UiT The Arctic University of Norway

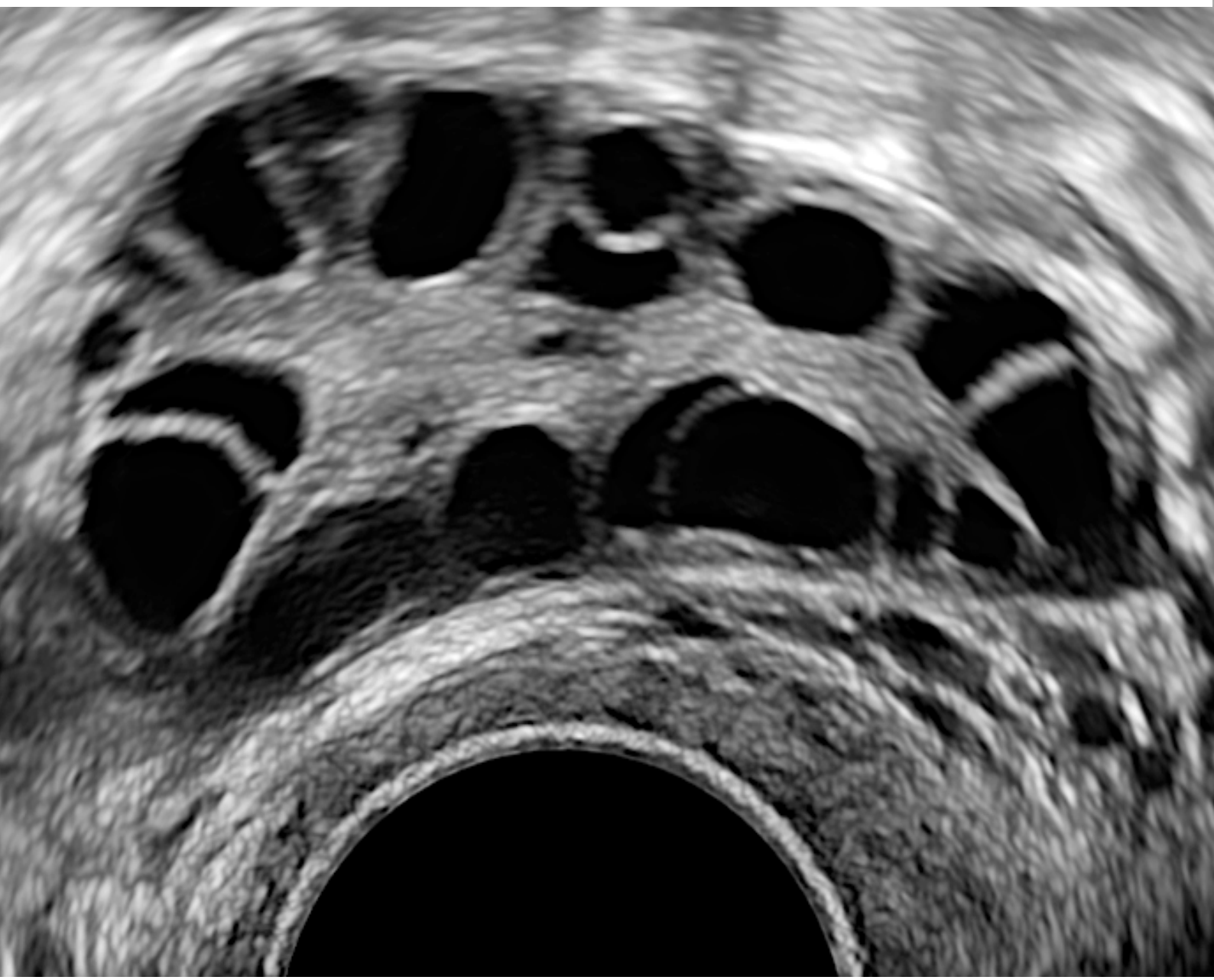
Faculty of Health Sciences

Women's Health and Perinatology Research Group

The role of ovarian reserve markers in fertility and fertility treatment

Priya Bhide

A dissertation for the degree of Philosophiae Doctor – January 2021




UNIVERSITY OF TROMSØ

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Acknowledgements

'To study the phenomenon of disease without books is to sail an uncharted sea, while to study books without patients is not to go to sea at all.'

This quote by Sir William Osler, from the Boston Medical Journal, 1901 was inscribed outside the library of my medical school. It has left a lasting impression on my mind and to date forms the cornerstone of clinical practice and clinical research.

The early years of my medical training in India, in obstetrics and gynaecology, a very hands-on speciality, was largely devoted to mastering technical and surgical skills and proficiency in these gave a profound sense of achievement. With passing time there came an awareness of the greater importance of a broader perspective to patient care, and eventually to be able to critically analyse evidence and base treatment decisions on current best evidence. Today, evidence-based medicine is the foundation on which all medical practice in the UK is based. In this continuing career journey, I then aspired to be one of those doctors who not only practices evidence based medicine but also contributes to generate the evidence for it! This was the start of my PhD journey.

I would like to thank my principal supervisor Professor Ganesh Acharya for being instrumental in my decision to register for a PhD and also for supporting me to complete it. I was very sceptical about completing this project especially as I was based in the UK, but Ganesh convinced me that it would not only be possible and worthwhile but also something I would never regret. Retrospectively, I have to completely agree with this. I have valued his every advice on my research projects and this will last beyond the doctorate for a lifetime. His critical comments and constructive suggestions have significantly improved all the manuscripts that I have submitted. His speed of correcting and returning documents has been phenomenal and unmatched and my co-PhD students have envied me and wished for a similar supervisor! I have enjoyed the academic discussions we have had on the projects for the thesis and on the broader topics of research and academic careers. In these years I have made several trips to Tromsø and I am grateful to Ganesh and Larissa, who are now like family to me, for their warm hospitality. I have enjoyed the social evenings and stunning views from the living room, not so much the deep snow and harsh winters!

I would like to thank my co-supervisor Professor Roy Homburg for introducing me to research when he took up the position of Head of Research at the Homerton Fertility Centre in 2010. His knowledge, research ideas and experience, especially in reproductive endocrinology and PCOS, were inspirational and taught me to develop my own research ideas and projects. I would also like to thank him for letting me pursue research independently whilst being supportive, thus allowing me to progress to being an independent researcher. He was instrumental in acquiring and setting up the AMH assay at the Homerton Hospital which was key to all the primary research done for this thesis. I am fortunate to inherit one of his great interests from him and carry this forward; PCOS and PCOS research. Unfortunately, not his other love; first class cricket and the Ashes!

I would like to thank my colleagues at the Homerton Fertility Centre. I would like to thank my colleagues Anil and Amit for their support to my degree at the management level. I would like to thank members of the research team Jyotsna, Abhijit, Sri, Merve and Liz, to name a few, for their help with all the research projects conducted.

In my time at Tromso, I have made friends, Purusotam, Martha and Åse who have been very warm and welcoming and I hope to continue these friendships.

My family have been my unshakable support system and this doctorate and thesis would not have been possible without their support. I have been very fortunate to have Amar as my in-house professional mentor for queries too stupid to ask my supervisors, help at odd hours and pre-submission in-house manuscript reviews. My grown-up daughters Malavika and Anuradha have been great, and supported this endeavour in many ways; from help with thesis formatting and technology issues to keeping up my spirits with the latest fashion and shopping advice!

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List of abbreviations

3D	3-dimensional
AFC	antral follicle count
AMH	anti Mullerian hormone
ANOVA	analysis of variance
ANCOVA	analysis of covariance
BMI	body mass index
CO	carbon monoxide
DCI	di-chiro inositol
DSL	Diagnostic Systems Laboratory
ELISA	enzyme linked immunosorbent assay
FAI	free androgen index
FSH	follicle stimulating hormone
Gen II assay	AMH Generation II Beckman Coulter assay
GnRH	gonadotropin releasing hormone
GRADE	Grading of Recommendations Assessment, Development and Evaluation
HA	hyperandrogenism
ICSI	intra cytoplasmic sperm injection
IOT	Immunotech Beckman Coulter
IR	insulin resistance
IVF	in-vitro fertilisation
LCMS	liquid chromatography–mass spectrometry

LH	luteinising hormone
MD	mean difference
mFG score	modified Ferriman Gallwey score
MI	myo-inositol
NHS	National Health Service
NOS	Newcastle Ottawa Scale
OA	oligo-anovulation
OCP	oral contraceptive pills
PCOS	polycystic ovary syndrome
PCOM	polycystic ovarian morphology
ROBINS-I	Risk Of Bias In Non-randomized Studies – of Interventions
RR	risk ratio
SHBG	sex hormone binding globulin
SMD	standardised mean difference
SPSS	Statistical Package for Social Sciences
T	total testosterone
WMD	weighted mean difference

1 Abstract

1.1 Background

Ovarian reserve is a key component influencing reproductive function and fertility. Serum anti-Mullerian hormone (AMH) and the antral follicle count (AFC) are established markers of ovarian reserve relevant for routine clinical practice. Additionally, AMH may also have a physiological inhibitory role in follicular recruitment and cyclical follicular growth and maturation. This may be heightened in polycystic ovary syndrome (PCOS) resulting in ovulatory dysfunction. The ovarian reserve is variable and may be affected by genetic, lifestyle and environmental factors, in addition to age. However, literature reports contradictory results for the effect of cigarette smoking on ovarian reserve parameters. As the antral follicle pool is a dynamic cohort of growing follicles it is responsive to the action of gonadotropins, ovarian steroids and other endocrine and paracrine influences. Hence there is biological plausibility to alter folliculogenesis with pharmacological agents acting through one of these pathways. In the context of in-vitro fertilisation (IVF), serum AMH and AFC are commonly used to estimate the quantitative response to controlled ovarian hyperstimulation. Their association with embryo quality is less well established and inconclusive.

1.2 Objectives

The overall aim of this thesis was to study the role of AMH and AFC on different aspects of fertility and fertility treatment. The thesis comprised of four work packages. Their specific objectives were:

1) To compare the per follicle AMH production (expressed as a ratio of serum AMH to the total AFC) in the various phenotypes of women with PCOS and with isolated polycystic ovarian morphology (PCOM). We also aimed to evaluate the factors which may be associated with variation in AMH production among these different clinical phenotypes.
2) To assess the effect of cigarette smoking on the quantitative ovarian reserve parameters in sub-fertile women, validating self-reported smoking behavior using biomarkers (breath carbon monoxide levels and urine cotinine levels).

3) To assess the effect of treatment with myo-inositol (MI)/di-chiro inositol (DCI) compared to no treatment, placebo or other treatment on markers of ovarian reserve in women with PCOS combining data from published literature. Our secondary aim was to assess the effect of these treatments on reproductive outcomes in women with PCOS undergoing IVF/ intracytoplasmic sperm injection (ICSI) treatment.
4) To assess the association between ovarian reserve markers and embryo quality in women undergoing IVF/ICSI treatment using time lapse imaging technology.

1.3 Methods

The work for the thesis included primary research and evidence synthesis. Our primary research comprised of three single-centre observational cross-sectional studies using a population of subfertile women seeking fertility treatment. For Paper I we measured serum AMH and total AFC in women with PCOS and PCOM. We calculated a ratio of serum AMH to the total AFC as a marker of AMH production per follicle and compared this in the different phenotypes of PCOS and PCOM. We also compared the secondary outcome measures, i.e. free androgen index (FAI), homeostatic model assessment (HOMA) and serum luteinising hormone (LH), in the comparison groups. For Paper II, we compared serum AMH and AFC in current smokers, ex-smokers and never smokers. We assessed smoking exposure using a self-reported questionnaire. We also measured biomarkers of smoking using a carbon monoxide (CO) breath test and a urine cotinine test. We compared these biomarkers between the groups to validate the results for the primary outcome variables and also evaluated the association between biomarkers of smoking and biomarkers of ovarian reserve. The pack years of smoking were compared between current smokers and ex-smokers and their correlation to serum AMH and total AFC was assessed. For Paper III, we conducted a systematic review and meta-analysis using standard methodology recommended by Cochrane to study the effect of the insulin sensitiser MI/DCI on serum AMH and AFC in women with PCOS undergoing IVF/ICSI. For Paper IV, we measured baseline serum AMH and assessed embryo quality using the time lapse incubators and a computerised known implantation data score (KID score) in women undergoing IVF/ICSI treatment. We compared serum AMH in the five KID score categories. We also compared the clinical pregnancy rate in the five KID score categories and explored the relative impact of serum AMH and the number of retrieved oocytes on the pregnancy rate.

1.4 Results

Per follicle AMH production was significantly higher in the anovulatory phenotypes of PCOS as compared to the ovulatory phenotypes and isolated PCOM. There was no substantial association between the per follicle AMH production and androgens, LH or insulin. No significant differences in serum AMH or AFC were found between current smokers, ex-smokers and never smokers. There was a good correlation between self-reported smoking history and biomarkers of smoking. No significant correlation between biomarkers of smoking or lifetime exposure to smoking and ovarian reserve parameters was found. There was no consistent direction or size of effect for a change in serum AMH or AFC after treatment with inositols. No significant differences between the inositol and control groups were seen for the number of retrieved oocytes, number of metaphase II oocytes, number of top-grade embryos, pregnancy rates and the rates of cycle cancellation due to the risk of OHSS in women undergoing IVF/ICSI treatment. The serum AMH levels were similar in women with different categories of embryo quality, although there was a significant difference in pregnancy rate between the KID score categories. Serum AMH however did not have a significant impact on pregnancy rates.

1.5 Conclusions

A greater per follicle AMH production in anovulatory phenotypes of PCOS may represent a heightened physiological role of AMH leading to ovulatory dysfunction. Exposure to cigarette smoking in women ≤ 35 years seeking fertility treatment did not significantly change the antral follicle pool and the biomarkers of ovarian reserve were not significantly associated with the biomarkers of smoking or lifetime smoking exposure.

Based on currently available data, evidence is lacking for an effect of inositols on altering ovarian reserve markers or subsequent outcomes following IVF/ICSI treatment. We found no significant association between ovarian reserve markers and embryo quality in women undergoing IVF/ICSI treatment. Their positive association with improved pregnancy outcomes following IVF/ICSI is most likely indirectly through the increased numbers of oocytes retrieved.

2 List of original papers

Paper I: Bhide P, Kulkarni A, Dilgil M, Dhir P, Shah A, Gudi A, Homburg R. Phenotypic variation in anti-Mullerian hormone (AMH) production per follicle in women with polycystic ovary syndrome (PCOS) and isolated polycystic ovarian morphology (PCOM): an observational cross-sectional study. Gynecol Endocrinol. 2017 Oct;33(10):801-806. doi: 10.1080/09513590.2017.1320377. Epub 2017 Apr 28. PMID: 28454499.

Paper II: Bhide P, Escriba M, Srikantharajah A, Joshi H, Gudi A, Shah A, Acharya G, Homburg R. Anti-Mullerian hormone (AMH) and embryo quality assessed by time-lapse imaging (TLI): a cross-sectional observational study. Arch Gynecol Obstet. 2017 Sep;296(3):583-587. doi: 10.1007/s00404-017-4453-2. Epub 2017 Jul 1. PMID: 28669060.

