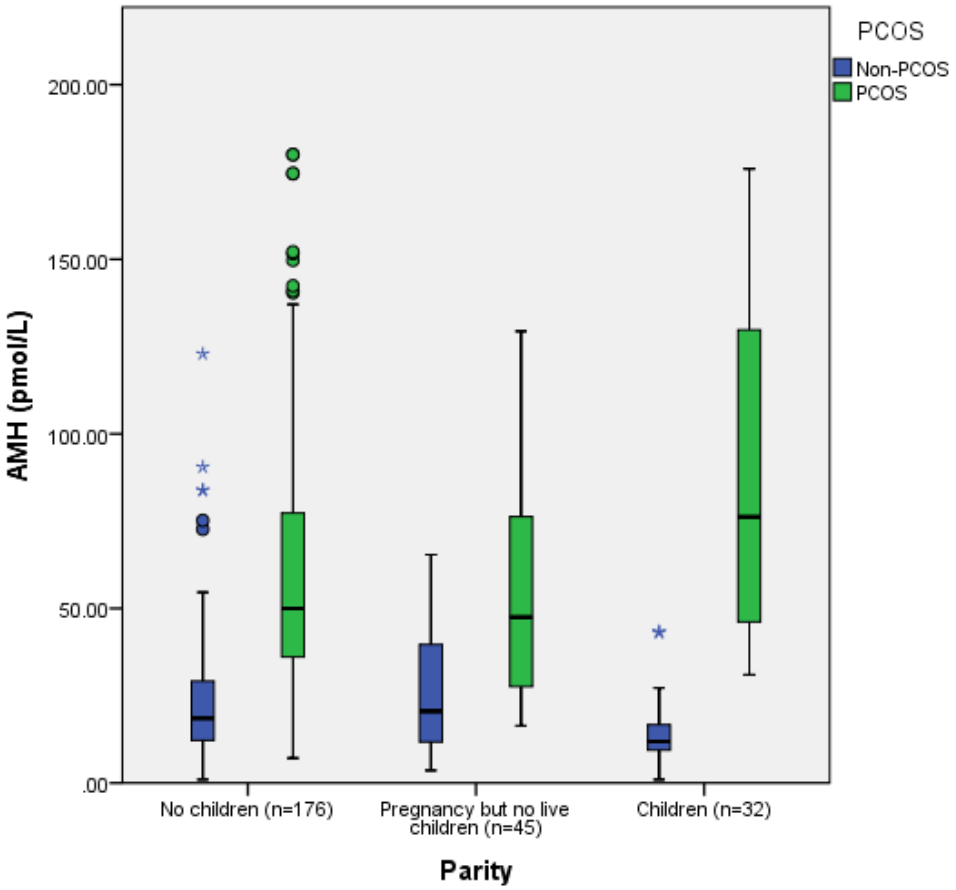


Figure 2-10 AMH concentration categorised by parity and Polycystic Ovary Syndrome.



2.3.3 Phenotypes of PCOS and corresponding AMH

Serum AMH level was significantly (p<0.01) associated with the different phenotypic presentations of PCOS (Figure 2-11). Those with all 3 components (PCOM, HA and OA) had the highest value when compared with those with HA and OA only (72.7 (61.0-86.7 95%CI))pmol/L vs 28.4 (6.0-134.9 95%CI)pmol/L; 0.4, 0.2-0.8, p=0.005). No difference was seen between those who had PCOM and either OA or HA (41.4 (31.4-54.5 95%CI) pmol/L vs. 44.7 (33.6-59.5 95%CI)pmol/L; 0.9, 0.6-1.4, p=0.69). The OA/HA group was too small (n=4) to draw a useful comparison between the non-PCOS group. Significant variation occurred across the groups in BMI and testosterone. Those with all 3 components had a heavier and more androgenic profile. Overall, any PCOS phenotype had an elevated BMI and testosterone compared with those without PCOS (Table 2-4).

Figure 2-11 AMH concentration by phenotype of Polycystic Ovary Syndrome

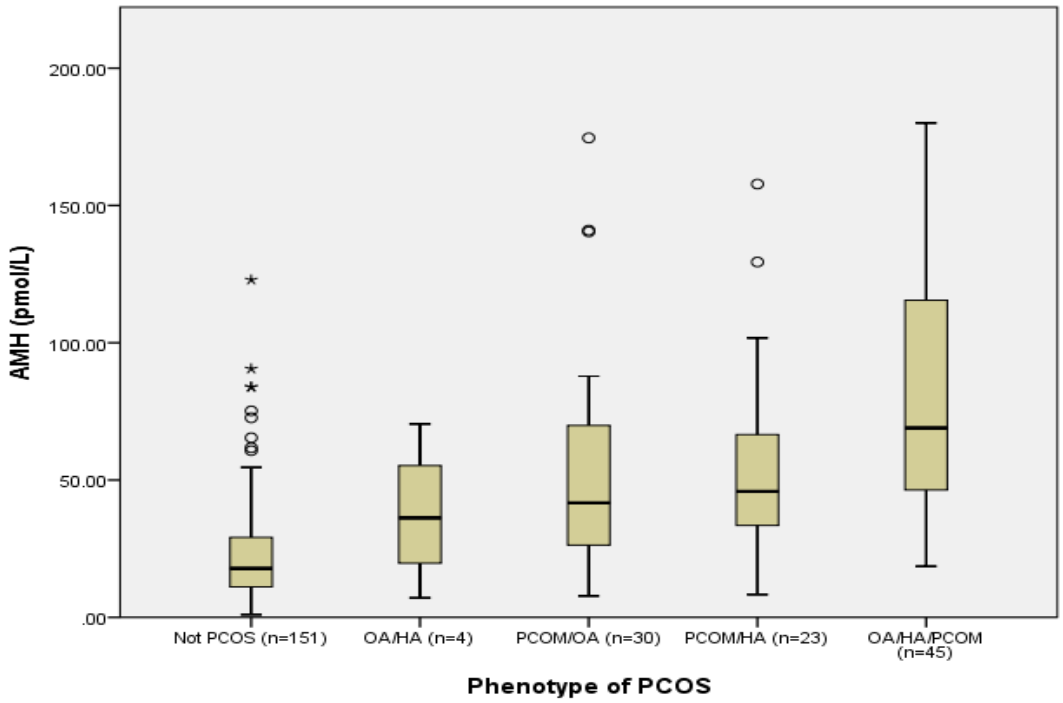


Table 2-4 Comparison of demographics by Polycystic Ovary Syndrome phenotype (OA: Oligo-anovulation, HA: Hyperandrogenism), PCOM: polycystic ovary morphology)

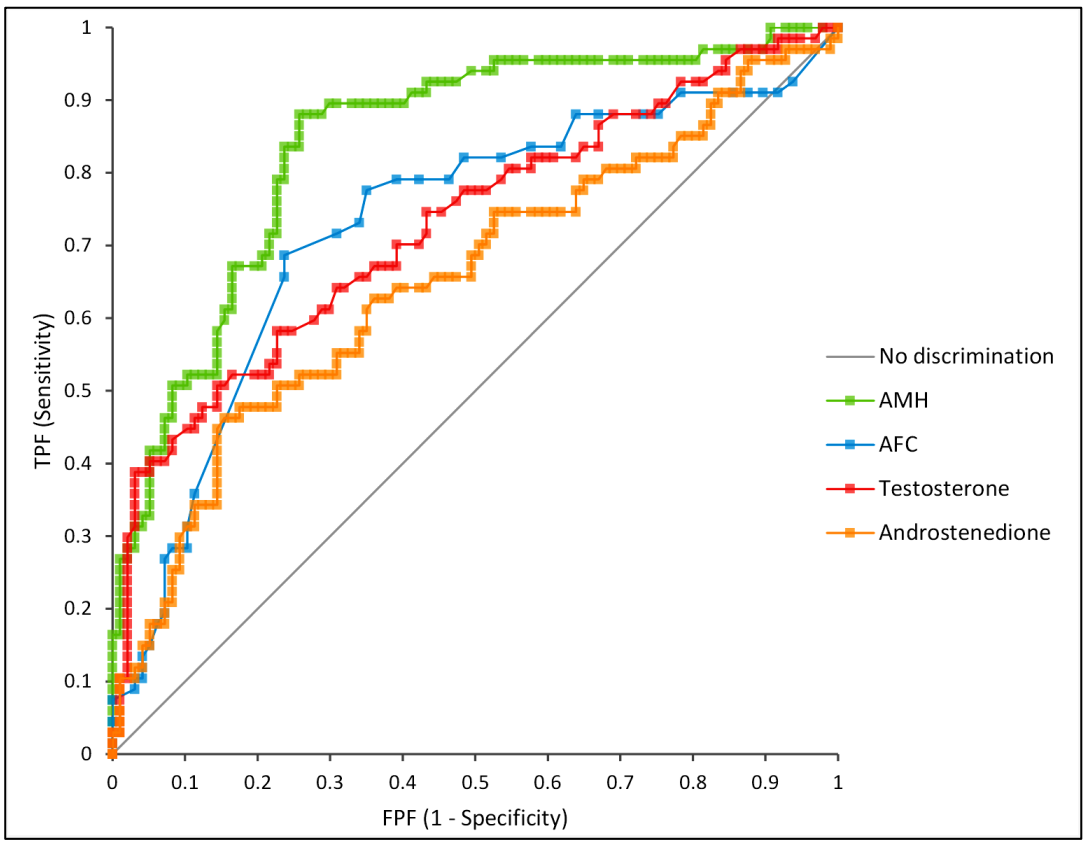
	Non-PCOS (n=151)	OA/HA (n=4)	PCOM/OA (n=30)	PCOM/HA (n=23)	PCOM/OA/ HA (n=45)	Between phenotypes *P*-value	Overall *P*-value
Age (Years)	32.7 ± 4.5	28.8 ± 5.3	31.0 ± 4.8	30.0 ± 5.3	28.6 ± 4.4	0.19[1]	<0.01[1]
BMI (kg/m²)	25.0 (22.0 – 27.5)	26.3 (21.3 – 32.0)	22.0 (21.0 – 28.1)	28.0 (24.0 - 30.0)	26.0 (24.0 – 29.8)	0.05[2]	<0.01[2]
FSH (iu/L)	7.2 (5.6 – 8.8)	7.0 (5.0 - 9.0)	6.1 (4.8 - 7.9)	6.3 (5.7 – 7.7)	6.3 (5.2 – 7.4)	0.78[2]	<0.01[2]
Testosterone (nmol/L)*	0.9 (0.8 - 0.9)	2.2 (0.5 – 10.1)	1.0 (0.9 – 1.2)	1.2 (1.0 – 1.5)	1.8 (1.5 – 2.0)	<0.01[1]	<0.01[1]
Androstenedione (nmol/L)*	4.1 (3.8 – 4.4)	6.4 (2.9 – 14.0)	5.4 (4.3 – 6.7)	5.4 (4.4 – 6.7)	6.3 (5.5 – 7.3)	0.50[1]	<0.01[1]
AMH (pmol/L)*	17.7 (15.5 – 20.1)	28.4 (6.0 – 134.9)	41.4 (31.4 – 54.5)	44.7 (33.6 – 59.5)	72.7 (61.0 – 86.7)	<0.01[1]	<0.01[1]

Mean ± SD, Median (IQR) or *Geometric mean (95% CI) [1]ANOVA [2]Kruskal-Wallis test

2.3.4 Diagnostic performance of AMH

Figure 2-12 presents a ROC curve using different hormonal tests to diagnose PCOS within the consecutive study population. When selecting AMH this has the best AUC of the group (0.84). This is in contrast to LH and an increased LH: FSH ratio (a variable traditionally used in PCOS diagnosis) which had an AUC of 0.64 which reflects a limited diagnostic value. Testosterone concentration was below AMH in terms of diagnostic value. This diagnostic performance for Testosterone will be artificially strengthened as it has been one of the criteria used in the PCOS diagnosis. Within this study population there was a higher than average prevalence of PCOS at 41% compared with that documented in the literature.

Figure 2-12 Diagnostic performance of AMH (0.84), antral follicle count (0.72), testosterone (0.74) and androstenedione (0.65) in PCOS using a receiver operator characteristic curve with the corresponding AUC values



TPF: True positive fraction FPF: False positive fraction

Further analysis of the different components of PCOS using the ROC curve, reflects the higher diagnostic potential of AMH compared with either Testosterone or FSH (Table 2-5). The AUC for AMH is >0.75 for cycles longer than 3 months, biochemical HA and PCOM. There was little difference between the AUC for clinical and biochemical HA when comparing AMH and Testosterone.

Table 2-5 Diagnostic performance using area under the curve values for AMH, Testosterone and FSH for components of Rotterdam criteria plus 95% confidence intervals

AUC	AMH	Testosterone	FSH
Hyperandrogenism	0.71 (0.62 – 0.79)	0.74 (0.66 – 0.82)	0.45 (0.36 – 0.54)
- **Clinical**	0.67 (0.58 – 0.76)	0.66 (0.58 – 0.75)	0.47 (0.38 – 0.56)
- **Biochemical**	0.85 (0.78 – 0.92)	0.99 (0.98 – 1.00)	0.45 (0.35 - 0.56)
Cycles			
- **>35 days**	0.62 (0.51 – 0.72)	0.59 (0.48 – 0.70)	0.45 (0.35 – 0.56)
- **>3 months**	0.75 (0.65 – 0.86)	0.72 (0.60 – 0.83)	0.43 (0.31 – 0.55)
PCOM	0.90 (0.84 – 0.95)	0.73 (0.65 – 0.81)	0.33 (0.24 – 0.41)
PCOS	**0.84 (0.78 – 0.90)**	**0.74 (0.66 – 0.82)**	**0.38 (0.29 – 0.46)**

2.3.5 Suggested AMH cut-off values

Using Youden's index (detailed within the statistical methods), the following tables (Tables 2-6 – 2-8) provide rationale for the best serum AMH threshold value to use to distinguish between women with normal ovaries, PCOM and PCOS.

Table 2-6 AMH cut-off values with corresponding sensitivity and specificity for normal and PCO morphology (orange shading=best value)

AMH cut-off (pmol/L)	Control (Normal Ovaries)	PCOM	Sensitivity	Specificity	Youden's Index (J)
10	51	27	0.96	0.26	0.22
15	37	27	0.96	0.46	0.42
20	18	26	0.93	0.74	0.67
22.5	12	24	0.86	0.83	0.69
25	8	22	0.79	0.88	0.67
30	5	20	0.71	0.93	0.64
35	5	18	0.64	0.93	0.57
40	4	18	0.64	0.94	0.58

Table 2-7 AMH cut-off values for distinguishing between PCOM and PCOS (orange shading= best value)

AMH cut-off (pmol/L)	PCOM	PCOS	Sensitivity	Specificity	Youden's Index (J)
25	22	60	0.90	0.21	0.11
30	20	58	0.87	0.29	0.16
35	18	55	0.82	0.36	0.18
40	18	49	0.73	0.36	0.09
42.5	16	45	0.67	0.43	0.10
45	12	45	0.67	0.57	0.24
47.5	11	40	0.60	0.61	0.21
50	10	35	0.52	0.64	0.16

Table 2-8 AMH cut-off values for distinguishing PCOS from non-PCOS (orange shading = best value)

AMH cut-off (pmol/L)	Sensitivity	Specificity	Youden's Index
25	0.90	0.69	0.59
28	0.89	0.72	0.60
29	0.88	0.73	0.61
30	0.87	0.74	0.61
31	0.84	0.76	0.60
35	0.81	0.76	0.57

Using the values of 29pmol/L and 45pmol/L as suggested AMH cut off values for diagnosis, a series of substitutions for PCOS criterion were made (see Table 2-9). An AMH value of 29pmol/L could be swapped with PCOM, without any significant changes ($p>0.05$) in the number of women diagnosed with PCOS. If 29pmol/L was used alongside PCOM for substitutions of either OA or HA PCOS was over diagnosed. Increasing the AMH limit to 45pmol/L eliminated this problem. Therefore a proposed 29pmol/L is optimal for PCOM and 45pmol/L for either OA or HA, if the other diagnostic criterion results are not available.

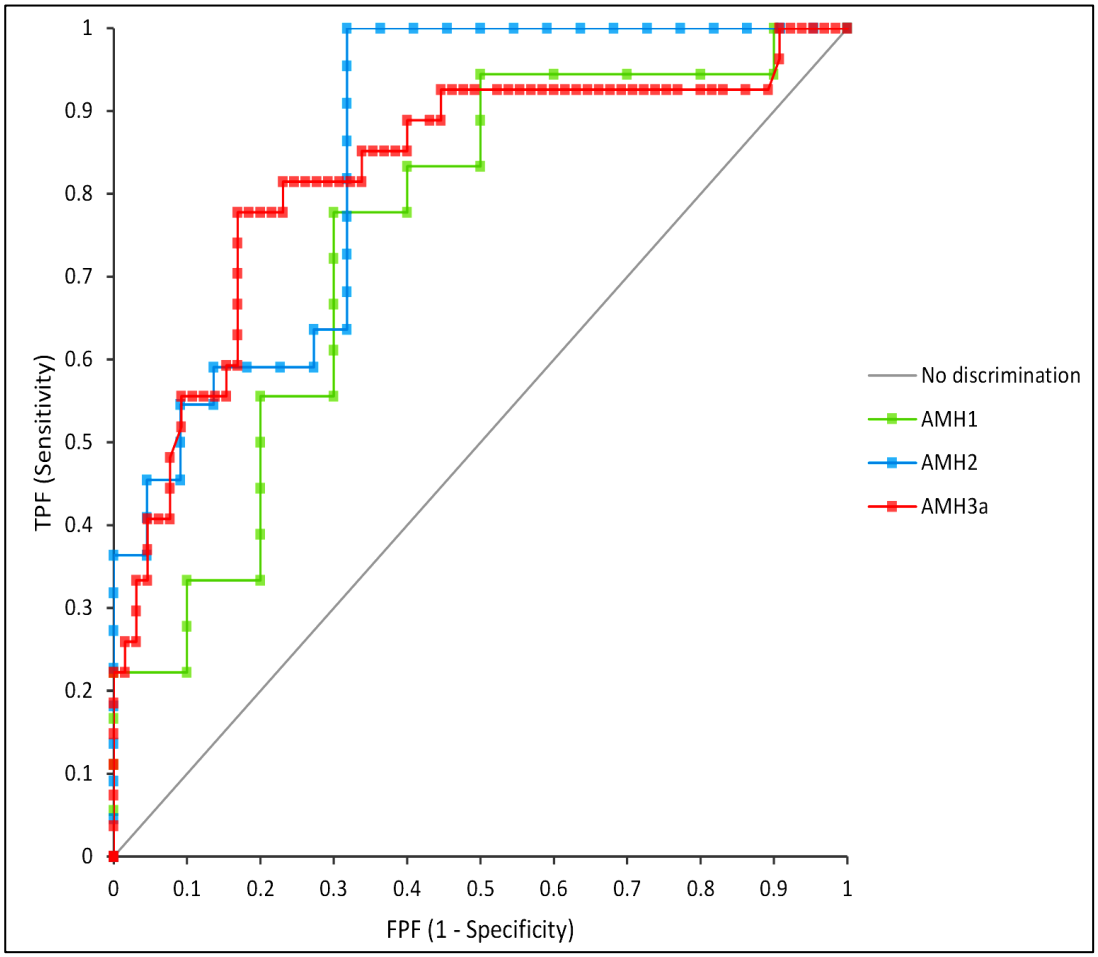
Table 2-9 Fisher's Exact: Substituting AMH for different PCOS criteria

Substitutions	Actual count (n=164)	Significance levels
OA/PCOM vs. OA/AMH (29pmol/L)	17 vs. 12	*p = 0.44*
HA/PCOM vs. HA/AMH (29pmol/L)	16 vs. 17	*p = 1.00*
HA/OA/PCOM vs. HA/OA/AMH (29pmol/L)	32 vs. 33	*p = 1.00*
BioHA/OA vs. BioHA/AMH (29pmol/L)	1 vs. 2	*p = 1.00*
OA/BioHA vs. OA/AMH (29pmol/L)	1 vs. 3	*p = 0.62*
PCOM/OA vs. PCOM/AMH (29pmol/L)	17 vs. 35	*P = 0.01*
PCOM/OA vs. PCOM/AMH (45pmol/L)	22 vs. 17	*p = 0.50*
PCOM/HA vs. PCOM/AMH (29pmol/L)	16 vs. 35	*p < 0.001*
PCOM/HA vs. PCOM/AMH (45pmol/L)	16 vs. 22	*p = 0.39*
PCOM/BioHA vs. PCOM/AMH (29pmol/L)	4 vs. 35	*p < 0.001*
PCOM/BioHA vs. PCOM/AMH (45pmol/L)	4 vs. 22	*P < 0.001*

2.3.6 Diagnostic performance of AMH for PCOS by age

In relation to patient age in women with PCOS, AMH had a higher diagnostic potential (Figure 2-13) in the older age groups. The youngest group has the lowest AUC. Overall there was a highly significant difference in AMH concentration between the non PCOS and PCOS group within each age bracket (as shown in Table 2-10). Although approaching significance (p=0.05), the downward trend in the non-PCOS AMH level was not significant across the age groups. There was no significant difference in AMH for those with PCOS in each age group, although the numbers in each group were small. There was also no difference in BMI by age or PCOS status. There was a significant difference (p<0.01) in testosterone levels across the groups.

Figure 2-13 Diagnostic performance of AMH for PCOS per age group (with the respective AUC beneath chart)



AMH1: age ≤ 25yrs (0.74), AMH2: age 26-30yrs (0.85), AMH3a: age ≥31yrs (0.83)
FPF: False positive fraction TPF: True positive fraction

Table 2-10 Comparison of AMH, BMI and Testosterone by age group and PCOS status

	Age group (Years)	Non–PCOS (n = 151)	PCOS (n =102)	Comparison between non-PCOS and PCOS by age group	Overall comparison by age non-PCOS *P* value	Overall comparison by age PCOS *P* value
AMH (pmol/L)*	≤25 (n=35)	28.1 (16.1 - 48.8)	65.3(48.1 -88.7)	0.4 (0.3 -0.8) <0.01[4]	0.05[3]	0.13[3]
	26-30 (n=73)	20.8 (15.3 - 28.4)	59.1 (47.4 - 73.6)	0.4 (0.2-0.5) <0.01[4]		
	31-35 (n=94)	16.1 (13.5 -19.2)	43.7 (33.3 – 57.4)	0.4 (0.3 - 0.5) <0.01[4]		
	>35 (n=51)	15.2 (11.8 – 19.5)	45.4 (29.6 – 69.6)	0.3 (0.2- 0.6) <0.01[4]		
BMI (kg/m²)	≤25	25.51 (21 – 30.4)	28 (24-32)	0.15[1]	0.27[2]	0.06[2]
	26-30	26 (22 – 29.1)	26 (23-29)	0.91[1]		
	31-35	24.6 (22 – 26)	24 (21-28)	0.89[1]		
	>35	24 (22.2 -27.1)	24 (21.8 – 31)	0.67[1]		
Testosterone (nmol/L)*	≤25	1.1 (0.8 – 1.4)	1.7 (1.3-2.2)	0.6 (0.4 – 1.0) 0.04[4]	<0.01[3]	0.05[3]
	26-30	1.0 (0.9 - 1.2)	1.5 (1.3 – 1.7)	0.7 (0.6 – 0.9) <0.01[4]		
	31-35	0.8 (0.8 - 0.9)	1.3 (1.1 – 1.5)	0.7 (0.6 – 0.8) <0.01[4]		
	>35	0.8 (0.7-0.8)	1.0 (0.7 – 1.4)	0.8 (0.6 – 1.1) 0.09[4]		

*Geometric mean (95% CI) or median (IQR) [1]Mann Whitney U test [2]Kruskal Wallis [3]ANOVA [4]T test (ratio of geometric mean (95% CI of difference), *p* value)

2.4 Discussion

This study has shown that a large proportion of women meet the Rotterdam criteria for PCOS within the infertility clinic setting. Women with PCOS often present at a younger age, with an increased androgenic profile. Isolated PCOM represents a group of women mid-way between those with normal ovaries and PCOS. This finding is reflected in the serum AMH concentration. Body mass index has little association with the value of AMH. For those with PCOS, AMH reflects the severity of the condition. The highest levels are seen in those with the full constellation of symptoms including OA, HA and PCOM. All of the Rotterdam diagnostic criteria for PCOS are independent predictors of the AMH value. Furthermore, AMH can be used as a substitution for any of these features. It has diagnostic value that is maintained with age.

2.4.1 AMH and hyperandrogenism

This study supports the correlation between anovulation (240, 453), hyperandrogenism (83, 151) and AMH. The significant differences ($p<0.01$) seen in AMH between elongated cycle length, particularly greater than 3 months apart and evidence of HA, whether clinical or biochemical, are symbolised in Figure 2-7. In this simple representation, the effect of each factor independently or together i.e. PCOM, OA and HA are not incorporated but provide a good starting position to evaluate AMH as a diagnostic tool in PCOS. Using multiple regression within the current study, Testosterone, the visualisation of PCOM and oligo-ovulation are all independent predictors of AMH when adjusted for BMI. This holds for the entire population and the PCOS group. This agrees with Eldar-Geva et al (217), who found an independent relationship between the AMH level and both the number of small antral follicles and serum androgen (testosterone) level. In contrast, Pigny et al (454), found that by using multiple regression analysis the association between serum androgen and AMH was no longer significant when controlling for the number of 2-5mm follicles. Further defence for the association between androgen concentration and serum AMH was

provided by Chen et al (455). These authors found via multiple regression with follicle number per ovary as the dependant variable, that only the free androgen index and AMH level maintained a significant independent relationship with follicle number. As outlined below it would seem reasonable to assume that the correlation is also true between AMH and serum androgen.

It is timely to remember the histological findings of earlier work (456). Women with PCOS have the same primordial pool size, with a larger number of growing follicles but also an increased population of atretic follicles. To explain this finding, AMH has been implicated in 2 stages of follicle activity leading to PCOS (151). Excessive follicle recruitment from the primordial pool is followed by increased attrition and ultimately defective selection of the lead follicle, culminating in anovulation. The inhibitory role of AMH in follicle recruitment (121) is deficient despite an elevated level of the hormone. New evidence, suggests that there is significantly less immunohistochemistry staining of AMH in the primordial and transitional follicles of anovulatory PCO women (222). This data supports the view that AMH operates in a paracrine fashion, whereby growing follicles locally inhibit the recruitment and growth initiation of adjacent primordial follicles. The relative deficiency of AMH surrounding the primordial follicles in anovulatory PCO women leads to reduced inhibition of primordial follicle recruitment. Furthermore, AMH reduces follicle sensitivity to FSH, inhibiting FSH-induced pre-antral growth (126). The theory of AMH acting as the 'gate keeper' of follicular oestrogen production should also be considered (113). The time of follicle selection corresponds with a declining intra-follicular expression of AMH in follicles >8mm (114). There is also a transition from a low to high oestrogen producing state. Oestrogen orchestrates this decline in AMH via the interaction between the oestrogen receptor β and the AMH promoter region (457). Evidence to support the gate keeper theory suggests that there is an inhibition of aromatase activity (113) by AMH. This ensures that there is limited oestrogen production from the small antral follicles, which allows direct communication between the ovary and pituitary in the regulation of follicle selection via FSH feedback mechanisms (115).

Although the exact causative factors leading to PCOS are unknown; the intra-ovarian environment, in particular the androgen concentration within it, is thought to play a role (2). Porcine work has demonstrated mitogenic effects of androgens on the granulosa cells via the androgen receptor, potentiating the follicle growth-promoting effects (458). A convincing argument for the role of androgens in this process, predating birth, is outlined by Homburg (459). Exposure to androgens *in utero* may re-programme the fetus, although these effects may not be apparent until later in life, as seen in studies using prenatally androgenised female rhesus monkeys (460). The gonadotrophin LH influences this androgenic environment by driving androgen production through the binding of thecal LH receptors. Granulosa cells from follicles >10mm (i.e. after acquisition of LH receptors) from a PCO cultured with LH generate a 4-fold increase in AMH production, augmenting the anovulatory problem (215). Granulosa cells cultured with LH from normal ovaries only produced a modest rise in AMH production. In addition, it has been suggested that granulosa cells from those with PCOS gain LH responsiveness at a smaller follicular size than normal ovaries (461). Further work on luteinised granulosa cells (post hCG), show an over expression of AMH and AMH-receptor II in anovulatory PCO women which is not seen in normal ovaries, in response to LH (129). The current study shows a significant correlation exists between AMH and LH ($p<0.01$) which may potentiate the effects of both of the hormones. Hyperinsulinaemia and insulin resistance (IR) may act as a 'second-hit' on the already dysregulated system by amplification of the intra-ovarian hyperandrogenism (2). Formal insulin resistance testing was not performed as part of this study.

A method to reconcile the opinions on PCOS definition (7, 462, 463) was suggested by Dewailly et al (83). These authors use principal component analysis to highlight that similar information can be captured by different variables. The outcome of this study aggregated AMH, AFC, LH, testosterone, androstendione and free androgen index (FAI). They suggest that a single ovarian anomaly can be summarised by ovarian androgen and follicle markers; therefore if OA or Hyperandrogenism (HA) are not present, then

AMH or AFC can act as a surrogate marker for either. The strong correlation seen between testosterone and AMH supports this theory. Interestingly, Dewailly et al (83) suggest a more appropriate androgen to use in general diagnosis is androstenedione over testosterone. This may be in part due to the secretion of androstendione by the theca cells and conversion to testosterone peripherally (464). Within the current study, both androstenedione and testosterone remained significant independent predictors of AMH during multiple regression. Further analysis helps with the often subjective topic of 'clinical HA'. Regression suggests that AMH is a better predictor than either testosterone or androstenedione.

The importance of the HA/AMH correlation is potentially relevant to the long-term sequelae of PCOS. It is thought that women with PCOS have an increased long term risk of cardiovascular and metabolic disease, often related to, if not caused by, obesity and insulin resistance. Shaw et al (465) outline an increased postmenopausal risk of angiographic coronary artery disease and reduced cardiovascular disease free survival if there is a positive history of irregular menses and biochemical evidence of HA. Furthermore, Wild et al (466) show that women with the metabolic syndrome prior to starting hormone replacement therapy (HRT) are more likely to experience adverse cardiovascular events during the course of treatment. This group of authors suggest individual risk stratification should be observed prior to commencing HRT. A raised AMH as a marker for HA could help counsel patients to make life adjustments to modify these long term risks. Conversely, Bleil et al (467) suggest that accelerated ovarian aging with a reduced AMH level may in fact predict an increased cardiovascular risk. The phenotypic presentations of PCOS may not all share the same cardiovascular risk (82, 468). It is suggested that those who have OA and PCOM but normal androgen profile have minimal increased risk to the normal population. The reduced AMH in this population compared with the most 'severe' form (OA/HA/PCOM) in the current data could reflect the reduced long term risks (Figure 2-11). As suggested later, the diagnostic performance of AMH with age increases. Further review of whether this is relevant to long-term health is needed.

2.4.2 AMH and PCOM

The well documented positive correlation between AFC (138, 454, 469) and AMH is clearly replicated within this trial (see Figure 2-7). This correlation is confirmed with the high AUC of 0.90 for AMH diagnostics for PCOM alone (see Table 2-5). This AUC is in fact the highest value for all separate criteria for PCOS. Such a significant parallel has led many to suggest that AMH can replace or act as an alternative for AFC (87, 232, 234, 455). As previously discussed, Dewailly et al (83) showed that AFC and AMH are strong markers of an ovarian anomaly. Sahmay et al (470) argued that PCOM is the most effective factor affecting serum AMH levels. The editorial by Raine-Fenning (471) summarises this argument, with the stance that AMH and an ultrasound scan (USS) 'are unlikely to offer mutually exclusive information'. Although the proposed cut-off values in our study were formulated on the current data, using an AMH of 29pmol/l as a substitute for PCOM, we found no difference via Fisher's exact analysis between the proportions of women diagnosed with different combinations of PCOS (Table 2-9). This finding continues when substituting AMH for OA or HA, when either of the latter two factors is present. If PCOM is the constant factor, there is a significant difference in proportions using an AMH of 29pmol/L. If the value is increased to 45pmol/L (as recommended to delineate between PCOM and PCOS in this study (Table 2-7) there is no longer a difference. The exception to this is if combining PCOM with biochemical HA or AMH. This raises a novel possibility in the diagnostic process; a 2-tier process for AMH substitution. For purely PCOM substitution the lower level could be used; but if substitution for either OA or HA when USS is the only feature, the higher threshold should be used. This would support the findings of Dewailly et al (83).

With improvements in ultrasound technology there have been recommendations to increase the threshold follicle number for the diagnosis of PCOM (76, 87, 472) to prevent over diagnosis. This is perhaps reflected in those exhibiting PCOM within the PCOS population studied here (98 of 102 women with PCOS had PCOM). A consensus on whether the new threshold should be 25 follicles (88) or even doubled to 28 follicles (473) remains to be validated. Problems related to inter-observer differences

may be eradicated by automated follicle counts (474). In the context of AFC, AMH quantification offers an attractive option to be used in conjunction with AFC data or individually for the diagnosis of PCOS, as long as ongoing assay stability problems can be resolved (173).

2.4.3 Phenotypic presentation

A formal gradation of PCOS severity has been postulated. The ability to do this can pose a problem due to the subjective nature of some of the components of diagnosis e.g. clinical hyperandrogenism. Although formal scoring systems are available for the latter issue, these may be academic when evaluating the patients' perception of the problem. Use of a simple diagnostic test in these situations may be of value to biochemically reflect the severity of condition and tailor treatments, such as gonadotrophin stimulation.

As seen in Figure 2-11, AMH is significantly affected by the different phenotypes of PCOS. Evidently those displaying all 3 factors have an increased AMH; this situation in itself could be deemed the most severe. This agrees with much of the published data (151, 229, 470, 475). Sahmay et al (470) found that those with HA/OA/PCOM showed a 3-fold increase in circulating AMH compared to those with OA/HA only. They found a non-significant marginal elevation in AMH in the PCOM/OA vs. PCOM/HA group. No difference was seen in BMI or testosterone across the 4 phenotypes. This differs from the current study where the reverse is seen, with a significant difference in BMI and testosterone by phenotype, but interestingly not androstendione. Although the data was not significant ($p>0.05$), having HA correlates with an increased AMH. Considerable variation was seen in both the BMI and testosterone levels, with the HA/OA/PCOM group being heavier and more androgenic than the other phenotypes. Piouka et al (151) agree with the current data and suggest that the lowest AMH level by phenotype (OA/PCOM) reflects the least severe form of the syndrome. Those in favour of this argument (476) agree that this phenotype has milder endocrine and

metabolic abnormalities. Controversially, some believe it not part of the PCOS spectrum (462). The conclusions surrounding the relative placing of the 'classical' OA/HA phenotype (73) from the current study are limited due to the small group size (n=4).

2.4.4 Timing of test

AMH variation within the menstrual cycle has been discussed in detail in chapter 1.3.4. Table 2-11 reflects this discussion providing the contradictory evidence surrounding intra-cycle variation. As suggested by Streuli et al, although individual variation will occur, this is unlikely to be clinically significant (141). Furthermore, it has been suggested that the intra-cycle variability in serum AMH concentration is less than that found in the antral follicle count (138). Two different AMH patterns related to AMH level, termed the 'ageing' or 'young' ovary, suggested by Sowers et al (144) may also be relevant. More recent studies provide further support for this phenomenon (145, 477). This theory is particularly pertinent to women with PCOS who are recognised to have elevated AMH levels and therefore may display more intra-cycle variation in their serum AMH levels. It should be noted that any true biological inter-cycle variability may also be eclipsed by assay measurement variation (173).

A diagnostic test should be easy to implement in the clinical setting. For example, it has long been recognised that FSH is menstrual cycle stage dependant and can have wide inter-cycle variability. This complicates the ease of use of the test, often necessitating repetition of samples to provide an estimate of ovarian reserve and tailor treatment protocols. From the current study data (Figure 2-8), no significant difference was seen between the AMH values whether taken in the early follicular (day 1-5), mid follicular (day 6-14) or secretory (day 15-35) phases (p=0.459). The similarity is maintained when either the PCOS population (p=0.735) or non-PCOS group (p=0.403) are individually reviewed.