Paper III: Bhide P, Pundir J, Gudi A, Shah A, Homburg R, Acharya G. The effect of myo-inositol/di-chiro-inositol on markers of ovarian reserve in women with PCOS undergoing IVF/ICSI: A systematic review and meta-analysis. Acta Obstet Gynecol Scand. 2019 Oct;98(10):1235-1244. doi: 10.1111/aogs.13625. Epub 2019 May 20. PMID: 30993683.

Paper IV: Bhide, P., Timlick, E., Kulkarni, A., Gudi, A., Shah, A., Homburg, R., Acharya, G. (2020) Effect of cigarette smoking on serum anti-Mullerian hormone and antral follicle count in women seeking fertility treatment. *Submitted for publication.*

3 Introduction

3.1 Ovarian development

The first anatomical description of the ovary can be credited to the Greek physician Soranus (98-138 AD). However, it was several centuries later that Fabricius (1533-1619), the chair of anatomy at Padua University, named the structure that contained eggs as the ovary. The origin of the word ovary is from the Greek word 'ovum' meaning egg. In 1672 the Dutch physician, Reinier de Graaf established the ovary as the source of the ovum in his work "A new treatise on the Female Reproductive Organs". His work earned him great recognition and the ovarian follicle is named after him as the Graafian follicle.

The female germ cells are ectodermal in origin and are present at the base of the yolk sac following fertilization (1). These germ cells migrate towards the genital ridges between 4-6 weeks of gestation to form the gonads (2). Besides the germ cells the gonad also contains somatic cells. The germ cells proliferate during migration and in the female fetus differentiate into oogonia directed by the sex chromosomes. There is a phase of rapid mitotic division of the germ cells between 6-8 weeks of gestation taking the final number of oogonia to 6-7 million by 16-20 weeks of gestation. From about 11-12 weeks of gestation, oogonia containing 46 chromosomes, are transformed to oocytes with 23 chromosomes, by the first meiotic division and arrest in prophase (3). At approximately 18-20 weeks when the ovarian cortex is vascularised, the process of follicle formation begins. Cells from the coelomic epithelium surround the oocyte to form the primordial follicle. The primordial follicles can then mature through various phases to develop in to primary, pre-antral and antral follicles. Germ cell loss takes place throughout this process of mitosis and meiosis resulting in a final number of about 500,000- 2 million germ cells/primordial follicles at birth (2, 4). Ovarian structure may be broadly divided in to the cortex and medulla with the cortex containing the primordial follicles. During childhood, despite low levels of gonadotropins, the ovary is not quiescent and follicular recruitment and growth from the primordial to the antral follicular stage continues. In adult life following puberty, under the influence of gonadotropins, antral follicles continue to follow a cycle of follicular maturation, selection for dominance and ovulation. An ongoing process of follicular atresia continues alongside these processes and contributes to the steady decline in oocyte numbers until the age of menopause.

3.2 Ovarian reserve markers

As detailed above, the human ovary establishes its full complement of primordial follicles in fetal life (2) which decreases over the reproductive lifespan up to menopause, at the average age of 51 years (5). This complete and non-renewable complement of primordial follicles comprises the true ovarian reserve and is the key determinant of the function and lifespan of the ovary. There is no currently known biochemical marker for estimation of the number of primordial follicles and their small size makes resolution for in-vivo imaging impossible. Hence, estimation of the size of the primordial follicle pool is difficult and impractical for routine clinical application. Primordial follicles are continuously recruited into a smaller cohort of growing follicles called the antral follicle pool (6). Antral follicles are sensitive to the actions of gonadotropins (7) and during the adult reproductive lifespan, are involved in cyclical follicular recruitment, selection of the dominant follicle and ovulation. Hence it is relevant to estimate the size of this pool of non-dormant follicles for clinical practice. Several biochemical and ultrasound markers and challenge tests have been developed to estimate the size of the antral follicle pool. These include serum AMH, AFC, basal serum follicle stimulating hormone (FSH) and basal serum inhibin.

The granulosa cells of the primary, secondary, pre-antral and small antral follicles secrete AMH (8). As AMH is secreted exclusively by the granulosa cells in the ovary, its measurement provides a direct quantitative reflection of the antral follicle pool. Literature reporting the influence of sex steroids on AMH expression and circulating AMH is contradictory (9-11). Serum AMH levels measured on day 3 of the menstrual cycle progressively decrease with age and are not detected after menopause(12). Serum AMH levels also show an excellent correlation with the number of retrieved oocytes during IVF treatment (9, 13). These indicate serum AMH to be a good marker of the antral follicle pool.

The antral follicle pool may also be estimated by in-vivo ultrasound measured AFC. AMH and AFC show an excellent correlation with each other as they measure the same biological entity (9). These ovarian reserve markers, representing the size of the antral follicle pool, have demonstrated a good correlation with the size of the primordial follicle pool in adult women (14). Hansen et al (2011), evaluated the relationship between ovarian reserve markers and primordial follicle counts assessed histologically. 42 women between 26-52 years were recruited to the study. After correcting for chronological age, serum AMH and AFC

significantly correlated with the primordial follicle count (r = 0.48 and r = 0.53 respectively) (14). Other studies using indirect methods have supported these findings. Observational data have demonstrated association between poor response to assisted reproduction treatments and early menopause (15) and prospective changes in ovarian reserve markers and age at menopause (16, 17). Hence AMH and AFC may be considered suitable indirect markers of ovarian reserve for clinical application in the adult female population.

Basal serum FSH is an indicator of pituitary function and may be considered relevant as an indirect marker of ovarian function in women of reproductive age with regular menstrual cycles. It is an inappropriate indicator of ovarian function in childhood and adolescence, states of pituitary suppression such as hypothalamic amenorrhoea or pituitary suppression due to long term treatment with oral contraceptive pills (OCP) or gonadotropin releasing hormone (GnRH) analogues. Serum FSH levels also show considerable inter-cycle and intra-cycle variability which make it less suitable as a test for ovarian reserve (18). Basal serum FSH has been used to estimate ovarian response and predict poor response to exogenous gonadotropins in the context of assisted reproduction treatment. In a systematic review, Broekmans et al (2006), reviewed 37 studies reporting the use of FSH to assess ovarian response to in-vitro fertilisation (IVF). They concluded that serum FSH did not show good clinical value as it could accurately predict a poor response only at very high values and there was a very low number of such abnormal tests reported (19). Fanchin et al (2003), investigated the correlation between the AFC on day 3 and other hormonal markers of ovarian reserve (9). Serum FSH levels showed only a weak correlation with the AFC ($r=0.29$, $p<0.001$) as compared to serum AMH ($r=0.74$, $p<0.0001$).

Serum inhibin may be considered a direct indicator of ovarian reserve as it is produced by the granulosa cells. Serum inhibin levels are however influenced by FSH stimulation with an increase in serum inhibin observed with increasing FSH levels in the follicular phase of the cycle (20). Serum inhibin levels also showed only a weak correlation with the AFC (9).

Hence for the purpose of this thesis we have included only serum AMH and AFC as markers of ovarian reserve for investigation.

3.3 Physiological role of AMH

AMH is produced in females exclusively by the granulosa cells of the ovary (21). Although its exact role is not clearly understood, an inhibitory role for ovarian folliculogenesis, and also for cyclical follicular maturation and ovulation has been hypothesised.

The exact mechanisms for the progression of follicles from the primordial to the antral follicle pool remain unknown. It has been proposed that the primordial follicles remain dormant due to a constant inhibitory influence (22). Movement to the growing or antral follicle pool may be due to removal of the inhibitory influence or presence of a stimulatory influence. Functional gonadotropin receptors have not been demonstrated in primordial follicles and hence it is unlikely that gonadotropins may influence the initial primordial follicle recruitment (23).The early growth of primordial follicles is independent of gonadotropins and is thought to be influenced by AMH. In-vitro studies on AMH null mice demonstrated a smaller number of primordial follicles and greater number of antral follicles as compared to control mice (24). Also, in-vitro treatment of neonatal mouse ovaries with exogenous AMH showed an inhibitory effect on the size of the antral follicle pool (25). This led to the hypothesis that AMH may have an inhibitory effect through the action of local paracrine factors on initiation of follicular growth in primordial follicles. This led to investigation of AMH expression in human ovaries. Weenen et al (2004), studied sections of human oophorectomy specimens by immunohistochemical staining for AMH. AMH expression was observed from the primary follicle stage, was maximal in the pre antral and small antral follicles and declined in larger antral follicles (8). AMH was not expressed by primordial follicles (8). AMH expression has been detected as early as 36 weeks of fetal life (26) and continues till the menopause. Stubbs et al (2008), in a study similar to Weenen et al (2004), confirmed their findings (27). This study also demonstrated AMH expression in some primordial follicles although the authors confirm that this was less prevalent and of lower intensity. This pattern of immunohistochemical staining and AMH expression in humans supports the animal model hypothesis for the inhibitory role for AMH in initial recruitment and early folliculogenesis.

AMH also plays an important role in cyclical follicular maturation for follicles in the antral follicle pool. In pre antral and small antral follicles, AMH inhibits FSH induced aromatase which promotes the conversion of androgens to estrogens. Pellatt et al (2007), measured AMH levels in follicular fluid and granulosa cells in antral follicles measuring between 2-10 mm

from normal ovaries. The concentration of AMH in follicular fluid and granulosa cells was high in small antral follicles, with maximum concentrations observed in follicles between 3-5 mm (28). An exponential decrease in AMH levels was observed with increasing follicular size. AMH levels were at the lowest level of detection (0.025 ng/ml) or undetectable in follicles greater than or equal to 10 mm (28). Similar findings were reported by Andersen et al (2010), where follicular fluid concentrations of AMH progressively decreased from a size of 3 mm to 9 mm (29). In-vitro treatment of rat granulosa cells with AMH resulted in reduced aromatase activity (30). Similar results were seen on studies involving human granulosa cells (31). These findings support the hypothesis for the inhibitory role of AMH in antral follicles and its subsequent decrease with increasing follicular size and selection of the dominant follicle.

3.4 Role of AMH in ovulatory dysfunction

Polycystic ovary syndrome (PCOS) is the commonest endocrinological disorder in women of reproductive age (32). It is diagnosed using the Rotterdam criteria (33) which requires at least two of three criteria to be met for diagnosis. These include oligo- or anovulation, clinical or biochemical hyperandrogenism and the polycystic appearance of ovaries on ultrasound scan. Women with PCOS have a higher number of antral follicles as compared to women without PCOS (34). The density of preantral and small antral follicles in the polycystic ovary is reported as six times that of the normal ovary (35). As AMH is expressed by the granulosa cells of the antral follicles, it is not surprising that serum AMH levels are higher in women with PCOS as compared to women without PCOS (36) (Figure 2-1). Also, women with PCOS have higher AMH levels as compared to asymptomatic women with isolated polycystic ovarian morphology (PCOM)(36) (Figure 2-1).

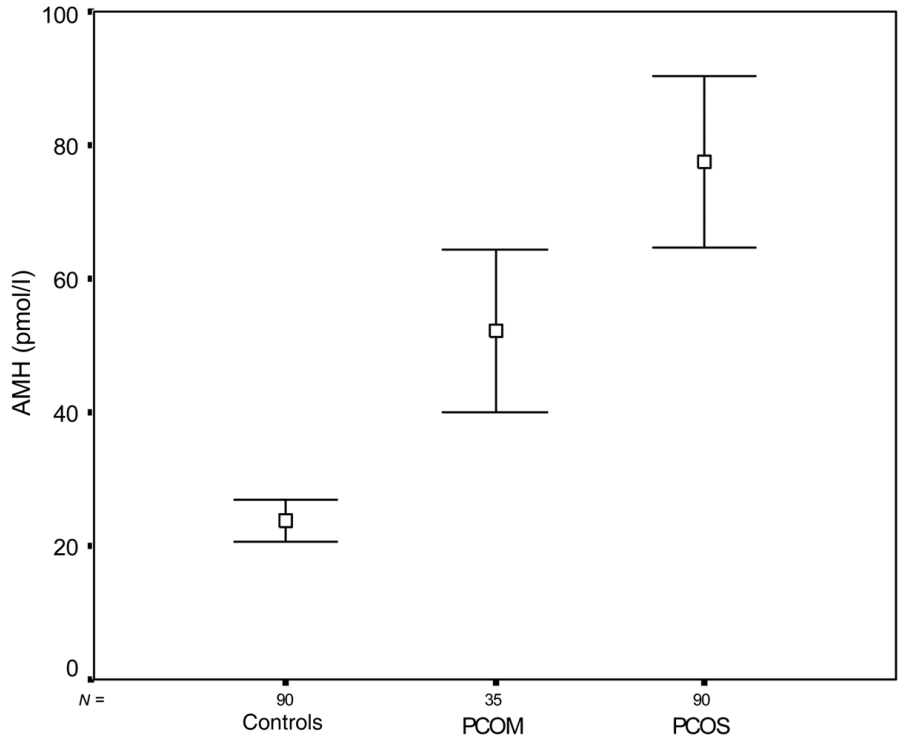

Figure 3-1 Serum AMH levels in women with PCOS, PCOM and controls.

Homburg R, Ray A, Bhide P, et al. The relationship of serum anti-Mullerian hormone with polycystic ovarian morphology and polycystic ovary syndrome: a prospective cohort study. Hum Reprod. 2013;28(4):1077-1083. doi:10.1093/humrep/det015 (reproduced with permission from the publisher)

PCOS is commonly associated with ovulatory dysfunction and accounts for 70-75% of all anovulatory infertility. As discussed earlier, AMH plays an important physiological role in recruitment to the antral follicle pool and cyclic follicular maturation and ovulation (37). Its actions are inhibitory to FSH-dependent follicular maturation and ovulation. Given this physiological role of AMH, we were keen to further explore the role of AMH in the pathogenesis of ovulatory dysfunction in PCOS.

The high levels of serum AMH in women with PCOS are due to an increased number of antral follicles. In our previous work we have demonstrated that these high levels of AMH are not only due to the presence of more follicles but also due to a higher per follicle production of AMH (38). A ratio of serum AMH to total AFC was calculated for 438 women with PCOS, isolated PCOM and women without PCOS, as a measure of AMH production per antral follicle. Women with PCOS had a significantly higher AMH/AFC ratio as compared to the other two

groups. The per follicle AMH production was not different in women with isolated PCOM and controls (Figure 2-2)

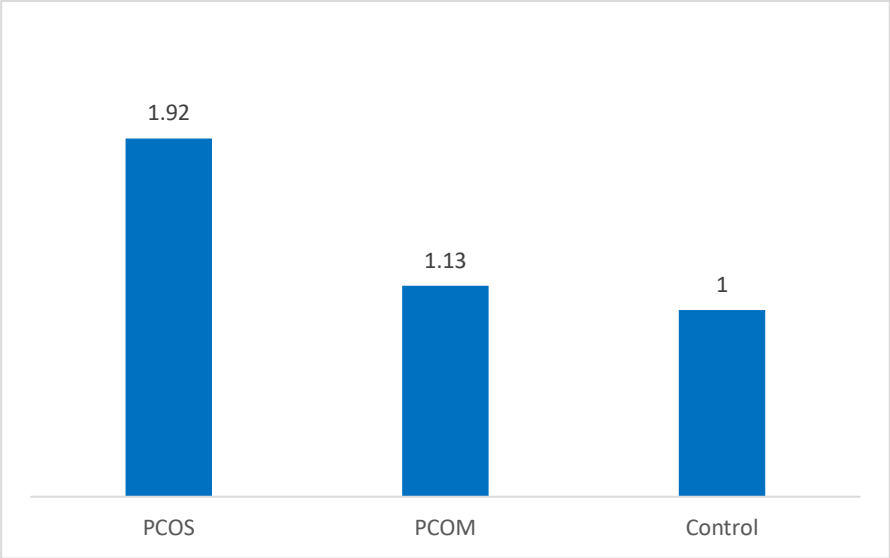

Figure 3-2 Median AMH/AFC ratios in women with PCOS, PCOM and controls

Based on data from previously published work - Ref 38

However, PCOS has a diverse reproductive phenotype. Based on the diagnostic criteria of oligo-anovulation (OA), hyperandrogenism (HA) and polycystic ovarian morphology (PCOM), PCOS may be divided into four phenotypes: Group A (OA+ HA+ PCOM), Group B (OA+HA), Group C (HA+PCOM) and Group D (OA+PCOM). Our previous study pooled all phenotypes of PCOS and did not evaluate the various phenotypes separately. Therefore, it remains unknown if this increased per follicle production is seen across all these clinical phenotypes.

Serum AMH concentrations are significantly higher in anovulatory PCOS as compared to ovulatory PCOS (37). We are unsure if this is due to an increased number of follicles or a greater per follicle production in anovulatory PCOS. In-vitro studies specifically compared women with anovulatory PCOS with controls. Pellatt et al (2007), demonstrated that the granulosa cells from women with anovulatory PCOS produce 75 times higher AMH than size-matched counterparts without PCOS (28). This is corroborated by other in-vitro studies which demonstrate higher AMH levels in follicular fluid of women with anovulatory PCOS (39) and

a greater expression of AMH mRNA in granulosa cells from women with anovulatory PCOS as compared to controls (40). AMH is thought to produce its inhibitory effect on follicular maturation by decreasing the sensitivity of the follicles to FSH through the inhibition of FSH induced aromatase expression. This is supported by in-vitro studies that demonstrate a reduced aromatase mRNA expression in granulosa cells treated with AMH (28, 31).

The above results led us to postulate that high intra-follicular concentrations of AMH due to a greater per follicle AMH production, may result in a heightened inhibitory response to follicular maturation and ovulation. This may result in the ovulatory dysfunction associated with PCOS. We hypothesized that the anovulatory phenotypes of PCOS have a greater AMH production per follicle as compared to the ovulatory phenotypes. If true, this would support the postulated mechanism of anovulation.

We were hence keen to explore the greater per follicle production of AMH amongst various phenotypes of PCOS. We also wanted to explore other factors that might be associated with a higher per follicle production of AMH, such as levels of luteinising hormone (LH), androgen and insulin.

3.5 Factors affecting ovarian reserve

Wallace et al (2010)(41) developed a model of human ovarian reserve (primordial follicles) from conception to menopause, combining histological evidence from literature (Figure 2-3). The simple peak model demonstrated a steady decline in primordial follicle numbers after 20-22 weeks of gestation and reported the main determinant of remaining ovarian reserve (81%) to be time, with environmental and genetic factors accounting for much of the remaining variation. When reporting on the ovarian reserve in a sub-section of the population from conception to 25 years, 95% of the variation in the ovarian reserve could be attributed to age alone. The remaining 5% variation was thought to be due to other factors. The authors speculated that as chronological age increased the role of factors other than age became more important in influencing ovarian reserve.

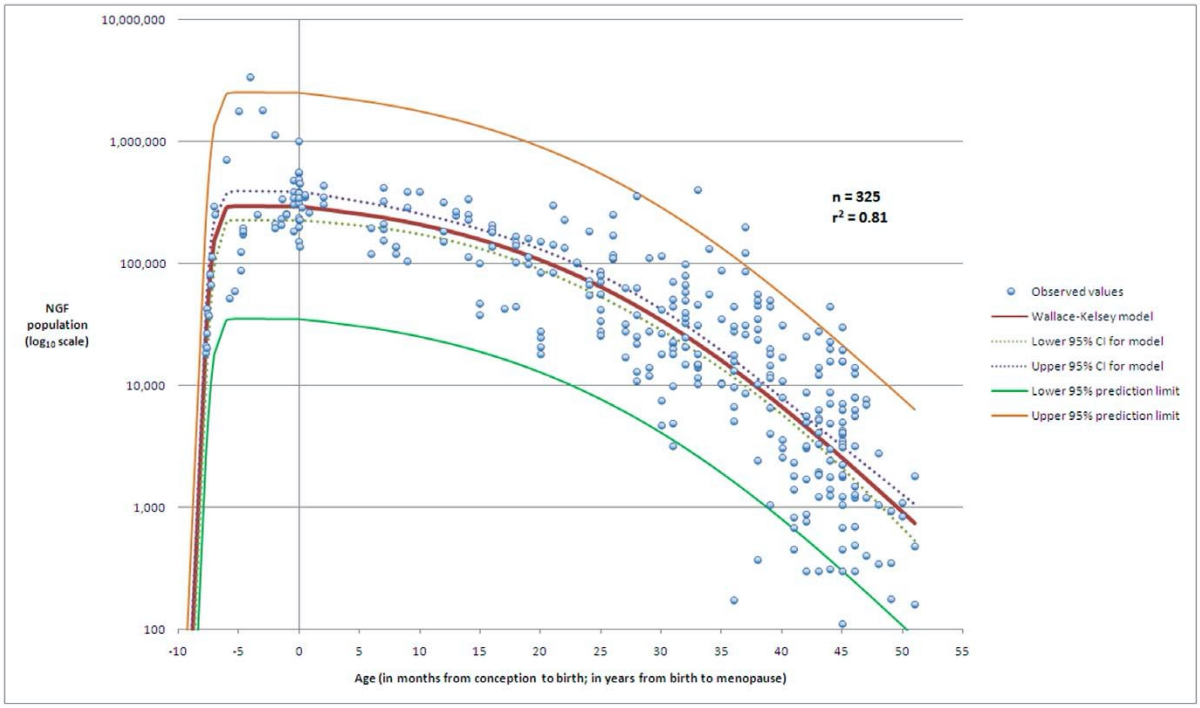

Figure 3-3 Model of human ovarian reserve from conception to menopause.

Wallace WH, Kelsey TW. PLoS One. 2010;5(1):e8772. Published 2010 Jan27. (Open access article)

Kelsey et al (2011), developed a model of serum AMH values from conception to menopause to represent the antral follicle pool (42) (Figure 2-4). This reported a peak in the neonatal period, an increase in serum AMH throughout childhood to young adulthood with a dip at puberty, followed by a steady decline to menopause. This model reported only 34% of the variation in serum AMH to be due to age with the remaining two third of the variation attributed to other factors.

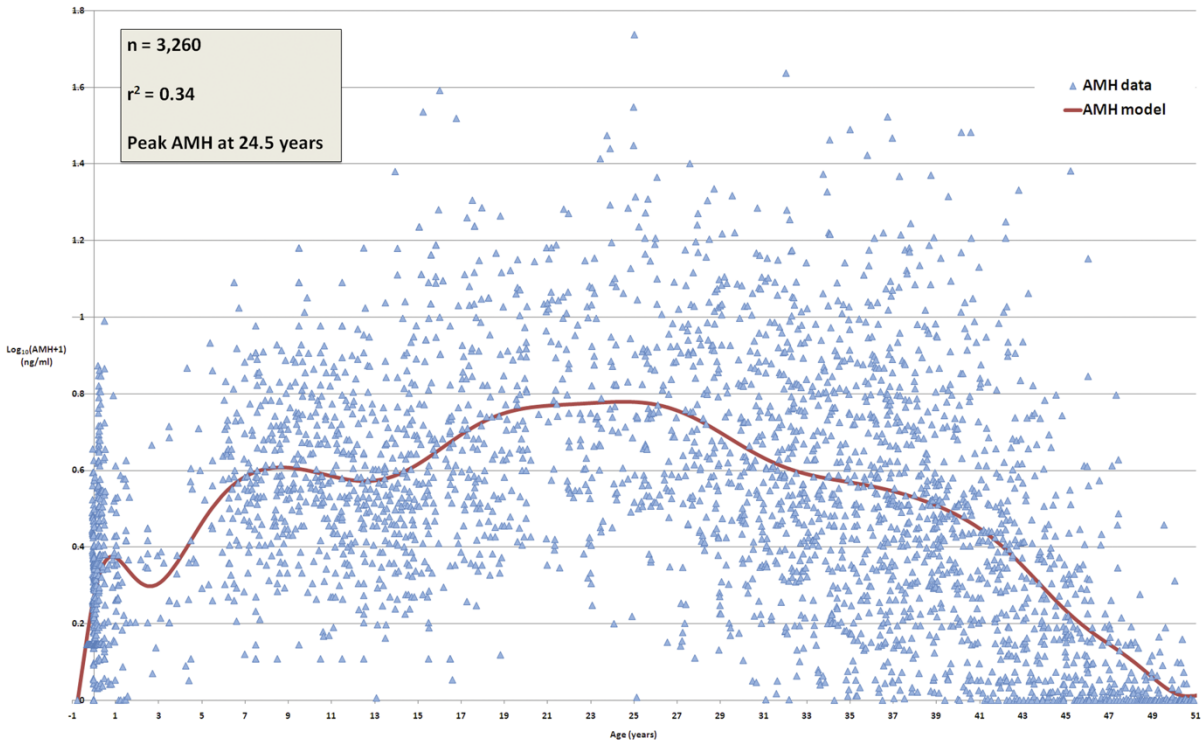

Figure 3-4 Model of serum AMH from conception to menopause.

Kelsey TW et al . PLoS One. 2011;6(7):e22024. (Open access article)

The size of the antral follicle pool is more dynamic than the primordial follicle pool. Its size is influenced by factors other than the size of the primordial pool. As well as time, genetics and environment, the complex endocrine and paracrine environmental changes associated with early life, puberty and peri-menopause may also affect AMH levels at a given age. Although there remains a steady decline in primordial follicles from 22 weeks of gestation to menopause, the rate of recruitment from the primordial to the antral follicle pool is variable. It increases from birth throughout childhood to peak at the age of 14 years, and then decreases to menopause (41). Hence, before 25 years, serum AMH and ovarian reserve are much less well correlated, with increasing serum AMH suggesting an increased recruitment and high ovarian activity rather than high ovarian reserve.

In the adult population, after peak serum AMH levels reached at the age of 24.5 years, there was a strong positive correlation (r = 0.96) between declining primordial follicles and declining serum AMH (43). This is supported by the study by Hansen et al detailed above (14). Hence after about 25 years of age, the trajectories for primordial follicles and serum AMH may be considered similar, with serum AMH thought to be a fairly accurate, although indirect marker of the primordial follicle pool.

From the above it is clear that in addition to age, genetic, lifestyle and environmental factors are recognised determinants of variation in ovarian reserve. It is hence reasonable to use available markers to estimate ovarian reserve in addition to age alone.

Literature has produced contradictory findings for the effect of genetic and lifestyle exposures on ovarian reserve. It is often difficult to ascertain the effect of a single factor as these factors may coexist and be difficult to separate. We are also unsure as to the mechanisms by which these factors may affect ovarian reserve and if their effects may be reversible. Self-reporting may also introduce a bias due to under-reporting. The literature reports conflicting results for the impact of ethnicity on ovarian reserve. Several studies report differences in serum AMH amongst women from different ethnicities (44-47) in contrast to others who report no differences (48, 49). These conflicting results may be attributed to heterogenous populations, small sample sizes, and confounding environmental factors. True biological variation in ovarian reserve due to ethnic differences may be confounded by varying environmental conditions the effect of which may be difficult to separate. Reporting bias due to self-reported ethnicity may also result in discordant results. Olcha et al (2016), assessed the relationship between genetic ethnicity using ancestry related markers and markers of ovarian reserve. They found no differences in serum AMH or AFC relative to genetic ethnicity (50). Conditions such as PCOS are more common in some ethnicities and may account for ethnic differences reported (48).

BMI is not thought to affect ovarian reserve markers. Although some studies reported differences, these were attributed to differences in age amongst the groups compared (51). Further studies demonstrate no effect of BMI on ovarian reserve markers (52-54).

Hawkins et al (2016),(55) reported lower levels of serum AMH with current, frequent binge drinking in a large population-based study. Other behaviours such as past history of alcohol intake, ever-drinking, number of drinks per day or daily consumption did not show a similar association. This was in contrast to other studies which did not demonstrate any differences (53, 56). Pregnancy, oral contraceptive pill (OCP) use, hypothalamic-pituitary suppression with GnRH analogues or states such as hypothalamic amenorrhoea are known to reversibly decrease levels of ovarian reserve markers such as serum AMH (57-61). However, studies on the effect of cigarette smoking on ovarian reserve parameters have reported contradictory findings.

In view of the contradictory results reported in published literature we decided to systematically review the effect of cigarette smoking on the quantitative ovarian reserve parameters, serum AMH and AFC.

3.6 Effect of smoking on ovarian reserve.

I have conducted a systematic review of the literature to assess the effect of cigarette smoking on the quantitative ovarian reserve parameters, AMH and AFC.

I searched for all types of published literature reporting the effect of smoking on ovarian reserve parameters and included all types of studies published as primary research involving humans published as full text manuscripts in English language. I assessed the quality of the included studies using the Newcastle-Ottawa scale (NOS) modified for observational studies (62). I further modified the scale as only non-interventional observational studies were included. I conducted a comprehensive search for eligible studies to minimise the impact of reporting bias. The outcome measures were markers of ovarian reserve; either serum AMH or AFC. I screened and identified relevant studies for the review using the electronic database MEDLINE from the inception of the database till 30/10/2019 and hand searching as described by Cochrane methodology (63). The search strategy included a combination of subject headings (MeSH) and text words relating to or describing the exposure/risk factor (smoking/cigarette smoking) and outcomes (ovarian reserve/egg reserve/AMH/AFC). The reference lists of the primary articles were searched for relevant citations not captured by the electronic searches.

After screening the titles and abstracts of articles retrieved from the search, I obtained and reviewed the full texts of potentially suitable articles. I extracted and collected the data from the selected articles on a bespoke data collection Excel spreadsheet. I collected data for study design, methodological characteristics, participant characteristics, methods for assessment of smoking exposure and outcomes. If a study was reported in multiple publications, I pooled these together under a single study ID.

The search of the MEDLINE electronic database retrieved 94 studies. Hand searching retrieved one additional study. After screening of the titles and abstracts, the full text of 22 studies were retrieved. 20 of these were selected for the review and two excluded.

All included studies were of an observational design. 19 studies were of a cross sectional design (53, 55, 56, 64-79) and one of a longitudinal study design (80). 11 studies were population based (53, 55, 64-71, 80) studies whereas the other nine were single centre hospital-based studies (56, 72-79).

Participants in population-based studies were volunteers recruited from larger cohorts. Those in single centre studies were infertile women undergoing fertility treatment. Ascertainment of smoking exposure in all studies was through self-reported smoking history. Some studies included an in-depth questionnaire to detail smoking exposure and quantify it; others only classified them into crude categories without details of intensity or duration of exposure. None of the included studies used objective methods to confirm exposure to smoking such as urine cotinine testing or a breath test for carbon monoxide (CO). Studies either dichotomized women into smokers and non-smokers or categorized them into three groups; current smokers, ex-smokers and never smokers. The inclusion or exclusion of passive smoking was also variable amongst included studies. 18 studies reported serum AMH levels, and six on AFC.