Table 2-11 Intra-cycle variation of serum AMH concentration

Author	Assay	Study group	Significant difference	No significant difference
La Marca et al (128) (2006)	IOT	n =12 18-24yrs regular cycle		No significant change throughout cycle
Hehenkamp et al (478) (2006)	DSL	n =44 fertile regular cycle		No consistent fluctuation throughout cycle compared with FSH, LH, E2
Tsepelidis et al (479) (2007)	DSL	n =20 18-35yrs regular cycles		No significant variation in cycle
Wunder et al (140) (2008)	DSL	n =36 20-32yrs regular cycles	Significant variation – highest in late follicular compared with time of ovulation or early luteal phase	
Streuli et al (141) (2009)	IOT	n =10 regular cycles. Mean age 24.1yrs	Small but significant drop in AMH in early luteal phase	Smaller than inter-cycle variability so not clinically relevant
Van Disseldorp et al (138) (2010)	DSL	n=44 regular cycles 25-46yrs		Less intra-cycle variation than antral follicle count
Sowers et al (144) (2010)	DSL	n =20 regular cycles 30-40yrs	Significant variation follicular phase in higher AMH levels 'young ovary'. Little variation in lower levels	
Rustamov et al (480) (2011)	DSL	n =186 during routine infertility investigations age 22-42yrs	Significant variation in intra-cycle variation which should be taken into account when producing algorithms for treatment	
Robertson et al (481) (2011)	DSL	2 groups: n=43 age 21-35yrs regular cycles n=45-55yrs variable cycles	In later reproductive life variable changes between follicular and luteal phase	No change in AMH levels in women mid-reproductive life
Overbeek et al (145) (2012)	DSL	n=44 regular menstruating women 25-46yrs	Substantial fluctuations in young individuals	
Hadlow et al (143) (2013)	GEN II	n =12 non-PCOS regular cycles 29-43yrs with at least 1 result 'adequate ovarian reserve'	Significant variation in AMH – negative trend from follicular to luteal phase. Maximum change in cycle 7.9pmol/l	
Randolph et al (477) (2014)	DSL / GEN II	n =20 age 30-40yrs regular cycles non-PCOS	Significant variation in higher AMH levels – biphasic peaks mid-follicular and mid-luteal	No variation lower AMH levels
Kissell et al (482) (2014)	GEN II	n =259 cycles 21-35 average age 27yrs	Significant variation in AMH in both ovulatory and sporadically anovulatory cycles. Drop at time ovulation.	Level of variation not clinically relevant – can test at any time point

A highly significant difference was seen if an AMH was taken outside of a regular 35 day cycle (p<0.01). As previously discussed this conforms to the correlation between

PCOS, in particular cycle length, and AMH. If the non-PCOS group are analysed, there is no significant difference (p>0.05) in AMH level irrespective of time to test (*p*=0.426), although the number of patients in the >35 day cycle group is small at only 4.

The ability to undertake AMH measurement without constraints of cycle timing is favourable for the diagnostic application of AMH. Many clinics currently rely on distant services to process the sample, requiring the sample to be taken at set locations. Add to this the geographical catchment area each clinic covers, and the convenience of opportunistic testing is clear. Although the individual variation in AMH value through the cycle has not been tracked within this study, the overall non-significant changes in mean AMH agree with the consensus of AMH stability within the cycle (128, 139). It is possible to speculate that the wider range of AMH seen in women with PCOS may also reflect intra-cycle variability. None of the aforementioned studies (Table 2-11) have focussed on this group of women, who may have a more exaggerated or stable intra-cycle variation. This, however, does not seem clinically relevant as suggested differences in AMH would not alter the ovarian reserve classification or indeed treatment options. For example, if 7.9pmol/L is the true intra-cycle variation, as suggested by Hadlow et al (143), this would have little impact on the median serum AMH 50pmol/l found in the current PCOS population.

2.4.5 Parity and ethnicity

It has been suggested that AMH could act as a predictor of LBR for IVF cycles or other assisted reproductive techniques (179, 483). Retrospective review of the parity of women enrolled, revealed no significant difference in mean AMH (see Figure 2-10). This supports the theory that there is only modest predictive value for live birth. Li et al (483) suggest that using a model including age and AMH versus using age alone, only a further 2% had an accurate prediction. They use logistic regression, with the outcome that neither AMH nor AFC are significant predictors of LBR. Similar diagnostic potential is seen using ROC curves. Two independent teams suggest an area under the curve of

0.65 (233) and 0.62 (179). Neither of these values shows strong diagnostic performance. An interesting trend was seen when the non-PCOS group were analysed separately with a difference in serum AMH concentration between those without living children/never pregnant and those with children (p=0.041). The latter group was on average >9pmol/L lower than the other 2 groups with a mean value of 15.3pmol/L. The majority of studies suggest that those with a higher AMH have an increased chance of live birth. The observation in this study is only likely to represent the case mix presenting to the fertility clinic, particularly as other factors have not been included in this analysis such as age and reasons other than PCOS for infertility.

Conclusions on the effect of ethnicity on AMH cannot be drawn from the current data, due to the relatively small numbers within the minority groups. The largest group, from Pakistan (n=22) had a mean AMH of 48.2pmol/l. When compared with the white Caucasian population mean of 41.0pmol/L, there was no significant difference in AMH value or the proportion of those presenting with PCOS in either group. These low numbers do not help to decipher the case selection presenting to an infertility clinic. For example, it may only represent a higher morbidity group from the smaller number minority accessing tertiary healthcare within the United Kingdom.

2.4.6 Recommended diagnostic level

The cut-off value for a test to define a condition is a repeated question. It is important to remember that the test performance will depend on the prevalence of the disease i.e. PCOS in the population studied. Using both ROC curves and Youden's index, suggested values have been generated from this data as outlined in Tables 2-6 – 2-8. The diagnostic ability of AMH in PCOS is high with an AUC of 0.84 as previously discussed. The best compromise between sensitivity and specificity suggest that values of 22.5pmol/L and 45pmol/L delineate the normal ovary from PCOM and PCOM from PCOS respectively. The group of patients used within this study are in some ways dissimilar to the other studies that suggest values with the generation II assay (228,

229). There are no selected or matched controls, representing an entirely consecutive group of women presenting to an infertility clinic. Case control studies have the potential to overestimate the performance characteristics of a diagnostic test (224). The proportion of women with PCOS is similar to that presented by Homburg et al (228). Emphasis is placed on creating 3 important subgroups, labelling PCOM as its own entity. As discussed in chapter 1.2.2 , there is a 2 sided argument as to whether PCOM is a silent form of PCOS or normal variant. If the current consecutive population is used, a suggested value of 29pmol/L could be used to diagnose PCOS from normal women. This is much lower than that suggested by either of the 2 studies using the generation II assay and may represent the case control effect. Meta-analysis produces a similar result (4.7ng/mL) to the current recommendation (4.06ng/mL) (224). The value suggested to separate PCOM from PCOS is very similar to Homburg et al (228) in sensitivity (67% vs. 60%). Unlike the latter trial, my suggested value also has a relatively low specificity (57%). This again is dissimilar to the other discussed trials which all maintain higher specificity (224, 228). Not all trials quote high specificity; for example Li et al (233) provide very similar values to our study. The lower specificity can be explained by the significant overlap of the PCOM population with the PCOS group as seen in Figure 2-5, which can create a higher number of false positives. If the PCOM population are removed the corresponding specificity of the test using 45pmol/L is significantly elevated (94.2%). The ROC curve is also improved with an AUC of 0.92. Although on paper this may look a better test, it has been produced from a selected cohort rather than representing the true population. Having a lower specificity is less concerning in the context of individualising fertility treatment for high risk women. Cautious use of gonadotrophins for those with increased AMH levels is a safe recommendation, universally accepted.

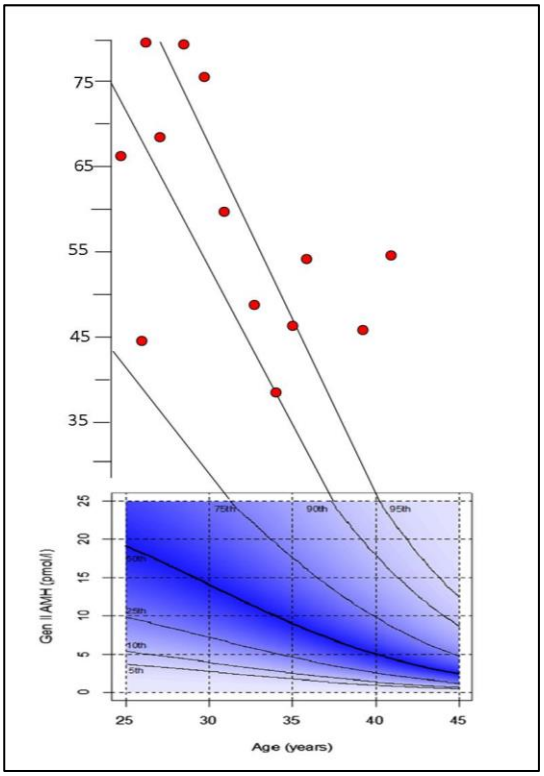
The combination of 2 tests in diagnostics i.e. AMH and LH has the potential to introduce more error by combining error rates alongside the true effects of the tests. Implementing the suggested combination of AMH (using my suggested 45pmol/L) and LH >6iu/L (228) produces a reduced sensitivity (43%) and improved specificity (94%).

For the purpose of diagnosis of PCOS, a balance between sensitivity and specificity is therefore the best compromise and would suggest using a simpler method of testing with a single test only.

2.4.7 Age effect on AMH in PCOS

The downward trend in AMH with age has been well documented reflecting reduced ovarian reserve (171, 444). The majority of the suggested age nomograms for AMH do not focus on PCOS. More recent literature focuses on this sub-group. Wiser et al (484) have produced a nomogram for the AFC in PCOS. Their retrospective review shows a slower decline in AFC with age in women with PCOS. They further quantify this decline as 0.8 follicles/year in PCOS and 1.7 follicles/year in non-PCOS (p<0.001). The data within my study, in agreement with others (485), shows a significant difference in all age brackets between those with PCOS and those without. Although not significant, the downward trend in the non-PCOS group is increased compared with the PCOS group, suggesting a more rapid decline in AMH. This correlates well with the aforementioned AFC nomogram (484). The limitation of my data apart from small numbers is the lack of longitudinal data. Limited longitudinal data is available on this topic. Tehrani et al (486) present a 10 year longitudinal dataset averaging 3 consecutive measurements on individuals. The decline in AMH in PCOS is more gradual than the consistent reduction in the non-PCOS group. They hypothesise that the average age of menopause is 2 years later due to this phenomenon. Hwang et al (475) agree with the slower decline in AMH with age. They found a maintained AMH until 35 years of age in PCOS, unlike those with normal menstruation. An estimated extrapolation from the AMH nomogram suggested by Nelson et al (436), allows the current mean serum AMH values to be plotted at different ages (see Figure 2-14). Although a formal nomogram has yet to be produced for those with PCOS, the general trend seems to tally well. It would be useful to evaluate this further with larger data sets.

Figure 2-14 Mean AMH concentration by age in those with PCOS plotted on an extrapolation of an AMH by age nomogram (from Nelson et al (436)) using the AMH Gen II assay



The statistical performance of AMH seemingly improves with advancing age, when ROC curve analysis is applied (see Figure 2-13). If the AUC for the <25 years group is compared with the >35 year group it is noticeably different (0.74 vs. 0.91). Although simple explanations can be assigned to this, it remains relevant when used for diagnosis of PCOS and the underlying pathophysiology. A significant positive correlation exists between AFC and AMH. Younger women have an elevated AFC which is not related to PCOM or PCOS. There is also a greater preponderance of PCOM without the other manifestations of PCOS. Referring back to Chapter 1.3.3, it is known that AMH increases through adolescence into the early 20's reaching a peak at ~25 years. This will muddy the scientific waters when using such an age dependant test within diagnostics.

The other relevant feature in the ROC curve finding is the cohort used in the study. The number of women presenting to our infertility clinic over 35 years old with PCOS was

low . The phenotypic presentation of PCOS alters with advancing age, with many women attaining a regular menstrual cycle (487-489) and a decline in HA (490). Women presenting with PCOS are often younger and many will have achieved a pregnancy before the advanced years (13). This speculates that those who do present later have a potentially more pronounced form of PCOS and therefore may have an increased serum concentration of AMH. All of these factors contribute to the strengthened diagnostic finding in older women. Supporting other studies (490-492), there is a significant decline in biochemical hyperandrogenaemia with age (see Table 2-10). Unlike other studies, there is no significant difference in BMI or presentation of clinical HA with age (χ^2=0.93, p<0.83). Formal testing for insulin resistance was not performed within this study.

2.5 Conclusion

In conclusion, AMH is a useful tool in the diagnostic armamentarium for PCOS. The serum concentration of AMH is closely related to antral follicle count, hyperandrogenism and anovulation. Anti-Müllerian Hormone can be on the whole interchanged with - and used as a surrogate for - any of the Rotterdam criteria, but perhaps most effectively for PCOM. It does reflect severity of disease with those exhibiting the full constellation of symptoms having the highest level. Further attempts to provide cut-off levels are provided; suggesting 29pmol/L for PCOS and a higher level of 45pmol/L to separate PCOM from PCOS. With advancing age the levels are maintained and increase in diagnostic ability, whilst care is needed when using it in young adults. The test can be used at any point in a regular menstrual cycle without a clinically significant effect. In conclusion, AMH lies within the foundations of PCOS and with further scientific work the exact contribution to the underlying pathophysiology of the condition will be further elucidated. In the meantime, it is a hormonal assay that has surmounted the traditional tests available and provides an easy to use tool in this reproductive field.

Chapter 3
Metformin And GnRH Antagonist Co-treatment In IVF For Women With Polycystic Ovary Syndrome

3.1 Introduction

Metformin has historically been a firm favourite with clinicians' treating all aspects of PCOS. More recently there has been a decline in metformin's popularity as an adjunct treatment during assisted conception, coinciding with several Cochrane reviews (338, 348) that suggest metformin's use is limited in ovulation induction, *in vitro* fertilisation cycles and symptom management e.g. hirsutism. A remaining positive association is the reduction in OHSS that metformin affords an IVF cycle (Table 3-1). Tso et al (348) included 6 of the main studies evaluating metformin and OHSS reduction. A significant reduction in OHSS was seen (OR 0.24 95% CI 0.12, 0.47), despite variables that may be assumed to aid this reduction revealing minimal change e.g. number of oocytes retrieved (mean difference (MD) -0.13, 95% CI -1.43, 1.17), stimulation days (MD 0.03, 95% CI -0.32, 0.37) and oestradiol level (MD 3.51, 95% CI -0.18, 7.19). The 2 most recent papers have opposing views (347, 350) with regard to OHSS. Palomba et al (350) agree with previous work that there is a reduced OHSS risk following metformin treatment (RR 0.28, 95% CI, 0.11–0.67). Their reasoning for this includes an overall reduced oestradiol level and reduction in number of non-periovulatory follicles, despite increased stimulation length and dose. Compared with earlier work (260), this group selected a higher risk population, who all had either a previous ART cycle cancelled or a cycle complicated by moderate to severe OHSS. Kjotrod et al (347) found no difference in OHSS rates following metformin treatment. A unique element of this trial was the implementation of 'coasting' (withholding gonadotrophin stimulation and trigger injection) with a trend towards increased coasting with placebo. Indeed, coasting has been suggested as a stand alone reduction strategy for OHSS (275)

(although not substantiated) and therefore this approach may mask any potential benefit from metformin.

The exact mechanism by which metformin exposure reduces OHSS has not been fully investigated. The ideas proposed (350) focus on 2 of the main features common to PCOS - namely insulin resistance and hyperandrogenism. Metformin appears to operate both directly on ovarian function and also indirectly via modulation of insulin excess. Evidence for the direct effect of metformin implicates both the granulosa and thecal cells as target cells (322, 493) for the drug. Within human ovarian thecal-like androgen-producing tumour cell culture (which has retained the ability to produce androstenedione and express steroid-metabolising enzymes under the control of cAMP) the addition of metformin (in doses higher than that achieved by oral administration) reduced androstenedione production (324). Furthermore, this inhibition was greater than that of 17–hydroxypregnenolone suggesting a preferential inhibition of 17-lyase activity compared with 17α-hydroxylase activity. This supports earlier work whereby a reduction in CYP450c17 activity was shown in women with PCOS treated with metformin (18, 494). In addition, Attia et al (324) speculate that the reduction in CYP450c17 activity is due to an increase in tyrosine activity in metformin-treated cells. This speculation was based on earlier work (495) from these authors where a significant increase in tyrosine kinase activity was demonstrated. A post-receptor defect has been suggested to underpin the peripheral hyperglycaemia seen in women with PCOS (37, 496). The defect leads to reduced tyrosine auto-phosphorylation and an increase in serine phosphorylation of the beta chain of the insulin receptor. Serine phosphorylation is thought to augment ovarian and adrenal CYP450c17 activity (497).

Table 3-1 The use of Metformin as an adjunct in *in vitro* fertilisation treatment cycles

Reference	Research type	Cycle	Study size & Participants	Duration / Dose	Endpoints measured
Stadtmauer et al (2001) (498)	Retrospective	Agonist	Clomiphene resistant n = 60 cycles (50% Metformin)	500mg BD/TDS BMI dependant. Day 1 preceding cycle until pregnancy test	Reduced number small follicles. Reduced IGFBP and increased IGF II
Fedorczak et al (2003) (499)	Randomised open-label cross-over trial	Agonist	Insulin resistant PCOS (NIH) n=17	500mg TDS 3 weeks prior to down regulation until hCG	Total dose FSH reduced <10% Increased number oocytes
Kjotrod et al (2004) (346)	Prospective double blinded randomised placebo controlled	Agonist	n = 73	16 weeks 1000mg BD until hCG	No difference in FSH, oocytes, fertilisation. BMI<28 had increased CPR
Onalan et al (2005) (500)	Prospective doubled blinded randomised controlled	Agonist	n = 110	850mg BD/TDS BMI dependant 8 weeks prior to cycle & until pregnancy test	No difference in stimulation or pregnancy outcomes. No difference in OHSS
Costello et al (2006) (349)	Systematic review	_	Includes 5 IVF studies		Metformin co-administration does not improve CPR (OR = 1.29; 95% CI = 0.84–1.98) or LBR (OR = 2.02, 95% CI = 0.98–4.14) rates but reduces risk of OHSS (OR = 0.21; 95% CI = 0.11–0.41, P < 0.00001)
Doldi et al (2006) (351)	Prospective randomised Metformin vs. no treatment	Antagonist	n = 40	2 month 1.5g/day prior to stimulation until embryo transfer	Reduced FSH. Increased oocyte number. Reduced OHSS
Tang et al (2006) (260)	Randomised double-blinded controlled trial	Agonist	n = 101	850mg BD from down regulation	No difference in FSH, oocytes retrieved or fertilisation. Significant increase in CPR beyond 12 weeks. Significant reduction in severe OHSS (MET = 3.8%, PLA = 20.4%; P = 0.023)
Tso et al (2009) (348)	Cochrane Review	-	6 studies included		No improvement clinical outcomes (LBR/CPR/miscarriage rates). No significant effect on ovarian stimulation (total dose FSH, stimulation days, oestradiol level, number oocytes collected, cycle cancellation rate) or fertilisation rate. Significant reduction in OHSS (0R 0.24 95% CI 0.12, 0.47)
Kjotrod et al (2011) (347)	Prospective double-blinded randomised placebo controlled trial	Agonist	n = 149 (intention to treat analysis)	Increasing dose to 2000mg/day for 12 weeks prior to stimulation until pregnancy test	Metformin pre-treatment improves the pregnancy (natural) rate in non-obese women with PCOS, but does not affect IVF/ICSI outcomes *per se*. No difference in OHSS
Palomba et al (2011) (350)	Prospective randomised double-blind placebo controlled trial	Agonist	n = 120	500mg TDS from down regulation until pregnancy test/bleed	OHSS and cancellation rates significantly reduced with metformin (RR 0.28, 95% CI, 0.11–0.67). With metformin, stimulation length and total amount of gonadotropins were significantly increased with significantly reduced oestradiol levels. No difference in CPR or LBR.
Tso et al (2014) (501)	Cochrane Review	-	9 studies included		Reduced OHSS (OR 0.29; 95% CI 0.18 to 0.49, eight RCTs, 798 women) No difference LBR (OR 1.39, 95% CI 0.81 to 2.40, five RCTs, 551 women) despite improved CPR (OR 1.52; 95% CI 1.07 to 2.15; eight RCTs, 775 women)

Evidence for a direct effect of metformin on granulosa cell function also exists (502). Adenosine 50 monophosphate-activated protein kinase (PRKA; formerly known as AMPK) is a key regulator of cellular energy homeostasis involved in cholesterol synthesis (502). Activation of PRKA has been shown to reduce progesterone secretion in immature rat granulosa cells and also reduce 3-beta-hydroxysteroid dehydrogenase (3BHSD) through the MAPK1/3 signalling pathway (503). Furthermore, metformin has been shown to activate PRKA and subsequently reduce basal and FSH-stimulated progesterone and oestradiol secretion via a reduction in 3BHSD, CYP11A1, CYP19A1 (aromatase) and steroidogenic acute regulatory (StAR) protein levels (502). This is consistent with La Marca et al (21) who demonstrated a reduction in CYP19A1 activity in response to FSH in human granulosa cells from women with PCOS following metformin use (21). Similarly, Mansfield et al (493) demonstrated a dose dependant reduction in progesterone and oestradiol production in luteinised human granulosa cells when co-cultured with metformin. Furthermore, a chronic activation of the PRKA pathway is involved in a potential anti-oxidant process at the ovarian level involving increased glutathione, nitric oxide synthase and prostaglandin production following metformin administration with DHEA (504). To enable steroid hormone production, there must be a translocation of cholesterol from the outer to the inner mitochondrial membrane. This process has been shown to be mediated by the StAR protein (324). Attia et al (324) demonstrated a drop in the StAR protein with metformin (but not an equivalent inhibition of StAR mRNA expression).

Excess androgens have long been considered detrimental to female reproductive health. This concept is challenged by work on androgen-receptor knockout mice, whereby a global reduction in fertility is seen if androgen deficiency exists (330). This effect has been subsequently pinpointed to the granulosa cells (505). Androgen receptors (AR) are expressed by the oocyte, thecal cells and cumulus cells but predominate in granulosa cells. The highest expression is in preantral / antral follicles with a gradual reduction with follicular maturation (506). Work with granulosa cell-specific AR knockout mice has shown increased follicular atresia, reduced cumulus

expansion and diminished oocyte viability (although not a global acceleration in follicle depletion) (505). Androgen priming is an increasingly 'hot' topic, particularly in low responders to IVF treatment (507). Paradoxically, PCOS is associated with an increased androgenic environment. An increase in the number of AR, AMHR2 and AMH expression in granulosa cells from women with PCOS may suggest an intrinsic granulosa cell dysregulation (216). Enhanced FSH-receptor expression (330, 508), increased number of FSH-responsive follicles and reduced follicle atresia (330) have all been implicated in the androgenic mechanisms used to regulate folliculogenesis. Androgens have been shown to promote *miR-125b* expression which suppresses pro-apoptotic proteins and subsequent follicle atresia (330). The expression of *miR-125b* is firstly regulated by extranuclear androgen signalling, before AR nuclear localisation and genomic androgen signalling. Sen et al implicate Paxillin as a critical regulator of this process (330). Furthermore, via the same Paxillin signalling process androgens can upregulate FSH receptor expression, enhancing the sensitivity to FSH stimulated follicular growth (330). In addition, in women with PCOS, there appears to be enhanced thecal cell androgen production in response to FSH, revealing a potential paracrine interaction between granulosa and thecal cells (509). Although the FSH receptor has not been located on the thecal cell, early work (510) supports Wachs et al (509) who suggested that FSH may augment androgen production via an unknown mechanism. An improvement in the intra-ovarian androgen environment with metformin may therefore attenuate the follicle response to gonadotrophins (350).

In a similar fashion, improving insulin resistance and hyperinsulinemia could marshall the aberrant response to gonadotrophins often seen in PCOS and the subsequent arrested follicle growth (511) (as discussed in Chapter 1). Insulin has been shown to directly stimulate several ovarian steroidogenic enzymes including CYP17, 3BHSD, P450 side-chain cleavage (P450scc) and StAR protein (37). Insulin also stimulates glucose uptake, by translocation of the GLUT4 transporter to the cell surface, via activation of the mediator PI3-K. In PCOS, reduced GLUT4 expression has been recognised (512). Metformin has been shown to increase GLUT4 and PI3-K activity

(513). Insulin-like growth factor 1 has been shown to have a synergistic effect with FSH via activation of aromatase and increased oestradiol production from granulosa and granulosa lutein cells (514). A key binding protein, IGFBP-1, is lower in women with PCOS and is inversely proportional to insulin concentration (515). Furthermore, this protein is implicated in the response to gonadotrophins, with an inverse correlation between the total amount of human menopausal gonadotrophin required during ovulation induction and serum IGFBP-1 (516). The putative mechanism for this may be the hyperinsulinimic hyperandrogenic environment leading to a direct reduction in hepatic IGFBP-1 production, with loss of the inhibitory control of IGF-1 in granulosa lutein cells. Metformin has been shown to increase plasma IGFBP-1, so reducing the IGF-1: IGFBP-1 ratio (43) and thereby modulating plasma androgen levels in PCOS. In addition, increased serum IGFBP-3 has been linked to an increased likelihood of pregnancy in women with PCOS (which is opposite to those women without PCOS) (517). The observed improvement in pregnancy rate occurs despite a reduction in circulating concentration of IGF-1 generating more immature oocytes. Insulin also augments the synthesis of a key growth factor, VEGF, that is known to be involved in the pathogenesis of OHSS and which is overexpressed by granulosa lutein cells from the polycystic ovary (259). Given this information, it is surprising that metformin has been shown to have little impact on reducing VEGF levels (261). The final target for metformin is ovarian morphology. A reduction in the number of antral follicles has been shown to be an acute response to metformin therapy, with some ovaries reverting to normal appearance (518, 519). The validity of the latter 2 trials is limited due to the extremely small sample size in both.

As shown in Table 3-1, the use of metformin alongside an agonist IVF cycle has been addressed. Some trials are powered to establish if a significant OHSS reducing effect exists (350) whilst others declare the effect within their secondary end points (260). Despite numerous trials, there is an absence of uniformity in the method of metformin treatment protocols. For example, the dosage and duration of metformin pre-treatment is highly variable. Dose escalation has been shown to be of limited value

(342). With the known side effect profile of metformin, achieving compliance with high doses and long courses of treatment can be problematic. Despite some basic differences in the methodologies of the trials, the overall outcomes are similar.

Research has not been conducted to the same depth for the use of metformin as an adjunct to antagonist assisted conception cycles. A single trial (12) has compared the stimulation outcome in a small sample population. There was no sample size calculation for this trial and it should therefore be considered a pilot study. It is unknown whether the use of the antagonist cycle alone provides as much OHSS protection as the addition of metformin. It is well recognised that an antagonist cycle reduces the risk of OHSS and that this strategy has a more pronounced effect in women with PCOS (RD -0.10 95% CI -0.14 to -0.07 $p < 0.00001$) (31). To address this knowledge deficit, there is need to conduct a sufficiently powered trial to evaluate the efficacy of metformin use in the antagonist treatment cycles, using OHSS as a primary end point to test the merit of metformin treatment as an OHSS reduction strategy for women with PCOS.

The aims of the current chapter were 2-fold:

(i) To conduct an appropriately powered RCT to evaluate the use of metformin as an adjunct in an antagonist IVF treatment cycle with the aim of reducing the risk of OHSS in women with PCOS.

(ii) To establish if metformin use had any impact on ovarian stimulation and embryological or clinical treatment outcomes within an antagonist cycle.

3.2 Materials And Methods

3.2.1 Eligibility criteria

Formal ethical approval (ethics reference 09/H1307/52) was granted for this project from Leeds West Ethics Committee. As a medicinal product was used, approval was sought and granted (EudraCT Number 2009-010952-81) from the Medicines and Healthcare products Regulatory Agency (MHRA). To be eligible for the study women had to have a known diagnosis of PCOS as defined by the Rotterdam criteria (449). All women therefore had at least 2 of the following characteristics: evidence of hyperandrogenism (clinical or biochemical), oligo/anovulation (cycles longer than 35 days) and PCOM on ultrasound with 1 or more ovaries with ≥12 follicles and/or volume ≥10cc^3.

Further inclusion and exclusion criteria for recruitment into the trial are documented within Table 3-2.

Table 3-2 Trial inclusion and exclusion criteria

Inclusion criteria	Normal Follicle Stimulating Hormone (FSH) <8.0iu/L (Day 1-5 menstrual cycle/following progesterone withdrawal) Age 20-39 years of age BMI<35 (maximum weight treated at clinic) Serum Testosterone <5.0nmol/L Prolactin <600mU/L Thyroid Stimulating Hormone 0.2-6.0mIU/L Normal renal, liver and haematological indices
Exclusion criteria	If taking any of following medication: - Oral-antidiabetic or blood glucose lowering preparations - Phenprocoumon, cimetidine, ketofen - Antivirals (Didanosine, Stavudine, Tenofir) Radiological examinations using contrast media within preceding 48 hours Recent myocardial infarction Known vitamin B12 deficiency

3.2.2 Recruitment process and subjects

Potential study participants were identified prospectively from the outpatient clinics and IVF starter list. Information surrounding the trial was supplied with further face to face discussion with the research doctor. After a reasonable time for reflection (at least 24 hours), if the patient wished to participate, written consent was completed.

Following written consent and confirmation of eligibility, patients were randomised to receive either placebo or active drug (metformin 850mg twice a day). Randomisation was done by the hospital pharmacy, using a random permuted blocks method with a 50:50 allocation ratio. This process was blinded to both patient and investigator by using identical over-encapsulation of the tablets (supplied and over-encapsulated by Bilcare Global Clinical supplies, Waller House, Crickhowell, UK). The allocation code was not broken until all women had been recruited and commenced treatment. Any patients who withdrew consent, continued with their routine IVF treatment. Their details were retained for analysis on an intention to treat basis unless the patient stipulated otherwise. Demographic and baseline data was recorded at the start of the treatment cycle (Table 3-3). Detailed information regarding the nature of the hormonal assays has been documented in Chapter 2.2.1.

Table 3-3 Demographic and baseline data

Demographics	**Blood tests**	**Ultrasound**
• Age • Number menses in preceding 6 months • BMI • Ethnicity • Duration of subfertility • Parity • Previous IVF cycles • Previous OHSS	• Anti-Müllerian Hormone (AMH) • FSH/LH/Oestradiol • Thyroid function tests • Prolactin • Testosterone/SHBG • Full blood count (FBC) • Liver function tests (LFT) • Urea, electrolytes and creatinine (U&E)	• Ovarian parameters - Volume - Antral follicle count - Exclusion of ovarian/uterine pathology

3.2.3 Treatment pathway

Following randomisation and consent, patients were supplied with the study medication and the routine ART drugs (Table 3-4). Due to drug availability there were different preparations used within the study time period. Within a 'nurse consultation' patients were instructed on how and when to commence their treatment.

Table 3-4 Standard ART drugs used

Generic Drug	Drugs used
Progestogen	Medroxyprogesterone Acetate (***Provera®***, Pfizer, Tadworth, Surrey, UK.) Progesterone • Vaginal - ***Cyclogest®***,Actavis UK Ltd, Barnstaple, UK • Intra-muscular - ***Gestone®***, Nordic Pharma Limited, Reading, UK; ***Prontogest®***, IBSA International, Pambio-Noranco, Switzerland.
Recombinant Follicle Stimulating Hormone (rFSH)	Follitropin alfa (***Gonal-F®***, Merck Serono Ltd, Feltham, Middlesex, UK) Follitropin beta (***Puregon®***, Organon, Hoddesdon, Hertfordshire, UK)
Gonadotrophin Releasing Hormone (GnRH) Antagonist	Ganirelix (***Orgalutron®***, Organon) Cetrorelix (***Cetrotide®***, Merck Serono)
Human Chorionic Gonadotrophin (hCG)	Chorionic gonadotrophin (***Pregnyl®***, Organon) Choriogonadotrophin alfa (***Ovitrelle®***, Merck Serono)

Metformin/placebo was started either 7 days prior to the patient's regular menstrual cycle (mid luteal) or day 1 of the period for women with an irregular cycle or using a progestogen (Provera®, Pharmacia) designed to induce a withdrawal bleed. Drug delivery was continued until the day before egg collection. At each subsequent visit patient compliance and side effects were documented. The IVF/ ICSI cycle followed the routine unit clinical practice for an antagonist cycle (286). For uniformity, recombinant FSH (rFSH) (no patients were treated with human menopausal gonadotrophin) was commenced on day 2 of the menstrual cycle, on a daily basis, at a dose adjusted for patient age, ovarian reserve and BMI (starting range 100-150iu). A GnRH antagonist

(250 micrograms) was added on day 6 of the cycle to prevent premature ovulation. A trans-vaginal ultrasound scan (TVUSS) was completed on day 8 of the cycle (6^{th} day of stimulation). Dependant on ovarian response, the dose of rFSH was adjusted. Participants were then reviewed as clinically indicated until 3 or more follicles were >17mm diameter. Follicular growth extrapolation was limited where possible. When this target was reached hCG of 5000-10000iu (dependant on follicle number) was administered 35-37 hours prior to the planned oocyte collection. If excessive follicular response was seen on scan (>30 follicles), consideration was given to stop the cycle prior to hCG administration.

On the day of oocyte collection, blood tests were repeated and a sample of serum stored where possible (including oestradiol, testosterone, FBC, U&Es, LFTs, and AMH). Oocyte retrieval was performed under conscious sedation using a combination of Fentanyl (up to 100µg) and Midazolam (up to 5mg). Trans-vaginal oocyte retrieval was performed using the standard approach. Oocytes were than inseminated (IVF) or injected (ICSI) within 4 hours of retrieval with either the partner's or donor sperm (520). The decision for insemination or ICSI was based on the WHO criteria for a normal human semen characteristics (520). Although individualised to patient and treatment cycle, if following sperm preparation the count was >4million/mL, grade a and b motility was >70% and yield was >10%, the intention was to use IVF. Samples for insemination were prepared using PureCeption sperm preparation media 40%/80% (Sage, Origio, Cooper Surgical company, Denmark) (521). For insemination, samples were adjusted to give a final motile concentration of $4x10^6$/mL sperm. Luteal phase progestogen support was commenced on the day of the collection. Type of progesterone (vaginal 400-800mg/day or intramuscular 50-100mg/day as detailed above) varied on patient/clinician preference.

Women who had more than 20 oocytes or 25 follicles aspirated were asked to return on day 3 and day 5 following collection to check for signs and symptoms of OHSS as shown in Table 3-7 (522). These included routine FBC and observations (weight change (kg), abdominal girth (cm), heart rate, blood pressure). All women were asked to

report if they had any symptoms suggestive of OHSS (shortness of breath, diarrhoea, nausea, vomiting, and inability to pass urine). Any women who were symptomatic of moderate OHSS had their embryos frozen without embryo transfer. The slow freeze technique was used rather than vitrification, using Quinn's Advantage (Origio, Denmark) embryo freeze kit, liquid nitrogen and the Planer freezer (Planer PLC, Middlesex, UK) set to Sage freezing programme (523). The time of presentation of symptoms dictated at what stage the embryos were frozen (between pro-nuclear and blastocyst stage).