Significant methodological heterogeneity was observed in the included studies. This related mainly to the participant population included and assessment of smoking exposure. All studies used validated measurements for the outcomes reported, considered the effect of confounding variables in either study design or analysis, and used appropriate statistical methods for analysis. The quality of evidence of individual studies assessed by the NOS was good to very good in 19 of the 20 studies. One study was considered satisfactory and none of the studies was considered unsatisfactory.

The included studies showed no consistent direction of effect for serum AMH. 10 studies reported no differences in serum AMH levels between smokers and non-smokers. The other eight reported a significant negative impact of smoking on serum AMH levels.

Bressler (2016) (55), in a large population-based study, including 1654 participants, with a detailed analysis of smoking history was unable to demonstrate significant differences in serum AMH levels between any groups. These conclusions were reiterated in studies by Dafopoulos et al (2010) (73), Freour et al (2010) (75), Freour et al (2012) (76), Jung et al (2017) (64), Kline et al (2016) (66), La Marca et al (2013) (69), Nardo et al (2007) (56), Szkup et al (2018) (68) and Waylen et al (2010) (79). In contrast, Dolleman (2013) (53) in an earlier population-based study in 2013, including 2320 participants, reported a significantly lower level of serum AMH in current smokers as compared to never smokers. No differences were noted between never

smokers and ex-smokers. Similar result was reported by Dolleman et al (2015) (81), Freour et al (2008) (82), Freour et al (2013) (77), Fuentes et al (2012) (78), Plante et al (2010) (71), Sowers et al (2010) (67) and White et al (2016) (70).

A significant negative correlation between intensity of smoking and serum AMH was reported by Dolleman et al (2013) and similarly by Freour et al (2008) and Fuentes et al (2012).

Two of the six studies reporting AFC reported a negative impact of smoking on AFC. Caserta et al (2013) (72) and Freour et al (2013) reported significantly lower AFCs in smokers as compared to non-smokers. Four other studies, Freour et al (2010), Freour et al (2012), Kinney et al (2007) (65) and Nardo et al (2008) could not demonstrate a significant difference between groups.

This review of literature showed no consistent direction of effect of smoking on serum AMH or AFC and hence for a quantitative change in the antral follicle pool following exposure to cigarette smoking. In relation to the effect of smoking on ovarian reserve it is important to assess serum AMH and AFC for two reasons. Firstly, these biomarkers are important as they allow estimation of response to ovarian stimulation and planning of fertility treatment protocols, which are crucial to success rates of treatment. Secondly, this may provide valuable insight into the possible mechanisms for the effects.

Animal studies have suggested adverse effects of cigarette smoke on ovarian reserve (83, 84). Several mechanisms have been postulated, which may affect quality, quantity or both. Gannon et al (2012),(85) hypothesised a mechanism of direct toxicity to ovarian follicles resulting in an accelerated follicle loss. An indirect effect on ovarian follicle numbers has been suggested through an action on the hypothalamic pituitary axis (86). The effects through either mechanism would mirror decreased levels of AMH and AFC. Suboptimal oocyte quality due to the presence of a greater concentration of reactive oxygen species has also been suggested (84). Impairment in granulosa cell proliferation and oocyte development were observed in animal models and these effects continued after cessation of exposure (87). These effects would be better reflected by outcomes such as embryo quality and pregnancy rates rather than serum AMH and AFC.

Published literature from clinical studies is contradictory and inconclusive as evidenced by this review. This may be attributed to several factors. Characteristics of participant populations in reported studies are heterogeneous in terms of age, fertility and ethnicity amongst other factors.

The natural decline of ovarian reserve with age does not follow a linear function but shows a rapid decline with increasing age(41). It may be more challenging to demonstrate significant differences amongst groups in younger populations with higher and less dispersed baseline values for serum AMH and AFC. This may account for discordance in the results of the two largest studies included in this review. The mean age of participants in the study by Dolleman et al (2013), which reported negative effects of current smoking was 37.3 (SD 9.2) years. In comparison, the mean age in the study by Bressler et al (2016), which was unable to find a significant association was only 29 (SD 4) years. It has also been suggested that ovarian follicles may differ in susceptibility to the effects of smoking at different ages with older oocytes being more susceptible to negative effects of smoking. Additional information in future studies may be obtained from the use of longitudinal data. Although ovarian reserve parameters are most relevant for fertility, the use of infertile/subfertile women may introduce an additional confounder. Hence population-based studies in a comparable age group may be considered most appropriate.

The second important factor contributing to contradictory results are differences in ascertainment of exposure to cigarette smoke. Several studies included in this review did not categorise ex-smokers separately and did not account for passive smoking. This leads to contamination of the two broad study groups; smokers and non-smokers. Ex-smokers and passive smokers included in the group of non-smokers may reduce mean differences between groups and the effect sizes of outcomes. This can lead to differences in significance levels of outcome measures reported. It is unknown if the possible negative impact of smoking affects all follicles in the ovary or only the antral follicle pool. If the effect is restricted only to antral follicles, it would explain the lower levels of AMH in current smokers but not in ex-smokers. This reinforces the need for categorising ex-smokers separately.

Studies have used variable definitions for current and non-smokers. Freour et al (2008) and Bressler et al (2016) include participants who quit smoking within one year as current smokers in contrast to Plante et al (2010) who has extended this definition to two years. As the effects of smoking on ovarian reserve are likely to be dose and duration dependant, it remains important to include details of the duration and intensity of smoking exposure. All of the above may be considered serious flaws in study design. Self-reported smoking histories may also be considered flawed due to inaccuracies of reporting. More objective measures of current smoking may be considered more robust for future study designs.

In conclusion, a review of the literature is unable to provide evidence of a quantitative change in ovarian reserve markers following exposure to smoking. Although the review does not provide definitive evidence of effect, it very clearly highlights the heterogeneity of existing literature. I consider this useful new information to direct future research.

Based on this systematic review we planned to assess the effect of cigarette smoking on the quantitative ovarian reserve parameters, validating self-reported smoking behavior using biomarkers.

3.7 Effect of insulin sensitisers on ovarian reserve

Since the seminal report by Burghen et al (1980) of the association of hyperinsulinemia and PCOS (88), and the significant publication by Dunaif et al (1989) which reported that women with PCOS have intrinsic insulin resistance (IR) independent of obesity (89), IR is widely reported at 50-70% in women with PCOS (90). Using the euglycemic–hyperinsulinaemic clamp method which remains the gold standard for assessment of insulin resistance, Dunaif et al (1989)(89) and more recently Stepto et al (2013)(91) supported the concept of intrinsic IR in PCOS. Intrinsic IR is independent of BMI but exacerbated by any increase in BMI. Stepto et al (2013) reported a 75% incidence of IR in lean PCOS and a 95% incidence of IR in obese PCOS. They reported a significantly increased incidence of IR in lean PCOS as compared to lean controls ($p = 0.038$). This concept still remains controversial but is supported by some studies hypothesising the mechanisms for intrinsic IR (92, 93).

The cause for intrinsic IR in PCOS remains unknown and several mechanisms have been postulated. Zhang et al (1995), proposed that a single factor that causes serine phosphorylation of the insulin receptor and serine phosphorylation of P450c17, the key regulatory enzyme controlling androgen biosynthesis, could produce both the insulin resistance and the hyperandrogenism characteristic of PCOS (94). Atypical insulin receptors and insulin signalling pathways have been proposed (92, 93). Inositols which are members of the vitamin B complex family are also hypothesised to play an important role in insulin and glucose metabolism as mediators in the signal transduction system. The major stereo-isomers d-chiro inositol and myo-inositol are hypothesised to play different physiological functions. D-chiro inositol is involved in insulin mediated androgen synthesis in the theca cells (95), whereas myo-inositol is involved in the FSH signalling pathway, glucose uptake and metabolism within

the cells. Myo-inositol is converted to d-chiro inositol by the action of the enzyme epimerase which is stimulated by insulin. Hence in states of hyperinsulinemia there is an increased production of d-chiro inositol and relative deficiency of myo inositol leading to increased androgen synthesis (96). Misso et al (2013), suggested that insulin sensitisers may be more effective in lean women with PCOS suggesting different mechanisms and hence treatment strategies for extrinsic and intrinsic IR (97). Weight loss interventions and decrease in visceral fat may help to reduce extrinsic IR, insulin sensitisers may be more effective in treating intrinsic IR (98).

Insulin resistance and hyperinsulinemia are thought to play a central role in the pathophysiology of PCOS through their actions on ovarian steroidogenesis. Syndromes of severe insulin resistance and hyperinsulinemia are associated with hyperandrogenism (99, 100). The defect in the action of insulin in these conditions of insulin resistance is thought to be selective for glucose metabolism but not for steroidogenesis (93). Insulin in high concentrations has shown to stimulate estrogen, androgen and progesterone secretion in-vitro (101) which has led to the hypothesis that hyperinsulinemia leads to hyperandrogenism. Although insulin receptors are present in ovarian tissue, it has been proposed that one of the mechanisms of action of insulin on ovarian steroidogenesis is mediated through its action on IGF-1 receptors (102). Insulin may also act through its own receptors on theca and granulosa cells to mediate hyperandrogenism by stimulation of ovarian steroidogenesis (95, 103) but also by stimulation of LH release by the pituitary. The actions of insulin on steroidogenesis are observed only in women with PCOS and not on normal women (104). This suggests that the pre-condition of polycystic ovaries must be present for hyperinsulinemia to produce hyperandrogenism.

Androgens produced by the theca cells stimulate ovarian folliculogenesis. Thus, an increased androgen production and hyperandrogenism secondary to hyperinsulinemia has the potential to increase folliculogenesis in women with PCOS.

Based on this background, treatment of insulin resistance with insulin sensitisers has the potential to reduce hyperandrogenism and subsequently affect folliculogenesis and the antral follicle pool. We planned to assess the effect of treatment with myo-inositol (MI)/di-chiro inositol (DCI) compared to no treatment, placebo or other treatment on markers of ovarian reserve in women with PCOS combining data from all published literature.

3.8 Prediction of natural fertility using ovarian reserve markers

Age remains one of the most important determinants of female reproductive function and fertility. A natural age-related decline in fertility is due to a decline in the number of oocytes and also a reduction in oocyte quality. Ovarian reserve markers such as serum AMH and AFC are able to demonstrate a decrease in the number of oocytes with increasing age. However, evidence for their role for prediction of natural fertility remains limited and contradictory. Steiner et al (2011)(105) in a prospective study, reported time to pregnancy in a community sample of 100 women trying to conceive for less than 3 months and with no history of infertility. As expected, age remained a strong predictor of pregnancy with women older than 35 years having a significantly lower probability of pregnancy than younger women (FR=0.42, 95% CI: 0.15, 0.85). They reported significantly reduced pregnancy rates in women with serum AMH levels of 0.7 ng/ml or less as compared to women with higher serum AMH levels even after adjusting for age (FR=0.38; 95% CI:0.08, 0.91). Hagen et al (2012)(106) recruited 430 couples who planned to discontinue contraception with a view to become pregnant and reported pregnancy rates in women with a low, medium and high level of serum AMH. They concluded that low serum AMH was not a good indicator of natural conception and pregnancy. They attributed this to be due to high oocyte quality inspite of a reduced ovarian reserve. The cohort of women in the study by Hagen et al (2012) were younger (19-35 years) as compared to the women in the study by Steiner et al (2011) (30-44 years). This along with the differing covariates analysed could account for these contradictory results.

It is difficult to study the effects of oocyte and embryo quality in the context of natural fertility. Age related decline in oocyte quality is thought to be related to abnormalities in meiotic spindle formation and chromosomal alignment resulting in an increased incidence of aneuploidy (107). Age related abnormalities in mitochondrial DNA are also reported to contribute to reduced oocyte quality (108).

In the absence of data and adequate evidence, the use of serum AMH and AFC for general fertility assessment and prediction of spontaneous conception in the general population is not recommended.

3.9 Ovarian reserve markers and outcomes of fertility treatment

A successful outcome following IVF treatment is strongly predicted by the quality of transferred embryos (109). Published literature is however unable to demonstrate a clear correlation between embryo quality and ovarian reserve/markers of ovarian reserve. Sunkara et al (2011) demonstrated a strong positive correlation between the number of oocytes retrieved at IVF and live birth rates (110). They analysed data from 400,135 fresh IVF cycles and demonstrated an increase in live birth with an increase in the number of retrieved oocytes, up to 15 oocytes with a plateau and decline thereafter across all age groups. Serum AMH and AFC correlate well with the response to controlled ovarian stimulation and the number of oocytes retrieved at IVF (111, 112). Further to this, a positive correlation between serum AMH and pregnancy rates following IVF has been reported (112-114). It may be argued that this positive relationship may be indirect and attributed purely to an increased probability of having good quality embryos for embryo transfer due to a greater number of retrieved oocytes. Some researchers report contradictory findings to the positive correlation discussed above (115, 116). Wang et al (2010) analysed 2712 fresh IVF cycles and concluded that the correlation between serum AMH and clinical pregnancy rates was modulated by age. The predictive value of serum AMH decreased at ages above 42 years where oocyte quality remained the more important predictor of outcome. Oocyte and subsequent embryo quality is negatively influenced by age due to an increase in the incidence of chromosomal abnormalities and aneuploidy (107, 108).

Variation in reported literature on the association between ovarian reserve markers and embryo quality may be attributed to differing ovarian stimulation protocols used, variable timings for embryo transfer and differing time points during IVF for measurement of serum AMH. Additionally, there may be variation due to different methods used for assessing embryo quality. Assessment of embryo quality in standard IVF treatments is through a morphological assessment of embryos using a light microscope at fixed time points following fertilisation. This assessment, although following a standardised system (117) is liable to inter-observer variability, can be subjective and may contribute to inconsistency in results (118). The use of a time lapse incubator with the use of morphokinetic parameters provides more objective and reproducible estimates of embryo quality as compared to standard morphological assessment.

Assessments of embryo quality done on day 3 following fertilisation have poorer correlation with outcomes as compared to extended culture and embryo assessment on day 5 (119). Assessment of embryo quality on day 5 rather than day 3 allows better embryo selection.

Based on this background, we were keen to assess the association between ovarian reserve and embryo quality using the newer technology of time lapse imaging. We planned to examine the correlation between baseline serum AMH levels as a marker of ovarian reserve and 'KID' (Known Implantation Data) scores of the developing embryos generated by time lapse imaging as an indicator of embryo quality also considering the effect of important confounders.

3.10 Aims and objectives

The overarching aim of this thesis was to study the role of the ovarian reserve markers, AMH and AFC, on different aspects of fertility and fertility treatment. The thesis comprised of four work packages. Their individual aims and objectives are as below.

1) To compare the per follicle AMH production (expressed as a ratio of serum AMH to the total AFC) in the various phenotypes of women with PCOS and with isolated PCOM. We also aimed to evaluate the factors which may be associated with variation in AMH production among these different clinical phenotypes.
2) To assess the effect of cigarette smoking on the quantitative ovarian reserve parameters in sub-fertile women, validating self-reported smoking behavior using biomarkers (breath carbon monoxide levels and urine cotinine levels).
3) To assess the effect of treatment with myo-inositol (MI)/di-chiro inositol (DCI) compared to no treatment, placebo or other treatment on markers of ovarian reserve in women with PCOS combining data from published literature. Our secondary aim was to assess the effect of these treatments on reproductive outcomes in women with PCOS undergoing IVF/ICSI.
4) To assess the association between ovarian reserve markers and embryo quality in women undergoing IVF/ICSI treatment using time lapse imaging technology.

4 Materials and methods

I conducted the work contributing to the thesis between 2014 and 2020. Although I was formally registered as a PhD student in January 2016, I started the planning, discussion and preparatory work in 2014 and continued it in the 18 months preceding registration. A timeline for the four work packages which make up the thesis is as shown below.

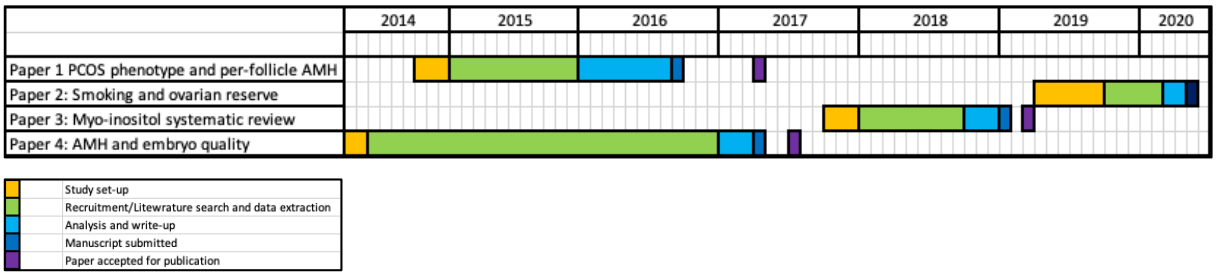

Figure 4-1 A timeline for the work packages for the thesis

I have used a combination of primary research and evidence synthesis for the different work packages. Three work packages were observational studies constituting primary research and one was a systematic review and meta-analysis.

For the purposes of the thesis I will refer to these as

Paper I: PCOS phenotypes and AMH

Paper II: Smoking and ovarian reserve markers

Paper III: Systematic review on inositols and ovarian reserve

Paper IV: Serum AMH and embryo quality

4.1 Primary research

4.1.1 Study design

We have used a cross-sectional study design for all the three primary research studies. The studies were observational without any active intervention, treatment or deliberate exposure.

4.1.2 Setting and population

All the primary research projects were single centre studies conducted at the Homerton Fertility Centre, London, UK. This is a tertiary referral fertility centre within a university teaching hospital. It sees more than 1500 new couples per year for investigations and treatment, offers

the full range of assisted conception treatments and performs approximately 1000 IVF/intra-cytoplasmic sperm injection (ICSI) treatments annually.

Figure 4-2 Homerton University Hospital, London, UK

The women included for all primary studies included in the thesis were patients at the fertility centre. Being an inner-city hospital in East London, this centre treats a wide range of multi-cultural and multi-ethnic populations. Fertility treatments here are predominantly funded by the National Health Service (NHS). As NHS funded fertility treatments are restricted based on factors such as age and BMI, this is reflected in the population of women seen in clinics and hence included for the research studies.

4.1.3 Inclusion and exclusion criteria

We have attempted to minimise the bias which is inherent to observational studies in the design stage by selecting appropriate inclusion criteria for each of the three studies.

Paper I: PCOS phenotype and per follicle AMH production: The inclusion criteria included all women with PCOS diagnosed by the Rotterdam criteria (33) or isolated PCOM. The Rotterdam criteria are globally accepted criteria with validated definitions for its individual components. The diagnosis of PCOS requires two of the three criteria to be satisfied.

1	Oligo-anovulation (OA)
2	Clinical or biochemical hyperandrogenism (HA)
3	Polycystic ovarian morphology on ultrasound (PCOM)

Table 4-1 Rotterdam criteria for the diagnosis of PCOS

Oligo-anovulation (OA) was defined as menstrual cycle length beyond of the range of 23-35 days (120). Clinical hyperandrogenism (HA) was defined by the presence of hirsutism (modified Ferriman Gallwey score (mFG) of ≥ 8) or acne (121). Biochemical HA was defined as a Free Androgen Index (FAI) of greater than 3.36 (122). PCOM was defined as the presence of at least 12 antral follicles, 2-9 mm, on at least one ovary (123)

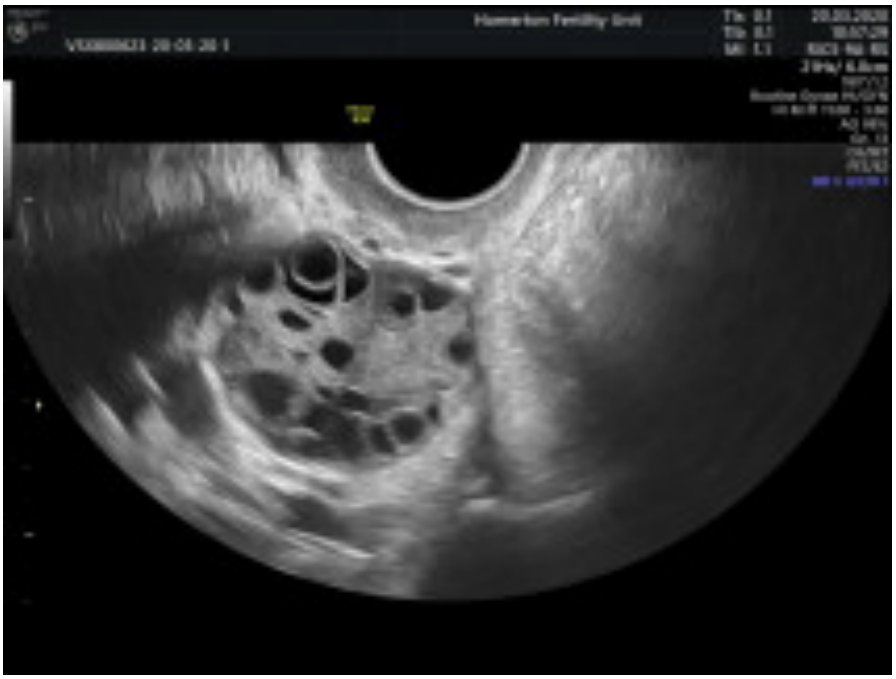

Figure 4-3 Polycystic ovarian morphology on 2-dimensional ultrasound scan

Women with PCOS were divided into four phenotypes based on the diagnostic inclusion criteria of OA, HA and PCOM. These included Group A (OA+ HA+ PCOM), Group B (OA+HA), Group C (HA+PCOM) and Group D (OA+PCOM).

	OA	HA	PCOM
PCOS-A	+	+	+
PCOS-B	+	+	
PCOS-C		+	+
PCOS-D	+		+

Table 4-2 Clinical phenotypes of PCOS

Paper II: Smoking and ovarian reserve markers: We included women ≤ 35 years attending the fertility centre for investigations and treatment. We excluded women on long term oral contraceptive pills or GnRH analogues, women not having both ovaries, those with previous chemotherapy, abdominal/pelvic radiotherapy or major ovarian surgery.

Paper III: AMH and embryo quality: We included all women undergoing IVF/ICSI, those with single and double embryo transfers and those with day 3 or day 5 embryo transfers. However, in order to allow a direct correlation of embryo quality to clinical pregnancy, a secondary outcome, only those women with known implantation data were included. This included all women with a single embryo transfer and those women with a double embryo transfer who either had a negative pregnancy test or a dichorionic twin pregnancy. We included only those women who had their embryos cultured and assessed in the time lapse incubators.

4.1.4 Screening, consent and the care pathway

We screened the medical notes of women attending the fertility centre for eligibility for all three studies. For papers I and IV consent was not required from individual participants but this was required for paper II, which assessed ovarian reserve markers and cigarette smoking. Potentially eligible participants were invited to participate in the study and informed consent was obtained from those who agreed. As the research did not involve any active intervention or treatment the standard care pathway for all participants remained unchanged.

4.1.5 Study procedure/intervention

Paper I: We measured serum AMH and a total AFC for each participant. A ratio of serum AMH to the total AFC was calculated as a marker of AMH production per follicle and expressed as the AMH/AFC ratio. We also collected data for other baseline and confounding variables. These included age, BMI, waist-hip ratio, mFG score, serum FSH, serum LH, T, SHBG, fasting glucose and fasting insulin.

Paper II: We assessed the participants for markers of smoking. This included a short self-reported questionnaire about the participant's current and past smoking history, a non-invasive breath test to detect the levels of CO and a urine test to detect the levels of cotinine. Based on the smoking history we classified participants into one of three categories; current smokers, ex-smokers and never-smokers. The smoking history also accounted for passive smokers and smoking details which allowed us to quantify the smoking exposure in terms of "pack years". We measured serum AMH and AFC as a part of the standard fertility work up done for all

fertility patients. We collected data for baseline and confounding variables such as age, ethnicity, BMI, presence of PCOM/PCOS and a history of ovarian surgery.

Paper IV: We measured baseline serum AMH before start of treatment for all participants. We assessed embryo quality using the time lapse incubators and the computerised KID score. We collected data for confounding variables such as age, BMI, smoking status and method of insemination (IVF/ICSI).

4.1.6 Devices, techniques and tools, laboratory procedures

Serum AMH measurement

The AMH assay has rapidly evolved over the last 30 years. Early years saw single laboratory versions of the AMH assay. This was followed by the development of two commercial assays, the Immunotech Beckman Coulter (IOT) assay introduced in 1999 and the Diagnostic Systems laboratory (DSL) assay in 2003. The primary antibodies used and the calibration standards were different for the two assays. Hence the reported values also differed. The IOT assay had antibodies directed towards the pro-region and the mature regions as compared to the DSL assay which had both antibodies directed to the mature region to minimise against proteolysis. The two assays were then consolidated to a single assay – the AMH Generation II Beckman Coulter (Gen II) assay. This used the antibodies from the DSL assay and the calibration standards from IOT. The AMH measurements for two studies in the earlier timeframes of the project (Paper I and Paper IV) used the Beckman Coulter Generation II assay. The results were presented in pmol/l. With an on-site laboratory, the samples were delivered, spun, and stored at -20°C, and analysed within 14 days. A further assay, the ultrasensitive ELISA assay (Ansh Labs) was also developed. This was followed by the introduction of two fully automated AMH assays, the Access assay (Beckman Coulter) and Elecsys assay (Roche). The values from the Access assay are identical to the Gen II assay. The Access and Elecsys assays use identical antibodies but differ in calibration. Hence, values generated by different assays differ and assay specific interpretation is required. The last study done for the thesis, Paper II, used the Beckman Coulter Access assay. Venous blood samples were obtained and delivered to the on-site laboratory immediately, centrifuged, stored at 2-8°C, and analysed every day.

Some issues have been reported for the Gen II assay following long term storage and transport of samples. However, this did not impact any of the measurements for our studies due to the availability of an on-site laboratory and quick processing of samples.

Measurement of serum AMH was not restricted to a particular time of the cycle due to low intra-cycle variability (124)

Measurement of AFC

Ultrasound measurement of the antral follicle count can be done by manual measurement using two dimensional ultrasound. This process is however labour intensive and prone to errors especially for smaller sized follicles and ovaries with a large number of antral follicles. Substantial intra-observer and inter-observer variability is reported for the use of this method.

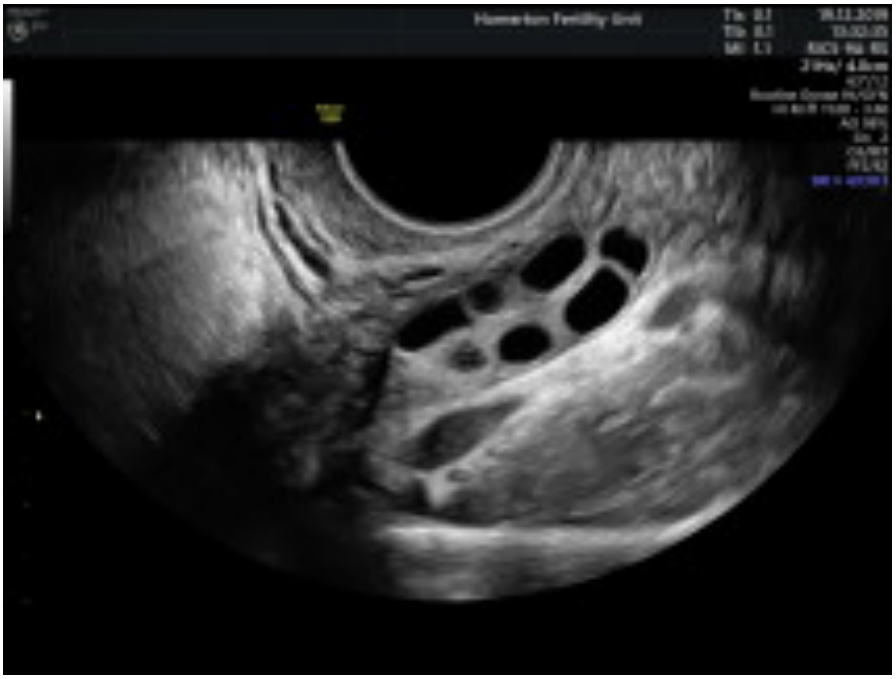

Figure 4-4 Two dimensional ultrasound image of the ovary

Three dimensional (3D) ultrasound coupled with an automated software which allows the assessment of the number and size of follicles reduces intra- and inter-observer variability and improves accuracy of measurement (125, 126).The software used identifies hypoechoic fluid filled structures as follicles and hence may sometimes detect structures other than ovarian follicles and interpret these to be antral follicles. Thus, image postprocessing is necessary to remove these artefacts.

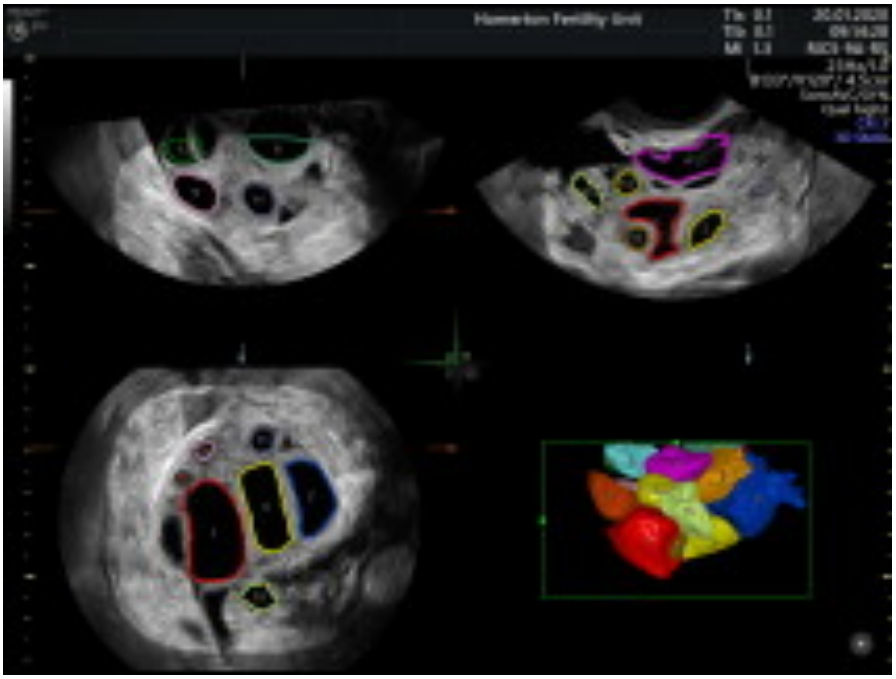

Figure 4-5 Three-dimensional ultrasound images of ovary demonstrating the technique of automated antral follicle count using sono-AVC software

All ultrasound measurements of the AFC for our studies was done by trained fertility doctors using 3D ultrasound with automated follicle counting software sono-AVC™ and manual image post-processing. We used the Voluson E6 diagnostic ultrasound system (GE Medical Systems) equipped with a multi-frequency transvaginal probe (RIC5-9-D: 4-9MHz) for the earlier study on PCOS phenotype. For the last study done in 2019-20 we used a similar but newer machine, the Voluson S10 diagnostic ultrasound system (GE Healthcare) equipped with a multi-frequency transvaginal probe (RIC5-9W-RS: 9-5MHz).