Fertilised oocytes were transferred into single drops of culture media (Medicult/Origio, Denmark) and overlaid with Origio oil. Embryos were cultured between 2 to 5 days in a Benchtop Incubator BT37 (Planer PLC, Middlesex, UK) in mixed gas 6% CO_2 and 5% O_2 at 37°C. Embryos that were cultured to day 5 underwent a media change on day 3. In fresh transfer cycles, ultrasound guided embryo transfer took place between day 2-5 following oocyte collection. A maximum of 2 embryos were replaced, the number being dependant on embryo quality and number. The grading system used for day 3 and day 5 embryos is shown in Table 3-5 and Table 3-6 respectively. An embryo was referred to as good quality if graded ≥ 6.3.3 on day 3 or ≥2Bb (524, 525).

Table 3-5 Embryo grading day 2 & 3 - Cell number / Size / Fragmentation

Blastomere number	
Blastomere size (526)	4 = regular, even division 3 = <20% difference 2 = 20-50% difference 1 = >50% difference
Fragmentation (527)	4 = <10% fragmentation by volume 3 = 10-20% 2 = 20-50% 1 = >50%

Table 3-6 Blastocyst grading - Expansion / Inner cell mass / trophectoderm

Expansion	**1**	Early blastocyst (blastocoel less than half volume of embryo, little or no expansion in overall size, thick zona pellucida (ZP)
	2	Blastocyst (blastocoel > half volume of embryo, some expansion in overall size, ZP thinning)
	3	Full blastocyst (blastocoel completely fill embryo)
	4	Expanded blastocyst (blastocoel volume larger than early embryo, ZP very thin)
	5	Hatched blastocyst (blastocyst has evacuated ZP)
Inner Cell Mass (ICM)	**A**	ICM prominent, easily visible, consisting of many cells tightly adhered together
	B	Cells less compacted, loosely adhered together, some individual cells visible
	C	Very few cells visible, may be difficult to completely distinguish from trophectoderm
	D	Cells appear degenerate or necrotic
	E	No ICM cells discernible in any focal pane
Trophectoderm	**a**	Many small identical cells forming continuous layer
	b	Fewer, larger cells, may not be completely continuous
	c	Sparse cells, may be very large, flat or degenerate

A serum pregnancy test was taken on day 14 (Siemens Advia Centaur & Siemens reagents, Siemens Healthcare, Camberley, UK) or a urine test on day 18 (Surestep^{tm}, Alere, UK). A serum hCG >2 IU/L was deemed positive but if a low reading was obtained a repeat hCG was completed 48 hours later to ensure an adequate rise occurred. If positive, a TVUSS was completed after ~7 weeks of gestation (5 weeks following oocyte retrieval). If a fetal heart was seen on this scan, it was deemed a clinical pregnancy. All outcomes were followed up in accordance with the Human Fertility and Embryology Authority (HFEA) guidance.

3.2.4 Primary and secondary trial end points

The primary end point for this trial was the incidence of moderate to severe OHSS within 6 weeks of completing the IVF cycle. OHSS was classified using the definition recommended by the Royal College of Obstetrician and Gynaecologists (RCOG) outlined in Table 3-7. The trial was appropriately powered for this end point. The secondary end points included the ovarian stimulation characteristics (number of oocytes collected and total stimulation doses), embryological measures (fertilisation rate, % of good quality day 3 embryos and % with day 5 embryo transfer) and cycle outcome including CPR and LBR.

Table 3-7 Classification of ovarian hyperstimulation syndrome as recommended by the Royal College of Obstetricians and Gynaecologists (522)

Grade	Symptoms
Mild	Abdominal bloating Mild abdominal pain Ovarian size usually <8cm*
Moderate	Moderate abdominal pain Nausea ± Vomiting Ultrasound evidence of ascites Ovarian size 8-12cm*
Severe	Clinical ascites (occasionally hydrothorax) Oliguria Haemoconcentration haematocrit >45% Hypoproteinaemia Ovarian size >12cm*
Critical	Tense ascites or large hydrothorax Haematocrit >55% White cell count >25000/ml Oligo/anuria Thromboembolism Acute Respiratory Distress Syndrome

*Correlation with ovarian size may not be useful if follicle aspiration has occurred

3.2.5 Statistical analysis and power calculation

This study was powered to assess metformin's ability to reduce OHSS as an adjunct in an ART cycle. To date there is only one small trial using the antagonist cycle and

metformin. Although the sample size was not predetermined, a trend to reduced OHSS was seen (351). Costello et al (349) performed a meta-analysis of metformin co-administration during IVF in women with PCOS. A significant reduction in OHSS was seen. Pooled analysis showed OHSS occurred at a rate of 12/216 in those patients on metformin and 44/210 in those on the Placebo. The odds ratio was 0.21 (95% CI 0.11-0.41). The chosen power for this study was 80% with a type 1 error of 0.05. From the meta-analysis data, the standardised difference (d) for reduction of OHSS following use of metformin was 0.454. Using the Altman nomogram (528), when d=0.454 and power=0.80, the projected sample size was 146 with 73 in each arm. The same outcome was found using a statistical package (Stata, Version 12, StataCorp LP, Texas, USA). This sample size calculation was confirmed by Dr Graham Law, senior lecturer in statistical epidemiology and Head of Biostatistics, University of Leeds. Intention to treat analysis was used. To allow for cycle dropout of recruited subjects, recruitment continued until the appropriate number of recruits had started the treatment cycle.

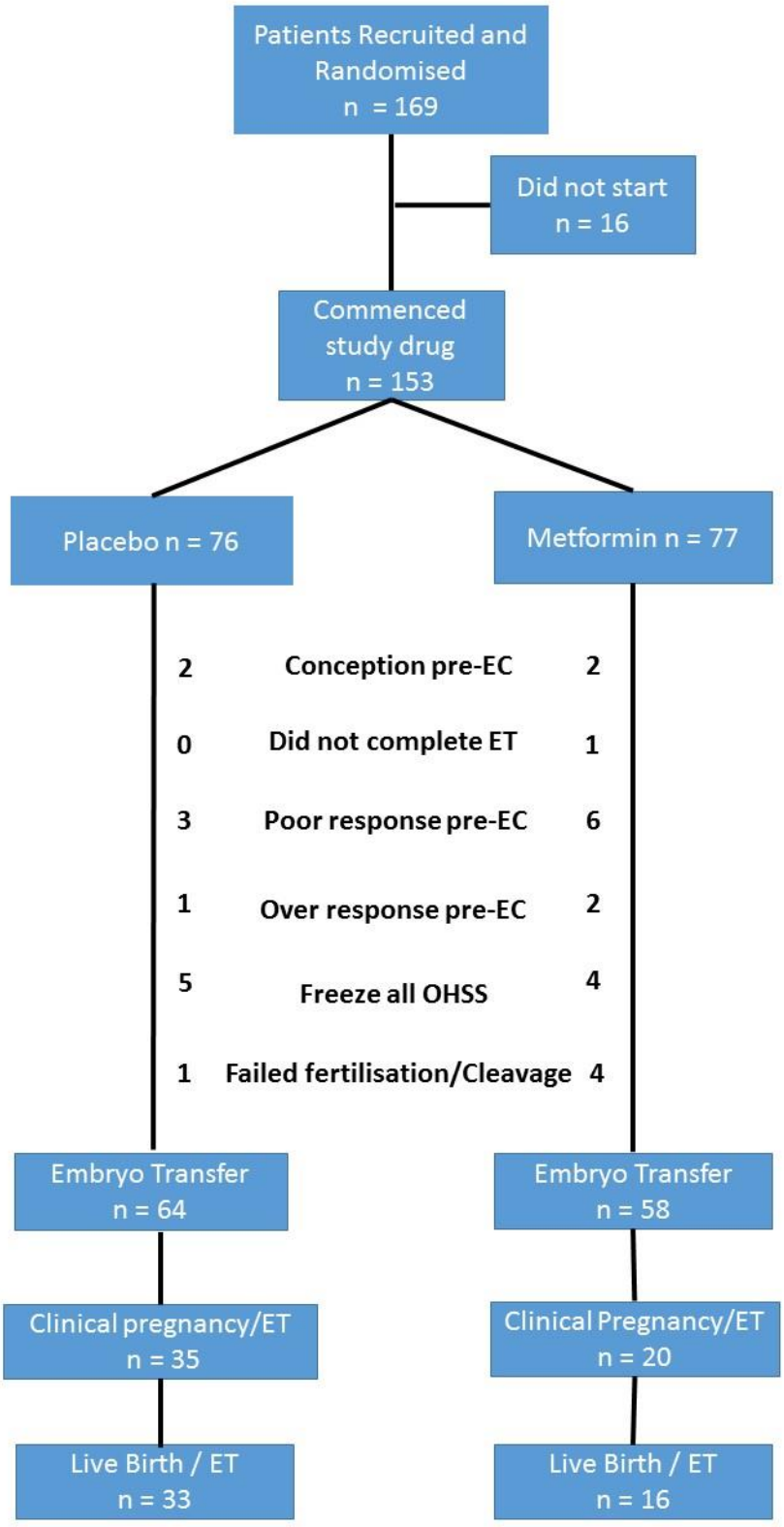
Statistical analysis was conducted using the IBM SPSS 21 statistics package (IBM Corporation, New York, USA). Descriptive statistics include mean and standard deviation for parametric data; median and IQR for non-parametric data. Unless stated otherwise the placebo group result was listed first in each comparison. Odds ratios were constructed where appropriate with 95% confidence intervals. Continuous data was tested for normality using the Kolmogorov-Smirnov test. Normally distributed data was analysed using Student's t-test (with Levene's test for equality of variance). This included the baseline demographics of age, BMI and fertilisation rate. This data was presented as mean ± standard deviation. Data that was non-parametrically distributed were analysed using the Mann-Whitney test. This included the ovarian stimulation characteristics, oocytes collected and baseline AMH values. They were presented as median (IQR). Contingency tables were employed were necessary with Chi-squared statistic (plus continuity correction) and Fisher's Exact tests. This was used for categorical data including OHSS rate, CPR and side effect profile. A p value of <0.05 was considered significant. Multiple regression analyses were used to assess impact of

independent factors on the dependant factors OHSS and clinical pregnancy rate. A ROC curve analysis was used to assess the ability of AMH to predict which women would experience moderate to severe OHSS. To establish correlation between ordinal variables (e.g. AMH and AFC) Spearman's Rank correlation coefficient was used. The Fisher r to z transformation was used to assess significance between 2 correlation coefficients.

3.3 Results

Between October 2009 and June 2014, 169 patients were recruited to the trial. A hiatus in recruitment occurred between August 2011 to August 2012, due to a change in principal investigator (initial ethical / MHRA approval and recruitment (*n*=98) completed by Dr Christopher Brewer). Of these, 153 patients commenced the study drug and were included in the analysis. The remaining 16 patients did not commence an IVF cycle or study medication for a variety of reasons (natural conception / lost to follow up / unable to take study medication for religious reasons/did not fulfil eligibility criteria). Figure 3-1 outlines the patient recruitment pathway and randomisation to metformin or placebo. A total of 122 patients had an embryo transfer, with an overall clinical pregnancy rate of 45.1%. For all cycles receiving gonadotrophins, the incidence of moderate-severe OHSS was 14.1% (21 women out of 149). Of these 21 cases, 9 had all embryos frozen with no fresh embryo transfer and 3 had cycles abandoned prior to the hCG trigger. Randomisation delivered the desired 50:50 split, regardless of non-starters. The study drug was taken for a median duration of 16 days (range 4-38) prior to oocyte collection. Due to work flow patterns and duration of recruitment, operator numbers were not restricted and the procedures were carried out routinely by the unit's personnel.

Figure 3-1 Recruitment and treatment pathway



3.3.1 Demographic data

There were no significant differences in baseline characteristics between the 2 groups of women (Table 3 8). Women included in the trial were on average under 30 years of age (29.7yrs ± 4.2) with a BMI on the upper limit of normal (25.1/m² ± 3.3). The patients' had on average entered their 3rd year of subfertility (3.6yrs ± 2.3) with an average 94% undergoing their first IVF cycle. Hormonal parameters revealed equally matched groups with respect to FSH and AMH; although non-significant the placebo group had an increased serum testosterone (p=0.05) and androstenedione level. Ultrasound features of PCOM were also equally matched with the average antral follicle count >30. There was a trend towards increased number of South Asian women in the metformin group but this was not significant (19.7% vs. 27.3%; p=0.364). The equal baseline characteristics demonstrate equality in risk of OHSS.

3.3.2 Study medication duration and dropouts

The 2 groups of patients had comparable durations of study drug prior to oocyte collection with a median duration of 16 days but the data included a large range of 4 - 38 days. Only 1 patient stopped the study drug after 4 days (metformin) due to physically not liking swallowing the tablet and for a further patient the drug dose was reduced to 850mg/day due to side effects. For women who had their cycle stopped due to poor response there was a non-significant (p>0.05) increased proportion on metformin (3 vs. 6) but in these groups the average duration of study drug consumption was similar to the entire population (20 vs. 18.5 days). The proportions were similar for cycles stopped for over response (1 vs. 2) and freeze all cycles due to risk of OHSS (5 vs. 4). Figure 3 1 highlights the patients who stopped their treatment before oocyte retrieval and embryo transfer.

Table 3-8 Patient demographic data*

	Placebo (n=76)	Metformin (n=77)	P value	Significance
Age (Years)	29.6 ± 3.9	29.9 ± 4.4	0.70[2]	*ns*
BMI	25.0 ± 3.3	25.3 ± 3.4	0.54[2]	*ns*
FSH (iu/mL)	5.1 ± 1.6	5.3 ± 1.4	0.41[2]	*ns*
Testosterone (nmol/L)	1.3 (0.9-1.7)	1.1 (0.8-1.5)	0.05[4]	*ns*
Androstendione (nmol/L)	5.1 (3.8-6.5)	4.2 (3.4-6.4)	0.19[4]	*ns*
AMH (pmol/L)	61.8 (40.8-98.6)	62.6 (45.4-84.2)	0.50[4]	*ns*
SHBG (nmol/L) **(limited numbers n=91)**	57.2 ± 29.6	54.8 ± 29.2	0.71[2]	*ns*
At least one ovary volume >10cm³ (%)	55.4	50	0.70[1]	*ns*
Antral Follicle Count	31.3 ± 9.7	30.0 ± 10.1	0.44[2]	*ns*
Women with cycles >3months (%)	18.4	23.4	0.58[1]	*ns*
Duration subfertility (Years)	3.7 ± 2.3	3.5 ± 2.3	0.57[2]	*ns*
1st treatment cycle (%)	92.9	95.5	0.70[3]	*ns*
% South Asian women (number)	19.7 ± 15	27.3 ± 21	0.36[1]	*ns*

[1]Chi squared with continuity correction [2]Independent t-test [3]Fisher's exact [4]Mann Whitney U
*Normally distributed data presented as mean ± SD and non-parametric data as median (IQR)
Non significant (ns)

3.3.3 Treatment outcome data

Stimulation characteristics of the 2 populations were similar (outlined in Table 3-9) with the same duration of stimulation and total dose of gonadotrophins. An equivalent number of follicles were punctured and oocytes retrieved. For those patients on metformin, there was a significantly reduced clinical pregnancy rate per cycle started (48.7% vs. 28.6%; p=0.02) and per embryo transfer (54.7% vs. 34.5%; p=0.04). This difference is perhaps more pronounced when expressed as an odds ratio (OR 0.436, 95% CI 0.210-0.906), which clearly shows an inferiority with metformin (Table 3-10). A similar disparity in live birth rate was seen in favour of placebo (51.6% vs. 27.6%; p=0.01). There was no significant difference in quality of embryo or day of transfer to support the difference in CPR/LBR. Using multiple logistic regression analysis (Table 3-11), metformin use and ethnicity of the patient were the main factors to exert an effect on CPR, when controlling for age and BMI. There was a significant difference between CPR per cycle started in the AMH concentration (No clinical pregnancy 71.3 (48.7-92.2) pmol/L vs. clinical pregnancy 53.5 (35.5-83.1) pmol/L; p=0.046). Those with an increased AMH had a worse CPR.

3.3.4 Side effects during trial

A highly significant proportion of women experienced at least 1 side effect whilst on metformin during the trial compared with controls (11.8% vs. 42.9%; p<0.001), of whom only 2 reduced or stopped the drug. The majority of these problems related to transient nausea, vomiting or diarrhoea (81.8%). The chance of experiencing a side effect is therefore high when taking metformin (OR 5.583 95%CI 2.436-12.796).

Table 3-9 Treatment outcome data*

	Placebo	Metformin	P value	Significance
% of ICSI cycles	55.3	44.2	0.23[1]	*ns*
Total days study drug	17(12-20)	16(12-20)	0.93[2]	*ns*
Stimulation days (including hCG day)	10 (9-11.8)	10(9-11)	0.89[2]	*ns*
Total dose gonadotrophins units	1200 (900-1500)	1200(1000-1375)	0.75[2]	*ns*
Total follicles entered	23 (12.8-30.3)	20 (14-26.5)	0.74[2]	*ns*
Total eggs collected	15(9.8-21)	14 (9-20)	0.66[2]	*ns*
Fertilisation rate (%)	60.7 ±22.2	53.3 ±25.4	0.07[3]	*ns*
Good D3 embryos (%)	52.3 ±31.9	47.1 ±32.9	0.37[3]	*ns*
D5 transfer (%)	59.4	48.3	0.30[1]	*ns*
ESET (%)	76.6	81.0	0.70[1]	*ns*
Implantation rate (%; gestation sacs/Number transferred)	51.9 *(41/79)*	34.9 *(24/69)*	0.14[4]	*ns*
Clinical pregnancy rate (%; FH/ET)	54.7 *(35/64)*	34.5 *(20/58)*	0.04[1]	*p<0.05*
CPR (%; FH/cycle started)	48.7 *(37/76)*	28.6 *(22/77)*	0.02[1]	*p<0.05*
Live birth rate (%; LB/ET)	51.6 *(33/64)*	27.6 *(16/58)*	0.01[1]	*p<0.05*
Side effect rate (%)	11.8	42.9	<0.001[1]	*p<0.01*
Moderate/severe OHSS (%)	12.2	16	0.66[1]	*ns*

[1]Chi squared with continuity correction [2]Mann-Whitney U [3]Independent t-test with Levene's test for equality of variances [4]Fisher's Exact. ESET: elective single embryo transfer.
* Normally distributed data presented as mean ± SD and non-parametric data as median (IQR)

Table 3-10 Odds ratio placebo vs. metformin

Metformin vs. Placebo	Odds Ratio	95% CI
Mod-severe OHSS	1.376	0.542-3.491
Clinical Pregnancy per ET	0.436	0.210-0.906
Live Birth per ET	0.358	0.168-0.762
Side effect profile	5.583	2.436-12.796

Table 3-11 Multiple Logistic Regression Analysis: Clinical Pregnancy Rate per cycle (Dependant Variable)

Independent variables	Coefficient	P value
Metformin/Placebo	-0.981	0.006
Age	0.035	0.427
BMI	-0.0223	0.663
Ethnicity	-1.066	0.025

3.3.5 Ovarian hyperstimulation outcome

There was no difference in the incidence of OHSS between the study medication groups (OR 1.376, 95%CI 0.542-3.491). This included the grouped moderate-severe OHSS and individual levels of OHSS (see Figure 3-2). There were no cases of critical OHSS. There were a total of 7 severe OHSS cases in each group. Of these only 1 patient with severe OHSS in each group had an embryo transfer. Of the remaining 12, 3 had an abandoned cycle pre oocyte collection and 9 had a freeze all cycle following collection. Of those who underwent an embryo transfer, there was an overall 6.6% incidence of moderate-severe OHSS and for severe alone 1.64%, with no significant difference between the groups (p=0.70). Furthermore, of the cycles started that achieved a clinical pregnancy following embryo transfer (n=55), there was no difference in OHSS between groups (p=0.82). When adjusting for age, BMI, total dose of gonadotrophin and study medication, there was no significant effect on the overall incidence of moderate-severe OHSS (Table 3-12). Of those patients who had OHSS, 54.2% achieved a clinical pregnancy per embryo transfer. There was a trend for increased AMH and incidence of moderate-severe OHSS but this did not reach significance (No OHSS 60.6(40.9-88.0) pmol/L vs. OHSS 73.7(55.7-114.0) pmol/L; p=0.16). This was not shown in the individual grades of OHSS as AMH levels in severe OHSS were lower than in mild or moderate OHSS.

Figure 3-2 Grade of OHSS between the placebo and metformin treatment group

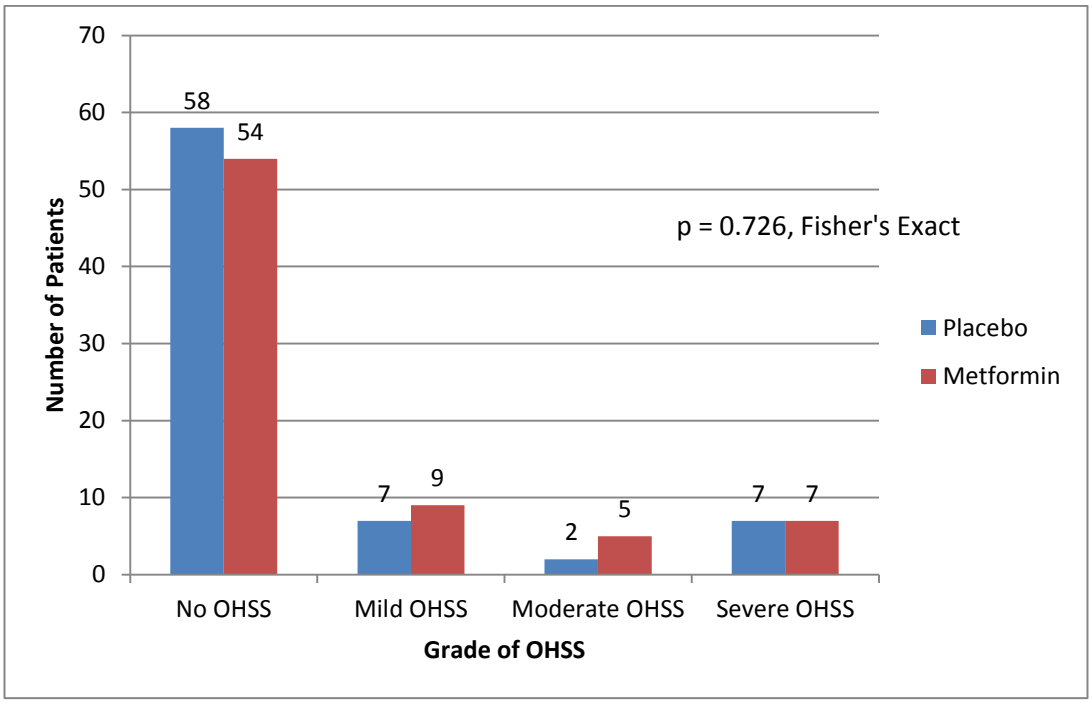
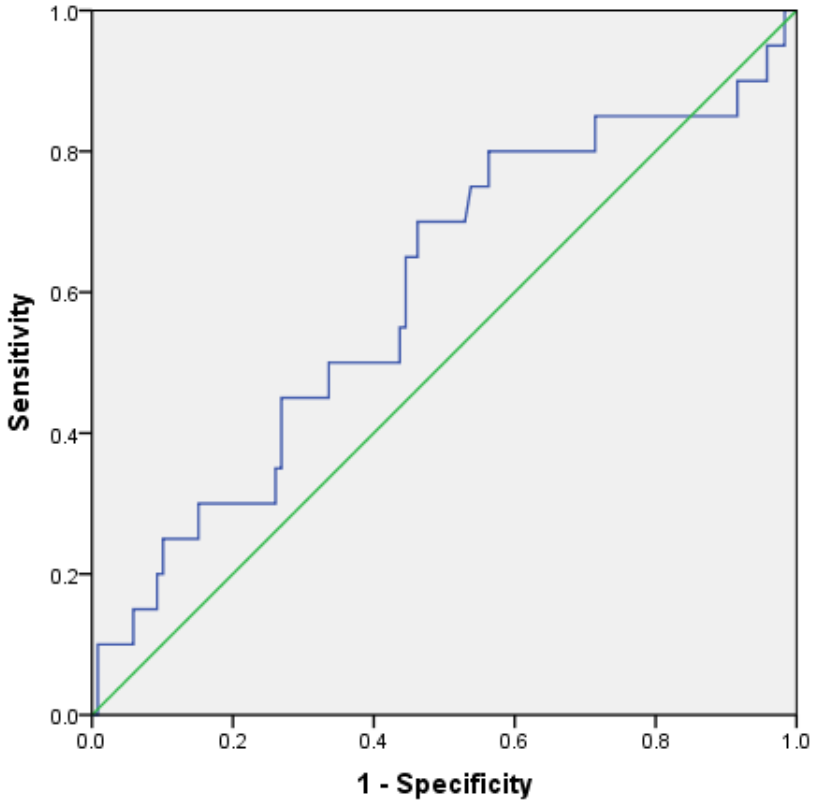


Table 3-12 Multiple logistic regression analysis: moderate-severe OHSS (dependent variable)

Independent variables	Coefficient	P value
Metformin/Placebo	0.352	0.465
Age	-0.056	0.327
BMI	-0.0973	0.160
Total dose of gonadotropins	0.00	0.935

A ROC curve was constructed to assess if baseline serum AMH levels predicted moderate-severe OHSS in a PCOS population (see Figure 3-3). This analysis showed poor predictive power of AMH in predicting OHSS (AUC 0.60). The AUC value was similar for any evidence of OHSS including mild symptoms (0.65).

Figure 3-3 A receiver operator characteristic curve testing AMH as a predictor for moderate-severe OHSS in PCOS population

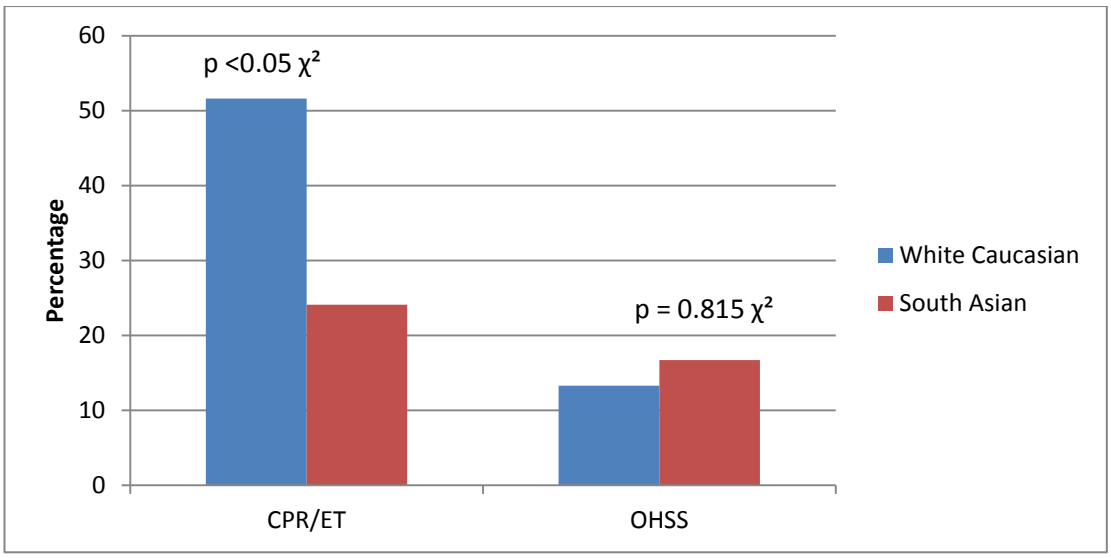


3.3.6 Ethnicity

Due to the population characteristics of the women attending LCRM, the only 2 ethnicities that fit the recruitment criteria were broadly categorised as White Caucasian or South Asian women. Between each study medication treatment group, there was no significant difference in pregnancy outcome within in each ethnic group (p=0.10) i.e. the CPR was similar for the South Asian group irrespective of study medication taken. When combining the study medication groups (see Figure 3-4), there was a significant reduction in pregnancy rate in the South Asian population (White Caucasian 51.6% vs. South Asian 24.1%; p=0.02). This difference was highlighted by multiple logistic regression where ethnicity and study medication remained significant for clinical pregnancy rate (Table 3-11). No significant difference

was seen in incidence of OHSS by ethnicity (White Caucasian 13.3% vs. South Asian 16.7%; p=0.82) or by ethnicity and study medication (p=0.42).

Figure 3-4 Clinical pregnancy rate and OHSS by ethnicity



To further compare the disparity in clinical outcome between the 2 ethnic groups, the data was re-evaluated combining the study medication groups by ethnic background (Table 3-13). Figure 3-5 further highlights some of these important differences. This was possible as within each ethnic group there was no difference between study arms. Thereafter there remained no significant difference in age, starting FSH level, ovarian morphology and degree of anovulation. There was a significantly increased ($p<0.05$) androgenic profile in the South Asian group. The South Asian group also had a longer duration of subfertility (4.6yrs ± 3.2 vs. 3.3yrs ± 1.9; p=0.03) and a reduced number were completing their first cycle (83.3% vs. 97.4%; $p<0.01$). There was also an increased proportion of the most 'severe' (OA/HA/PCOM) form of PCOS within the South Asian cohort (26.5% vs. 38.9%; p=0.127). There was an equal distribution of those with this phenotype on metformin or placebo. The South Asian population had a significantly increased BMI (24.9 ± 3.4 vs. 26.0 ± 2.8; p=0.05).

Table 3-13 Comparison between White and South Asian baseline demographics, stimulation characteristics and outcome*

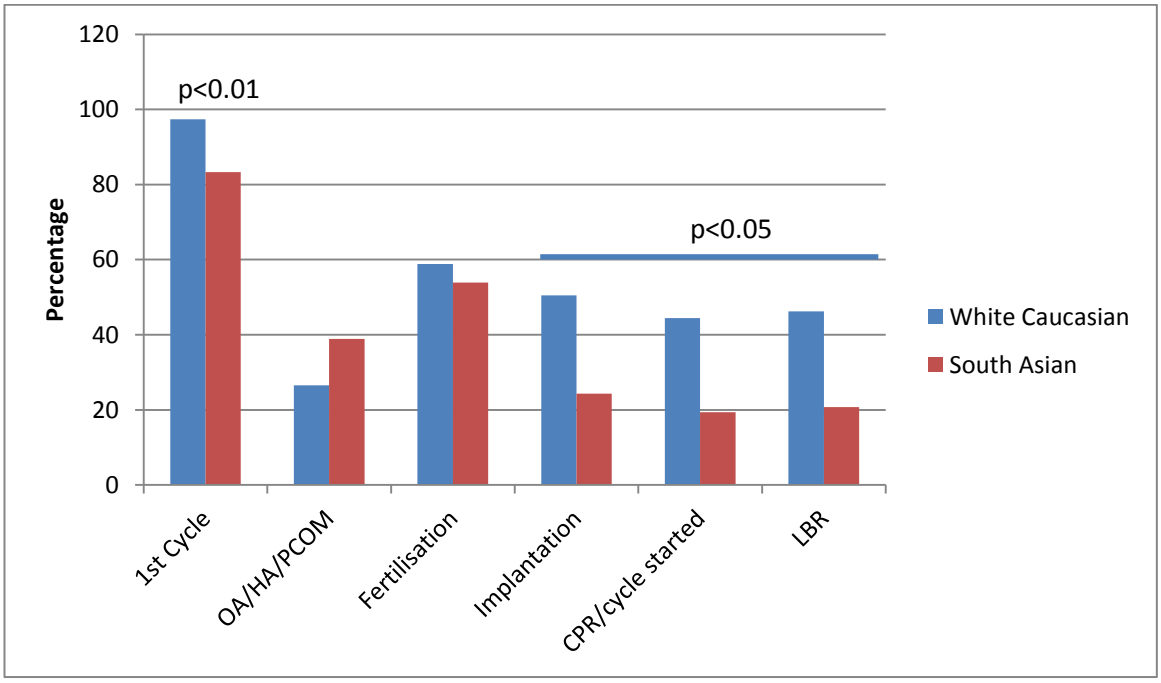
	White (n=117)	Asian (n=36)	P value	Significance
Age (Years)	30.0 ± 4.1	28.9 ± 4.2	0.15[3]	ns
BMI	24.9 ± 3.4	26.0 ± 2.8	0.05[3]	p<0.05
FSH (iu/mL)	5.3 ± 1.5	4.9 ± 1.7	0.28[3]	ns
Testosterone (nmol/L)	1.1 (0.8-1.5)	1.4 (1-1.9)	0.02[2]	p<0.05
Androstenedione (nmol/L)	4.5 (3.4-6.3)	6.1 (4-7.8)	0.02[2]	p<0.05
AMH (pmol/L)	61.0 (39.8-92.3)	68.1 (48.4-87.2)	0.56[2]	ns
At least one ovary volume >10cm³ (%)	57.1%	52.94%	0.81[1]	ns
Antral Follicle Count	30.8 ± 10.2	30.3 ± 8.8	0.80[3]	ns
Women with cycles >3months (%)	21.4%	19.4%	0.99[1]	ns
Duration subfertility (Years)	3.3 ± 1.9	4.6 ± 3.2	0.03[3]	p<0.05
1st Cycle (%)	97.4% (114/117)	83.3% (30/36)	0.01[4]	p<0.01
% of ICSI cycles	48.7%	52.8%	0.81[1]	ns
Total days study drug	16.5 (12-19)	20 (13-22)	0.06[2]	ns
Stimulation days (including hCG day)	10 (9-11)	11 (9-13)	0.05[2]	ns
Total dose gonadotrophins	1200 (900-1350)	1300 (1100-1631.3)	0.06[2]	ns
Total follicles entered	21 (14-30)	19.50 (13-28)	0.65[2]	ns
Total eggs collected	15 (10-20)	13 (7.3-20.8)	0.68[2]	ns
Fertilisation rate (%)	58.8 ± 24.8	53.9 ± 21.5	0.38[3]	ns
Good D3 embryos (%)	48.4 ± 31.0	54.6 ± 36.6	0.37[3]	ns
D5 transfer (%)	53.8%	55.2%	1.00[1]	ns
ESET (%)	81.6%	72.4%	0.49[1]	ns
Implantation rate (%; gestation sacs/Number transferred)	50.5% (56/111)	24.3% (9/37)	0.01[4]	p<0.05
Clinical pregnancy rate (%; FH/ET)	51.6% (48/93)	24.1% (7/29)	0.02[1]	p<0.05
CPR (%; FH/cycle started)	44.4% (52/117)	19.4% (7/36)	0.01[1]	p<0.05
Live birth rate (%; LB/ET)	46.2 (43/93)	20.7% (6/29)	0.03[1]	p<0.05
Side effect rate (%)	27.4% (32/117)	27.8% (10/36)	1.00[1]	ns
Moderate/severe OHSS (%)	13.3% (15/113)	16.7% (6/36)	0.82[1]	ns

[1]Chi squared with continuity correction [2]Mann-Whitney U [3]Independent t-test with Levene's test for equality of variances [4]Fisher's Exact

* Normally distributed data presented as mean ± SD and non-parametric data as median (IQR)

Although there was a trend in the South Asian population for increased stimulation and total gonadotrophin dose with fewer oocytes collected, this remained non-significant. There was no difference in fertilisation rate, quality of embryos or number of cycles having a single embryo transfer. As already stated, there was no difference in OHSS rate, nor any difference in reported side effects. There was a significant inferiority of the South Asian population with respect to implantation rate (24.3% vs. 50.5%; *p*=0.01), clinical pregnancy rate (per cycle started 19.4% vs. 44.4%; *p*=0.01) and live birth rate (20.7% vs. 46.2 %; *p*=0.03).

Figure 3-5 Highlighted differences between the White Caucasian and South Asian populations



If the South Asian cohort is removed from the dataset, and the data reanalysed by study medication, the significant differences in clinical pregnancy and live birth rate are lost. There remains a trend in favour of the placebo arm of the study across multiple parameters assessed (placebo data vs. metformin data): fertilisation rate (61.1% vs. 54.5%; *p*=0.18), CPR per cycle started (52.5% vs. 35.7%; *p*=0.10), CPR per

embryo transfer (58.8% vs. 42.9%; *p*=0.19) and live birth rate (54.9% vs. 35.7%; *p*=0.10). There remains no difference in incidence of OHSS (22.0% vs. 24.1%; *p*=0.97). This was similar if the South Asian population alone were also analysed according to study medication.