Figure 4-6 Diagnostic ultrasound systems (GE Voluson S10) used for imaging ovaries

Hormonal profiles

We assessed the detailed hormonal profiles of women included in Paper I which included women with PCOS. We measured levels of pituitary gonadotropins, serum FSH and serum LH using enzyme linked immunosorbent assay (ELISA). These were measured in the early follicular phase of the menstrual cycle when they are at their lowest level. Measurement of androgens is an important part for the diagnosis and characterisation of women with PCOS. It is recommended that either the calculated free testosterone, free androgen index or calculated bioavailable testosterone should be used to assess biochemical hyperandrogenism for the diagnosis of PCOS. Assays such as liquid chromatography–mass spectrometry (LCMS) and extraction chromatography immunoassays are recommended for the most accurate assessment testosterone (127, 128). We measured serum total testosterone using mass spectrometry (MS) coupled with liquid chromatography (LC)(LCMS) and results expressed as nmol/l. Serum insulin and SHBG were measured using chemiluminescent microparticle immunoassay.

Measurement of other variables

Body-mass index (BMI): This was calculated using a standardised formula: BMI=weight(kg)/height(m)^2 The weight of the woman was measured in light clothing without shoes using an electronic scale and the height was determined using an altimeter.

Waist-hip ratio: The waist measurement was done at the mid-point between the lower rib margin and the iliac crest. The hip measurement was taken at the widest point of the hips. All measurements were expressed in centimetres and a waist-hip ratio calculated.

Modified Ferriman Gallwey (mFG) score: This was assessed as a measure of clinical hyperandrogenism for women with PCOS. This score assesses excessive hair growth in nine body regions on a scale of 1-4. A maximum score of 36 is possible. A score of ≥ 6-8 is considered to represent clinical hyperandrogenism (129). We used a self-reported assessment by participants asking them to use information prior to cosmetic measures taken to treat hirsutism. It may be argued that objective physician assessment may be more accurate than a patient-reported assessment. Although theoretically correct, women regularly use several cosmetic measures such as shaving, waxing, epilating, bleaching, threading and laser treatment for this. Hence assessment carried out in the centre is more likely to be an inaccurate measure of the extent of hirsutism. We have used a cut off of a score of ≥ 8 for our study.

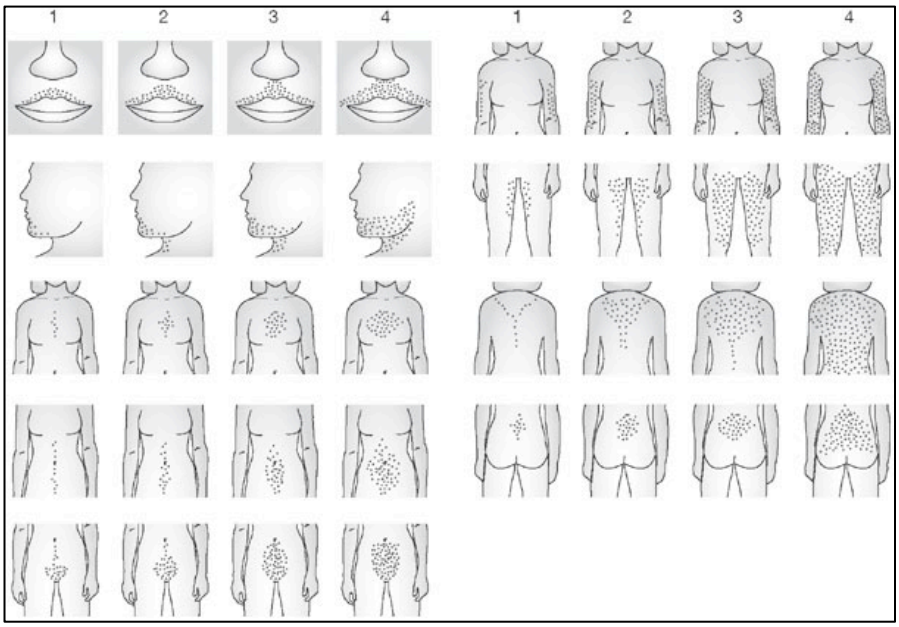

Figure 4-7 Modified Ferriman Gallwey scoring chart (British Hair and Nail Society, bhns.org.uk)

Questionnaires for smoking assessment

We used a bespoke questionnaire to obtain a self-reported smoking history for this study. We designed the questionnaire with the input of clinical and research members of the team to ensure content validity and reliability. We then tested this on a pilot sample of the target population. This highlighted deficiencies and allowed improvements in the final questionnaire used.

Carbon monoxide (CO) measurement

We measured breath CO as a biomarker of smoking as a part of the study to assess the effect of smoking on ovarian reserve markers. The device used to measure the breath CO (Smokelyser) is a CE marked, commercially available, non-invasive CO breath test that uses an electrochemical sensor to measure the breath concentration of CO with a concentration range of 0-150 ppm with a sensor sensitivity of 1 ppm and an accuracy of ±2 ppm. The instrument was used within the specified warranty period and used and serviced according to manufacturer's specifications.

Figure 4-8 Smokelyser used for breath CO measurement

Urine cotinine measurement

We also measured urine cotinine as another biomarker of smoking. The urine cotinine was measured using the DRI®Cotinine assay (Thermo Fisher Scientific). The DRI® Cotinine Assay is an in vitro diagnostic medical device intended for the qualitative and semi-quantitative determination of cotinine in human urine at a cut off level of 500 ng/mL. The accuracy of the assay has been confirmed by gas chromatography /mass spectrometry. According to

manufacturer, the sensitivity, defined as the lowest concentration that can be differentiated from the negative urine calibrator with 95% confidence, is 34 ng/mL.

Calculation of pack years

We calculated pack years using the standard formula: Number of pack-years = (packs of cigarettes smoked per day) × (years smoked). One pack equalled 20 cigarettes.

Embryoscopy and calculation of KID scores

Embryo incubation and assessment are important steps in the IVF treatment process. Standard incubators provide stable and controlled incubation. Embryo assessment is done after physically removing embryos from the incubators and examining them under the light microscope. This constitutes a snapshot assessment of the embryo at a fixed timepoint. Newer time lapse incubators incorporate a camera within the incubator allowing images of developing embryos to be taken at intervals of every 5-15 minutes during development. This creates a time lapse video of embryo development. The timing of important events in embryo division and development called morphokinetic parameters are noted. This information cannot be obtained in a standard snapshot assessment. Computerised software allows morphokinetic parameters to be collated into a single score which is indicative of embryo quality. Along with the standard morphological assessment of embryos this additional information is hypothesised to improve the predictive accuracy of embryo selection. Time lapse imaging provides a more reproducible and objective method of embryo assessment as compared to standard methodology (130).

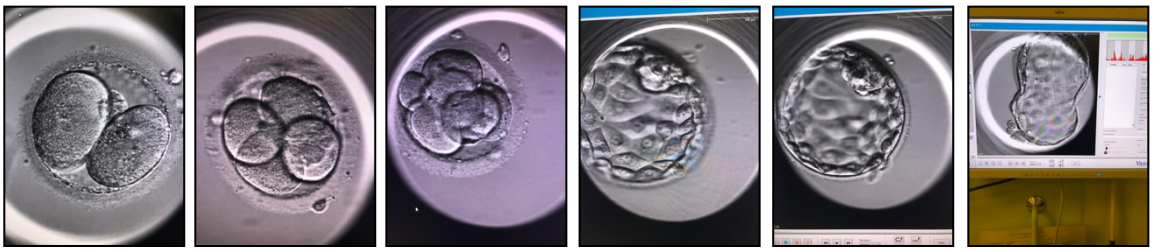

Figure 4-9 Embryo development from day 2 to day 6 demonstrated by time lapse imaging in an incubator

We used the Embryoscope time lapse incubators (Vitrolife, Sweden) for our study.

Figure 4-10 Embryoscope time lapse incubator

Embryologists placed embryos in the Embryoscope following IVF fertilisation check/ICSI and assessed them on day 3 of embryo culture. Five morphokinetic parameters related to timing and synchronicity of cell division were noted and these were combined to generate a composite score – the KID score. The annotations were, time of pronuclear fading-tPNf, time of first cell division to two cells – t2, time of 3 cells – t3, time of 4 cells – t4 and time of 5 cells – t5. The scores ranged between 1-5 with 5 denoting the best embryo quality and 1 a poor embryo quality.

4.1.7 Outcome measures and comparison groups

Paper I: The primary outcome measure was the AMH/AFC ratio as a marker for per follicle AMH production. This was compared in the four phenotypes of PCOS and isolated PCOM. The secondary outcome measures were the FAI, homeostatic model assessment (HOMA) and serum LH. These were compared in the four clinical phenotypes of PCOS and isolated PCOM and their correlation to the AMH/AFC ratio was assessed.

Paper II: The primary outcome measures were serum AMH and total AFC. These were compared between current smokers, ex-smokers and never smokers. The secondary outcome measures were lifetime smoking exposure (pack years) and biomarkers of smoking (CO and urine cotinine). Biomarkers of smoking were compared between current smokers, ex-smokers and never smokers and used to validate the group stratification and the results for the primary outcome variables. The pack years of smoking were compared between current smokers and ex-smokers and their correlation to the primary outcome measures, serum AMH and total AFC assessed.

Paper IV: The primary outcome measure was serum AMH. We compared this in the five KID score categories. The secondary outcome measure was clinical pregnancy which was also compared in the five KID score categories. We also explored the relative impact of serum AMH and the number of retrieved oocytes on the pregnancy rate.

4.1.8 Data collection

We collected all data from medical notes and electronic patient records and entered them on secure, bespoke excel databases.

4.1.9 Statistical considerations and sample size

All statistical analyses for this thesis were performed using the Statistical Package for Social Sciences (SPSS V 20-26). Before analysis all data were assessed for descriptive statistics and normality of data distribution was checked. Skewed data were log transformed before analysis. The ANOVA test, Chi-squared test or a Kruskal-Wallis test were used for univariate analysis to assess differences between baseline variables and also outcome variables. A p-value of <0.05 was considered to be statistically significant in all tests. Analysis of covariance (ANCOVA) was used to test for differences while controlling for confounding factors. Either a Pearson correlation (Paper II) test or a non-parametric correlation using Spearman rho (Paper I) was used to explore the relationships between variables of interest. In Paper IV, we performed a logistic regression analysis in order to explore the relative impact of serum AMH and the number of retrieved oocytes on the pregnancy rate. We used pregnancy as a binary outcome variable and serum AMH and number of oocytes as explanatory variables.

For Paper IV, the sample size calculation was based upon the primary outcome of serum AMH. To detect an absolute decrease in AMH from 28.28 to 10.28 pmol/l with 80% power at a 5% significance level with an enrolment ratio of 0.5, we would require 96 participants (32

smokers/ex-smokers and 64 non-smokers). We planned to recruit approximately 100 participants to compensate for dropout and loss to follow up.

4.1.10 Ethical and regulatory approvals

For Papers I and IV, ethics committee approval was not required as the study was based on routinely collected clinical data. The process of data extraction was consistent with the data protection rules. The study was approved by the Research and Development office of the Homerton University Hospital NHS Foundation Trust, London, UK. For Paper II, the study was approved by Health Research Authority and Health and Research Care Wales- Central Research Ethics Committee on 10/Apr/2019. (REC reference: 19/WA/0089).

4.2 Evidence synthesis

We conducted a systematic review and meta-analysis to study the effect of myo-inositol/di-chiro-inositol on markers of ovarian reserve in women with PCOS undergoing IVF/ICSI.

Standard methodology recommended by Cochrane was used (63). We first formulated the research question in terms of PICO (Participants, Intervention, Comparators and Outcomes).

Participants	Women with PCOS
Intervention	Treatment with either myo-inositol (MI), di-chiro inositol (DCI) or a combination of the two
Comparators	No treatment, folic acid, placebo or other treatment
Outcomes	
Primary outcomes	Serum AMH and AFC
Secondary outcomes	Number of retrieved oocytes, number of mature (metaphase II) oocytes, number of top grade embryos, pregnancy rate, live birth rate, risk of OHSS

Table 4-3 Research question in terms of PICO

We did a literature search to identify relevant studies which could answer the research question. Studies were identified by a search of electronic databases and hand searching. We formulated

the search strategy which included a combination of subject headings (MeSH/emtree) and text words relating to or describing the participants (polycystic ovary syndrome, PCOS, PCO polycystic ovar*) and intervention (inositol, myo inositol, di-chiro inositol). We searched the electronic databases MEDLINE, EMBASE, CENTRAL and CINAHL from inception till 31/12/2017. We searched review articles and guidelines, reference lists of primary and review articles and trial registries for ongoing and unpublished studies. We included all published literature; randomised trials, non-randomised comparative studies and observational studies which were published as full-length manuscripts in the English language.

After initial screening of abstracts, we obtained the full text of potentially suitable studies and assessed them for suitability. Two authors independently extracted data on a bespoke data sheet. We collected data for participants, intervention and outcomes and tabulated in 2x2 tables for dichotomous outcomes and 1x2 tables for continuous outcomes. We contacted authors of original articles when there was missing or unclear data in published manuscripts.

We presented a narrative summary of results for all outcomes listed. We assessed included studies for clinical and methodological characteristics to determine if they were sufficiently similar to allow meta-analysis and a pooled estimate of individual outcomes to be studied. We analysed the data per participant for all outcomes except for the number of top grade embryos where the unit of analysis was embryos. We presented the pooled estimates for outcomes as risk ratios (RR) for dichotomous variables and Mean Difference (MD)/Standardised Mean Difference (SMD) for continuous variables with 95% confidence intervals using the random effects model and inverse variance method. Statistical significance was assumed when $p<0.05$. Where sufficient data were available we planned subgroup analyses for the two different types of inositols: MI and DCI.

We assessed the included studies for study quality and the risk of bias. As we included randomised and non-randomised studies we used different tools for assessment of the risk of bias. We used the Cochrane risk of bias assessment tool to evaluate randomised trials. We evaluated for bias in several domains such as sequence generation and allocation concealment, adequacy of blinding of participants, assessors and outcome assessors, completeness of outcome data, risk of selective reporting of outcomes and other potential sources of bias. We used the Risk Of Bias In Non-randomized Studies – of Interventions (ROBINS-I) assessment tool for evaluation of non-randomised studies.

We evaluated the overall quality of evidence for all outcomes using GRADE criteria (risk of bias, consistency of effect, imprecision, indirectness and publication bias). We used the Guideline Development Tool software to prepare a summary of findings of the quality of evidence. We reported the judgements about strength of recommendation (high, moderate. low or very low) based on these findings.

We prospectively registered the systematic review on the International Prospective Register of Systematic Reviews (PROSPERO) (Registration: CRD42017082275)

5 Results

5.1 Paper I: PCOS phenotypes and AMH

262 women were recruited to the study. 199 had PCOS and 63 had isolated PCOM. These were divided based on their phenotype into five groups as below

PCOS A (OA+ HA+ PCOM): 91

PCOS B (OA+HA): 7

PCOS C (HA+PCOM): 59

PCOS D (OA+PCOM): 42

PCOM (PCOM): 63

The AMH/AFC ratios as a marker of per follicle AMH production was compared in the five groups. The median AMH/AFC ratios in PCOS A, PCOS D, PCOS C and PCOM were 1.5, 1.6, 1.2 and 1.1 respectively.

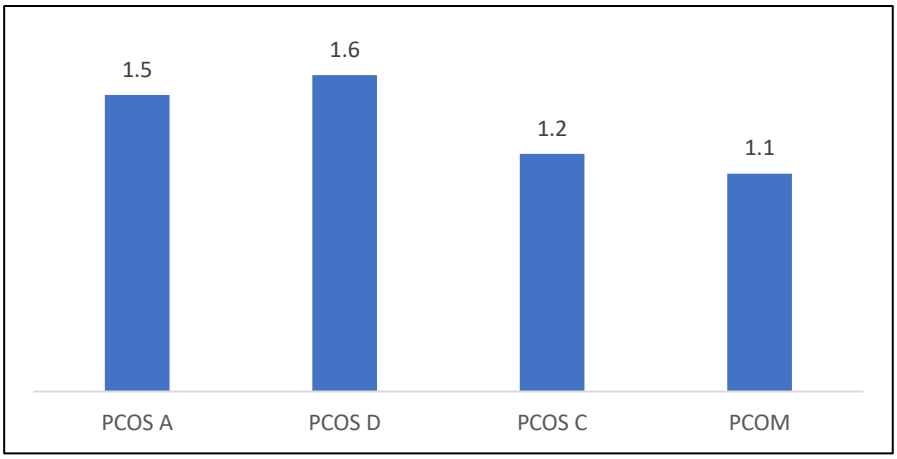

Table 5-1 Median AMH/AFC ration in various phenotypes of PCOS and PCOM (based on data from Paper 1)

Univariate analysis showed AMH/AFC ratios in the anovulatory phenotypes A and D to be significantly higher than in women with isolated PCOM (p=0.004 and 0.002 respectively). There was no significant difference in the AMH/AFC ratios between the ovulatory PCOS phenotype C and isolated PCOM (p=0.59). These findings remained unchanged after accounting for differences in age and BMI in a multivariate analysis.

Non-parametric bivariate correlation using Spearman rho showed a significant but weak positive correlation between the AMH/AFC ratio and the FAI (r =0.274, n=249, p=0.001) and LH (r =0.280, n= 249, p<0.001). No correlation was seen between the AMH/AFC ratio and insulin (r =0.154, n=249, p=0.123).

5.2 Paper II: Smoking and ovarian reserve markers

101 women were recruited to the study from July 2019 to February 2020. Based on a self-reported smoking history women were classified into three comparison groups: current smokers, ex-smokers and never smokers. The baseline clinical characteristics of the participants such as age, BMI, ethnicity, history of ovarian surgery, infertility diagnosis and diagnosis of PCOS were compared in the three groups. There were no significant differences seen in the baseline variables amongst the groups.

Pack years of smoking, quantifying exposure to cigarette smoking, were not significantly different between current and ex-smokers (F(1,25) = 0.547, p=0.467). The breath CO levels

were significantly different amongst current, ex- and never smokers (F(2,97) = 33.32, p<0.0001). Urine cotinine levels were also significantly higher in current smokers as compared to ex-smokers and never smokers. (p< 0.001). Current smokers reported to be more exposed to passive smoking (75%, 9/12) as compared to ex-smokers (20%, 5/25) and never smokers (25%, 16/64) (p=0.001).

No significant difference was observed amongst current, ex- and never smokers either for serum AMH (F(2,91) = 1.19, p=0.309) or total AFC (F(2,81) = 0.403, p= 0.670). When comparing baseline variables, age showed borderline non-significance between the groups (p=0.057). Hence, we performed an analysis of covariance (ANCOVA) to explore the impact of smoking status on serum AMH using age as a covariate. No significant difference was demonstrated among the three groups (F(2,90) = 0.398, p = 0.673).

No significant correlation was demonstrated between the pack years of smoking and serum AMH (r= -0.212, n=23, p=0.166) or total AFC (r= -0.276, n=19, p=0.126). No significant correlation was found between breath CO and serum AMH (r= 0.082, n=94, p=0.216) or total AFC (r= 0.096, n=83, p=0.195). Similarly, no significant correlation was found between urine cotinine levels and serum AMH (r= 0.146, n=83, p=0.095) or total AFC (r= -0.027, n=77, p=0.386).

5.3 Paper III: Systematic review on inositols and ovarian reserve

18 studies were selected for the review. Seven studies assessed changes in either AMH or AFC or both. Twelve studies evaluated outcomes following IVF/ICSI treatment. One study evaluated both categories of outcomes.

5.3.1 Included studies:

Of the studies assessing changes in AMH and /or AFC, only two of the seven studies were randomised controlled trials and five were non-randomised studies. All studies were single centre studies. A total of 415 women were recruited to these seven studies. A majority of the studies, five of the seven, were conducted in Italy. Although all participants in the included studies were diagnosed with PCOS, there were varying restrictions in the inclusion criteria based on age, body mass index and insulin resistance in some studies. We have included a study (Pkhaladze 2017(131)) which recruited participants between 13-19 years of age based

on a consensus opinion from the international paediatric subspecialty societies. There was a wide variation in the preparation of inositol used (MI and DCI), doses administered (ranging from 1 gm to 4 gm daily) and duration of administration (between 12 weeks to 6 months) with no explanations for these variations. Four studies assessed the effect on serum AMH and three assessed its effect on AFC.

10 of the 12 studies assessing reproductive outcomes after IVF/ICSI were RCTs and only two were non-randomised studies. All studies were single centre studies. Again, most of the studies were conducted in Italy (10 of the 12). A total of 1225 women, all with a diagnosis of PCOS were included in the 12 trials. Some studies restricted inclusion of participants based on age, body mass index and insulin resistance. Heterogeneity in the preparation of inositols used, dose (1-4 gm /300-1200 mg daily) and duration of treatment (2 weeks to ongoing treatment) were observed. Details are described in the manuscript.

5.3.2 Risk of bias and quality of evidence in included studies

Of the randomised trials, 75% of trials were at low risk for selection bias for random sequence generation but only 17% were considered low risk for allocation concealment. Similarly, only 25% of studies were considered at low risk of performance bias due to blinding. The risk of attrition bias due to incomplete outcome data was low in 42% of studies. None of the trials were considered at low risk of reporting bias.

All the non-randomised studies were considered at a high risk of selection bias due to the presence of confounders or inclusion of participants into the study. 83% were low risk for bias in classification of interventions and 67% were at low risk of bias due to missing data. 50% of studies did not provide information on nonconformities in the planned interventions and all of the studies were at high risk of reporting bias.

The quality of evidence contributing to the review was considered to be very low across most domains. These included inconsistency, indirectness, imprecision and a high risk of bias.

5.3.3 Synthesis of the results:

5.3.3.1 Primary Outcomes:

Anti-Mullerian Hormone

The studies assessing change in serum AMH before and after treatment with inositols showed significant clinical and methodological heterogeneity. Hence it was deemed unsuitable to

include these results for a meta-analysis. Five studies with 172 participants were included in this section of the review. The results for a standardised mean difference in levels of serum AMH before and after treatment for the five individual studies showed was no consistent direction or size of effect. This is graphically represented in the published manuscript.

Antral follicle count

We did not perform a meta-analysis for the studies which assessed changes in the antral follicle counts after treatment with inositols due to clinical and methodological heterogeneity of the included studies. Four studies with 143 participants were included in this section of the review. The SMD show a high heterogeneity in effect size for AFC. This is graphically represented in the published manuscript.

Overall, the review is unable to demonstrate a clear change in AMH or AFC values following treatment with inositol.

5.3.3.2 Secondary Outcomes:

Number of retrieved oocytes

Eleven studies reported the number of oocytes retrieved. Seven RCTs comprising 722 participants were included in the meta-analysis. We were unable to find a statistically significant difference between the intervention and control arms (MD -0.39, 95% CI -1.11, 0.33). Similar results were seen for a subgroup analysis for MI (MD -0.76 95% CI -2.04, 0.52) and DCI (MD -0.18 95% CI -1.11,0.74).

Data from two studies (Ciotta 2011(132) and Lesoine 2016(133))could not be included in the meta-analysis as data were presented in an unsuitable format suitable for meta-analysis. These studies reported contradictory results. Ciotta 2011 reported a significantly higher number of oocytes following treatment with MI as compared to control ($p<0.05$) in contrast to Lesoine 2016 who reported a higher number of oocytes in the control group as compared to the MI group. Alviggi 2016 (134) in a non-randomised study reported no significant difference between the MI and control groups ($p=0.23$) similar to Unfer 2011 (135) who compared treatment with MI to DCI.

Number of metaphase II oocytes

10 studies reported on the number of metaphase II oocytes retrieved. Three RCTs with 207 participants were included in the meta-analysis. No statistically significant difference was seen between the intervention and control arms (MD 0.29, 95% CI -0.83, 1.40). Similar results were seen for a subgroup analysis for MI (MD -0.32 95%CI -1.49, 0.86). Seven other trials could not be included in the meta-analysis as data from these studies were in an unsuitable format for inclusion in the meta-analysis. They reported contradictory results with no consistent direction of effect. Piomboni 2014 (136) reported significantly higher number of MII oocytes, with a moderate effect size, following treatment with DCI as compared to control (MD 1.30 95%CI 0.15, 2.45) in contrast to Isabella 2012 (137) who reported a significant decrease in the number of MII oocytes following treatment with DCI as compared to control. Artini 2013 (138) reported a significant increase in the percentage of top quality oocytes in the group treated with MI as compared to control ($p<0.05$). Pacchiarotti 2016 (139) and Ciotta 2011 reported no significant differences between the groups. The results from Lesoine 2016 are unclear. Alviggi 2016 in a non-randomised study reported a significant increase in the number of MII oocytes following treatment with MI as compared to control ($p=0.03$). Unfer 2011 compared treatment with MI to DCI and reported a significant increase in the number of MII oocytes following treatment with MI as compared to DCI ($p<0.05$).

Number of top-grade embryos

Four RCTs comprising 957 participants and reporting number of top-grade embryos were included in the meta-analysis. We were unable to find a significant difference between the groups (RR 1.02, 95% CI 0.93-1.12). Four studies reporting this outcome had data in an unsuitable format for inclusion in the meta-analysis. These studies did not show a consistent direction of effect. Pacchiarotti (2016) reported no significant difference in the number of top-grade embryos between the MI and control groups. Isabella (2012) reported a significant decrease in the number of top-grade embryos following treatment with DCI ($p<0.01$) in contrast to Lesoine (2016) who reported a significantly higher number of top-grade embryos following treatment with MI as compared to control ($p<0.05$). Unfer (2011) reported a significantly higher number of top-grade embryos following treatment with MI as compared to treatment with DCI ($p<0.01$).

Clinical pregnancy rate

Three RCTs comprising 488 participants and reporting clinical pregnancy rates were included in the meta-analysis. We were unable to find a significant difference between the groups (RR

1.16 95% CI 0.87, 1.53). Three studies (Schillaci 2012 (140), Alviggi 2016 and Wdowiak 2016 (141)) did not detail if the pregnancies were biochemical or clinical and were hence not included in the meta-analysis. These studies reported no significant differences between the groups. When comparing treatment with MI to DCI Unfer (2011) reported no significant difference in clinical pregnancy rates between the two groups.

Live birth rate

Only one trial (Artini 2013) reported live birth rate. This reported a significant improvement following treatment with MI compared to placebo ($p<0.05$).

Risk of cycle cancellation due to the risk of OHSS

Six studies reporting cycle cancellations due to the risk of ovarian hyperstimulation syndrome were included in the meta-analysis. We were unable to demonstrate a significant difference between groups (RR 0.73, 95% CI 0.39, 1.37). Similar results were seen for a subgroup analysis for MI (RR 0.70 95% CI 0.34, 1.42) and DCI (RR 0.85, 95% CI 0.22-3.29).

5.4 Paper IV: Serum AMH and embryo quality

198 participants and 304 embryos were included for analysis. Embryos were categorised into five groups based on the KID scores. For the primary outcome, serum AMH, univariate analysis demonstrated no significant differences between the five KID score categories ($F(4,293) = 1.769$, $p = 0.135$). Univariate analysis however showed significant differences in age and method of insemination for the different KID score categories ($p=0.049$ and $p=0.033$ respectively). Hence and ANCOVA test was done to test for differences in AMH levels among KID scores while controlling for significant confounding variables. This showed no significant differences ($p = 0.305$).

There was a significant difference for the secondary outcome of pregnancy rate between the KID score categories. Serum AMH however did not have a significant impact on pregnancy rates.

6 Discussion

6.1 Main findings

Paper I evaluated the per follicle AMH production in the various phenotypes of PCOS and isolated PCOM. The study showed a significantly increased per follicle AMH production in the anovulatory phenotypes of PCOS as compared to women with isolated PCOM. No significant difference was found between the ovulatory phenotype of PCOS and isolated PCOM. The study was unable to show a substantial role for androgens, insulin or LH in the increased per follicle AMH production.

Paper II examined the association between cigarette smoking and ovarian reserve parameters. We did not find a statistically significant difference in either serum AMH or AFC between current, ex and never smokers in our study population. By demonstrating significant differences in breath CO and urine cotinine levels among the groups we confirmed that self-reported smoking correlates well with quantitatively measured markers of smoking. We did not find a significant correlation between the pack years smoked and serum AMH and AFC. We did not find a significant association between biomarkers of smoking and biomarkers of ovarian reserve.

Paper III assessed the impact of pharmacological intervention with the insulin sensitiser, inositol, on ovarian reserve markers using evidence synthesis. Our systematic review was unable to demonstrate a consistent direction or size of effect for a change in serum AMH or AFC after treatment with inositols. Thus, we are unable to provide adequate evidence for a quantitative change in the antral follicle pool subsequent to treatment with inositols. We were unable to perform a meta-analysis for the primary outcome due to substantial clinical and methodological heterogeneity of the published studies. No significant differences between the inositol and control groups were seen for any of the secondary outcomes of the review. These included the number of retrieved oocytes, number of metaphase II oocytes, number of top-grade embryos, pregnancy rates and the rates of cycle cancellation due to the risk of OHSS.

Paper IV assessed the association between ovarian reserve and embryo quality in women undergoing IVF/ICSI treatment. There was no significant association between baseline serum AMH at the start of IVF treatment and embryo quality assessed by time lapse imaging. Serum AMH positively correlated to pregnancy rates due to the increased number of oocytes retrieved

during the treatment rather than an impact of serum AMH on embryo quality. Embryo quality showed a significant positive association with clinical pregnancy rates. These results remain important for counselling women who plan to start IVF treatment.