3.3.7 PCOS phenotype

There was no significant difference in incidence of OHSS by PCOS phenotype (Table 3-14, p=0.50) or clinical pregnancy rate (p=0.89). When study medication was taken into account there remained no significant difference between phenotypes for OHSS (p=0.93) or clinical pregnancy rate (p=0.17). There was no significant difference in AMH by phenotype (*p*= 0.08, Kruskal Wallis).

Table 3-14 OHSS by PCOS phenotype

		AMH pmol/L (median/IQR)	>=Moderate OHSS		Total
			no	yes	
PCOS Phenotype	OA/HA	112.7	1	0	1
	OA/PCOM	69 (49.0-87.5)	54	7	61
	HA/PCOM	55.0 (33.8-75.8)	35	4	39
	OA/HA/PCOM	65.5 (46.1-107.8)	38	9	47
Total			128	20	148

OA: Oligo-anovulation HA: Hyperandrogenism PCOM: Polycystic ovary morphology

It should be noted that all recruited patients except 1 had PCOM. Table 3-15 shows the significant increase in AMH with AFC in the whole cohort. These results should be viewed with caution as for some recruited patients, the documentation was 12+ follicles/per ovary. Therefore the results are likely to be skewed favouring the lower AFC categories, which may falsely elevate the median AMH values in these categories.

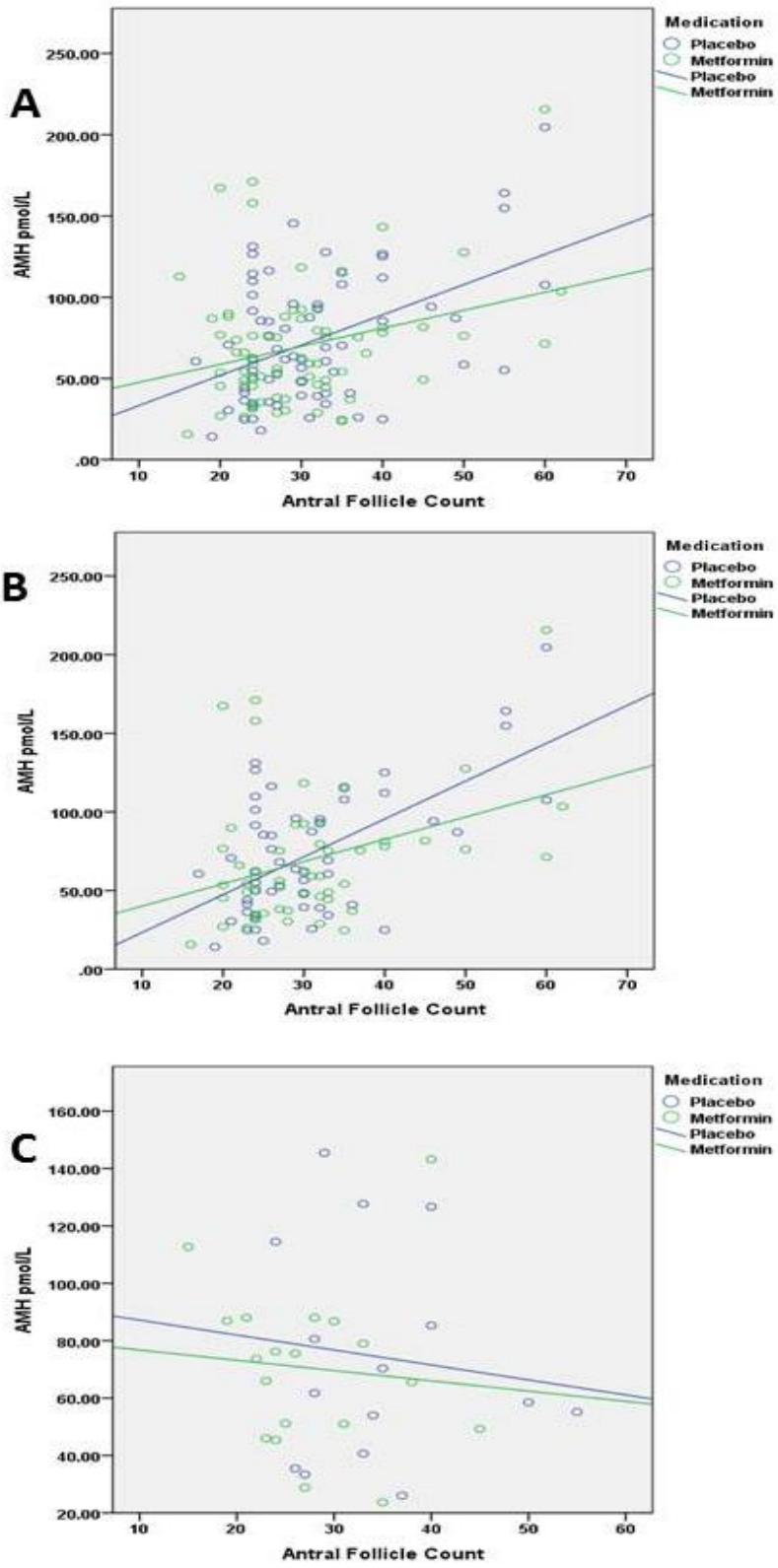
Table 3-15 Anti- Müllerian Hormone and antral follicle count

Antral follicle count	Total AMH pmol/L (n=141)	Placebo (P) (n=69)	Metformin (M) (n=72)	p-value between P & M
<24 (n=25)	53.5 (33.3 – 75.2)	38.8 (26.2 – 56.4)	66.0 (45.6 – 87.5)	$p < 0.05^2$ (0.03)
24 < 36 (n=88)	59.8 (41.6- 87.9)	63.0 (46.0 -95.6)	55.2 (38.0 -79.1)	$p = 0.18^2$
36 < 48 (n=16)	79.7 (43.0 – 107.6)	89.7 (29.7 – 121.9)	76.8 (53.3 – 81.6)	$p = 0.72^2$
48 < 65 (n=12)	105.3 (72.6 – 161.8)	107.7 (58.5 – 164.2)	103.5 (73.8 – 171.6)	$p = 1.00^2$
p-value across age range	$p < 0.01^1$ (0.002)	$p < 0.05^1$ (0.01)	$p = 0.06^1$	

[1]Kruskal Wallis [2]Mann-Whitney U. Values expressed as median (IQR)

Further evaluation of the relationship between AMH and AFC is shown in Figure 3-6 including between the placebo and metformin group. A significant correlation was found ($p<0.01$) between AMH and AFC when the entire group and White Caucasian population were reviewed. There was no significant correlation in the South Asian population although this is likely due to small numbers (n=15). There was no significant difference in correlation coefficients between the placebo and metformin arms of the study.

Figure 3-6 Correlation between AMH and AFC in placebo and metformin group. A: all recruited patients B: White Caucasian patients C: South Asian patients



3.3.8 Biochemical parameters

On the day of oocyte collection, only 20 patient serum samples were stored due to an early methodological oversight. This limited the evaluation of the medication (placebo/metformin) on baseline values of AMH, oestradiol and testosterone (see Table 3-16). There was a trend for reduced serum AMH, testosterone and oestradiol with metformin although this did not reach significance. There was a significant reduction from baseline in median AMH in each medication group but not between medication groups.

Table 3-16 Biochemical parameters comparing baseline tests to those on day of oocyte retrieval

		Placebo	**Metformin**	**p value**	**significance**
AMH pmol/L (n=20)	Baseline (B)	80.6 (46.4-128.8)	75.26 (48.9-81.2)	0.50[1]	ns
	Oocyte retrieval (OR)	37.9 (21.4-54.3)	24.8 (17.9-37.3)	0.30[1]	ns
	Difference between B & OR	35.9 (19.4-93.5)	44.3 (23.0-57.3)	0.66[1]	ns
Oestradiol pmol/L (n=23)	Baseline	146 (83-247)	141 (124-225)	0.69[1]	ns
	Oocyte retrieval	5217 (3039-10638)	3922 (2068-5914)	0.18[1]	ns
	Difference between B & OR	5012 (2861-10499)	3660 (1947-5689)	0.18[1]	ns
Testosterone nmol/L (n=20)	Baseline	1.2 (1-1.6)	1 (0.8-1.3)	0.30	ns[1]
	Oocyte retrieval	3.7 (1.6-4.8)	1.5 (1.4-2.7)	0.18[1]	ns[1]
	Difference between B & OR	2.5 (0.35-3.5)	0.7 (0.6-1.7)	0.26[1]	ns[1]

[1]Mann-Whitney U *all results expressed as median (IQR)

3.4 Discussion

3.4.1 Ovarian hyperstimulation syndrome and metformin

Ovarian hyperstimulation remains a serious risk of ART despite numerous advances in the treatment of infertility, especially for those at high risk of hyper-response. The overall reported incidence of clinically relevant OHSS in the literature varies between 1-60% (248, 264, 269, 529).The current study is the largest randomised placebo controlled trial to-date to assess the adjuvant effect of metformin on the risk of OHSS in an antagonist IVF cycle in patients with PCOS. Disappointingly, unlike the promising trend of reduced OHSS suggested by Doldi et al (351), metformin had no impact on the incidence of OHSS when used in this appropriately powered study. The overall occurrence of moderate-severe OHSS (14.1%) was in keeping with published data (529, 530). Similar to many of the published agonist trials, there was no change in the stimulation characteristics including total FSH dose, duration of stimulation and number of oocytes retrieved (260, 348, 500). It has been suggested that metformin may attenuate the risk of OHSS by reducing the number of small follicles (350). Although not formally assessed, a trend of reduced follicle entry and smaller follicle size range was seen in the metformin group, but this did not affect the overall oocyte yield. Alongside this in the small number of patients who had a hormonal profile taken on the day of oocyte collection, there was a trend towards reduced oestradiol increase following metformin treatment (5012 (2861-10499) vs. 3660 (1947-5689)pmol/l n= 23; *p*=0.18) and suppression of serum testosterone relative to controls with stimulation (median change in paired samples with metformin 0.7 (0.6-1.7) nmol/L vs. placebo 2.5 (0.4-3.5) nmol/l n=20, p=0.26). A reduction in oestradiol and improvement in intra-ovarian androgenic environment have been proposed as methods by which metformin may achieve its effect (350). Had metformin been used for a longer time prior to commencement of gonadotrophin stimulation, this effect may have been more pronounced. This idea is supported by work from Fleming and colleagues (242) who

suggest that with protracted use of metformin (>4 months) a cohort of follicles may develop without the stimulatory effects of insulin. It has been observed to take approximately 3 months from primary follicle to the antral stages of follicle development (531). Thus, with protracted metformin use the follicle cohort can be replaced with a smaller cohort formed under a 'normalised' insulin drive. These authors (242) accept flaws in this theory as there is only an apparent difference following a minimum 4 months treatment, with little change in insulin resistance or testosterone parameters. This contrasts with Kjotrod et al (346) who found after 16 weeks of metformin, there was no improvement in ovarian response or total number of oocytes retrieved. A mechanism for this finding involves endogenous LH (499). During down-regulation in an agonist cycle, LH is suppressed. Insulin potentiates the effect of LH on steroid synthesis via a synergistic relationship by binding to its own receptor on thecal cells and increasing androgen synthesis, although the effect of insulin alone is small (532). If the basal LH is low then metformin may not exert enough action to modulate the androgenic response. If this theory is right, then the antagonist's shorter duration of action and limited basal LH downregulation, should aid metformin in reducing the excessive response to stimulation. A further suggestion from Onalan et al (500) is that longstanding hyperinsulinaemia may down regulate the insulin receptors in the ovary, reducing the effect of insulin on the granulosa cells. This raises the possibility that short term use of insulin sensitisers may have the potential to augment insulin's effect in the ovary by altering the number of insulin receptors and in so doing be detrimental in the immediate short term (such as the short use of metformin in the current trial).

A limitation of the current trial is the lack of formal insulin resistance testing. Sex hormone binding globulin has been suggested as a surrogate marker for insulin resistance in PCOS (533). Although levels were not obtained for all in the trial, there was no significant difference in mean value between the placebo or metformin group (57.2 ± 29.6 vs. 54.8 ± 29.2, p=0.71, n=91). Furthermore, there wasn't any difference in SHBG for those with different levels of OHSS, although the numbers were small. Finally,

the mean values for SHBG were in the entirely normal range and would not be suggestive of insulin resistance in this cohort. Recommended use of metformin in women with PCOS aside from during an ART cycle, is confined to those with glucose intolerance (311) related to the biochemical parameters of the patients. Unfortunately, hormonal parameters including oestradiol, testosterone and AMH were not investigated on the day of oocyte retrieval in the early cases in this trial. These measures were included in the later cases, providing limited data on the potential impact, if any, of short term use of metformin on the outcome parameters measured.

A recent web-based survey of IVF clinic treatments suggested that only 46% of clinics opted to use the antagonist protocol for PCOS patients (534). At first glance, this is perhaps a worrying finding given the superiority that an antagonist has in reducing the risk of OHSS (298). The survey was completed in September 2010. Since that date, the updated Cochrane review and other papers published are in favour of antagonist use in women with PCOS (181, 297). The initial reduction in live birth rate seen with the earlier antagonist work has subsequently been refuted (181). The assumption is that the antagonist would be favoured if the study was repeated now. The authors (534) recognise the limitations of their survey, in particular not acknowledging the use of an agonist trigger in an antagonist cycle when questioning how to manage a hyperstimulated patient. There has been a resurgence of interest in using the GnRH agonist trigger following the implementation of successful antagonist cycles (535). The GnRH agonist has the ability to competitively displace the bound antagonist from receptors in the pituitary, generating a flare response. Improved luteal support with either a modified hCG dose (536) or addition of extra steroid hormones (oestradiol/progesterone) (537) reduce the risk of miscarriage following a deficient corpus luteal response seen with the agonist and standard luteal support (progesterone alone). Further work supporting the use of the antagonist in the luteal phase of severe OHSS cases, shows enhanced luteolysis and reduction in VEGF (538), which is a known factor that can impact on the severity and duration of OHSS (539).

Achieving a pregnancy following assisted conception can perpetuate the problem of OHSS and increase the incidence of the condition. The hormonal drive from the sustained corpus luteum and developing pregnancy can lead to a protracted course of ovarian hyperstimulation (250). Within this study there was a reduction in CPR and LBR for those who had metformin. This may have impacted on the reported rate of OHSS, falsely suggesting that there was no difference between the 2 groups. Indeed, if the pregnancy rates had been equal we may have seen an increase in OHSS for those on metformin.

3.4.2 Pregnancy outcome and metformin

Earlier studies on agonist cycles failed to reach consensus on the effect metformin has on clinical and live birth rates. Some publications suggest improvement (260, 346) but the majority (348-350, 500) suggest no difference including a Cochrane review. The current study suggests that metformin may actually have a negative impact on pregnancy rate in an antagonist cycle. This outcome is difficult to explain and it seems that ethnicity may play a larger role in the outcome of this trial than was previously expected (see next section). Even when controlling for ethnicity, there was a trend of reduced fertilisation, CPR and LBR with metformin (see 3.3.6). Although the evidence is limited, there are publications that positively evaluate implantation, endometrial receptivity and the use of metformin (540, 541). It is suggested that to achieve and maintain a successful pregnancy, it is beneficial to have a type 2 T helper (Th2) dominant cytokine profile; whereas a type 1 T helper (Th1) profile, is more likely to result in miscarriage (542). Progesterone is a key promoter of Th2 dominance, with its action mediated by a number of proteins including *progesterone-induced blocking factor (PIBF).* This factor inhibits the release of arachidonic acid, usually converted to prostaglandins via the *enzyme cyclooxygenase 2 (COX2)* and controls natural killer cell activity (543). Mice treated with DHEA, created to mimic PCOS in women, show an increased risk of miscarriage with reduced PIBF (544). Work on mice has also shown

that metformin could counteract fetal resorption in an androgenic environment (541). The mechanisms to account for this observation include restored progesterone production, reduced ovarian oxidative stress and improved insulin resistance (545). It has also been shown that metformin restores PIBF expression in DHEA treated mice, whilst avoiding the reduction of interleukin-6 (IL-6; a Th2 type cytokine). Unfortunately, there was no reduction in interleukin-2 (IL-2; a Th1 type cytokine). In contrast, Luchetti et al (541) found that metformin treatment alone regardless of DHEA treatment could increase IL-6, which aids the generation of protective asymmetric antibodies in pregnancy (546). Metformin also prevented the increased COX-2 expression seen with DHEA treatment. These factors may explain why some authors (547), have found a reduced risk of miscarriage with metformin treatment (OR 0.50, 95%CI 0.30-0.83). It should be noted that the doses of metformin given to these mice far exceeded any human dose. Luchetti et al (541) gave 2 oral doses of 240mg/kg, proceeding implantation. Although a variety of human doses have been used clinically (see Table 3-1), these do not exceed 2000mg/day. Equivalent doses to the murine studies (an 8 fold increase of the usual maximum human dose for a 70kg human) would be unlikely to be tolerated in humans given the high side effect rate of these drugs (42.9% in the current study). In contrast, Maruthini et al (548) showed a reduction in *in vitro* granulosa cell progesterone production with metformin pre-treatment which was lower than either the PCOS counterparts or control subjects. *In vivo* there was no apparent alteration in progesterone production in granulosa cells from women with PCOS following metformin pre-treatment. Reduced progesterone bio-synthesis may have implications on pregnancy outcome for women with PCOS (549). The negative impact from metformin exposure on progesterone production may counterbalance positive effects such as improved pyruvate production essential for oocyte nuclear maturation and developmental capacity (550).

Endometrial receptivity as assessed by the endometrial (551) and sub-endometrial (540) blood flow has shown to be impaired in women with PCOS. Lam and colleagues (551) found that this measurement was more pronounced in hyperandrogenic women.

The addition of metformin for 6 months was found to improve the blood flow in overweight PCOS women (540). This study (540) did not promote weight loss or exercise during the study duration and metformin use alone did not lead to weight reduction. This is in agreement with Tang and colleagues (339). Although the limited number of studies surrounding implantation and metformin are on the whole positive, an interesting finding by Germeyer et al (552) may support the current study's findings of reduced clinical pregnancy with metformin. Following short term use (in this case 48 hours) a substantial increase in chemotactic interleukin 8 (IL-8) occurred which may have deleterious immune effects by fending off embryo implantation. With long-term treatment this surge is neutralised. It has been suggested that parameters such as insulin resistance may be improved by 4 weeks of treatment (553). This still remains in excess of the current study duration of use and therefore will have had negligible effect on the trial outcome.

A recognised occurrence in ART is the ability to conceive whilst the patients are waiting to start their treatment cycle. One large cohort study found on average 9.1% of women conceived within a year of awaiting IVF treatment (554). It is also recognised that although time to conception may be increased, the final family size is seldom affected in women with normo-gonadotrophic anovulation (12). This raises the question of when ART interventions should be used, given the high cumulative natural conception rate (555) and associated risks of OHSS, imprinting disorders (556) and potential obstetric complications (557, 558). The occurrence of conception in the lead up to the start of treatment within this trial was 2.61% (4/153) once the study medication had started. Although lower than other published work, for example Kjotrod et al (347) who had an overall 15.4% (23/149) incidence, the current observation is not surprising given the relative short duration of metformin/placebo consumption (average duration 17 days compared with >12 weeks (347)).

3.4.3 Ethnicity

One of the most significant findings from the present study is the wide gap in clinical outcome between the White Caucasian and South Asian populations. This divide has been well documented in reproductive medicine (559) as well as in wider medical fields (560, 561). Whilst a simplistic view of this difference may be case selection, it raises an uncomfortable question regarding the application of generic treatments for all women regardless of their ethnic origin. It may be that what is seen in a primarily White Caucasian British clinic does not reflect the ethnic minority as a whole, but rather reflects a bias due to the cohort enlisted into the current trial. Examples of bias that could exist include language interpretation and religious practice. Although interpreters were used where necessary, it may be inherently more difficult to enrol patients if there is any difficulty in interpretation of the benefit of a clinical trial. An example specific to the South Asian Muslim population within this trial, was the withdrawal of 2 patients before commencing the study medication due to the porcine encasing of the drug. Due to their religious views, they did not wish to take part in the trial even if there might have been a potential benefit to them. An interesting finding from an American survey shows racial difference in those accessing services, despite states mandating insurance to cover fertility treatment (562). The reasons behind this are multifactorial including educational level, economics and clinician referrals to tertiary fertility care. A highlighted deficiency in much of the published data, is the lack of reporting of ethnicity or the loose term of 'ethnicity unknown' which may affect the validity of the conclusions (563).

The difference in clinical outcome between the South Asian and White Caucasian population in an assisted conception setting has been previously documented (559, 564, 565). Although the reason behind the inferior outcome in the South Asian population is not clear, it has been suggested that reduced embryo quality may be causative (564). Shahine and colleagues further considered this hypothesis by comparing cycles transferring comparable blastocysts in an Indian and Caucasian population (564). A significantly reduced CPR (36% vs. 52%, P=0.02) and LBR (24% vs.

41%, p<0.01) was observed in the Indian population. This result persisted when age, stimulation protocol and evidence of PCOS was considered in multivariate analysis (lower LBR OR 0.56 95%CI 0.40-0.79). In the current study, there was no difference in good quality day 3 embryos or cycles that reached blastocyst transfer between ethnic groups. Furthermore, in contrast to Palep-Singh et al (559), there was no increased sensitivity to gonadotrophin stimulation or increased oocyte number retrieved. Similarly to the latter author and Shahine et al (564) an equivalent ratio of reduced CPR and LBR was seen. A large study including >1500 South Asian women confirmed the reduced outcome with respect to both clinical pregnancy and live birth rate (566). The current South Asian population have a longer duration of infertility, raised BMI and worse phenotypic presentation of PCOS. Each of these points can be viewed as poor prognostic indicators for clinical outcome. Obesity *per se* has also been shown to impair fecundity, endometrial development and implantation (567). Obesity can also worsen the phenotypic presentation of PCOS. Importantly, classification of obesity by BMI varies according to ethnicity. The WHO suggest that a BMI >25kg/m² in South Asian Indians is equivalent to a BMI>30kg/m² in White Caucasians (55). As the current study's South Asian population exceeds this value (26.0±2.8kg/m²) this may explain in part the negative clinical outcome. There is also a greater propensity for insulin resistance in the South Asian population which as previously discussed can harm clinical outcome (51). Speculation on dietary/environmental factors that could affect IVF outcome in the South Asian population include methyl mercury, a reproductive toxin found in Seafood (568) and levels of vitamin D. Evidence suggests that those who are vitamin D deficient, have a worse assisted conception outcome with the South Asian population over represented in this group (569). It has also been suggested that those who have PCOS and vitamin D depletion have increased obesity and endocrine disturbance (570).

3.5 Conclusion

There are a number of limitations within this study. Firstly, metformin was used for too short duration, which although may have had effect on some aspects of metabolism, is unlikely to have optimised metformin's potential. Secondly, formal insulin resistance testing was not carried out. The surrogate measure of SHBG suggested that this population was not overtly insulin resistant and therefore may not be the best cohort to test. If this trial was to be repeated, it may be more appropriate to select women with evidence of disordered glucose metabolism and insulin resistance. In summary, the current trial does not support the use of metformin in an antagonist cycle to treat women with PCOS. There is neither reduction in OHSS with short term use of metformin nor any improvement in clinical pregnancy rate. The rather puzzling outcome of reduced CPR and LBR is likely to be explained by chance rather than true effect. The pertinent point of the trial is the inequality in clinical outcome between the South Asian and Caucasian populations.

Chapter 4
Clinical Application Of *In Vitro* Maturation for the treatment of PCOS

4.1 Introduction

In vitro maturation is the process of immature oocyte collection and laboratory maturation prior to fertilisation. It has been considered a possible low risk alternative to standard IVF assisted conception regimens for more than 15 years. For those patients with PCOS, who are at high risk of over response and OHSS, the concept of minimal gonadotrophin exposure is appealing. Despite the theoretical advantages of IVM, low success rates have precluded IVM from routine use. Alongside the low success rate, IVM requires more intensive laboratory time and planning of cycles. For the patient on the other hand, IVM has been marketed as a 'patient-friendly', low cost approach (571).

It is clear from the literature that a consensus on the optimum protocol for IVM treatment has yet to be reached. Whilst not exhaustive, Table 4-1 and Table 4-2 provide an overview of the published protocols and outcomes for the majority of IVM treatments published worldwide for women with PCOS. From this summary, it is apparent that the majority of centres use hCG priming prior (usually 36 hours) to oocyte pick-up with fewer adopting a truncated ovarian gonadotrophin stimulation before oocyte collection. Priming with one or both of these agents (hCG or gonadotrophin), has been shown to improve the maturation rate of oocytes in some studies (359, 395, 397) and may improve implantation (396). The LBR within Table 4-2 ranges from 7-45.2%, highlighting the inconsistency of the treatment outcome. It also highlights the limited number of cycles completed in comparison to conventional ART cycles. Although some may view IVM sceptically, the more recent trials have published success rates that equal the centres standard IVF & ICSI treatments (359, 572).

Table 4-1 Clinical trials of in vitro maturation in patients with Polycystic Ovary Syndrome – clinical protocols

Name	Number cycles	FSH prime	hCG prime	Day to OR	Endo priming	ET / time	Oocytes
Cha et al.(573) (2000)	n=94	x	x	10-13	OR 4mg E2, OR+2 100mg IM P	If <7mm cryopreserved	13.6±7.5
Chian et al.(397) (2000)	n=24	x	10000 36[3] / x[4]	10-14	OR 6-10mg, from ICSI 400mg BD V P	8-13mm on transfer	7.8±3.9[3]/7.4±5.2[4]
Mikkelsen& Lindenberg (396) (2001)	n=36	150 day 3 rFSH 3 days[1]/x[2]	x	[1]8-9/[2]endo 5mm	OR 2mg TDS E2, OR+2 100mg TDS V P	UK	7.5 median
Child et al. (413) (2002)	n=107	x	10000 36hrs	<10mm DF (9-14)	Or 6-10mg E2, from ICSI 200mg BD V P	If <7mm on transfer - cryopreserved	10.3±7.6
Lin et al.(400) (2003)	n=68	rFSH 75iu 6days[1]/x[2]	10000 36hrs	10_14	OR 6-10mg E2, from ICSI 800mg V P	Day hCG 8.1±1.5, 7.8±1.0	[1]21.9±9.4 /[2]23.1±11.0
Le Du et al.(574) (2005)	n=45	x	10000 36hrs	Fo.=7mm	OR 6-10mg E2, V P	8-13mm on transfer day	11.4±6.9
Soderstrom-Anttila et al.(412) (2005)	n=18 IVF:10 ICSI	x	x	endo 5mm DF 10mm	OR 600mg/day V P & 6mg E2	5.5±1.0/6.1±0.5 on OR	14.7±7.0 / 13.9±6.0
Son et al.(575) (2007)	n=82	x	10000 36hrs	ET (not stated)	OR+1 6mg E2, OR/OR+1 100mg IM P	UK	24.4±9.9
Benkhalifa et al.(576) (2009)	n=350	x	10000 36hrs	ET>6mm (9-11)	OR E2 if ET >7mm 6mg if <7mm 8-10mg. From ICSI V P 200mg TDS or SC P 100mg	No transfer if ET <7mm	9±UK
Zhao et al.(577) (2009)	n=152	x	x	DF <10mm (9-14)	D3-5 if ET<5mm 4mg E2, OR if ET<7mm 6-10mg otherwise 4mg,from ICSI 60-80mg IM P	8.72mm at transfer	18.2 ± 8.6
Bos-Mikich et al.(398) (2011)	n=34	x	yes - dose UK 36hrs	endo≥7mm	OR 6mg E2, OR+1 800mg V P	UK	16.3±UK
Gremeau et al.(358) (2012)	n=97	x	10000 35/40hrs	Fo.<14mm (8-16)	OR 4mg E2 BD, OR+1 400mg V P	UK	15.8±7.2
Junk & Yeap (359) (2012)	n=66	day 3, 3 days 100-150	x	Fo.10-12mm	2 days pre-OR 2mg E2 / 3mg TDS E2 +patches 50µg/day based ET 6mm. From OR 90mg P V BD	All ≥8mm at OR	8.8 ±4.4 (PCO) 14.4 ±6.4 (PCOS)
Roesner et al.(578) (2012)	n=215	3 days 125	10000/250 33-38hrs	trigger last day FSH/+1	From OR 6mg/day E2 + from OR+1 600mg V P	UK	8.9
Shalom-Paz et al.(360) (2012)	n=310	150hMG if endo <6mm	10000 8hrs	endo 6mm/Fo.10-12mm	hMG 150iu/day if ET<6mm for 3-5days from day 7-10	UK	17.1 ± 13.6
Zheng et al.(572) (2012)	n=82	x	10000 36hrs[3] / x[4]	endo 6mm. No Fo.>10mm	From OR 6mg E2 /60mg IM P	UK	13.8-14.35

[1]FSH prime [2]No FSH [3]hCG prime [4]No hCG. UK-unknown, OR-oocyte retrieval, DF- Dominant follicle, ET-endometrial thickness, Fo.-follicle, endo- endometrium, E2- oestradiol P- progesterone, V- vaginal

Table 4-2 Clinical trials of in vitro maturation in women with Polycystic Ovary Syndrome - laboratory protocols

Name	Number cycles	Culture (Hrs)	Culture additions	Maturation %	IVF/ICSI	Fertilisation %	Transferred	CPR (%)	LBR (%)
Cha et al.(573) (2000)	n=94	48	TCM-199 plus 20% bovine serum, 10iu/ml FSH/LH, 10iu/ml hCG, pyruvate, penicillin G & streptomycin	62.2	ICSI	75.1	4.9±2.5	27.1 (23/85)	17/23
Chian et al.(397) (2000)	n=24	up to 48	TCM-199 plus 20% bovine serum, 25mol/l pyruvic acid, 75miu/ml FSH/LH	84.3[3]/69.1[4]	ICSI	90.7[3]/83.9[4]	2.8±0.9[3]/2.5±1.1[4]	38.5[3]/27.3[4]	25.0
Mikkelsen & Lindenberg (396) (2001)	n=36	28-36	TCM-199 plus pyruvate, penicillin G, streptomycin, 0.075iu recFSH, 0.5iu hCG, 10% serum	[1]59 / [2]44	ICSI	[1]70 / [2]69	UK	[1]33 [2]0	[1]14.3 / [2]0
Child et al. (413) (2002)	n=107	24-48	TCM-199 plus 20% serum, 0.25mmol/L pyruvate, penicillin/streptomycin, 75mIU/ml FSH/LH	76	ICSI	78	3.2±0.9	21.5	15.9
Lin et al.(400) (2003)	n=68	48	TCM-199 plus 20% serum, 75mIU/mL hMG, o.2mmol/l pyruvate	[1]76.5/ [2]71.9	ICSI	[1]75.8 / [2]69.5	3.8±1.0	[1]31.4/[2]36.4	29.4
Le Du et al.(574) (2005)	n=45	24-48	20% serum, 0.75iu FSH/LH	54.2 by 24	ICSI	69.5 by 24	2.5±UK	22.5 by et	UK
Soderstrom-Anttila et al.(412) (2005)	n=18 IVF:10 ICSI	24-36	10% patient serum, 0.075iu/ml rec FSH, 0.1iu/ml hCG	54.3/53.2	IVF/ICSI	43.8/78.4	1.7±0.5 / 1.8±0.4	52.9/22.2	UK
Son et al.(575) (2007)	n=106	UK	30% human follicular fluid, 1 iu/ml FSH, 10 iu/ml hCG, 10ng/ml rec human EGF	78.2	ICSI	80.5	Blast 2.95 ±0.2	51.9	40.6
Filali et al.(579) (2008)	n=49	24-48	20% patient serum, 0.75iu/mL FSH/LH	60.6	ICSI	56.8	2.2 ± 0.1	28.6	UK
Zhao et al.(577) (2009)	n=152	48	20% FBS. 0.25 Pyruvic acid, 75mIU/mL FSH, 0.5iu/mL hCG	68.6	ICSI	70.28	3.2 ± 0.7	40.0	UK
Bos-Mikich et al.(398) (2011)	n=34	28-30	75mIU/ml FSH , 0.1iu/mL hCG	63	ICSI	62	3.29±UK d3	32.0	UK
Gremeau et al.(358) (2012)	n=97	48	75iu/L FSH/LH high humidity	65.01	ICSI	62.9	1.9±0.4	19.6	16.5
Junk & Yeap (359) (2012)	n=66	24-26	0.1iu/mL FSH, 0.5iu/mL hCG, 10% patient serum	69.7	ICSI	71.4	single blast	46.7 (per et)	45.2
Roesner et al.(578) (2012)	n=215	24+	Nil	64	IVF/ICSI	45.1	2.1±UK	15.3	7.3
Shalom-Paz et al.(360) (2012)	n=310	24-52	75mIU/mL FSH/LH	~61.4	ICSI	70.2	3.4±0.8	48.0	29.0
Zheng et al.(572) (2012)	n=82	32	0.075iu/mL FSH/LH	55.43[3] 42.29[4]	ICSI	63.4[3] 65.49[4]	2±UK	44.12[3] 52.50[4]	26.5[3] 32.5[4]

[1]FSH prime [2]No FSH [3]hCG prime [4]No hCG. UK-unknown, OR-oocyte retrieval, DF- Dominant follicle, ET-endometrial thickness, Fo.-follicle, endo- endometrium, E2-oestradiol P- progesterone, Vag- vaginal, EGF-epidermal growth factor, rec-recombinant

Both studies are on young (30-31 years) PCOS women and both use ICSI. However, the protocols that have been employed pre-oocyte collection use significantly different approaches. Junk & Yeap (359) used a 3 day course of FSH (100 or 150iu Puregon, Merck Sharp & Dohme Limited, Hertfordshire, UK) stimulation from day 3 of the cycle without hCG, whilst Zheng et al (572) only used hCG (10000iu Profasi, Merck Serono, Darmstadt, Germany) priming in part of their study group. The latter study concluded that the hCG made no difference to the clinical outcome or live birth rate. The 2 studies also used different IVM and embryo culture conditions. The 1st group (359) use G2-Plus media (Vitrolife, Sweden AB) with the addition of 10% of the patient's heat inactivated serum, 0.1iu/mL FSH (Puregon) and 0.5iu/mL hCG (Pregnyl). While the 2nd group (572) use SAGE (CT, USA) IVM maturation media with the addition of 0.075 iu/mL FSH/LH (Menopur, Ferring, Germany) before switching to a different cleavage medium (G-M, LifeGlobal, CT, USA). Both groups used oestradiol and progesterone supplementation for endometrial priming, but Junk and Yeap (359) commenced oestradiol 2 days prior to oocyte retrieval based on endometrial thickness, with the addition of vaginal progesterone following retrieval while Zheng et al (572) started both oestradiol and intramuscular progesterone from the day of oocyte retrieval. Due to the similar outcomes of CPR and LBR, this suggests either minimal impact from these aspects of the treatment protocols on oocyte maturation and quality or that neither centre had a fully optimised protocol. The question of which stimulation regimen and culture media should be used, has been raised previously with regard to IVM (580, 581) and there has been considerable discussion on whether the media should be supplemented with gonadotrophins and/or serum (582, 583). The differing approaches but similar outcomes of these trials, emphasises that the perfect culture and treatment protocol for IVM is yet to be reached.

The wide variation in clinical pregnancy and live birth rate observed after IVM could in part be related to the variability in the average number of embryos transferred between centres and countries. The legal boundaries within the UK, for example, limit a maximum of 2 embryos for transfer in the populations concerned with a strong

emphasis towards single embryo transfer, whilst many studies outside the UK transfer more embryos. From Table 4-2, only 4 of the studies had an average embryo transfer of ≤2 (358, 359, 412, 572). One study transferred up to 5 embryos per IVM cycle (573).

A good quality RCT is clearly needed, to address whether IVM can add anything further to the treatment of women with PCOS. The majority of published literature cites retrospective observational data. With the advances in assisted conception techniques, that limit the risk of OHSS for these patients (e.g. such as the antagonist cycle and the use of then GnRH agonist trigger) the validity of continuing to offer IVM is questioned. A direct comparison of IVM with a conventional assisted conception cycle (centred on controlled ovarian stimulation and IVF/ICSI) is needed in an appropriately powered study to answer this question. A secondary outcome of such a trial should be assessment of patient welfare and assessment of the relative cost of treatment.

The aims of this chapter were therefore 2-fold:

(i) To conduct a pilot study to optimise conditions for the use of IVM as a treatment strategy in women with PCOS, as a prelude to conducting a full RCT to evaluate the efficacy of IVM as a strategy to treat women with PCOS or PCOM.
(ii) To conduct a cost analysis of IVM as a treatment for PCOS compared with a conventional antagonist protocol.

4.2 Materials And Methods

Women identified with either PCOM alone or PCOM with PCOS using the Rotterdam criteria (7) were offered the opportunity to have a research funded cycle of IVM. Candidates were identified from the pool of women about to commence an assisted conception treatment cycle at LCRM. Once eligibility was confirmed by review of clinical notes, an appointment with the research doctor was made to discuss the clinical details and participation in the trial. Time for reflection (minimum 24 hours)

was given and if the couple wished to proceed on to treatment written consent was completed.

Inclusion criteria apart from PCOM, included: age 23-39yrs, BMI ≤30, easily accessible ovaries on trans-vaginal scanning and a normal FSH (≤8iu/L). A negative infection screen for HIV/Hepatitis B/Hepatitis C/ Syphilis/ Chlamydia was a pre-requisite. Only women due to commence an IVF treatment cycle were approached after less invasive treatments had failed or were not suitable for them. Couples with severe male factor infertility (<1x10^6 sperm/mL) or those who required the use of frozen sperm were not eligible.

Informed consent outlined the nature of the treatment. Information included: first time the clinical treatment would be completed at LCRM, clinical pregnancy rates (16% (394)) from the literature, that the same laboratory method practiced by Oxford Fertility Unit (only UK centre to regularly offer IVM) (358) would be used and that ICSI would be used in all cases. Patients were made aware that IVM treatment was uncommon and that <2000 children have been born worldwide from the treatment. It was highlighted that the safety profile for the children was less robust compared with standard IVF/ICSI due to reduced numbers and age of children born following IVM to date. From the literature there was no significant increased risk to the unborn children either physically or mentally.

Ethics approval was granted from Leeds West Ethics Committee (09/H1307/51) to complete an RCT comparing a standard antagonist assisted conception cycle with an IVM cycle. A preliminary 30 patients were recruited and treated using the same clinical protocol and laboratory method to ensure the smooth operation and integration of the known clinical treatment into the LCRM work setting. Patients were consented in the same written manner to ensure that all aspects of the trial were outlined and that they were happy for their data to be analysed.

4.2.1 Treatment pathway

Prior to the onset of treatment all baseline investigations were completed for each patient which included a day 1-3 hormone profile (FSH/LH/oestradiol/testosterone /SHBG/TFTs/prolactin; see Chapter 2.2.1 for the assay details) and a TVUSS to assess accessibility, as well as ovarian volume and AFC for each ovary. Due to the extra laboratory time required per treatment cycle a limited number of cycles were completed each week with a maximum 3 a week / 1 per day. Women were programmed with the oral contraceptive pill (OCP; microgynon 30®, Bayer Healthcare, Germany) to allow optimum cycle planning. The maximum number of days on the OCP was 21. If used for longer than 1 week, a 5 day break was given following OCP withdrawal prior to commencing the IVM cycle. For women with amenorrhoea or oligomenorrhoea (cycles >35 days apart), a progesterone induced withdrawal bleed was incurred using medroxyprogesterone (Provera®, Pharmacia, Ltd, Kent, UK) 5mg 3x/day, 5 days prior to starting treatment.

All women had a TVUSS on day 2 of their treatment cycle (day 0 = 1st day menstrual cycle/progesterone withdrawal bleed ± OCP programming) to ensure no ovarian cysts were present. If the ovaries were 'quiet' (no follicles >10mm), the first dose of FSH 150units was given subcutaneously (Fostimon®, Pharmasure, Hertfordshire, UK). A further self-administered Fostimon 75units was given on days 4 and 6. A TVUSS was completed on day 7 or 8 of the cycle prior to the administration of low or high dose hCG (Pregnyl®, Organon, Hertfordshire, UK) either 24 hours or 36 hours prior to the oocyte retrieval according to the details in Table 4-3. Minor optimisation modifications were made to the clinical treatment pathway through the pilot study with the intention of improving the pregnancy outcome. The changes were sequentially made following the outcomes of the preceding recruited patients. These were based on the varying common practices published in the most recent trials (outlined in Table 4-1), with each strategy having a published clinical pregnancy rate. These changes are outlined by group in Table 4-3. The laboratory protocol remained constant throughout the pilot study. All women received a course of vaginal clindamycin 2% (Dalacin®,

Pharmacia Ltd, Kent, UK) prior to oocyte collection to reduce the risk of infection at oocyte retrieval. The majority of ultrasounds (>90%) were carried out by a single operator (Dr Susan Nicholas).

Table 4-3 Clinical treatment protocols (methodology groups)

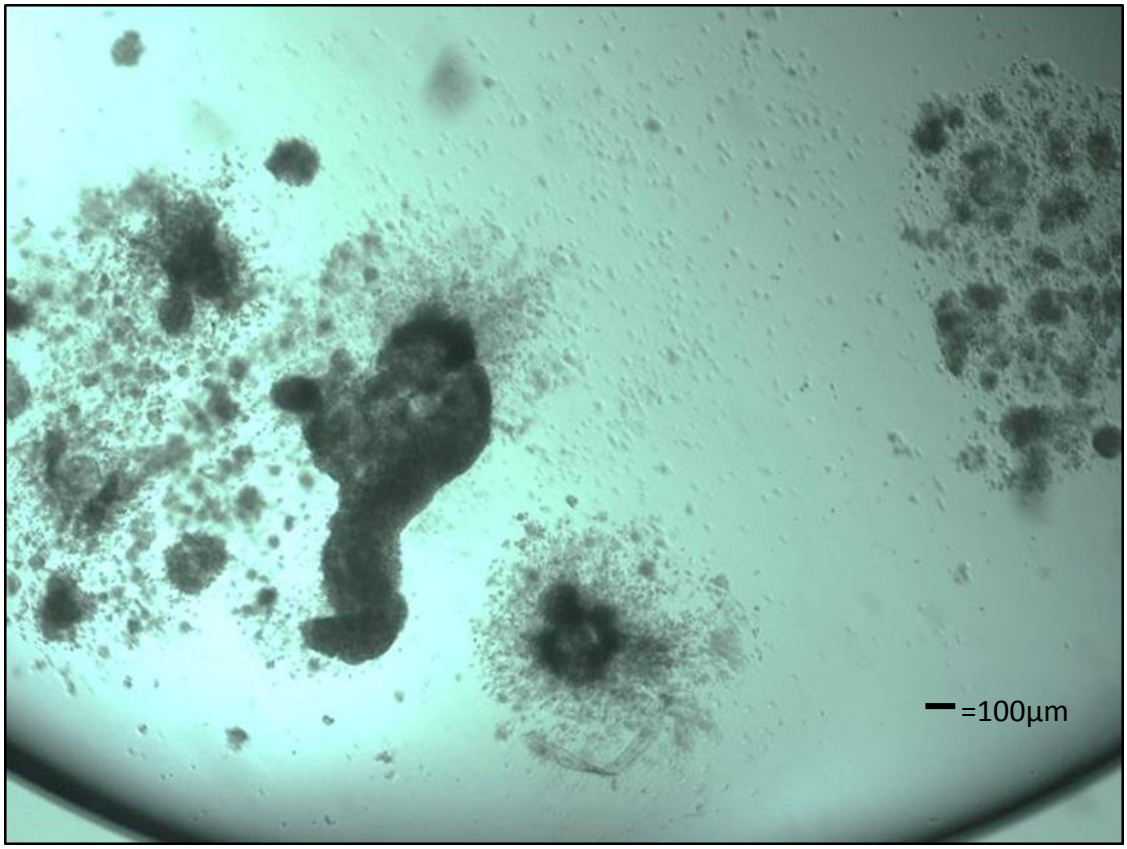
	Details of Treatment
Group 1	5000 hCG[1] given day 8, 24 hours prior to day 9 oocyte collection. 6-8mg Oestrogen[2]/400mg Progesterone[3]
Group 2	10000 hCG[1] given day 8, 24 hours prior to day 9 oocyte collection. 6-8mg Oestrogen[2] / 800mg Progesterone[3]
Group 3	10000 hCG[1] given day 9 prior to day 10 oocyte collection. Luteal phase as group 2
Group 4	As group 3 but embryos frozen if possible with frozen embryo transfer cycle
Group 5	10000 hCG[1] given 36 hours prior to collection, determined by lead follicle/endometrial thickness (aim follicle>10 &/or endometrium >6mm). Luteal phase as group 2

Sequential allocation of recruited patients into outlined methodology groups based on recognised clinical treatment strategies (Table 4-1). [1]Pregnyl (Organon) [2]Progynova (Bayer Healthcare) [3]Cyclogest (Actavis)

On the day of oocyte retrieval (OR), patients were fasted from midnight prior to the collection. An anaesthetist was present to administer conscious sedation under LCRM's normal conditions using a combination of Midazolam (up to 5mg) and Fentanyl (up to 100µg) ± Propofol (up to 20mg). Patient's had an empty bladder prior to oocyte collection. Using a double lumen flushing Clarendon needle (Casmed International Ltd, Epsom, Surrey, UK) set to a vacuum aspiration pressure of 80mmHg, the ovaries were

punctured trans-vaginally. Multiple follicles were drained per collection tube. Each follicle was flushed at least 3 times using Vitrolife heparinised flushing media (Vitrolife AB, Göteborg, Sweden) before moving onto the next follicle. An example of a pooled follicular sample is shown in Figure 4-1. If more than 1 puncture per ovary was required a dose of intravenous antibiotics were given according to the clinic's standard guidelines (1.2g Augmentin®, GlaxoSmithKline, Middlesex, UK). Haemostasis was confirmed at the end of the procedure. Patients were discharged home later the same day.

Figure 4-1 Example of a pooled sample at an *in vitro* maturation collection showing oocytes, cumulus and cellular debris



On the day of the oocyte collection, a fresh sperm sample was obtained from the male partner and prepared ready for the ICSI procedure according to the standard clinical protocol. Following production, the sperm was allowed to liquefy at room temperature, before adding 1.0mL of semen onto a PureCeption gradient (40 & 80%, Sage, Origio Ltd, Reigate, UK) and centrifuged at 300g for 20 minutes. The sperm pellet was then washed twice in 2mL of Quinn's Sperm Washing Medium (Origio Ltd). Sperm samples were then stored at room temperature until it was required for insemination. From the day of oocyte collection, patients received an increasing dose of oestradiol valerate (Progynova®, Bayer Healthcare, Germany) of 6mg to 10mg and also commenced vaginal pessary progesterone support of either 400mg or 800mg dependant on treatment group (cyclogest®, Actavis, Dublin, Republic of Ireland) to prepare the endometrium for the embryo transfer. Patients were given information regarding which OHSS symptoms to be vigilant for and were asked to report to the Unit if they experienced any of them e.g. shortness of breath, extreme bloating, diarrhoea and nausea.

For women undergoing an IVM cycle, fertilised embryos were cultured to Day 3. Optimally, 2 embryos were selected for double embryo transfer if available. Embryos were used where possible from the Day 0 or Day 1 matured oocytes. If not possible or obviously inferior quality graded embryos were produced following insemination then Day 2 embryos were used for transfer. An embryo was referred to as good quality if graded ≥ 6.3.3 on day 3 or ≥2Bb (524, 525). Classification of embryos for day 3 and day 5 are shown in Table 3-5 and Table 3-6. For simplification of displaying results any ungradable embryo was classified as abnormal and those below <6.3.3 or <2Bb were referred to as poor quality.

Embryo transfer took place using a Cook catheter (Cook Medical Inc., Bloomington, USA) and directed with abdominal ultrasound. Remaining embryos were cultured to blastocyst stage and graded prior to discarding the embryos. There was no freezing of embryos for an additional frozen embryo transfer cycle, apart from group 4. This group of women had cleavage stage embryos frozen before undergoing a standard frozen

embryo transfer cycle. The slow freeze technique was used rather than vitrification, using Quinn's Advantage embryo freeze kit (Origio), liquid nitrogen and the Planer freezer (set to Sage freezing programme). The hormone replacement cycle included down-regulation with a GnRH analogue (Prostap 3.75mg SC, Takeda UK, High Wycombe, UK) before preparation with 12 days of oestradiol valerate 4-6mg. Following an USS on day 12 of oestradiol, if the endometrium was >6mm, vaginal progesterone 800mg was added for 4 nights prior to the day 3 transfer. All embryos were thawed at once and cultured to day 3 and the best embryos selected for transfer.

Confirmation of pregnancy test took place on day 14 using maternal serum hCG as an index of implantation (Siemens Advia Centaur & Siemens reagents, WHO 5th IS 07/364). A positive test was confirmed if hCG was >2 iu/L. If the test was positive a further test was conducted 48 hours later. If this was positive a TVUSS was booked for 7 weeks gestation to confirm a viable intrauterine pregnancy. The oestradiol and progesterone commenced at oocyte collection was planned to continue until 10 weeks gestation. Any adverse reactions or symptoms of OHSS were recorded throughout the treatment cycle.

4.2.2 Laboratory protocol for IVM

The full laboratory protocol is outlined in Figure 4-2 – 4-4, which includes the preparation required for an IVM cycle and the steps involved during the retrieval, maturation and culture.

Figure 4-2 IVM laboratory protocol before the day of oocyte retrieval (day -1)

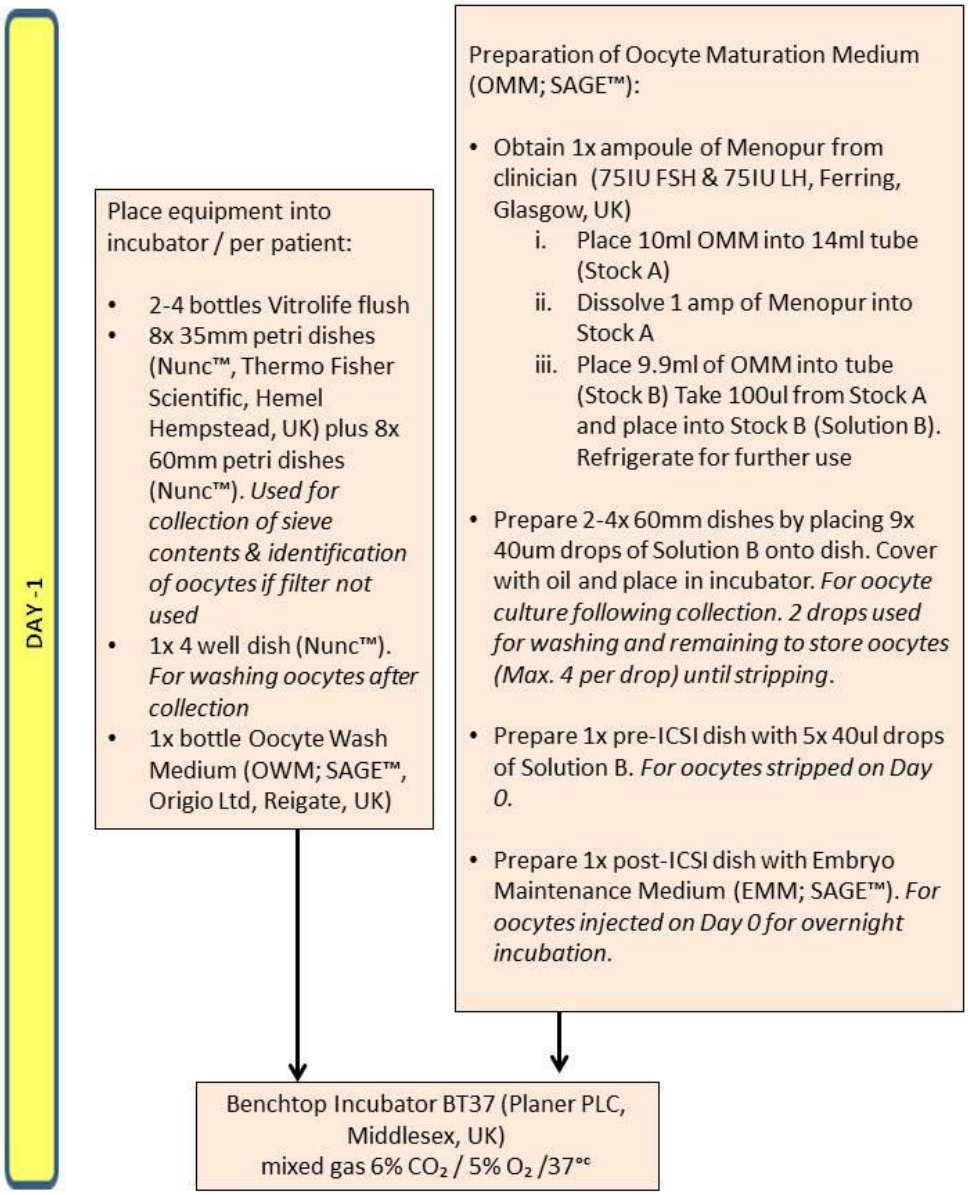


Figure 4-3 Laboratory protocol for IVM on day of oocyte retrieval (day 0)

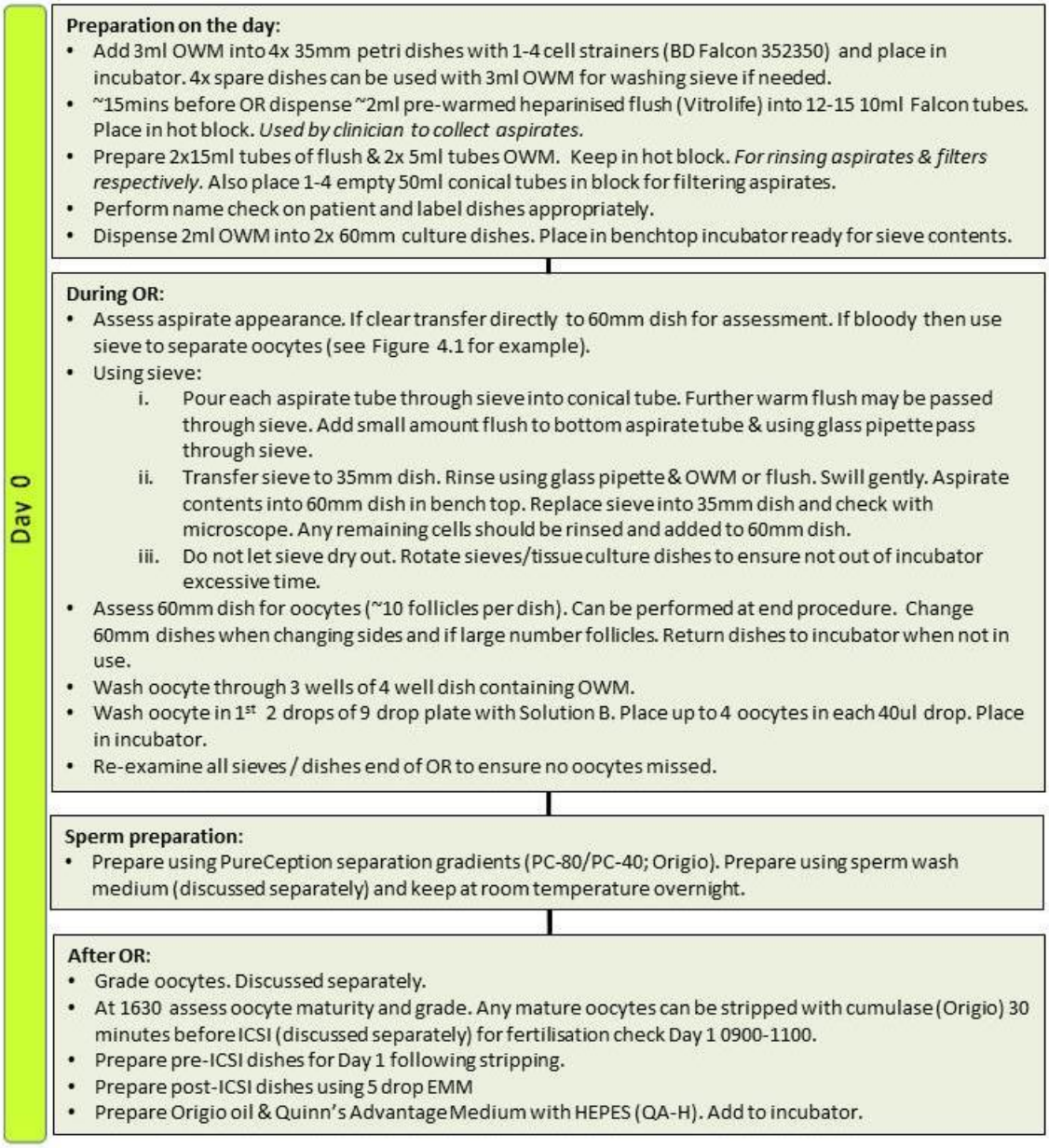
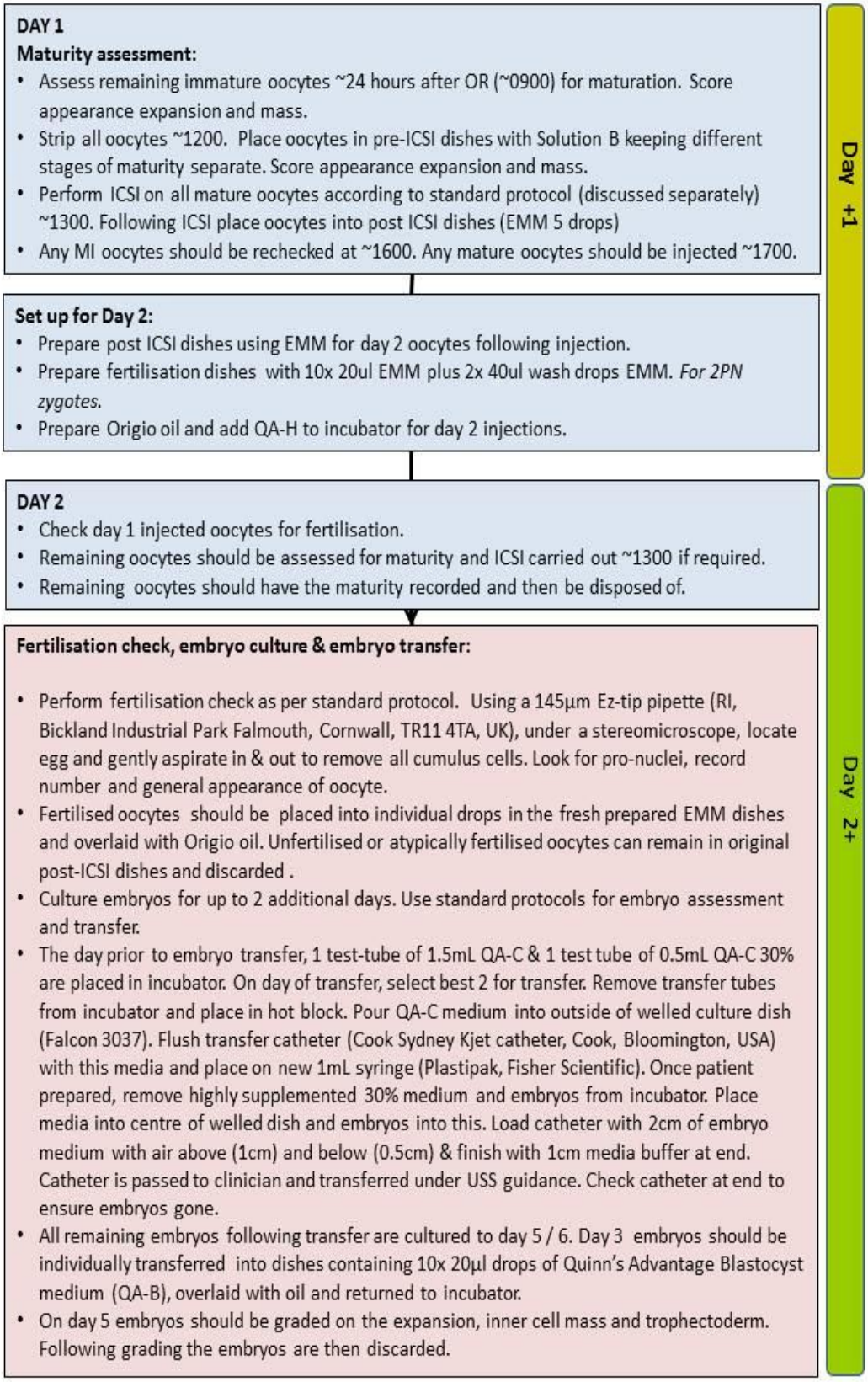


Figure 4-4 IVM laboratory protocol following oocyte retrieval (day 1 & 2)



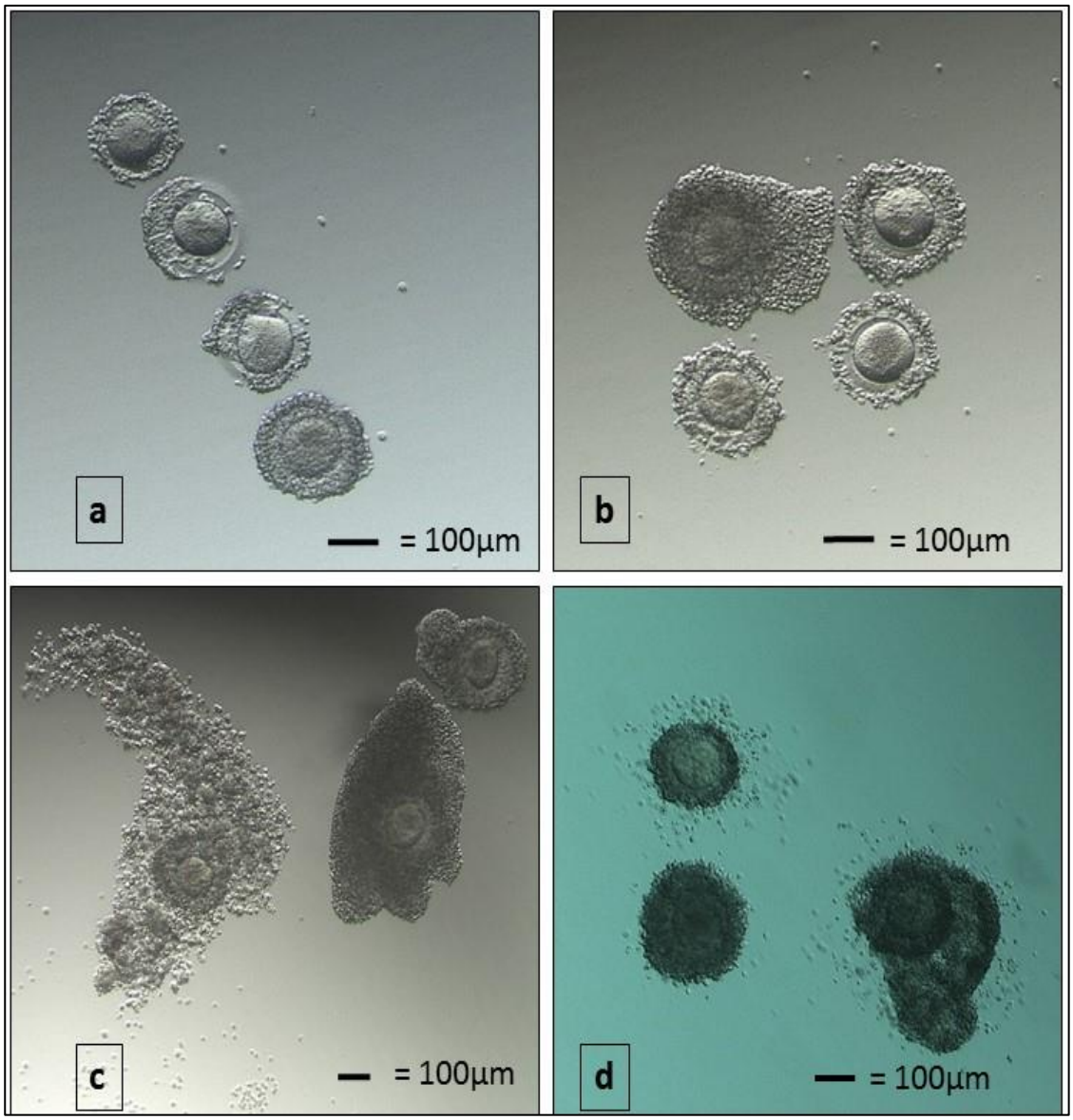
Oocytes were scored according to Wynn et al (395) to reflect/predict the oocytes' maturation potential. This occurred at collection, 24 hours following collection (Day 1) and immediately prior to stripping at the end of cumulus oocyte complex (COC) culture (termed strip). Due to the technical difficulty of assessing nuclear maturation with surrounding cumulus intact, all oocytes were stripped on day 1. A score was generated for both cumulus-oocyte coverage (mass) and cumulus expansion (see Table 4-4 and appendix 1). Examples of oocytes and their corresponding mass and expansion scores are given in Figure 4-5.

Table 4-4 Cumulus mass and expansion score

	Cumulus Mass	**Cumulus Expansion**
Grade	C0: ≤3 layers cumulus cells	E0: tight, dense corona cells
	C1: >3<10 layers	E1: moderate expansion
	C2: ≥10 layers	E2: fully expanded cells

Intra-cytoplasmic sperm injection was used for all cases in this pilot study using the Unit's standard protocol. In preparation, 13mL of liquid paraffin oil (Origio Ltd) per 10 eggs was placed into 15mL tubes in incubator to warm overnight. Approximately 2 hours before stripping, cumulase (Origio Ltd) and Quinn's Advantage medium with HEPES (QA-H, Origio Ltd) was removed from the fridge and placed in the warming oven, alongside pre-gassed sterile Nunc (Thermo Fisher Scientific, Hemel Hempstead, UK) culture dishes (150270) and sterile Nunc ICSI dishes (150265). Polyvinylpyrrolidone (PVP; Sage, Origio Ltd) was placed in hot block set at 37°C 20 minutes before use. Oocytes were denuded of cumulus layers to assess maturity using a range of sterile Pasteur pipettes or EZ tips (Research Instruments Ltd, Cornwall, UK). Metaphase II oocytes were injected with sperm according to the standard ICSI protocol used by LCRM.

Figure 4-5 Oocyte grading based on cumulus mass (M) and expansion (E) from different patients a) top to bottom: E0 M0, E0 M0, E0 M0, E0 M1 b) clockwise from top left: E0 M2, E0 M1, E0 M0, E0 M1 c) left to right: E2 M2, E0 M2, E0 M2 d) clockwise from top left: E1 M1, E1 M2, E0 M2



Cumulase and wash drops of QA-H were dispensed into a sterile NUNC culture dish then overlaid with pre-warmed oil with a maximum of 4 COCs eggs per dish. The COCs were transferred into the cumulase and aspirated vigorously before moving through successively smaller pipettes in the QA-H drops. All denuded oocytes were transferred into the pre-ICSI dishes (solution B). To prepare the ICSI dish, 5µl drops of QA-H and

PVP were overlaid with oil and kept in the un-gassed desktop incubator until needed. To the PVP drop 1-5µl sperm was added to the upper section with the fastest sperm collected from the bottom. Metaphase II oocytes were added to the QA-H drops and the dish transferred to a pre-warmed microscope stage at 37°C. A motile, normal sperm was picked up and brought to the side of the oocyte. Using a holding pipette the oocyte was grasped at 9 o'clock with the polar body at 6 o'clock. Using an ICSI pipette, the zona and oolemma were gently pierced half way in to the cytoplasm at 3 o'clock and the sperm deposited. The injected oocytes were returned to the post-ICSI dishes containing EMM and returned to the incubator for culture.

4.2.3 Cost analysis

Cost analysis of IVM treatment was based on cycle costings produced for the time period of the trial by Leeds Teaching Hospitals NHS Trust. These were based on the 2010 pay scales for employees of the trust. A comparison was made between an IVM cycle and a standard antagonist cycle using 10 days of gonadotrophin stimulation (150 units/day) appropriate for women with PCOS. The comparison with the antagonist protocol was made due to the recommendation to use such a protocol in women with PCOS. The analysis included the average time for carrying out a procedure and the cost of the member of staff completing the procedure. The analysis also included consumable costs such as the medication required for an average treatment cycle. The price of the medication was the market cost price.

4.2.4 Primary and secondary end points

The primary end point of this pilot study was to achieve a clinical pregnancy. This was defined as ultrasound evidence of a fetal heart beat. The secondary end points were:

1) oocyte maturation rate
2) fertilisation rate
3) embryo quality
4) OHSS (or rather the elimination of OHSS).

The pilot study had a set number of participants based on the recommendation by the Ethics committee that a clinical pregnancy should be achieved within this number of patients.

4.2.5 Statistical analysis

Statistical analysis was conducted using the IBM SPSS 21 statistics package (IBM Corporation, New York, USA). Continuous data was tested for normality using the Kolmogorov-Smirnov test. If the test was significant ($p<0.05$) normality was not assumed. Normally distributed data was analysed using the parametric tests including Student's t-test (with Levene's test for equality of variance) and ANOVA. Normally distributed data included the baseline demographics of age, BMI, FSH and number of oocytes retrieved. This data were presented as mean ± standard deviation. Non-parametric data was analysed using Mann-Whitney and Kruskal-Wallis tests. This type of data included the number of oocytes that matured and fertilised. These data were presented as median and IQR. Contingency tables were employed where necessary with Chi-squared statistic (plus continuity correction) and Fisher's Exact tests (if any cell had an expected count less than 5). This was used for categorical data including percentage of oocytes matured, fertilised and CPR/LBR. A *p* value of <0.05 was considered significant. To establish correlation between ordinal variables (e.g. oocyte

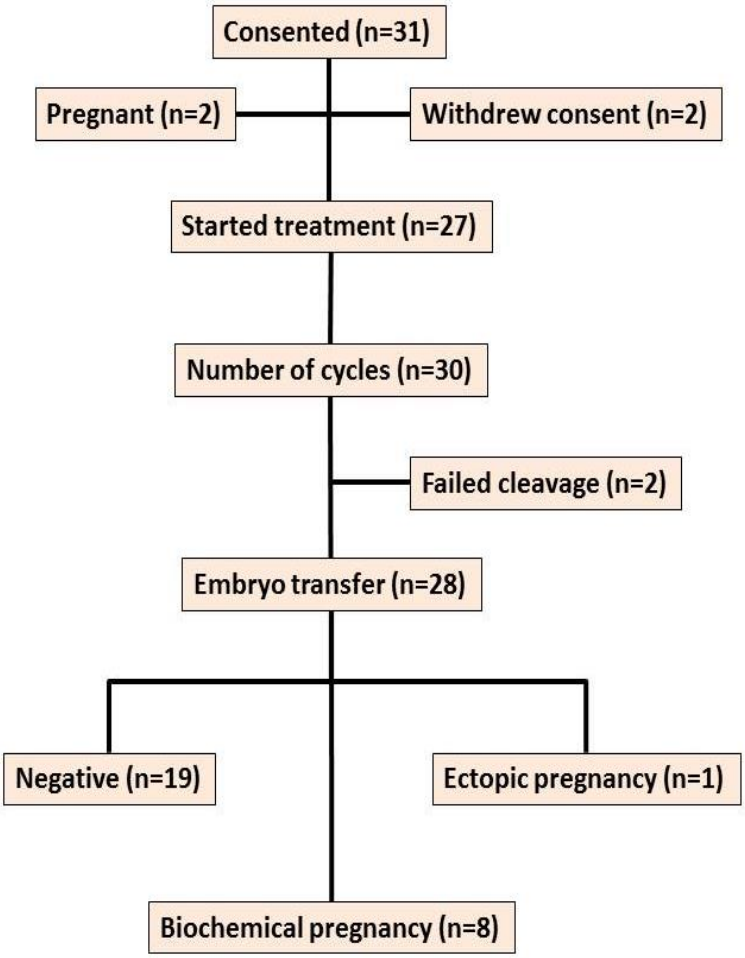
grading score and oocyte maturation) Spearman's Rank correlation coefficient was used.

4.3 Results

4.3.1 Patient recruitment and outcome

Between September 2012 and October 2013, 31 patients consented to undergo IVM treatment within the pilot group at LCRM (see Figure 4-6). Of the 31 women, 27 women underwent 30 IVM oocyte collections (3 of whom had 2 cycles each). Of the remaining 4, 2 became pregnant before commencing treatment and 2 women withdrew consent after further reflection. At oocyte collection the average oocyte number retrieved was 17 ± 8 (total number 503). The transition of oocytes from GV to MII is highlighted in Figure 4-8. No oocytes were found to be mature on day 0 (day of retrieval). Of the 30 oocyte retrievals, 28 cases had an embryo transfer and 2 women had no embryo to transfer. Twenty-six women had a double embryo transfer. All embryos transferred except 1 were created from day 1 matured oocytes. The first 2 patients in the pilot study had a single cleavage stage embryo transferred on day 2. Of the embryos transferred, 87.0% were deemed good quality (47 embryos). The overall embryo quality is shown in Figure 4-8. Embryos created from oocytes maturing on day 1 had an increased cleavage and blastocyst formation rate. The majority of day 3 embryos were classified as poor quality. Twenty-one blastocysts were created of which 23.8% were deemed good quality (5 blastocysts from 4 patients). The endometrial thickness was not recorded in all cycles prior to transfer.

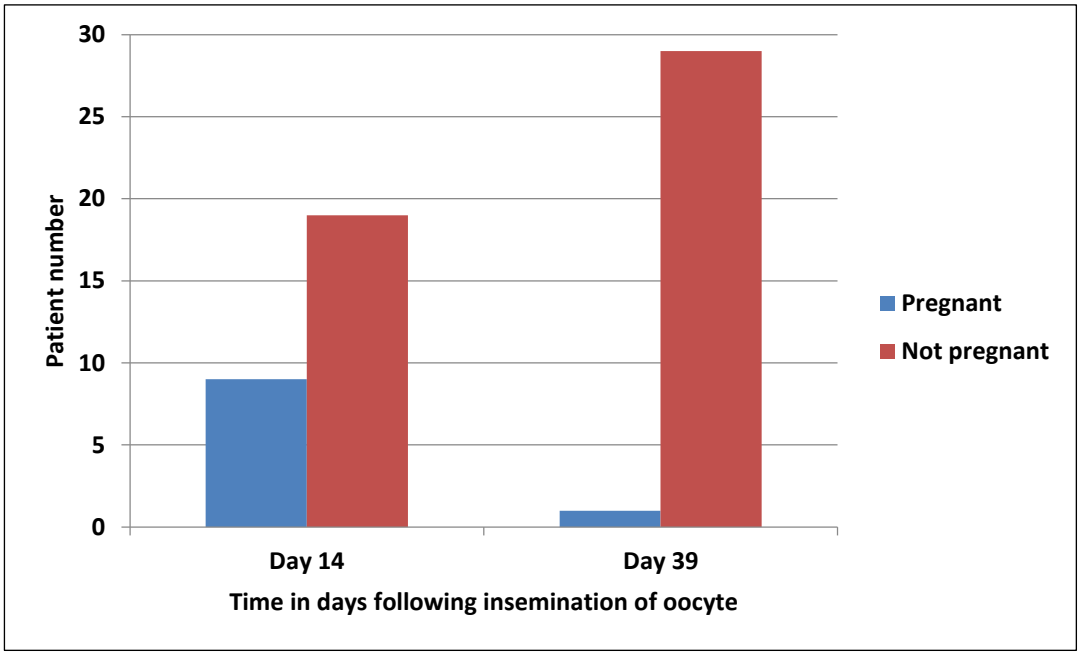
Figure 4-6 Patient recruitment and treatment pathway



Of the 28 embryo transfers, 9 (32.1%) had a positive serum hCG on day 14. Eight of these resulted in a biochemical pregnancy (with low hCG readings) and 1 an ectopic pregnancy (see Figure 4-7). The ectopic was managed medically with Methotrexate and required two repeated doses (each dose 50mg/m² based on actual body weight). Ten IVM treatment cycles yielded blastocysts from the remaining embryos produced. Only 2 of these 10 cycles had a positive day 14 serum hCG. There were no reported cases of OHSS. One patient had significant discomfort on day 2 post-oocyte collection but only required simple analgesia. All 27 women had PCOS and not PCOM alone. The average age was 30.5 ± 4.2 years (range 23-38) and body mass index 25.0 ± 3.1kg/m²

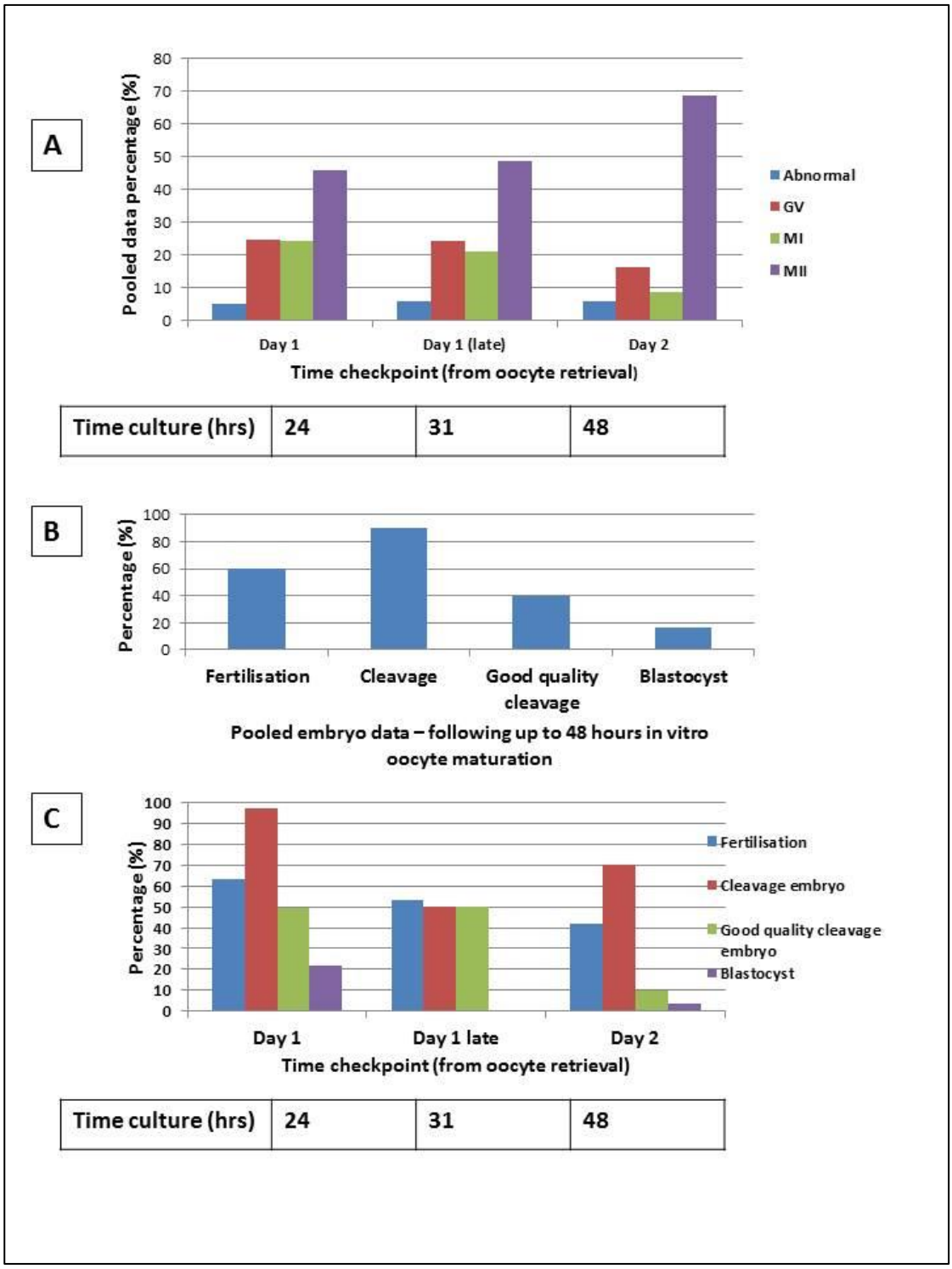
(range 20-30.5). Following the unsuccessful IVM treatment, 4 women achieved a natural conception within 4 months following their treatment cycle. All were confirmed as a clinical pregnancy with unfortunately 1 patient miscarrying around 8 weeks of gestation. Fifteen women subsequently underwent a conventional IVF cycle with or without a frozen embryo transfer cycle of whom 9 achieved a clinical pregnancy. Twelve had not undergone any further treatment at LCRM to date.

Figure 4-7 Clinical outcome of pilot IVM study at 14 and 39 days post insemination of mature oocyte



From 30 started cycles of IVM, 9 positive hCG results were achieved on day 14 but by day 39 only 1 patient remained pregnant (ectopic).

Figure 4-8 Outcome of oocytes from oocyte retrieval*. A: Cumulative data regarding level of oocyte maturation with reference to time in culture. B: Pooled data of the outcome of all MII oocytes regardless of day of maturation. C: Embryo outcome rates based on when oocyte maturation reached and insemination completed.



* Fertilisation rate = Number of 2PN zygotes/Number metaphase II oocytes. Cleavage rate = Number cleaving embryos / Number of 2PN zygotes. Blastocyst rate = Number of Blastocysts / Remaining cleavage embryos following embryo transfer. 'Good quality' cleavage embryos ≥6.3.3 ≤10.4.4

4.3.2 Oocyte grading system and maturation

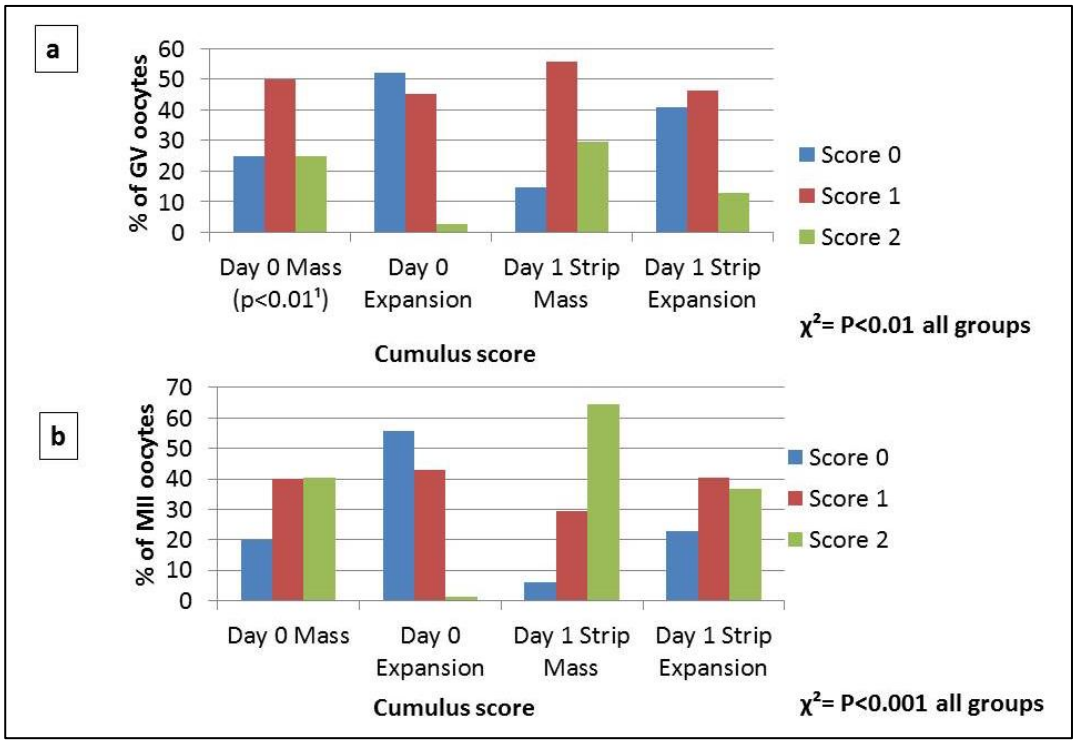
The cumulus-oocyte coverage (mass, M) and expansion (E) scores assigned to the different time points were correlated with day 1 maturation (excluding the late day 1 oocytes) and total maturation. This included the grouping of oocytes classified as abnormal (by morphology or degeneration), as this did not affect the correlations. The stronger correlations were seen between the scores assigned at the point of cumulus stripping and maturity (see Figure 4-9). In particular, the expansion score with day 1 maturation and the coverage score with total maturation. Although these were the strongest links, the coefficient value was well below 1, revealing a less than perfect correlation.

Figure 4-9 Correlations between cumulus-oocyte grading scores relating to mass and expansion with oocyte maturity

Oocyte score	Maturation	Spearman's Rank coefficient	Significance
Day 0 M	Day 1	0.133	P<0.01
Day 1 M	Day 1	0.216	P<0.01
Day 1 Strip M	Day 1	0.247	P<0.01
Day 0 E	Day 1	0.080	ns
Day 1 E	Day 1	0.315	P<0.01
Day 1 Strip E	Day 1	0.407	P<0.01
Day 0 M	Total Maturation	0.171	P<0.01
Day 1 M	Total Maturation	0.243	P<0.01
Day 1 Strip M	Total Maturation	0.305	P<0.01
Day 0 E	Total Maturation	-0.14	ns
Day 1 E	Total Maturation	0.150	P<0.01
Day 1 Strip E	Total Maturation	0.231	P<0.01

Figure 4-10 represents the scores assigned for oocytes that were either GV or MII by day 2. Although there are significant differences between the scores and mass / expansion, when you compare the charts, the predictive capacity of oocyte maturity appears limited especially on day 0. On day 1, a score of 2 for either mass or expansion was more likely to result in a MII oocyte.

Figure 4-10 Cumulus score for mass and expansion following oocyte retrieval and prior to cumulus stripping a) Germinal vesicle oocytes by day 2 b) Metaphase II oocytes by day 2



4.3.3 Comparison by treatment methodology group

The baseline demographics for these women are shown in Table 4-5. There were no significant differences (p>0.05) between treatment groups (as detailed in Table 4-3) in age, BMI and FSH. There was also no difference in predicted numbers of oocytes and endometrial thickness prior to hCG (p>0.05).

Table 4-5 Baseline patient demographics ordered by treatment methodology group

	Group 1 (n=5)	Group 2 (n=4)	Group 3 (n=6)	Group 4 (n=5)	Group 5 (n=10)	Total (n=30)	Significance (p-value)
Age (years) 23-38	32.6 ± 2.3	32.8 ± 2.2	32.5 ± 6.0	27.4 ± 3.4	29.0 ± 3.4	**30.5 ± 4.2**	**ns[1] (0.08)**
BMI (kg/m²) 20-3	23.8 ± 2.6	24.6 ± 2.2	25.4 ± 2.5	27.2 ± 3.7	24.6 ± 3.5	**25.1 ± 3.1**	**ns[1] (0.50)**
FSH (iu/L) 1.8-8	5.2 ± 1.1	5.2 ± 2.1	4.9 ± 2.3	6.4 ± 1.3	5.2 ± 2.0	**5.4 ± 1.8**	**ns[1] (0.72)**
Number of follicles on day 7/8 USS	34.8 ± 14.8	36.8 ± 9.6	27.5 ± 2.9	33.0 ± 10.7	31.0 ± 8.0	**32.0 ± 9.3**	**ns[1] (0.57)**
Endometrial thickness (mm) prior to hCG	6.0 ± 1.4	5.0 ± 1.9	3.8 ± 1.1	6.8 ± 3.9	4.9 ± 1.0	**5.2 ± 2.1**	**ns[1] (0.14)**

[1]ANOVA [2]Kruskal-Wallis Test [3]Fisher's Exact Test [4]Chi squared statistic *Parametric data presented as mean ± SD; non-parametric data as median (IQR)

Table 4-6 outlines the different oocyte, embryo and outcome results per treatment group as outlined in the methodology section. There was no significant difference in baseline demographics including age, BMI and FSH level. A similar number of oocytes were collected from each group of patients. There was no significant difference ($p>0.05$) in maturation by individual patient or cumulative total for each group. There was a significant difference in favour of group 3 for total fertilisation of day 1 matured oocytes and total fertilisation of all matured oocytes. Despite this finding, there was a significant difference ($p<0.05$) in good day 3 embryos, in favour of group 1 and 2. Furthermore, there was a trend for an improved occurrence of positive pregnancy test in first two groups, although this was not significant (p=0.72). Overall, there was a poor development of embryos to the blastocyst stage. For the full results per patient please see Appendix 2. It is evident from Figure 4-11 that irrespective of treatment group, the better quality embryos were produced from day 1 matured oocytes, but these resulted in very limited blastocyst formation.

Figure 4-11 Embryo quality analysed according to patient treatment group: a) Day 3 embryos from day 1 matured oocytes b) Day 3 embryos from day 2 matured oocytes b) Blastocysts from all remaining non-transferred embryos

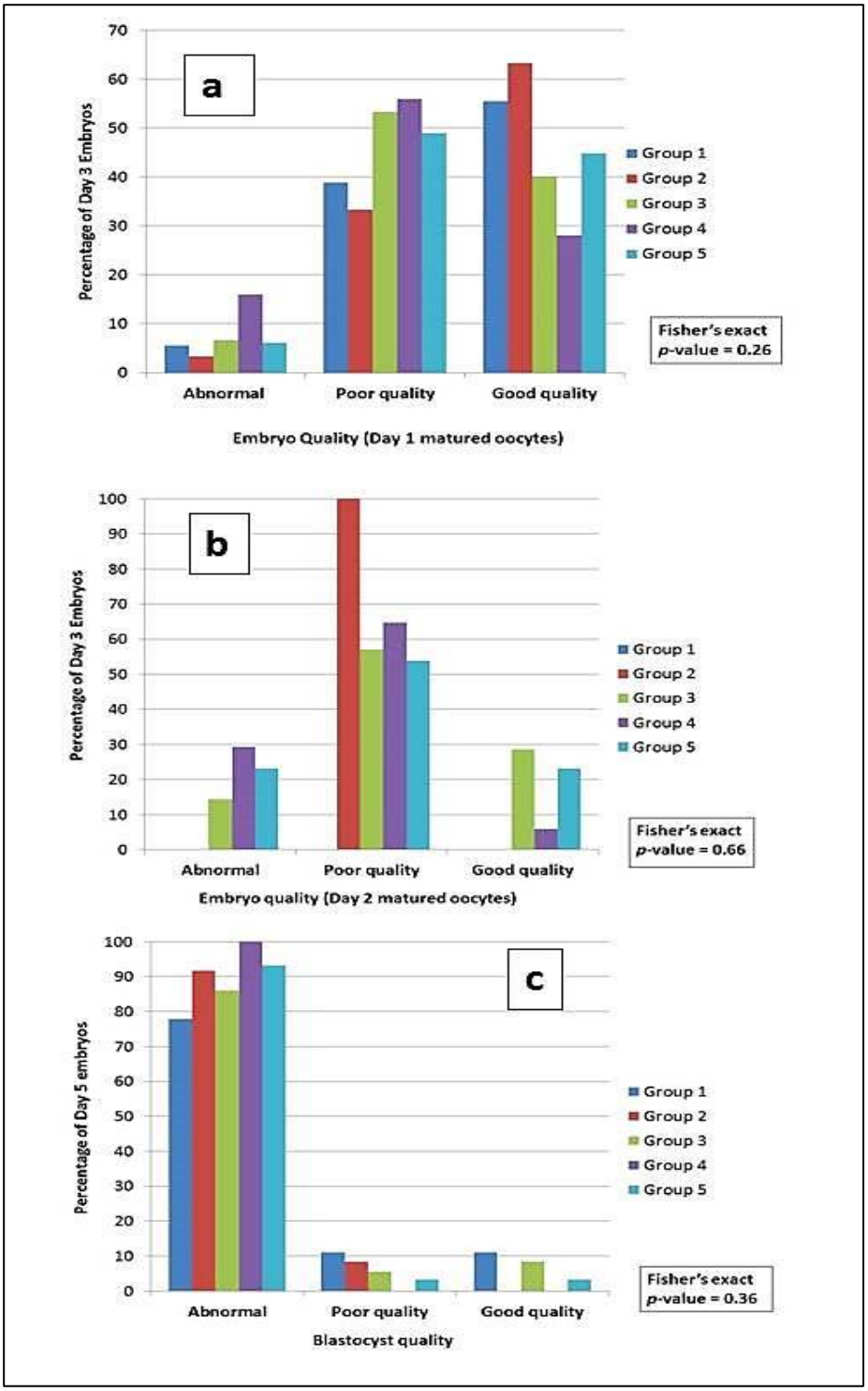


Table 4-6 Oocyte, embryo and pregnancy outcome results by treatment methodology group*

	Group 1 (n=5)	Group 2 (n=4)	Group 3 (n=6)	Group 4 (n=5)	Group 5 (n=10)	Total (n=30)	Significance (p-value)
Oocytes per patient	10.8 ± 3.7	21.0 ± 9.4	15.3 ± 6.2	18.8 ± 10.9	17.9 ± 7.5	**16.8 ± 7.9**	**ns[1] (0.32)**
Oocytes per group	54	84	92	94	179	**503**	**na**
Mature oocytes day 1 per patient	5 (2-7.5)	8 (5.5-20.3)	9 (5.3-12.8)	9 (3.5-12)	8.5 (5.8-10.8)	**7.5 (5-10.3)**	**ns[2] (0.43)**
Mature oocytes day 2 per patient	1 (0-3)	4 (0.5-0.8)	1 (0-4.3)	4 (1.5-10)	3 (2-4.8)	**2 (1-4.5)**	**ns[2] (0.21)**
Total mature oocytes day 1 (%)	24 (44.4%)	45 (53.5%)	54 (58.7%)	40 (42.6%)	82 (45.8%)	**245 (48.7%)**	**ns[4] (0.14)**
Total mature oocytes day 2 (%)	7 (13.0%)	16 (19.1%)	13 (14.1%)	27 (28.7%)	39 (21.8%)	**102 (20.3%)**	**ns[4] (0.08)**
Fertilised oocytes day 1 matured per patient	2 (1-5)	5 (2.5-12)	6 (4.3-11.8)	5 (1.5-8.5)	4.5 (3-7.3)	**5 (2.8-7)**	**ns[2] (0.39)**
Fertilised oocytes day 2 matured per patient	0 (0-2)	1.5 (0-3.8)	1 (0-2.5)	2 (1.5-6)	0.5 (0-2.8)	**1 (0-3)**	**ns[2] (0.28)**
Total oocytes fertilised D1 (% of D1 mature)	14 (58.3%)	26 (57.8%)	44 (81.2%)	25 (62.5%)	47 (57.3%)	**156 (63.7%)**	**P<0.05[4] (0.04)**
Total oocytes fertilised D2 (% of D2 mature)	4 (57.1%)	7 (43.8%)	8 (61.5%)	17 (63.0%)	15 (38.5%)	**51 (50%)**	**ns[3] (0.29)**
Total cumulative mature oocytes by day 2 (%)	31 (57.4%)	61 (72.6%)	67 (72.8%)	67 (71.3%)	121 (67.6%)	**347 (69.0%)**	**ns[4] (0.30)**
Total fertilised of mature oocytes (%)	18 (58.1%)	33 (54.1%)	52 (77.6%)	42 (62.7%)	62 (51.3%)	**207 (59.7%)**	**p<0.01[4]**
D3 embryos/patient	3.4 ± 2.3	8 ± 4.6	8 ± 4.9	6.6 ± 4.0	5.6 ± 3.1	**6.2 ± 3.8**	**ns[1] (0.28)**
Good D3 embryos/ patient	2.0 ± 0.7	4.8 ± 3.5	3.3 ± 2.9	1.6 ± 1.1	2.5 ± 2.0	**2.7 ± 2.3**	**ns[1] (0.25)**
Total good quality D3 embryos of graded ones	10 (10/17; 58.8%)	19 (19/32; 59.4%)	20 (20/48; 41.7%)	8 (8/33; 24.2%)	25 (25/56; 44.6%)	**82 (44.1%)**	**P<0.05[4] (0.04)**
Patients achieving a blastocyst per group (of remaining cleavage stage embryos after D3 transfer)	1 (1/5;20%)	1 (1/4; 25%)	4 (4/6;66.6%)	1 (1/5; 20%)	3 (3/10; 30%)	**10 (33.3%)**	**ns[3] (0.52)**
Positive pregnancy test (per collection)	60% (3/5)	50% (2/4)	33.3% (2/6)	20% (1/5)	10% (1/10)	**30%**	**ns[3] (0.72)**
Max. mean serum hCG level (range)	12.1 (3.1-22.3)	1489.5 (9.5-2976)	22.6 (11.2-34)	3.9	8	**na**	**na**

[1]ANOVA [2]Kruskal-Wallis Test [3]Fisher's Exact Test [4]Chi squared statistic *Parametric data presented as mean ± SD; non-parametric data as median (IQR)

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4.3.4 Comparison by age

To compare the effect of age on the results, the patients were regrouped into those under 35 years and those 35 years or more (see Table 4-7). This was based on the common understanding of declining fertility after the age of 35 years. Age group 2 had a combination of treatment groups 1-3 within it but not treatment groups 4 or 5. Due to the significant findings related to fertilisation and embryo quality by treatment methodology group, these points have not been analysed by age category. There was no significant difference ($p>0.05$) in BMI or FSH by age group. There was a trend for reducing oocyte number collected per patient with age but this was not significant. Although individually, maturation did not differ by group, a significantly higher proportion ($p<0.01$) of oocytes from the oldest age group (age group 2) matured by day 1. In contrast, significantly more oocytes from group 1 matured by day 2. Using Spearman's rank, there was no significant correlation between age and total maturation of oocytes. There was no significant difference ($p>0.05$) in positive pregnancy test by age, despite age group 2 having no positive results.

Table 4-7 Results by age group*

	Age group 1 <35yrs (n=25)	Age group 2 ⩾35yrs (n=5)	Total (n=30)	Significance (p-value)
Age (Years)	29.3 ± 3.4	36.6 ± 1.3	30.5 ± 4.2	-
BMI (kg/m²)	25.2 ± 3.2	24.4 ± 2.5	25.1 ± 3.1	ns[1] (0.58)
FSH (iu/L)	5.2 ± 1.8	6.2 ± 1.1	5.4 ± 1.8	ns[1] (0.31)
Oocytes per patient	17.7 ± 7.8	12.2 ± 7.4	16.8 ± 7.9	ns[1] (0.16)
Total oocytes retrieved per age group	442	61	503	-
Mature oocytes D1 per patient	8.1 ± 3.6	8.6 ± 9.1	8.2 ± 4.8	ns[1] (0.91)
Mature oocytes D2 per patient	3.9 ± 3.4	1.0 ± 1.3	3.4 ± 3.3	p<0.01[2] (0.004)
Total mature D1 by age group (% of collected oocytes)	202 (45.7%)	43 (70.5%)	245 (48.7%)	p<0.01[2] (0.000)
Total mature D2 by age group (% of collected oocytes)	97 (21.9%)	5 (8.2%)	102 (20.3%)	p<0.05[2] (0.02)
Cumulative total Mature oocytes (%)	299 (67.6%)	48 (78.7%)	347 (69.0%)	ns[2] (0.11)
Positive pregnancy test (per collection)	9 (36%)	0 (0%)	30%	ns[3] (0.29)

[1]Independant t-test [2]Chi-squared statistic [3]Fisher's Exact test *Parametric data presented as mean ± SD

4.3.5 Comparison by ovulatory status and phenotype

The patients were regrouped by ovulatory status (see Table 4-8). The proportion of patients who were ovulatory, was similar in each of the original 5 treatment groups, hence all the data was re-evaluated including fertilisation and embryo development. Those with a menstrual cycle consistently >35 days were termed an-ovulatory (based on the definition of oligo/anovulation in the Rotterdam criteria for PCOS). The reason for this analysis was to determine any characteristics that may influence IVM outcome and therefore future patient selection. Women who are anovulatory may often have a more pronounced form of PCOS than their ovulatory counterparts and may therefore represent a group of women who may benefit more from IVM as a treatment.

There was no significant difference in baseline demographics of age, BMI or FSH when data were analysed according to ovulatory status. A similar number of oocytes were retrieved from each patient group. Although no difference in individual maturation rates were recorded, an increased proportion of the oocytes matured in the ovulatory group. There was no difference in fertilisation rate or number of good quality day 3 embryos ($p>0.05$). A significantly increased proportion of blastocysts formed from oocytes harvested from anovulatory patients ($p=0.05$). There was no difference in the number of positive pregnancy tests between the ovulatory or anovulatory women. If the anovulatory group was further split into those with cycles <6months or ≥6 months, the group with the longer cycles had a significantly lower maturation (ovulatory & cycles <6months (n=23) 75.5% vs. cycles >6months (n=7) 53.4%; $p<0.01$) but no significant difference in fertilisation rate, proportion of good day 3 embryos formed or positive pregnancy test rate were detected.

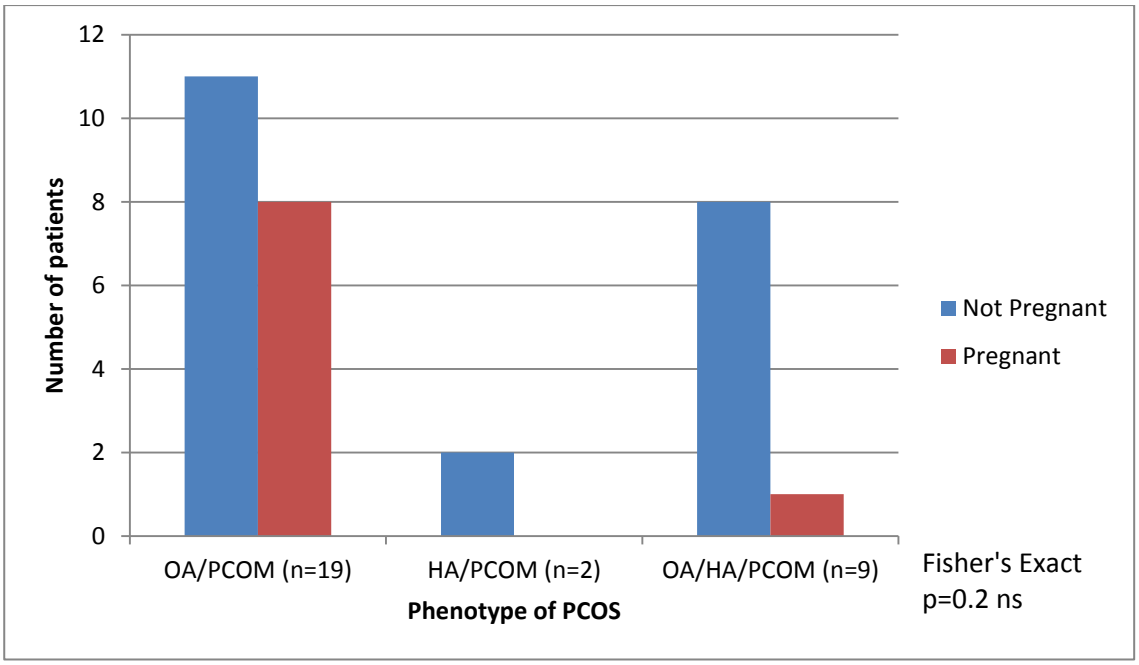
Table 4-8 Comparison of results by ovulatory status

	Ovulatory (n=11)	Anovulatory (n=19)	Significance (p-value)
Age (Years)	30.1 ± 4.5	30.8 ± 4.1	ns[1] (0.67)
BMI (kg/m²)	24.3 ± 3.1	25.5 ± 3.0	ns[1] (0.28)
FSH (iu/L)	5.6 ± 2.2	5.3 ± 1.5	ns[1] (0.64)
Oocytes per patient	14.5 ± 6.8	18.1 ± 8.3	ns[1] (0.23)
Oocytes per group	159	344	-
Mature oocytes day 1 per patient	7 (5-12)	9 (5-10)	ns[2] (0.83)
Mature oocytes day 2 per patient	3 (1-4)	2 (1-7)	ns[2] (0.58)
Total mature oocytes day 1 (%)	84 (52.8%)	161 (46.8%)	ns[3] (0.25)
Total mature oocytes day 2 (%)	36 (22.6%)	66 (19.2%)	ns[3] (0.44)
Total mature oocytes (%)	120 (75.5%)	227 (66.0%)	P<0.05[3] (0.04)
Fertilised mature oocytes day 1 /per patient	4 (2-8)	5 (3-7)	ns[2] (0.58)
Fertilised mature oocytes day 2 / per patient	1 (3-7)	1 (0-3)	ns[2] (0.40)
Total fertilised oocytes day 1 (% of mature)	51 (42.5%)	105 (46.3%)	ns[3] (0.58)
Total fertilised oocytes day 2 (% of mature)	13 (10.8%)	38 (16.7%)	ns[3] (0.19)
Total fertilised of mature oocytes (%)	64 (53.3%)	143 (63%)	ns[3] (0.10)
D3 embryos/patient	5 (3-7)	7 (4-9)	ns[2] (0.22)
Good D3 embryos/ patient	2 (1-3)	2 (1-3)	ns[2] (0.78)
Total good quality D3 embryos (%)	26 (26/58; 44.8%)	56 (56/128; 43.8%)	ns[3] (1.00)
Patients achieving blastocyst per group (of remaining cleavage stage embryos after D3 transfer) (% of cycles)	1 (1/11; 9.1%)	9 (9/19; 47.4%)	P=0.05[4]
Positive test (per collection)	3 (3/11; 27.3%)	6 (6/19; 31.6%)	ns[4] (1.00)

[1]Independent samples t-test [2]Mann-Whitney U test [3]Chi squared with continuity correction [4]Fisher's exact test

The classification of ovarian morphology as PCOM was an essential criterion for this pilot study; therefore 3 of the 4 main phenotypes of PCOS were sampled (according to the Rotterdam criteria combinations). There was no significant difference in spread of phenotypes across the original 5 methodology treatment groups ($p>0.05$). There was also no significant difference in positive pregnancy test by phenotype ($p>0.05$) (see Figure 4-12). There were too few patients in the HA/PCOM group to warrant making further comparisons.

Figure 4-12 Positive pregnancy test by phenotype of PCOS



4.3.6 Cost analysis

Table 4-9 provides a cost breakdown of a standard antagonist IVF with ICSI treatment cycle compared with an IVM treatment cycle. A cycle of IVM is marginally cheaper, with a £204.76 reduction in apparent costs. The difference may have been more marked if an agonist cycle had been used due to additional scans and potential increased gonadotrophin costs. This cost breakdown is per cycle started and does not take into account hospital admissions for OHSS or clinical pregnancy rate. The cost of medication (NHS cost prices kindly supplied by C. England, Pharmasure) for a standard antagonist IVF/ICSI cycle, based on 10 days stimulation using 150units/daily of FSH was £624.94 compared with an IVM cycle of £165.90. Although this favours IVM, the additional costs for IVM sequential media, laboratory time and clinician time reduces the medication cost advantage. The prices given in Table 4-9 are based on an optimised IVM cycle which involves a single ICSI procedure on day 1 matured oocytes. This decision was made on the data provided in this study, which suggests that embryo quality is poor if the day 2 matured oocytes are used.

Table 4-9 Cost per IVF/ICSI cycle compared with a modified IVM cycle up to day 3 transfer (based on 2010 staff pay scales)

	Procedure Specific Cost	IVF-ICSI	Modified IVM
HFEA fee per cycle	£75 per treatment	75	75
Nurse consultation	1.5hr x £20.83	31.25	31.25
counselling	1hr x 16.70	16.7	16.7
Documentation	Info leaflet/consents/plan/notes	2.8	2.8
Pre-stim/Day 2 scan	Doctor 0.33hr x £28.52	9.41	9.41
	Equipment (Condom, gel, gloves, linen, Scanner)	5.38	5.38
Teach Injection	Nurse time 0.25hr x £20.83	5.21	5.21
	Needles, syringes & sharps bin	0.9	0.9
Day 7 scan either	Doctor 0.33hr x £28.52	9.41	9.41
	Equipment as Day 2 scan	5.38	5.38
Day 8-9 scan IVF	Doctor 0.33hr x £28.52	9.41	
	Equipment as Day 2 scan	5.38	
Egg collection	Admission & recovery nurse 1.5hrs x 20.83 plus equipment	31.95	31.95
	Laboratory set up embryologist 0.2 x 17.91	3.58	7.16
	Theatre set up Nurse 0.75 x 10.26	7.7	7.7
	Registrar 0.66 x 28.52	18.8	28.52
	Embryologist 0.66 x 20.83	13.8	27.8
	Nurse 0.66 x 10.26	6.8	10.26
	Anaesthetist & anaesthetic drugs	100.48	100.48
	Egg collection pack inc. cannula, pudendal, equipment, sterilisation	18.95	18.95
	Egg collection needle	35.02	28
	Hospitality	3	3
	Theatre garments (staff/patient), gloves, linen	39.26	39.26
Laboratory	Laboratory miscellaneous inc 60mm dishes, plate pipettes	28.96	28.96
	Additional for IVM 35mm dishes, filters, tubes		19.98
Sperm prep	Witness 0.1x 13.18	1.32	1.32
	Embryologist 0.5 hr x 20.83	10.42	10.42
	Equipment (5ml culture tube, 15ml conical sperm tube, pipettes, tips, microscope, stage, flow hood)	9.32	9.32
	Suprasperm	3.47	3.47
Medium	IVM medium pack	na	234
	IVF/ICSI medium	10.6	
	SAGE oil	7.2	7.2
	Vitrolife flush	10.2	20.4
IVM/ICSI procedure	Embryologist (inc. OCC wash, strip and inject) hr 20.83	40.62	40.62
	ICSI equipment & hyaluronidase etc	57.7	57.7
	Electronic witnessing	16.2	16.2
Fertilisation check	Embryologist 0.5 x 20.83	10.42	10.42
	Witness 0.1x 20.83	2.08	2.08
Embryo grading D2	Embryologist 0.2 x20.83	4.17	4.17
	Culture equipment	18.75	18.75
Embryo transfer	Registrar 0.5 x28.52	14.26	14.26
	Nurse 0.67x 20.83	13.96	13.96
	Embryologist 0.5 x 20.83	10.42	10.42
	Accessories theatre (Tissues/Glove/clothing/scanner/sterilisation)	44.42	44.42
	Laboratory equipment inc. Catheter, medium	31.36	31.36
Pregnancy test	Serum hCG	5	5
	Nurse 0.33 x 20.83	6.87	6.87
Pregnancy scan	Registrar 0.50 x 28.52	14.26	14.26
	Equipment	5.38	5.38
Cycle documentation	Registrar/nurse/embryologist	76.76	76.76
OHSS monitoring	Nurse 0.33 x 20.83	8.25	
Trust overheads	Lighting/heating/buildings/maintenance	590.35	590.35
Medication	Standard antagonist IVF cycle (based on 10 days stimulation)	624.94	
	Standard IVM cycle		165.9
Total		**2133.23**	**1928.47**

4.4 Discussion

The ability to collect, mature and fertilise immature oocytes has been confirmed within our laboratory. The maturation and fertilisation rates replicate earlier work by different groups (358, 359, 398). No incidence of OHSS following an IVM treatment cycle was an ideal outcome, but is perhaps arbitrary when no clinical pregnancies were achieved in this study. Embryos, including blastocysts were generated but overall embryo quality was poor. This was reflected by the substantial number of low level biochemical pregnancies that did not yield clinical pregnancies. Never the less, it is important to evaluate and reflect on the positive outcomes alongside the short comings of this pilot study. To do this each stage of the treatment pathway should be addressed including patient selection, oocyte quality, stimulation and the *in vitro* environment. The poor outcome could finally also be attributed to chance, whereby if the trial had continued pregnancies could have been achieved. Discussing the outcome with an expert at the Oxford Fertility Unit reached the same conclusion, where they have had similar periods of poor outcome despite no change in a functioning protocol (unpublished data).

4.4.1 Patient factors and oocyte quality

The majority of published data on the clinical application of IVM relates to patient's with PCOS (see Table 4-1). The belief is those with PCOS have poorer quality oocytes with a lower developmental potential. This raises the question as to whether those with PCOS are the most suitable for the more challenging IVM laboratory process. Despite this, it had been shown that those with PCOS can achieve the same LBR as normal ovulatory controls but with a higher oocyte yield and lower fertilisation rate (294). Studies have examined factors integral to the oocyte, surrounding cumulus cells and extra-ovarian factors, in an attempt to establish markers and cause of differences seen in patients with PCOS (329, 584).

The oocyte has an uncoupled transcription and translation process, with a meiotic cell cycle that stops and starts twice during the lifespan of the gamete (585). Due to the uncoupling and long half-life of individual mRNAs, there are a wealth of maternal mRNAs in the oocyte cytoplasm, that are needed for completion of the meiotic cell cycle, the 1st embryonic mitotic cycle and activation of the embryonic genome (584, 586). Any disruption in the transcription process or mRNA instability may impact on the oocyte's growth, maturation and its developmental competence post fertilisation (584). Microarray analysis demonstrates an increased content of mRNA in oocytes from women with PCOS compared with normal oocytes (584); these include maternal-effect genes (producing mRNA and/or protein required around the time of zygotic genome activation) and genes involved in the meiotic/mitotic cell cycle. Specifically, microarray analysis revealed a 3-fold increase in transcripts of *Mater/NALP5*, which is required for developmental progression past the 2-cell stage. Also, a 4-fold increase in *Basonuclin 1* and *Formin 2* was shown, genes which are involved in transcription regulation and spindle dynamics during oogenesis. Murine work has revealed the necessity for timely degradation of the RNA, likely signalling the transition from the maternal to zygotic genome, to ensure appropriate embryonic development (587). Furthermore, work in Zebrafish has shown that impaired RNA degradation leads to disordered embryogenesis and can result in compromised development including neural tube defects (588). These facts lead Wood et al (584) to speculate that increased mRNAs disrupt the timely maternal-to-zygotic transition and contributes to the embryo developmental failure in oocytes from women with PCOS. Furthermore, these authors have identified putative binding sites for androgens and Peroxisome Proliferating Receptor (PPAR) gamma receptors. These receptors have been implicated in normal follicular development and ovulation (589). Also, insulin has been shown to phosphorylate PPAR-γ resulting in its activation (590). Wood et al concluded (584) that the genes involved in spindle dynamics and centrosome function contain these putative nuclear receptor binding sites. With an increased intra-follicular testosterone level and circulating insulin concentration seen in their patients with PCOS undergoing IVF, the altered endocrine and metabolic environment surrounding the PCOS oocyte

may negatively affect the meiotic cell cycle of the oocyte and/or early embryo. Indeed, the list of follicular fluid factors involved in folliculogenesis is long; including members of the numerous growth factor families, cytokines and inhibins (329, 511). Any imbalance in these factors can alter the oocyte competence.

Further support for the concept of an altered follicular microenvironment in PCOS patients is provided by Desforges-Bullet et al (591). These authors describe a disproportionately high level of AMH in the follicular fluid (FF) of women with PCOS which was accompanied by a lowered FSH concentration. No relationship was shown between LH or androgen levels that had been previously considered integral to the increased FF AMH (215, 217, 592). A lower availability of FSH may lead to a reduced inhibitory impact of FSH on AMH secretion in those with PCOS. This supports Baarends et al (127) who suggested that FSH may play a role in AMH down-regulation *in vivo*. Furthermore, Pellatt et al (215) has shown that FSH inhibits AMH production in the polycystic ovary but not in the normal ovary. A negative relationship between pregnancy rate and FF AMH was also shown, supported by Cupisti et al (593), suggesting high FF AMH may be harmful to oocyte maturation and development potential. Conversely, others (594, 595) suggest the opposite with improved fertilisation and implantation. In early folliculogenesis, a relative deficiency of AMH protein has been reported in the primordial and transitional follicles in women with PCOS, implicating AMH in the accelerated progression of follicles from the primordial pool (222).

The layers of cumulus cells surrounding the oocyte are known to play a crucial role in supporting the growth and development of the oocyte and of inhibiting or supporting meiotic progression in a timely manner. Up until the LH hormone surge, the corona radiata cells provide a transport link between cumulus and oocyte allowing exchange of substances such as GDF–9 and kit ligand, thought necessary for oocyte maturation (596). In good quality MII oocytes that were either transferred or vitrified (if a high grade blastocyst), no significant differences were seen in gene expression between the normal or PCOS cumulus cells (597). This was not the case if the entire pool of oocyte

cumulus was evaluated i.e. no knowledge of the oocyte developmental capacity, where an altered gene expression profile were shown in those with PCOS including members of the epidermal growth factor-like and IGF-like families (598), both groups of genes are involved in the acquisition of oocyte developmental competence. In addition, there was a deregulation of mRNA transcripts involved in steroid metabolism in PCOS cumulus cells (598). Further transcriptomic analysis of cumulus, showed up-regulation of pathways involved in DNA replication and cell cycle, in the cumulus cells from women with PCOS (597). Usually, a distinct down-regulation of these pathways occurs at the LH/hCG surge (599). Wissing et al (597) speculate that the up-regulation in PCOS reflects a delayed response to the maturation LH trigger. The theory of increased proliferation within the cumulus of PCOS oocytes is supported by a small study by Polzikov et al (600), who show increased ribosomal RNA expression and number of ribosomes. Proliferation and protein synthesis correlates with ribosomal synthesis (601). Furthermore, within lean and overweight patients with PCOS, there is a differential expression of genes within the cumulus (602), supporting potential differing pathophysiological pathways of PCOS related to weight. Indeed, it has been well documented that lean and overweight PCOS women often differ in respect to the underlying pathogenesis of PCOS. Lean women have higher basal LH levels with increased LH responsiveness to GnRH (603). In contrast, overweight women with PCOS exhibit increased insulin resistance, reduced IGFBP-1 and increased testosterone to SHBG ratio (604). Overweight women with PCOS share similar cumulus gene expression profiles with overweight normal ovary controls. One exception is the up-regulation of genes involved in the insulin signalling system including *INSR, IRSI* and the fat mass and obesity associated gene *FTO* previously associated with PCOS (66). Lean PCOS women had the most pronounced number of differentially expressed genes in the cumulus cells. One set of down-regulated genes included the Wnt/β-catenin and MAPK signalling pathways which have been shown to work together in regulating and aiding folliculogenesis and ovulation (602). An upregulated set of genes in lean PCOS augment the extracellular matrix assembly, a key factor in folliculogenesis. An example of this includes the hyaluronan and proteoglycan link protein 1 (*HAPLN1*) involved in

cumulus expansion in response to gonadotrophins. Within the current study, there was a clear correlation between the crude categorisation of expansion and coverage of cumulus around the oocyte with oocyte maturity. This is in agreement with Wynn et al (395). Although the gross appearance of the expanded cumulus may look similar between oocytes, underlying transcriptomic analysis may reveal a further perturbed system within the oocyte or cumulus cells from women with PCOS during IVM cycles.

The extra-ovarian endocrine environment common in those with PCOS can interrupt and disrupt the complex developmental process of oocyte maturation and embryo development. Similar to previous discussion of follicular fluid hormone levels, this can be extended to incorporate serum levels found in the complete individual. The key factors are FSH, LH, oestrogen, androgens and insulin. Follicular recruitment and growth is dependant on FSH, where there is a fine balance between recruitment and atresia. Prior to the acquisition of aromatase activity and increasing oestrogen levels, small human antral follicles (2-5mm) become responsive to FSH and are recruited (605). As oestrogen rises, FSH falls towards the end of the follicular phase with only the most advanced follicle proceeding to ovulation. Women with PCOS have been shown to have lower serum FSH levels (606) and an accumulation of small antral follicles, of which many may undergo premature growth arrest (511). Furthermore, it has been suggested that high oestrogen levels demonstrated during assisted conception cycles in patients with PCOS, may be detrimental to oocyte maturation and embryonic development (607). Alongside an FSH deficiency, a tonic hyper-secretion of LH in the follicular phase is recognised in women with PCOS (608). High LH levels have been implicated as a cause of the reduced oocyte maturation, fertilisation, embryo quality and the higher incidence of miscarriage associated with PCOS (329, 609). Premature luteinisation and follicular atresia may occur, due to suppressed FSH function, culminating in premature oocyte maturation and terminal differentiation of the granulosa cell layer (511, 610). Impaired endocrine control of meiosis and extrusion of the polar body may contribute to chromosomal and embryonic aneuploidy and elevated miscarriage rate (329). Hyperandrogenaemia is common in PCOS. It is

primarily ovarian in origin with substantial contributions from adrenal and adipose tissues (611). Like LH, high levels of thecal androgen have been shown to have a negative impact on oocyte development (612). *In vitro* work has shown a strong inhibition by testosterone on cumulus-free oocyte meiotic maturation (327). This evidence suggests that cumulus cells may protect the oocyte during the final stages of meiotic maturation. Elevated testosterone levels surrounding the oocyte, may reduce calcium oscillations, so inhibiting oocyte cytoplasmic maturation (329). The mechanism for this has not been fully elucidated but appears to be non-genomic and related to the ratio of oestradiol to androgen, rather than the individual levels of steroids *per se.* Calcium oscillations initiated by oestradiol are limited by treatment with androgens (613). Insulin resistance and compensatory hyperinsulinemia, has been widely implicated in reduced oocyte competence summarised by Qiao and Feng (329). These mechanisms include upregulation of LH receptor mRNA expression (37), inhibition of FSH-induced aromatase activity, local induction of androgen production (614) and altered molecular spindle dynamics and centrosome function (584).

Extra-ovarian and intra-ovarian mechanisms inherent to PCOS can generate poor quality oocytes that may enter but falter in an *in vitro* system, leading to poor outcomes in an IVM treatment. There is limited clinical data on the patients in the current study. The group of women were young (30.5 ± 4.2yrs) with a normal BMI (25.1 ± 3.1kg/m^2). Limitations of the study include unknown systemic androgen, AMH levels and also their insulin resistance status of the recruits. The latter is particularly relevant when considering potential causes of IVM failure inherent to those with PCOS. It can be assumed that all of these factors are elevated compared with normo-ovulatory women without PCOS, especially as more than half of the population have ovulatory dysfunction with prolonged menstrual cycles. A judgement on enhanced patient selection for IVM cannot be made. The only difference by ovulatory status (Table 4-8) was a reduction in oocyte maturation rates in women who were either ammenorhoeic or who had cycles of >6months. This is in agreement with Barnes et al (392). The finding of improved blastocyst formation in the anovulatory group is

difficult to explain, unless removal from the detrimental PCOS follicular environment into a more favourable *in vitro* culture environment even for for such a short period of time could be considered beneficial. Despite many reasons for women with PCOS being poor candidates for IVM including compromised oocyte quality, the overriding fact is that IVM offers a way to eliminate OHSS in those individuals at high risk, thus making the compromise in clinical outcome acceptable in this population. With respect to the poor outcome of this pilot study, patient selection was seemingly not the reason given the CPRs achieved in other identical PCOS populations, outlined in Table 4-1.

4.4.2 Stimulation protocol and endometrial preparation

It is clear from the literature, that many different stimulation protocols have been used for IVM. The use of gonadotrophin stimulation has been shown to improve maturation rates (395) but there is still a debate as to whether both FSH and hCG are needed for stimulation (359, 400, 401). In the current pilot study, minor changes were made to the initial stimulation protocol to establish the optimum working protocol for use in the planned RCT. The main change was the duration of time from hCG trigger to the time of oocyte retrieval. Initially time to retrieval was 24 hours (which appears unique to our study). Group 5 followed a more classical IVF pathway with 36 hours to retrieval. Group 3 (see Table 4-3), where hCG was administered 24 hours before oocyte retrieval on day 10 of the cycle, had a significantly improved fertilisation by 48 hours. In contrast, there were significantly more good quality day 3 embryos in Groups 1-2. Furthermore, there was a trend for positive pregnancy tests in favour of Groups 1-2. Overall there was a better IVM outcome for those patients who had a shorter exposure to hCG. This questions the benefit of including an hCG trigger pre collection of oocytes during an IVM cycle. In support of this notion, 2 of the most recent studies have omitted use of hCG prior to oocyte retrieval (359, 572) with good results. Zheng et al (572) showed that although maturation was improved with hCG priming this was not translated into improved fertilisation, implantation or pregnancy rates. Hence,

although nuclear maturation may be triggered *in vivo* by hCG, the impact on oocyte cytoplasmic maturation *in vitro* remains unclear. The impact of hCG priming in IVM cycles on oocyte maturation may also be confined to oocytes from women with PCOS, whereby LH receptor expression in cumulus and granulosa cells from PCOS follicles may occur in smaller follicles compared with normal women allowing hCG action (615, 616). The mechanism for this may be related to the stimulating effects of LH on AMH expression and loss of the down-regulation of the AMH-II receptor in anovulatory PCOS women but not ovulatory normal or PCOS women (129). Similar maturation benefit from hCG priming has not been shown in non-PCOS women (617). Better outcomes in the earlier recruited patients in the pilot study, suggests that if the study was to be run again hCG should either be omitted or used closer to the planned time of oocyte retrieval.

It is reassuring that the maturation parameters were equal with another UK centre (see Table 4-10) highlighting that gross errors within the oocyte retrieval and maturation process were not occurring, particularly as it was the first time that IVM had been offered as a clinical treatment at LCRM. To compare our data with Oxford Fertility Unit (from whom the laboratory protocol is modelled), data was extracted from their most recent publication (358). This included 97 IVM cases conducted between January 2007 and March 2010. Table 4-10 summarises the comparative results. Despite a younger cohort of patients enrolled at LCRM, there was no difference in baseline FSH, BMI or ovulatory status. There was no significant difference between oocyte number retrieved, maturation by 48 hours or fertilisation of mature oocytes. A direct comparison of embryo development was not possible from the results. The closest comparison was number of cleaving embryos (6.4 ± 4.8; Oxford) and day 3 embryos (6.4 ± 3.8; LCRM), for which there was near identical findings. No significant difference was seen in biochemical pregnancy rate (although day 16 urine hCG was reported by Oxford compared with day 14 serum hCG at LCRM). There was a significant difference in clinical pregnancy rate, with unfortunately no ongoing pregnancy at LCRM. There were no reported cases of OHSS at either Leeds or Oxford.

Table 4-10 Comparison between Oxford Fertility Unit and Leeds Centre for Reproductive Medicine*

	Oxford Fertility Unit (n=97)	LCRM (n=30)	Significance
Age (Years)	32.4 ± 3.8	30.5 ± 4.2	*P<0.05¹*
BMI (kg/m²)	24.2 ± 4.6	25.1 ± 3.1	*ns¹*
FSH (iu/L)	5.5 ± 1.4	5.4 ± 1.8	*ns¹*
Ovulatory PCO/PCOS % (n)	46.4 (45)	43.3 (13)	*ns²*
Anovulatory PCOS % (n)	53.6 (52)	56.7 (17)	*ns²*
Oocytes collected	15.8 ± 7.2	16.8 ± 7.9	*ns¹*
Total maturation 48 hours % (n)	65.0 (1087)	69.0 (348)	*ns²*
Average mature oocytes	11.2 ± 7	11.6 ± 5.8	*ns¹*
Fertilisation mature oocytes %	62.9	59.7	*ns²*
Biochemical Pregnancy % (n)	28.9 (28)	30 (9)	*ns²*
Clinical Pregnancy % (n)	19.6 (19)	0 (0)	*P <0.01²*
OHSS cases	0 (0)	0 (0)	*ns²*

ns = non-significant; significance level <0.05 unless specified ¹Student's t test ²Chi squared statistic with continuity correction *results are presented as mean ± SD or % (number)

The high biochemical pregnancy rate and its lack of translation into ongoing pregnancies in the present pilot study were disappointing. It has been suggested that women undergoing IVM have a higher miscarriage rate (618). Although this may be related to the underlying PCOS aetiology rather than IVM *per se* (619), it has also been shown that a higher rate of aneuploidy is present in embryos derived from IVM (425). Furthermore, confocal microscopy had shown a higher frequency of abnormal meiotic spindles and chromosomal alignments in IVM human oocytes (427). More recent data refutes these suggestions (620-622) and has demonstrated comparable morphological parameters between *in vivo* and *in vitro* matured human oocytes. It is important to note that the serum hCG levels were extremely low in the current study (see Table 4-6). The half life of hCG 10000 units subcutaneous injection has been shown to be 32hours (623) with <10% detectable bioactivity by 8 days. From these results, it is highly

unlikely that the hCG detected at day 14 in the patients was due to residual hCG from the trigger injection.

Endometrial preparation is a key factor for successful IVM. Asynchrony between endometrium and embryo would lead to implantation failure regardless of embryo quality. A major criticism of this pilot study was the failure to record endometrial thickness on the day of embryo transfer. Endometrial thickness was only recorded sporadically either within clinic before hCG or at oocyte retrieval. This omission would have provided valuable basic information on how the endometrium performed through the IVM process. The endometrial thickness prior to hCG was 5.2 ± 2.1mm with no significant differences between treatment groups. The endometrium would be expected to increase in thickness following hCG and during the priming phase before embryo transfer. Many of the published studies used a policy based on endometrium thickness with no embryo transfer with an endometrium <7mm (Table 4-1). A thin endometrium has been suggested as a poor prognostic factor for IVF outcomes, although a recent meta-analysis suggested that both the prognostic and predictive capacity of endometrial thickness is limited (624). Child et al (625) have shown no difference in endometrial thickness on the day of oocyte retrieval between successful and unsuccessful pregnancy cycles, but by embryo transfer successful cycles have a considerably thicker endometrium. Attempts to improve the endometrium, has seen the trial of low dose oestrogen prior to oocyte retrieval (393), which was detrimental to oocyte maturation although not overall fertilisation rates. Minimal stimulation followed by the administration of oestrogen and progesterone for endometrial priming has been the general approach used by all groups who practice IVM. Oral oestradiol and vaginal progesterone from retrieval was used in this study, with doses in keeping with the majority of trials (see Table 4-1). Interest in an endometrial 'implantation window' has gained momentum (626), whereby the endometrium shifts from a pre-receptive to receptive state. It has been suggested that ovarian stimulation in ART can hasten the onset of this implantation window compared with natural cycles (627). Indeed, both transcriptomic and proteomic technologies have shown a change in gene expression and regulation dependant on the controlled ovarian stimulation strategy

(626). IVM techniques perhaps sit somewhere in between the natural cycle and stimulated cycle. It would be useful therefore to classify the optimum transfer window for IVM using these technologies.

4.4.3 *In vitro* environment and media

As alluded to in the introduction to this chapter, various types of media have been used in IVM treatments. This comparison was drawn between the most recent trials with successful outcomes in which 2 different commercial medias were used (359, 572). Certainly within this country, an IVM medium with a known composition is needed to gain ethical approval for clinical research treatments, preventing the addition of some serum additives known to improve reproductive outcome (628). This enables the marketing and use of standardised commercial media which has undergone rigorous quality control, rather than in-house specialist media as has been used previously in our IVM research program. In the current study, commercially available and *Conformité Européene* (CE) marked Sage IVM media was used, which provides a sequential series of media that the oocyte moves through during the maturation process *in vitro*. The cost of this medium per cycle (£234/cycle) is higher than standard IVF culture medium (£10.60/cycle) and it has a far shorter shelf life. This is an important consideration when IVM cycles are infrequently completed. Recent murine and human work has suggested that there is little difference between using conventional blastocyst culture medium instead of IVM-specific medium on embryo development (580, 629). Kim et al (629) show that the only difference in a blastocyst culture medium (BMI, Suwon, South Korea) compared with a conventional IVM medium (Sage) is the presence of taurine and calcium lactate (although not specified in the paper one assumes that it is the maturation medium in the sequential IVM media pack these authors use in comparison). These authors speculate that these additives may improve outcome through the provision of energy substrates and enhanced anti-oxidant capacity. Taurine is a by product of hypo-taurine following free radical scavenging. Increased oxidative stress can impair *in vitro* embryo development and

thus by supplementing culture medium with taurine it is hypothesised that, oocyte meiotic maturation and embryo development can be improved (630). During oocyte maturation, mitochondria redistribute to the areas of the cell that require high energy (629, 631), namely the perinuclear and subcortical regions of the gamete. It has been suggested that increased free calcium is needed to ensure that appropriate mitochondrial redistribution occurs to prevent blastomere formation with low energy and reduced mitochondrial number (632). Interestingly, Kim et al (629) found that Tissue culture medium – 199 (TCM-199) was inferior to blastocyst media for maturation and blastocyst development. They even suggest that blastocyst media outshines the standard IVM media (Sage) in blastocyst development. Kim et al (629) compare the content of these media but do not make reference to the quantity of each constituent. A set 1ml drop is used to culture cumulus-oocyte-complexes which may not contain the same comparative volume of each constituent altering the oocytes maturation ability. TCM-199 was the media preferentially used in earlier work by Cha et al (368) and Child et al (413). Due to its undefined IVF specification, this media is no longer regularly used in human clinical treatment (579). In contrast to Kim et al (629), Filali et al (579) reported no significant difference in overall maturation or implantation between TCM-199 and a commercial IVM media supplied by Medicult Ltd.

Despite much research, the success rates for IVM on the whole remain inferior to conventional IVF (see Table 4-1) which perhaps reflects its minimal uptake into clinical practice over the last 20 years. A potential method to improve the synchronous nuclear and cytoplasmic maturation of oocytes (633) has recently been presented by Albuz et al (634). The recognition of cyclic-AMP (cAMP) as an important signalling molecule within the oocyte has provided a base to improve laboratory treatments. Limited amount of this second messenger is made within the oocyte, with the greater majority being supplied via gap junction connections from the cumulus cells (635, 636). Elevated levels of oocyte cAMP maintain meiotic arrest. Phosphodiesterase is responsible for degrading cAMP. The activity of PDE is inhibited by cyclic GMP supplied to the oocyte from the surrounding somatic cells (637). *In vivo,* following the ovulatory

surge a cascade of events occur allowing oocyte maturation to proceed. A drop in cGMP is seen within the oocyte and follicle (383, 638). There is also a release of epidermal growth factor (EGF)-like proteins within the follicle required for maturation (639, 640). The further discovery that PDE subtypes are compartmentalised within the ovarian follicle, allows targeted treatment of cumulus cells or oocyte PDEs (641, 642). Oocytes will resume meiosis spontaneously, once removed from the controlling follicular environment, in the absence of the aforementioned endocrine and paracrine control, following a drop in endogenous cAMP (634). This has been termed 'precocious' oocyte maturation, as meiotic progression is reinitiated but in the absence of the gamete gaining its full *in vivo* developmental competence. There is also a breakdown of the gap junction connections between oocyte and cumulus cells, preventing exchange of beneficial nucleotides and nutrients (642). The concept of biphasic IVM has been previously documented (634, 643, 644). This prevents meiotic resumption, using high concentrations of cAMP analogues or PDE inhibitors following oocyte retrieval for a period of time (24 - 48 hours), before these factors are removed by media change allowing maturation to occur. Unfortunately, this strategy has had a largely disappointing impact on improving IVM outcome. Albuz et al (634) present a system they term as simulated physiological oocyte maturation (SPOM) in mice. It includes a pre-IVM phase that rapidly generates an increased cAMP within the cumulus oocyte complex followed by an extended IVM phase with enough FSH to drive meiosis in the presence of a PDE inhibitor. The mice were primed with hCG prior to oocyte retrieval but subsequently only FSH was used in culture of the oocytes, which seems contrary to the usual physiological trigger of hCG/LH. The authors qualify this with evidence suggesting that oocytes will mature spontaneously regardless of *in vitro* gonadotrophin stimulation (634). In contrast, it has been suggested that *in vitro* maturation without gonadotrophin presence hampers the developmental potential of the oocyte (645, 646). Furthermore, FSH uses the EGF receptor in these authors SPOM system. Firstly, over 1-2 hours the COC's are exposed to cAMP modulating agents (Forskolin, a potent adenylate cyclase activator and Isobutylmethylxanthin (IBMX), a non-specific PDE inhibitor) which drives up cAMP levels and extends communication

time via the gap junctions. Within the extended IVM phase, continuous exposure to a PDE3 (Cilostamide) oocyte specific inhibitor occurs (unlike the biphasic systems). They speculate that this enhances the G2 to M-phase cell cycle resumption which has also been benefited by the extended gap cell cumulus communication. Furthermore, Albuz et al (634) confirm that the EGF receptor is implicated in oocyte maturation following gonadotophin stimulation/surge, requiring an ERK 1/2-mechanism previously documented (639, 640, 647, 648). It is important to note that clear species differences exist. In mammalian systems EGF was found to aid oocyte maturation *in vitro* (649), however microarray analysis did not identify EGF as significant in human granulosa cells. More recently, other members of the Regulin family that includes EGF have been implicated in both sheep (645) and human work including amphiregulin and epiregulin (650). The SPOM system for IVM was been shown to improve the blastocyst rate following the IVM over 24 hours of murine and bovine oocytes. Cattle breeding programmes usually generate blastocyst rates of <40% (651), where as using the SPOM system achieved 69%. The SPOM system certainly seems compelling, particularly when minimal change has occurred in conventional IVM systems; nor improvement in success rates over the last 2 decades. Whether this can be transferred effectively into the human clinical setting is yet to be seen but perhaps it will provide a basis to improve the clinical IVM systems in the future. It may transpire that SPOM is only relevant to animals that have a shorter oocyte maturation time *in vitro* i.e. mouse and cow oocytes mature in IVM over 24 hours vs. humans 36 hours post hCG. Encouragingly, there seems to be no ill effect from having IBMX in the culture medium on chromosomal and aneuploidy rate (620) but this compound has yet to be approved for clinical use in human cells.

4.4.4 Embryo quality and embryo selection

The inherent characteristics of poor oocyte quality in those with PCOS continues to underpin embryo development. The increased numbers of oocytes retrieved in PCOS

patients can compensate for these deficiencies culminating in normal cumulative pregnancy rates (294, 652). Although it has been suggested that increased embryonic fragmentation and decreased blastocyst formation occurs in patients with PCOS (653); there is contrasting work that suggests similar fertilization and achievement of good quality embryos (654, 655). In the current study, although 87% of transferred embryos were regarded as good quality; only 43% of all day 3 embryos reached the same good category. The conversion to blastocyst was also poor. As the 'best' embryos were transferred on day 3, a lower blastocyst rate could be expected. If this is compared to unpublished work from the LCRM at the same time in women with PCOS, the percentage of good quality day 3 embryos was ~50%. Although lower, this is unlikely to be significant. It raises the question of impaired implantation following IVM rather than poor embryo grade.

The optimum timing for ICSI in an IVM cycle would seem critical to ongoing embryo development as it is essential to allow for oocyte nuclear and cytoplasmic maturation to be completed *in vitro*. The nature of IVM cycles mean that oocytes may not all reach MII at the same time and therefore performing ICSI at a set time point may be detrimental for some oocytes. For example, it has been shown that delaying insemination of oocytes after MII has been reached, rather than immediate insemination leads to more embryos developing to advanced preimplantation stages (656). To perform insemination / ICSI at exact time points from MII arrest in IVM cycles would be near impossible in the clinical setting due to work force demands. In contrast, it is also recognised that there is impaired fertilisation if oocytes take longer to mature (657). De Vos et al (658) showed that embryo development was similar in 'rescue- IVM' oocytes compared with MII oocytes from a stimulated IVF cycle (47% vs. 52%). It should be noted that these oocytes may have had enhanced development due to *in vivo* culture prior to retrieval and the limited 4 hour *in vitro* culture, making comparison difficult. Furthermore, given the short IVM incubation in this study(only 4 hours) the oocytes would almost certainly have not been at GV at the start of culture but rather at late MI. Jaroudi et al (387) show the embryo grading from cleaved

embryos by day 3. They classified 69.8% embryos as ‘good’ or ‘fair’ (which included all embryos with <30% fragmentation). Direct comparison with the current data set is not possible as good embryos were <20% fragmentation, but the overall impression suggests the current study did not reach the IVM embryo quality levels achieved by other centres using this technology. Unfortunately, the more recent trials did not publish the % of good quality embryos generated, making further conclusions impossible surrounding the current study’s embryo quality.

Optimum embryo selection is critical for a successful IVM cycle. This has led to many selection strategies being developed, of which some are more successful than others (659). The current study used only a conventional classical grading system (526, 527). After fertilisation had taken place, there were only 2 further embryo checks on day 2 and 3 prior to transfer. This allowed only a snapshot of the embryo development to be assessed. So although 87% of transferred embryos were between the 6-8 cell stage with <20% fragmentation, any other aberrant behaviour the embryo may have displayed was unknown. It should also be noted that the better quality blastocysts produced from the remaining embryos, came from embryos that would have been classified as poor using this system on day 3. During the preliminary trial, the LCRM had acquired and were regularly using the Embryoscope® (Vitrolife, Unisense Fertilitech, Denmark) time-lapse system. This tool provides an uninterrupted culture environment whilst capturing multiple images of the embryos fine morphological development. It would have been beneficial for this trial to use this technology to understand if the embryos created from IVM performed in a grossly different way to embryos from conventional IVF. It has been suggested that time-lapse imaging should improve the clinical outcome (660). An RCT comparing Embryoscope® with a morpho-kinetic selection model with a standard incubator and morphological selection revealed a significantly improved on going pregnancy rate and reduced miscarriage rate (661). A Cochrane review (662) on the subject, which includes the last RCT (661), concludes that there is insufficient evidence particularly on live birth rates to instigate time lapse imaging in routine treatment. Interestingly, time-lapse imaging has revealed

further information pertinent to IVM (663). Increased fragmentation was noted in embryos from oocytes that had a detectable meiotic spindle later (>36.2hrs) following hCG injection. In the IVM stimulation protocols, there is a range of durations of hCG prior to collection and the *in vitro* process. This may inadvertently predispose the oocytes to increased fragmentation related to stimulation. Furthermore, it has been shown that FSH stimulation can alter the chromosomal alignment in metaphase 1, increasing aneuploidy in *in vitro* matured mouse oocytes (664). A focus on PCOS embryos has also shown that in embryos from hyperandrogenic women, delayed cleavage is seen compared with regularly cycling women, using the Embryoscope® (665). This last point highlights the differences that can occur in women with PCOS and the developmental competence of their embryos.

Apart from time-lapse imaging, proteomic and metabolomic measurements have been considered potential biomarkers for embryo selection (659). Once again these technologies were not implemented in this study, but may have provided more information on failings of the trial and ways to improve IVM in general. Harris et al (666) have shown that oocytes from women with PCOS have a higher consumption of glucose and pyruvate (the key energy substrate used by human oocytes), which can be attenuated by prior use of Metformin. Although the chromosomal constitution of *in vitro* matured oocytes was no different to controls, a higher pyruvate turnover was associated with abnormal oocyte karyotypes. Amino acid profiling has been shown to have a predictive capacity for determining embryos that have a developmental capacity to reach blastocyst stage, in particular increased take up of Leucine (667). Furthermore, amino acid turnover has been related to aneuploidy and embryo sex (354). This phenomenon has been further investigated in the oocyte; with oocytes that arrest at the GV stage after 24 hours of *in vitro* culture displaying differences in amino acid profiling compared with oocytes that progress through to MII (668). The latter data was achieved from oocytes that had failed to mature during routine IVF stimulation, which could have impacted on the results due to the limited capacity of the oocytes already. It would be worthwhile evaluating this finding in a true IVM

system with a known clinical outcome (i.e. where the embryo's viability was assessed during culture and transfer).

4.4.5 Outcome following an unsuccessful IVM cycle

Although the outcome from this pilot study was disappointing, an interesting point to note was the natural conception of 4 couples within 4 months of completing an IVM cycle. A similar finding was published by Frantz et al (669), whereby 3 natural conceptions occurred shortly after an IVM treatment cycle. Whilst a causal link cannot be proved or indeed ruled out, it is possible that ovarian puncture during an unstimulated IVM treatment cycle may have similar properties to that seen with "ovarian drilling". Three of the conceptions occurred in couples where repeated cycles of conventional IVF had been unsuccessful, resulted in an exuberant abandoned attempt or in 1 couple failed fertilisation. Two of the 4 women were amenorrhoeic. These factors depict a group of women that were hard to treat and perhaps had a more pronounced form of PCOS. Troude et al (670) reviewed rates of natural conception following unsuccessful IVF treatment cycles in a retrospective survey of patients. They found that the LBR following a natural conception was a cumulative 24% over a 7 year period (slightly higher than the 17% in previously successfully treated couples). The majority of births happened by the end of the 2nd year following treatment, with only 6% of the 24% happening within the 1st year. This suggests that our cohort have had a better than average conception rate within the 1st 4 months following IVM treatment. It should be noted that the reason for initial IVF treatment is often not recorded consistently, making direct comparison difficult. The long term benefit of IVM has been suggested by Agdi et al (671). Their study was designed to assess the effect IVM had on subsequent IVF cycles. An increased proportion of retrieved MII oocytes and fertilised oocytes were seen following IVM. An earlier pilot study by Ferraretti et al (672) had drawn the same conclusion. Women who had 2 previous failed IVF treatment cycles, had 'trans-vaginal ovarian drilling' within a 6

month period prior to a repeat IVF cycle. The outcome was an increased requirement of FSH stimulation but significantly improved fertilisation and cleavage of embryos. The overall pregnancy rate was similar to a normo-ovulatory patient with tubal pathology. Ferraretti et al (672) proposed that this technique provided a safer and lower cost alternative to laparoscopic ovarian drilling with electro-cautery. Furthermore, (673) it has been shown that IVM can transiently alter hormonal markers including AMH and Testosterone levels. This alteration is short lived, especially in relation to AMH. A shortcoming of this study (673) was the limited follow up of patients, relying on testing patients who chose to return at 3 months to complete the study. From the small number who completed the study, the group hypothesise that the benefit seen did not exceed 3 months. The long term benefits of laparoscopic ovarian drilling with electro-cautery are recognised and exceed what has been seen with IVM (674). The exact mechanism that ovarian drilling uses to improve ovulation remains unknown. One theory suggests that local ovarian trauma interrupts the intra-ovarian androgen synthesis pathway, reducing androgen levels and the inhibitory control on follicular maturation (675). Furthermore, the decreased androgen levels may reduce peripheral conversion to Oestrogen, therefore impacting on the feedback system of LH secretion. Destruction of the ovarian tissue through drilling, may remove these inhibitory factors, allowing a new cohort of follicles to be recruited and develop (672). Moreover, the reduction in AMH levels seen particularly with electro-cautery may restore ovulatory function by inducing a new wave of activation of primordial follicle growth and by improving follicular sensitivity to the circulating FSH (676). Although not as effective as laparoscopic ovarian drilling (LOD), IVM/ trans-vaginal ovarian drilling are likely to operate on a similar level. As it is less invasive, the patients' underlying steroid synthesis and folliculogenesis will resume quickly, restoring the pre-treatment ovulatory pattern for the individual (673). The validity of trans-vaginal ovarian drilling as a treatment in its' own right is limited, although 1 RCT suggests that it is equally as effective as LOD and has potential as an 'office' procedure (677). The most recent Cochrane review for LOD (678) concludes that there is no difference in rates of pregnancy in clomiphene-resistant PCOS patients compared with other

medical treatment. As the effects of IVM are short lived on ovarian function, there seems little point in championing its use for natural conception. Despite this conclusion, it seems churlish not to accept that there may have been some benefit for the 3 patients who now have a child following this study.

An interesting point is raised by Shalom-Paz et al (360) regarding the learning curve in clinical and laboratory practice when introducing a new technique within a clinic. These authors show a progressive improvement in cleavage, implantation and live birth rate over a 5 year time period, with no change in their conventional IVF results in the same time period. They acknowledge that the learning curve is hard to quantify with multiple factors contributing to the improvement (679). Although LCRM have previously run research trials centred on IVM, this was the first time IVM was practiced as a clinical treatment at the LCRM. Personally, as I completed all oocyte retrievals, the practical procedure did become easier as my dexterity with the procedure improved. If the same occurred for all steps of IVM, it should be hoped that our clinical pregnancy rates would have improved likewise if we had continued the trial. As the fertilisation and embryo quality reduced through the trial, perhaps the minor changes to stimulation protocol had more impact than perhaps accounted for.

A further factor to take into account is the performance of the LCRM as a whole. During the time period of the preliminary trial, a slight downturn in results occurred across the board at LCRM. Results published on the HFEA website include data from 2012. The CPR and LBR per cycle started were 28.3% and 25.4%; which were both below the national average of 36.1% and 32.9% respectively. The results for the duration of the trial for IVF-ICSI was a CPR of 32.2% and LBR of 28.09%, which although improving remained below the national average. Although the effect of this downturn will not have directly attributed to the poor outcome of the IVM pilot study, it would seem plausible that it may have contributed to it.

4.4.6 Cost analysis of IVM

In vitro maturation is marketed as a lower cost alternative compared with conventional IVF due to the minimal use of expensive gonadotrophins. Despite this assertion, there is minimal data on the exact costings of an IVM cycle in comparison with a short antagonist IVF cycle (358). An estimate costing between the 2 types of treatment up to a day 3 transfer is shown in Table 4-9. The cost of an optimised IVM cycle is marginally cheaper when compared with a standard short antagonist IVF cycle (£1928.47 vs. £2133.23). The suggested 'optimisation' is based on the outcome of the pilot study with reference to the poor quality of embryos generated from day 2 matured oocytes. It is proposed that only oocytes matured by day 1 should be inseminated and used for treatment, thereby reducing laboratory costs. Despite increased gonadotrophin costs with standard IVF (£624.94 vs. £165.90); the cost difference is marginalised by the increased costs of commercial sequential IVM media packs and increased clinician / embryologist time. This costing has not taken into account unplanned hospital admissions sometimes required for OHSS management. Nor has it balanced the treatment cost to pregnancy ratio. Rose et al (571) describe a 91% price reduction in medication costs for IVM. Overall they calculate that an IVM cycle is 45.9% cheaper than an IVF cycle (antagonist cycle) but unfortunately do not include with this the outcome data. These authors calculate the results based on, in part, the insurance company pay-outs for treatments. This makes comparison with the UK system difficult as these prices are based on one company alone (the NHS). Although Rose et al (571) agree with the current study that more laboratory and technical expertise is needed for IVM, this is not reflected in the price that is attributed to what they term 'procedure and anaesthesia costs'. In fact they suggest that the mean cost of this part is less than for IVF ($7532 ± 1275 vs. $8681 ± 1874; p=0.02). The authors reason that this is due to IVF procedures generating more oocytes for more expensive ICSI, less time is needed in the incubators as embryos are transferred at day 2 or 3 and finally, less stripping is needed on the cultured oocytes making ICSI easier.

These theories seem fundamentally flawed and would likely be revealed if the clinical pregnancy rate and cumulative pregnancy rate (including frozen cycles) was included.

Apart from financial costs, other costs such as time and visits for the patient should be considered. Within this pilot study there were a reduction in visits before (reduced follicular monitoring) and after oocyte retrieval (no OHSS monitoring). Importantly, patient's who experienced both an IVF and IVM cycle, subjectively reported that they preferred IVM if it had generated a pregnancy. Reasons given included a reduced number of self given injections, fewer visits and overall felt better through treatment. The latter may be partially explained by taking part in a clinical trial and continuity of care, but reveals the potential advantage for patients not easily quantified.

4.5 Conclusion

Drawing conclusions on a study that has failed to achieve even one clinical pregnancy is a difficult task. The successful oocyte retrieval, maturation and fertilisation to create embryos suitable for transfer was an encouraging finding. The necessity to close this trial following the pilot study before embarking on the full RCT to compare IVM against the standard antagonist protocol was hugely disappointing. In the initial ethical approval for the trial, if a clinical pregnancy was not confirmed following the treatment of 30 patients, the investigators were obliged to stop the trial. Although only the preliminary group, the team felt it was appropriate to stop the trial following the same principal.

Oocytes from those with PCOS may predispose the IVM treatment cycle to achieve a less successful outcome. Minimal change in clinical IVM treatment despite low CPR/LBR's also suggests that the momentum for this treatment has faltered. Although there is a continued interest in culture conditions, e.g. SPOM (634), it may be that this is confined to the animal industry, for example cattle breeding, where successes seem more abundant. The advances in clinical care for these high risk women, such as the

antagonist cycle, agonist trigger and minimal stimulation IVF, provide a treatment pathway that reduces the risk of OHSS without compromising the pregnancy outcome. Due to this the merits of IVM now appear limited and supports why the treatment is not offered on a large scale worldwide. With respect to the current trial, there are obvious limitations (e.g. minimal baseline data recorded and unknown endometrial thickness) and new technologies that may improve future attempts (e.g. Embryoscope). For now though, I do believe that IVM for those with PCOS may have had its time unless dramatic improvements occur in the IVM process.

Chapter 5
General Discussion And Future Directions

5.1 Summary

Polycystic ovary syndrome remains the most common endocrine disturbance to affect women during their reproductive years. The complex pathophysiological process remains superficially understood. Insulin resistance, hyperinsulinemia and hyperandrogenism are central players in the pathophysiology and reproductive dysfunction. Obesity and metabolic disturbance have significant impact on the reproductive outcome but also the long term health of many women with PCOS. For those with PCOS, although successful fertility may be the patient's goal, these individuals provide a challenge for the clinician. Large cohorts of antral follicles can remain refractory to gonadotrophin stimulation, until a threshold is reached and an exuberant follicular response may then occur in an unpredictable fashion. This response predisposes the individual to both the risk of multiple pregnancy and OHSS, with its attached morbidity and occasional mortality. Whilst the risk of OHSS is relatively low with ovulation induction for anovulatory infertility, it is in the context of superovulation for assisted conception techniques such as IVF or ICSI and related treatments that the risks are much greater. It is reassuring that in the most recent confidential enquiry into maternal deaths, OHSS was not cited as a cause of death. Indeed, only 3.1% of the 321 women who died between 2009-2012 had undergone assisted conception technologies (680). Although encouraging, this fact does not leave room for complacency. The incidence of severe OHSS ranges from 0.5-2.0% and mild-moderate OHSS upwards of 30% (681). In 2013, 46 instances of severe OHSS requiring hospital admission were reported to the Human Fertilisation and Embryology Authority (682).

Based on the impact of gonadotrophin stimulation for those at high risk of over response, the objective of this thesis was to evaluate 3 ways of reducing the risk for women undergoing superovulation. The research looked at women with PCOS and integrated appropriate diagnostic testing with methods to attenuate or eliminate the incidence of OHSS. Three key hypotheses were outlined from the beginning and provided the foundation for the research presented in this thesis.

Aim 1

'AMH has clinical utility as a diagnostic test for PCOS and can be used as a marker of severity of the condition'

The work presented in this thesis has clearly shown that AMH provides a 4th dimension to the diagnostics of PCOS in women. The clear correlation of AMH levels with AFC (138) has been replicated in the present study. In agreement with the work of many (87, 232, 234), the subjective morphological description of the polycystic ovary can be replaced or enhanced by the measurement of AMH. Whether this is appropriate in assisted conception is debatable given the extra anatomical information a high resolution ultrasound can generate, but certainly quantification of the AMH level adds significant weight to the simple AFC description of PCOM and in doing so AMH can distinguish between those women with isolated PCOM and those with the full syndrome of PCOS. This corroborates data reported by Homburg et al (228). With respect to the other factors named in the Rotterdam criteria, the current study supports an independent correlation with both anovulation and hyperandrogenism (83, 217). Within the current study, these links remained irrespective of BMI, which is often reported to have a negative relationship with AMH level (151). Based on the current findings, the suggested cut off values for PCOM (29pmol/L) and oligo-anovulation or hyperandrogenism (45pmol/L) using the AMH generation II assay support other proposed values (224). Using these suggestions on the data from chapter 3, Table 5-1 compares the categorisation of PCOS phenotypes against the cohort of women recruited within the metformin trial. Each substitution led to a statistically significant

(p<0.01) alteration in phenotype categorisation with up to 11% of the cohort being deemed not to have PCOS. Evidently further work is needed to optimise the ideal surrogate values, especially when substituting for OA or HA. However, the substitution for PCOM may reflect the need to increase the follicle number in the PCOM definition, to prevent over diagnosis of PCOS.

Table 5-1 Comparison of phenotype categorisation using suggested AMH levels as surrogate markers for PCOM, OA and HA using data from chapter 3

PCOS Phenotype	Original data (Chapter 3)	Replace PCOM with AMH 29pmol/L	Replace OA with AMH 45pmol/L	Replace HA with AMH 45pmol/L
OA/HA	1	3	1	1
OA/PCOM	61	57	48	22
HA/PCOM	34	30	24	21
OA/HA/PCOM	46	41	56	83
Not PCOS	0	10	13	15
Comparison against original		p = 0.01*	p < 0.001*	P < 0.001*

* Fisher's exact test.

Phenotypic presentation of PCOS is perhaps a little more difficult to discern from a single AMH value. From the current study, the phenotype that could be classed as the most 'severe', which possesses all 3 of the Rotterdam criteria, had a significantly elevated AMH value compared with the others. This reflected a heavier and more androgenic subset of subjects. Importantly, the elevated AMH value (72.7pmol/L (61.0-86.7 95%CI)) may identify a subset of women who may be the hardest to treat during ART and who present the greatest risk of OHSS following ovarian stimulation. It would be interesting to continue this research to ascertain if this is indeed true when women with this PCOS phenotype undergo an assisted conception IVF cycle. Finally, with

respect to age, although AMH is recognised as having an inverse relationship with increasing age, for those women with PCOS the AMH concentration followed a slower decline. This may in part be due to the trend of women to present to an infertility clinic at a later age rather than a true reflection on the age related decline of AMH in women with PCOS. None of these associations are surprising given the integral role AMH plays both in initial follicle recruitment and selection of the dominant follicle (113). The current study has therefore provided additional data on the biological importance of AMH to PCOS using the current generation II AMH assay and the data suggests a diagnostic benefit of AMH assays in the diagnosis of PCOS.

Aim 2.

'Metformin treatment can significantly reduce the risk of OHSS in women with PCOS undergoing a GnRH antagonist treatment cycle'.

Individually, metformin treatment and the GnRH antagonist cycle are known to reduce the incidence of OHSS in women with PCOM/PCOS who are undergoing superovulation for assisted conception. Evidence supports the use of metformin as an adjunct in the GnRH agonist cycle to decrease the risk of OHSS (260, 501). The antagonist cycle is the recommended approach for those with PCOS due to the reduction in OHSS risk (181). Metformin is believed to act directly on the ovary and indirectly via attenuation of insulin resistance and hyperinsulinemia (322). In stark contrast to the pilot study by Doldi et al (351), the current RCT has demonstrated no difference in OHSS risk with metformin use (placebo 12.2% vs. metformin 16%, p=0.66). Similar to our team's earlier work with metformin in the agonist cycle (260), there was no change in total gonadotrophin dose, duration of stimulation or number of oocytes retrieved after drug exposure. There was minimal increase in systemic testosterone level from baseline to oocyte retrieval in the metformin group, unlike the placebo group. Furthermore, there was a trend for a greater increase in oestradiol in the placebo group by the time of oocyte retrieval. These changes reflect the rapid effect metformin may have on biochemical parameters and suggests that with a more sustained approach, metformin

may have had greater effect. This finding highlights the greatest limitation of the current study design, which was the short use of metformin prior to oocyte retrieval. An intriguing outcome of the current study is the trend for reduced clinical pregnancy and live birth rate with metformin use. This may be due to factors such as altered progesterone production suggested in earlier work by our group (Maruthini et al (548)). A reduction in progesterone production may alter and impede endometrial receptivity, thereby reducing the implantation and ongoing pregnancy rate. A clear disparity in clinical outcome between the South Asian and White Caucasian population, unrelated to metformin consumption, has once again been found (559, 564). Despite obvious markers of potential poor outcome (raised BMI, longer duration of subfertility and worse phenotypic presentation) it remains disappointing that such an ethnic gap persists. Further directed research into narrowing this discrepancy is needed, with the possibility of ethnic specific protocols for assisted conception.

Aim 3.

'IVM is an effective alternative to a standard IVF assisted conception cycle for those with PCOS who wish to avoid OHSS'.

The ability to collect, mature and fertilise oocytes in an *in vitro* environment is not questioned. The current study has shown that this is possible and that a high maturation and fertilisation rate can be attained, in agreement with the work of other groups (358, 359, 572). The poor embryo quality and lack of clinical pregnancy rate in the pilot study does however raise the question as to whether IVM is an effective alternative to standard IVF cycles. The variable CPRs in the literature (see Table 4-2, chapter 4) despite decades of research support the concept that IVM is a more difficult procedure in a clinical ART setting and fully optimised clinical and laboratory protocols have yet to be established . With advances in other risk reduction strategies such as the adaptation of the antagonist cycle for the treatment of PCOS with vastly reduced OHSS rates, it does seem that IVM has perhaps had its time as a viable option for treatment of these women. Indeed women with PCOS may not be the best candidates

for IVM, despite its obvious advantage in reducing the risk of OHSS. Reduced oocyte quality, an altered hormonal microenvironment and impaired implantation are a small selection of the suggested reasons why oocytes from women with PCOS are not ideal candidates for IVM (329, 584). Conversely, this patient group have the most to gain from a cycle of IVM. Elimination of the risk of OHSS may offset reductions in clinical outcome per cycle started. Financially, it has been reported that IVM provides a low cost alternative to a long treatment protocol in assisted conception (571). Our study has challenged that concept and shown that the cost of IVM is approximately similar to a short antagonist cycle of treatment. Even using an optimised cost plan, whereby oocytes are only injected at one time point (due to limited benefit of later maturing oocytes contributing to the best embryos for transfer) only £200 separates the cost of an antagonist treatment cycle from IVM, in favour of the later. More expensive culture media with limited shelf life and extra clinician and embryologist time particularly around oocyte retrieval, limit IVM's clear advantage of reduced gonadotrophin costs. Although the costing hasn't taken into account costs of hospital admission for OHSS, this is immaterial when no pregnancy has been achieved. If the pilot study had been continued, it would seem appropriate to discontinue hCG priming. The addition of this trigger *in vivo* appears to have been detrimental to oocyte developmental competence in the current study and as such supports the findings of other groups (359, 572). The use of the Embryoscope would have been interesting as a vehicle to monitor fertilisation and early preimplantation development of IVM derived embryos but is unlikely to have altered the outcome. To conclude, IVM does not appear to provide either a clinically efficient or a cost effective alternative to the antagonist IVF protocols despite eliminating OHSS.

5.2 Future Research

Optimisation of the current successful strategies to reduce OHSS should form the basis of future research. Lowest effective gonadotrophin doses based on validated

investigations such as AMH and individualised treatment plans will target those most at risk of OHSS. Perhaps it is time to revisit the type of gonadotrophin used within superovulation. It is well recognised that FSH exists in a number of different isoforms dependant on the number of branching carbohydrate moieties in particular sialic acid residues found on the molecule (683). Within the menstrual cycle, the more acidic isoform predominates in the early follicular cycle with a switch to the less-acidic form around ovulation (684). The follicle controls this switch via oestradiol. A rising oestradiol level downregulates glycosyltransferase, the enzyme responsible for incorporating sialic acid residues into FSH within the pituitary (685). *In vivo* acidic isoforms are shown to have a longer half-life and increased biological activity. Furthermore, the acidic type provokes a less pronounced steroidogenic response with a gradual rise in oestradiol and selective follicle growth (686).This contrasts with the less-acidic isoform which induces exponential growth of the follicles (mainly related to granulosa proliferation), in a less selective manner (687). There is a difference in the ratio of acidic to less acidic isoforms of FSH within the various urinary and recombinant FSH preparations used in ART today; with the purified human preparation exhibiting a more acidic profile (683, 688). It has been suggested that use of acidic isoforms for controlled ovarian stimulation may therefore reduce peak oestradiol levels and reduce the risk of OHSS. In practice, no difference in oestradiol level has been demonstrated. Although Abate et al (689) found a reduction in total gonadotrophin dose and stimulation length, this was not replicated by others (690, 691). A recent Cochrane review concludes that there is no difference in OHSS rate between human or recombinant FSH in controlled ovarian stimulation (692). The lack of difference in clinical trials to date may in part be related to the type of GnRH analogue used (all studies use the agonist). In the classic GnRH agonist cycle, the pituitary is down regulated with suppression of FSH and LH secretion. Theoretically, residual FSH secretion may tend towards the acidic isoform due to a low oestrogen environment. In the antagonist cycle, no such pituitary suppression occurs. Adding a less acidic FSH stimulation to the normal pituitary FSH may therefore alter the natural dynamics of menstrual cycle FSH isoforms. This alteration may provoke a higher oestradiol

environment and predispose to OHSS. No studies as yet address this question in the antagonist cycle.

Intuitively, the next step is to consider how best to trigger oocyte maturation and ovulation. The traditionally employed hCG marks the end of controlled ovarian stimulation, yet can create a cascade of events that culminate in the sequelae of OHSS. Kisspeptins and the connected neuronal network of Kisspeptin-neurokinin-B-dynorphin (KNDy) have provided insight into how upstream modulation of the GnRH signal can be harnessed to improve reproductive outcome (693). Encoded by the *KiSS1* gene, Kisspeptin is a 54 amino acid peptide that can undergo further cleavage; and are collectively known as the kisspeptins (694). The function of these peptides has been identified in regulation of puberty and sex-hormone mediated secretion of gonadotrophins (695). Direct signalling to the GnRH neurone via the kisspeptin receptor, allows pulsatile release of GnRH into the portal circulation. In turn this stimulates pituitary gonadotrophin release, with a preferential secretion of LH and to a lesser extent FSH (696). Variation between the male and female is recognised; in the former the hypothalamic clock can be effectively reset by exogenous use of kisspeptin, unlike the latter in which the menstrual cycle exerts an effect (697). Kisspeptin is under both positive and negative feedback control from progesterone and oestradiol (693). Due to co-localisation of neurokinin-B and kappa opioid peptide receptor within the hypothalamus, auto-synaptic control of kisspeptin secretion occurs (698). Neurokinin-B is stimulatory and dynorphin inhibitory. The ability to induce an LH surge capable of triggering ovulation provides an interesting alternative to the classic hCG. Jayasena and colleagues (699), provide results from an eloquent proof of concept study using kisspeptin as the ovulation trigger in an antagonist IVF cycle. Fifty-three patients underwent a standard antagonist cycle with a single subcutaneous dose of kisspeptin-54, 36 hours before oocyte retrieval. Maturation, fertilisation and embryo formation were all achieved. The clinical pregnancy rate was 23%. Maximum peak oestradiol levels were seen 12 hours following injection reaching 16000pmol/L ± 2000 but fell rapidly by 36 hours. Previously, either direct stimulation of LH receptors on the ovary

or GnRH receptors on the pituitary has generated ovulation. Kisspeptin adopts a physiological approach using the hypothalamic endogenous GnRH reserve, which was reflected in the lower peak levels of LH when compared with a GnRH agonist trigger (700). Furthermore, it is recognised that hCG has a long half-life and sustained effect on ovarian stimulation (701). In contrast, kisspeptin exposure is short lived with a half-life of only 28 minutes (702). Within this study, women with PCOS were excluded (although the median AFC was still 20-30). It would be interesting to see within larger scale studies whether kisspeptin is useful for women with PCOS; in particular if it is capable of inducing ovulation in a greater cohort of oocytes without the effects of OHSS. In addition, the dose that kisspeptin should be used, given the increased pulse frequency of LH and basal LH levels (703) plus the finding of an elevated kisspeptin level in those with PCOS (704).

Whilst kisspeptin may be in the fledgling stages of clinical use, the agonist trigger should now be considered a viable, if not mainstream, option for high responder individuals such as those women with PCOS. Although OHSS is not eliminated using the agonist trigger, the rate is far below that seen using hCG as the trigger (705, 706). Unlike hCG which maintains the corpus luteum and progesterone production, optimised luteal phase support is necessary. Following the LH surge and oocyte maturation, direct pituitary down regulation provides insufficient LH to maintain the corpus luteum, leading to a potential luteal phase insufficiency and reduced clinical pregnancy outcome (707). With optimised luteal phase protocols including combinations of exogenous hCG, oestradiol and progesterone, the success rates should silence the sceptics of the agonist trigger (708). Two recent retrospective reviews (681, 709) reveal clinical pregnancy rates (40-41.8%) that equal if not exceed those seen with the conventional approach. It is encouraging that an ongoing survey suggests that clinics are tending towards the antagonist cycle and case based selection for the GnRH agonist trigger approach (710). This is a marked improvement on a previous survey (534) and perhaps reflects a paradigm shift in the clinician's support of such treatment strategies.

5.3 Overall Conclusion

Irrespective of risk reduction strategy employed for those with PCOS, the underlying focus should be to use superovulation strategies only when necessary. Rapid resort to IVF with only limited duration of trying to conceive exposes patients to both known (i.e. OHSS risk) and unknown risks (e.g. epigenetic changes) that are potentially unnecessary and unjustifiable (555). For those with PCOS, emphasis should continue to be placed on lifestyle modification (339), weight reduction and ovulation induction strategies. When assisted conception is necessary optimum diagnostics before proceeding to treatment can help to tailor safe treatment. Anti-Müllerian Hormone has an undisputable literature base correlating the systemic circulating level firstly with AFC but foremost with elements of PCOS. Work in this thesis has strengthened the knowledge surrounding AMH as a diagnostic tool for PCOS using the AMH Gen II assay, suggesting reference levels that can be adopted by primary and tertiary care providers. The significance of elevated AMH and the impact of PCOS on the outcome of assisted conception technologies should focus the clinician's choice of a safe treatment strategy. Attempts to further attenuate risk, with the addition of metformin has proven unsuccessful. No benefit was seen in the antagonist cycle in stimulation, embryological or clinical outcome parameters. Empirical metformin use for those with PCOS should therefore not be advocated, although metformin may have a role for those with impaired glucose tolerance and type 2 diabetes mellitus. Finally, it would seem that the theoretical advantage of IVM has been superseded by advances in OHSS reduction approaches seen with the antagonist cycle, agonist trigger and optimised luteal phase support. The latter strategy has been replicated by many with good pregnancy outcomes together with limited risk for the individual, unlike *in vitro* maturation as a vehicle to treat PCOS. Perhaps IVM should remain confined to the successful commercial ART sheep and cattle market and no longer in the treatment of women with PCOS.

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Appendix 1

The Leeds Centre for Reproductive Medicine

The Leeds Teaching Hospitals NHS
NHS Trust

IVM CUMULUS OOCYTE COMPLEX SCORING AND DATA RECORD SHEET	Emb091

Patient Id No_____ Date of Egg Recovery_____ Page ___/ ___

Follicle No & diameter at hCG_____

Grading Scores for cumulus expansion at the start and end of culture (see Wynn et al 1998 for images)

	Cumulus mass (Mass)		Cumulus expansion (Exp)	
Grade:	C0:	≤3 layers of cumulus cells	E0:	tight, dense cells
	C1:	>3<10 layers of cells	E1:	moderate expansion of cells
	C2:	≥10 layers of cells	E2:	fully expanded cells

Day 0 Cumulus score after OR		**Day 0** Cumulus score 16.30		**Day 0** Stripping 16.30		**Day 0** ICSI		**Day 1** Cumulus score only 09.00		**Day 1** Cumulus score 12-12.30. Strip all remaining		**Day 1** Maturity after stripping	**Day 1** ICSI		**Day 1** Recheck of MI's -16.30		**Day 2** Recheck-0.900		Transfer (Y/N). Cell stage and number. Comments
By- Date-																			
Mass	Exp	Mass	Exp	Strip Y/N	Stage	Y/N	Fert from day 0	Mass	Exp	Mass	Exp		Y/N	Fert from day 1	Stage/ICSI Y/N	Fert from day 1	Stage/ ICSI Y/N	Fert from day 2	

Appendix 2

Number	Age	BMI	PCOS Phenotype	Cycles	FSH:LH	Day OR	HCG duration	ET (plan)	Lead foll	Oocyte (predict)	Oocyte (collect)	Mature D1	Fertilised (D1)	Mature D2	Fertilised D2	Outcome	ET (OR)	ET (plan)	ET (Transfer)	Transferred	Good D3 embryos	Total gradable D3 embryos	Blast
1	32	22	PCOM/OA/HA	6mo	4.4:4.9	9	24	8.26	8	58	13	3	1	0	0	Bio22.3		8.26		4.3.3 (d1)	1	1	no blast only single embryo
2	35	22	PCOM/OA	32-3mo	5.1:6.7	9	24	5.97	8	31	6	1	1	3	3	Negative		5.97		5.3.3 (d1)	2	4	no blasts
3	33	23	PCOM/OA	28-35	6.8:6.9	9	24			19	8	5	3	0	0	Bio 3.1		5.55		8.4.4/7.3.4	2	3	no blasts
4	29	28	PCOM/OA	6mo	3.9:2.5	9	24	4.59	7	27	12	7	7	1	1	Negative		4.5		10.3.4 x2	3	7	2 blasts d6 4bc/5Bb
5	34	25	PCOM/HA	32	5.7:7.9	9	24	5.8	8.5	39	15	8	2	3	0	bio10.8		5.8		3.3.4/4.3.4	2	2	no other embryos
6	32	26	PCOM/OA	amen	05:10.3	9	24			41	33	9	6	8	3	Ectopic (2976)		4.2		8.4.4/8.3.3	3	9	no blasts
7	32	27	PCOM/OA	30-37	2.6:1.7	9	24			23	14	7	2	6	4	Negative		2.68		4.3.4 x2	3	5	no blasts
8	31	23	PCOM/OA	8wks	7.6:11.8	9	24			45	13	5	4	2	0	Bio9.5		6.1		8.4.4/7.3.3	3	4	no blasts
9	36	23	PCOM/OA	3mo	5.6:4.2	9	24	2.21	7	38	24	24	14	0	0	Negative		7		8.3.4/8.4.3	10	14	3 blasts 2dc-4cb
10	36	25	PCOM/OA	2mo	08:09.5	10	24			31	15	9	6	1	1	Negative		3.45	4.1	6/3/4:6/3/3	2	7	d6 4bc/3cc
11	31	24	PCOM/OA/HA	amen	1.8:<.3	10	24			30	22	15	14	0	0	bio 34		3.37	6	8/4/4:8/4/3	8	13	3 blasts 4bb
12	38	25	PCOM/OA	34+	6.8:7.2	10	24			23	8	6	5	0	0	Negative		5.67	7	6.3.3/6.3.2	1	5	2 blasts 3dc
13	38	28	PCOM/OA/HA	30-37	7.4:4.6	10	24	4.33	8.5	28	8	3	2	1	1	negative		4.3	7.2	4/2/2:6/4/4	1	3	no blasts
14	29	22	PCOM/OA	26-36	4.9:3.6	10	24	2.39	9	27	21	12	11	8	4	Negative		2.39	7	8.3.4/9.3.4	6	15	4dc
15	23	29	PCOM/OA	28-42	3.8:5.8	10	24			26	18	9	6	3	2	bio 11.2		3.6	10	8/4/4:7/3/4	2	5	no blasts
16	28	28	PCOM/OA	90	5.8:6	10	24	5.02	8	36	18	6	3	9	7	negative (fet)		7.3		10.2.3/8.3.3	1	8	no blasts
17	33	30	PCOM/OA	3mo	6.9:11.8	10	24			28	19	13	10	2	2	negative (fet)		9.8		9.3.3/8.3.3	3	10	no blasts
18	24	30	PCOM/OA	amen	4.6:5	10	24	5.6	7	50	23	9	5	4	2	bio3.9(fet)		9.19		8/4/4 7/3/3	2	6	no blasts
19	26	21	PCOM/OA/HA	28-29 (upto 7 wks)	08:06.4	10	24			29	2	1	0	1	1	no transfer		nd		failed cleavage	0	0	
20	26	27	PCOM/OA/HA	3mo	6.9:6.4	10	24	nd	nd	22	32	11	7	11	5	Negative		7.67		6.3.3/7.3.4	2	9	2 poor blasts

																(fet)							(2cc/3cc)
21	30	20	PCOM/OA	28-49	7.9:4.5	13	36		10	27	18	6	5	4	0	Negative	6.1	5.64		8.4.4/8.3.4	3	5	no blasts
22*	26	27	PCOM/OA/HA	3mo	6.9:6.4	15	36		9.5	28	24	10	7	7	5	Bio 8	5.4	4.5		8.4.4/7.3.4	5	9	2 poor 1cc/5dc
23	32	28	PCOM/HA	28-31	7.1:4.0	12	36		13	22	10	7	4	2	0	Negative	6.8	4		8.4.4/7.4.4	4	4	no blasts
24	25	31	PCOM/OA	amen	5.4:5.8	15	36		7	40	29	10	3	1	0	Negative	5.9	5.24		8.4.4/7.4.4	3	3	1 4cc
25	30	22	PCOM/OA/HA	28-35+	1.8:3.9	15	36		9	33	24	13	8	4	1	Negative	6.1	4.2		9.4.4/9.3.4	3	9	no blasts
26*	29	28	PCOM/OA	90	5.8:6	18	36		7.5	27	22	5	3	11	6	Negative	7.7	5.51		8.3.4/9.3.4	5	9	1 5aa
27	27	25	PCOM/OA	30-3mo	2.8:3	15	36		10	30	9	6	3	2	0	Negative	8.5	4.42		6.2.3/10.2.3	0	3	no blasts
28*	33	23	PCOM/OA/HA	6mo	4.4:4.9	16	36		7	45	16	10	6	2	1	negative	7.8	7.05		7.3.3/7.3.3	2	7	no blasts
29	34	21	PCOM/OA	4mo	06:04.6	12	36		10.5	20	6	2	0	2	2	no transfer	4.3	3.75		failed cleavage	0	0	na
30	24	23	PCOM/OA/HA	28-42	51:11.4	14	36		9	38	21	13	8	4	0	negative	nd	4.75	nd	5.2.3/5.3.3	0	7	no blasts

PCOM: Polycystic ovary morphology, OA: Oligo-anovulation, HA: Hyperandrogenism, ET: endometrial thickness, OR: oocyte retrieval, *2nd cycle

Group 1
Group 2
Group 3
Group 4
Group 5

Appendix 3

Alere Limited
Admin Office Bio-Stat House,
Stockport,
Cheshire
SK7 5BW

Actavis UK Ltd,
Barnstaple,
Devon,
UK

Bayer Healthcare,
Sellerstraße 31,
13353 Berlin,
Germany

Beckman Coulter (UK) Ltd
Oakley Court
Kingsmead Business Park
London Road
High Wycombe
HP11 1JU

Bilcare Global Clinical supplies,
Waller House,
Crickhowell,
UK

Bio-Rad Laboratories Ltd.
Bio-Rad House
Maxted Road
Hemel Hempstead
Hertfordshire HP2 7DX

Casmed International Ltd,
Epsom,
Surrey,
UK

Cook Medical Headquarters,
P.O. Box 489,
750 Daniels Way
Bloomington, IN 47402-0489, USA

Eppendorf UK Limited
Eppendorf House
Arlington Business Park
Stevenage
SG1 2FP

GlaxoSmithKline,
Middlesex,
UK

Heidolph UK - Radleys
Shire Hill
Saffron Walden
Essex, CB11 3AZ

IBM Corporation,
New York,
USA

IBSA International,
Via del Piano 29,
6915, Pambio-Noranco,
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Pharmacia Ltd,
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Pharmasure,
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Planer PLC,
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Sage, Origio,
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