6.2 Interpretation of results

For Paper I, the finding of a greater per follicle production of AMH in anovulatory PCOS supports the hypothesis for a role for AMH in the mechanism of ovulatory dysfunction. High intra-follicular concentrations of AMH due to an increased per follicle production are postulated to cause a greater inhibition of FSH induced aromatase expression. This results in a heightened inhibitory response to follicular maturation and ovulation resulting in the ovulatory dysfunction associated with PCOS.

The mechanism for this increased per follicle AMH production remains unknown. Although the FAI showed a weak significant correlation with the AMH/AFC ratio other indicators were unable to support a role for androgens. The high per follicle AMH production was not different in the androgenic (A) and non-androgenic (D) phenotypes. Also, it was similar in the androgenic phenotype C and isolated PCOM which is normo-androgenic. It may be suggested that the positive correlation of serum AMH and androgens (142) may be due to increased folliculogenesis rather than an increased per follicle production. LH levels were not significantly different amongst PCOS phenotypes and LH showed only a weak correlation with the AMH/AFC ratio. It is unclear if the positive correlation between serum AMH and LH in women with PCOS (36) is due to an independent action of LH or indirectly through the androgen pathway. Similarly, our study indicate a limited role insulin as a factor leading to an increased per follicle AMH production. The correlation of insulin with serum AMH is likely to be similar to androgens as it may increase serum AMH through androgen mediated folliculogenesis rather than increasing the amount of AMH produced per follicle. The absence of a substantial association between androgens, LH and insulin and the per follicle AMH production, points to an intrinsic granulosa cell dysfunction. A recent meta-analysis of genome wide association studies suggests a similar genetic architecture for phenotypically distinct subtypes of PCOS (143).

For Paper II, although biological plausibility exists for the effect of smoking on ovarian reserve and ovarian ageing, these effects were not evident in our study population of younger women based on serum AMH and AFC. Animal studies have suggested adverse effects of cigarette smoking on ovarian reserve (83, 84). Several mechanisms have been postulated, which may affect quality, quantity or both. Gannon et al (2012),(85) hypothesised a mechanism of direct toxicity to ovarian follicles resulting in an accelerated follicle loss. An indirect effect on ovarian follicle numbers has been suggested through an action on the hypothalamic pituitary axis (144).

The discordance of our results with some previously published studies may be attributed to several reasons. The natural decline of ovarian reserve with age does not follow a linear function but shows a rapid decline with increasing age (41). It has also been suggested that ovarian follicles may differ in susceptibility to the effects of smoking at different ages with older oocytes being more susceptible to negative effects of smoking. Hence, the effects may not evident in our study population of younger women. The effect of smoking may be dose related. The pack years of smoking in our study population was relatively low at 2.13 pack years. It is possible that the deleterious effects are evident only at higher levels of smoking exposure or lower levels of smoking may be associated with smaller magnitude of reduction in ovarian reserve markers. Although it may be possible to demonstrate such small differences with a larger sample size, the clinical implications of such findings would be questionable. Serum AMH and AFC are largely used in young women in the context of fertility treatment, to predict ovarian response to treatment and pregnancy rates. Hence in younger women seeking fertility treatment, a clinically relevant decrease in ovarian reserve may be considered one which significantly reduces the probability of the most important outcome for this group of women; the pregnancy rate. Significantly lower pregnancy rates have been reported in the lowest quartile of AMH below 10.28 pmol/l (111). Pregnancy rates in women with serum AMH in the upper three quartiles are not statistically different from each other (111). The absence of an association between smoking and serum AMH and AFC also argues for a mechanism against follicular atresia. This is strengthened by the finding of no association between ex-smokers and lower AMH values in our study and also in other studies such as Dolleman et al (2013)(53).

For Paper III, based on the included evidence we are unable to recommend treatment with inositols with the aim of changing the antral follicle pool/ovarian reserve markers or improving outcomes following IVF/ICSI treatment. Although ovarian reserve markers are not endpoint clinical outcomes, they remain important for several reasons. Firstly, the antral follicle pool is

dynamic and as discussed before is amenable to change due to the influence of external factors. Inspite of a normal primordial follicle pool, serum AMH and AFC are diminished in states where ovarian steroidogenesis and subsequent folliculogenesis has been suppressed such as long term treatment with oral contraceptives, GnRH agonists or states of hypothalamic amenorrhoea. Similarly, the antral follicle pool is greater in women with PCOS. Initial assessment and subsequent alteration of the antral follicle pool may result in improved reproductive outcomes for these women.

Inositols have shown to significantly reduce androgen levels in women with PCOS (145). Hence, due to their biological plausibility to alter ovarian folliculogenesis, changes in serum AMH and AFC following treatment with inositols would support the mechanism of action of inositols through the insulin-androgen-folliculogenesis pathway.

Lastly, knowledge of changes to the antral follicle pool would help to plan fertility treatment and IVF/ICSI protocols. Reduction in the quantitative antral follicle pool would reduce the risk of OHSS which remains an important iatrogenic complication of IVF in women with PCOS.

From Paper IV, our results demonstrate that serum AMH and AFC remain excellent markers of quantitative ovarian reserve but show no association with the quality of embryos. Although pregnancy rates may relate positively with serum AMH and AFC, this effect is indirect through the increased number of oocytes retrieved and hence the possibility of better quality of embryos being available for transfer. These results remain very important for counselling women embarking on fertility treatment.

6.3 Comparison with previous literature

For Paper I, the findings of our study agree with those of Alebic et al (2015)(146). Their in-vivo study also analysed the per follicle AMH production in women with PCOS and PCOM and found a higher per follicle AMH production in the anovulatory phenotypes of PCOS. In vitro studies show agreement with our findings (28).

For Paper II, our results agree with those of Bressler et al (2016) (55). They were unable to demonstrate an association between smoking exposure and serum AMH in a population based cross-sectional analysis. The age of their study population was women aged 23-35 years which is similar to that of our study. However, exposure ascertainment was done using only a self-

reported questionnaire. Similarly, Kline et al (2016) reported no association between AMH and smoking in a cross-sectional study using self-reported smoking to ascertain exposure. Dolleman et al (2013) in a large population based study reported lower serum AMH in current smokers (but not in ex-smokers) as compared to never smokers. The study population was however significantly older (mean 37.3, SD 9.2) than our study population, which may explain a difference in the results. It has been suggested that the increase in follicular decline may be accelerated and more evident with advancing age(144). Also, the smoking exposure in pack years was higher in this population (mean 10.2, SD 9.1) as compared to our study (median 2.13 (IQR 0.59-3.48)) which could account for the differences. Dolleman et al (2013), reported a threshold after which the linear association of pack years and serum AMH was significant. They reported this at 10 pack years of smoking below which there was no significant association with serum AMH. Hence, these results could be considered to be in agreement with our study.

For Paper III, to our knowledge, this is the first review to assess the effect of inositols on ovarian reserve markers. Other systematic reviews have reported the effects of inositols on outcomes following IVF/ICSI treatment (147-150). The first of these reviews by Unfer et al (2016)(147) included only five studies and reported an improvement in oocyte and embryo quality. The findings of this review are in disagreement with those of our review. The review however was a narrative systematic review rather than a meta-analysis. The discordant results may be due to positive outcomes in earlier studies included in this review, in contrast to contradictory findings in some recent studies included in our review resulting in a pooled estimate showing no significant difference in outcomes. The review does not discuss the quality of evidence presented or the risk of bias and there may be concerns about publication bias. Similar positive outcomes were reported by Gateva et al (2018) (148). Being a narrative review rather than a systematic review and being published by the same group of authors lends it to similar criticisms. A more recent review by Mendoza et al (2017)(149) reports findings broadly in agreement with our review. However, the findings of our review may be considered more robust as we have added 4 more studies with 206 participants. We have reported on ovarian reserve markers which was not considered and discussed by Mendoza et al (2017). We were also the first to review the effect of inositols on the risk of OHSS in IVF/ICSI treatment and provide a pooled effect estimate. This is an important safety outcome for any trial intervention on women with PCOS who are at a greater risk of OHSS following IVF/ICSI. Our review has also provided a formal and objective assessment of the quality of evidence included. Morley et

al (2017), in a Cochrane review include a section on the effect of inositols in women with PCOS (150). Due to very stringent inclusion criteria only two studies and none of the outcomes of this review are included. Similarly, a very recent Cochrane review discusses the effects of inositols on outcomes following IVF/ICSI treatment (151). The two outcomes investigated by this review (live birth and clinical pregnancy) are in broad agreement with those reported by our review.

For Paper IV, our results are in agreement with those of Smeenk et al (2007), which demonstrated a positive correlation between serum AMH and the number of oocytes and embryos but was unable to show a predictive capacity with respect to embryo quality. Embryo grading was done by morphological assessment on day 3 of embryo culture (152). In a study by Fong et al (2008), participants were allocated to receive either standard stimulation or mild stimulation. In the standard stimulation group there was no correlation seen between serum AMH at start of treatment and embryo morphology. These participants also had pre-implantation genetic screening of embryos. There was no correlation seen between baseline serum AMH and aneuploidy rate (153). This supports the results of our study. When serum AMH was measured on the day of hCG trigger, a significant decrease in serum AMH was seen in the standard stimulation group but not the mild stimulation group. In the mild stimulation group, baseline serum AMH showed a positive correlation with embryo quality. The authors have hypothesised this to be due to the mild stimulation protocol, which allows recruitment of only the most FSH sensitive follicles which constitute the better quality oocytes. However, no correlation between serum AMH and aneuploidy rates were demonstrated even in the mild stimulation group. Although invasive, PGS is a more objective assessment than morphology alone and hence in that sense, the results of this study would agree with our findings. Silberstein et al (2006)(154) reported similar results to Fong et al (2008). Higher serum AMH concentrations on the day of hCG trigger, with levels greater than 2.7 ng/ml were associated with better embryo quality. Some studies have measured and reported follicular fluid AMH and its positive correlation to embryo quality (155). The pragmatic value of the measurement of serum AMH on the day of hCG or follicular fluid AMH may be considered academic as it is impossible to change management, delay or stop treatment at this point for an individual participant.

More recent studies confirm the findings of our study. Morin et al (2018), reported results agreeing with our findings. In women < 38 years, no differences were seen in blastulation rates, aneuploidy rates and live birth rates despite differences in baseline serum AMH levels (156).

A more recent study which assessed morphokinetic parameters in normal and low responders based on baseline serum AMH levels found no difference in embryo quality between the two groups (157).

6.4 Strengths and limitations

We have used a cross-sectional study design for all the three primary studies. As none of these studies had an active intervention, treatment or deliberate exposure and aimed to identify an association between two or more variables of interest, we considered this to be the most suitable design to answer the research questions.

For Paper I, a cross-sectional design allowed identification of an association between PCOS phenotype and the per follicle AMH production. Although unable to infer causality, as both variables (exposure and outcome) would be assessed at the same time point, it could be useful to support a hypothesis for causation of ovulatory dysfunction based on biological plausibility.

For Paper II, which aimed to assess the impact of smoking on ovarian reserve markers, a cross-sectional design was suitable to identify this association, especially as the exposure (smoking) could not be deliberate. Again, although a causal relationship cannot be established, associations in different smoking categories such as current and ex-smokers could support hypotheses for causality. A cross-sectional design was also appropriate for Paper IV to understand the association between ovarian reserve markers and embryo quality. However, with this design it was not possible to derive a causal relationship between the two.

Bias is inherent to all observational study designs. Hence, we have carefully considered factors which may introduce bias and adjusted for these as far as possible in either in the design or analysis phases of the work packages.

In the design phase we carefully selected the inclusion criteria appropriate for each study. For Paper I on women with PCOS, we included all eligible women with PCOS and isolated PCOM in the defined study period. As individual patient consent was not required this minimised volunteer bias. Using well defined objective inclusion criteria allowed appropriate characterisation of the participant groups. The women with PCOS were in the reproductive age group and came from diverse social and ethnic backgrounds making the study group representative of the population to be studied. For Paper II on smoking and ovarian reserve,

the participants included an unselected population of women attending the clinic for various investigations and treatments. There were wide variations in the baseline characteristics of participants such as ethnicity, cause of infertility and diagnosis. By using a wide-ranging unselected population of women, we have attempted to improve the generalisability of the results. Age remains a major determinant of ovarian reserve. We have included only women 35 years and younger to reduce bias due to the impact of advancing age. The participants included only sub-fertile women with a limited range of BMI and age. This is because fertility treatment within the UK and funded by the National Health Service is restricted by limits on age and BMI. Therefore, caution should be exercised when extrapolating these results to other populations. For Paper IV related to embryo quality and ovarian reserve in women undergoing IVF, we attempted to reduce selection bias using wide inclusion criteria and hence consider the population studied to be representative of the wider population of women undergoing IVF/ICSI treatment. As no systematic criteria were used to place individual participant embryos in either standard incubators or time lapse incubators, it is unlikely that these populations would be sufficiently different to introduce bias and affect results. Volunteer bias was minimal as informed consent of individual participants was not required and all eligible participants were included.

As discussed earlier, all women included for the work packages in the thesis were patients at the fertility clinic. Hence, caution should be exercised when extrapolating the results to the general population. However, as the research questions were related to fertility and fertility treatment, we agreed that this would be an appropriate population for conducting the studies.

We have collected data for confounding variables which may affect the direction and strength of the associations assessed and accounted for these during statistical analysis for all our studies. Ascertainment of all our exposures and outcome measures were well-defined, objective and validated, thus reducing observer bias.

Serum AMH and AFC were assessed with standardised measurements to reduce variability. All hormonal measurements were conducted using standardised assays. Androgens were measured using mass spectrometry (MS) coupled with liquid chromatography (LC) as recommended by international guidelines (128).

We have used breath CO and urine cotinine as biomarkers of smoking to validate self -reported smoking history. This is in agreement with previously reported studies. Marrone et al (2010), report significantly higher breath CO and cotinine levels in smokers compared with non-

smokers (P<0.001), with 100% specificity and sensitivity at a concentration of 5ppm (158). Similarly, MacLaren et al (2010), reported a strong agreement between self-reported smoking and breath CO levels with a sensitivity of 96% and specificity of 93.3% using a cut off of 7ppm (159).

A major strength of our study on smoking is that we used a comprehensive and detailed self-reported questionnaire to assess smoking exposure, which allowed estimation of lifetime smoking exposure in terms of pack years and also accounted for passive smoking. Furthermore, we also used breath CO and urine cotinine concentrations to validate our study groups. The CO breath test shows the amount of CO in the breath (ppm), as an indirect, non-invasive measure of blood carboxyhaemoglobin (%COHb). CO leaves the body rapidly and the half-life is about 5 hours. Within 24 to 48 hours of not smoking, smokers will be at non-smoker levels. Cotinine is the predominant metabolite of nicotine. It has a half-life of 20 hours and is detectable for up to one week after the use of tobacco. This is useful to identify smokers who have abstained from smoking for several hours. Our study was powered only to detect differences in ovarian markers of relatively large magnitude that we considered to have a clinical significance in the management of young women seeking fertility treatment. However, a much larger sample size would be required to detect statistically significant differences of smaller magnitude which may be relevant to different study populations and research questions.

The use of the time lapse incubators for embryo grading and assessment may be considered a major strength of this study. The use of this system, which provides greater detail and allow a more objective and reproducible assessment of embryo quality has eliminated ascertainment/observer bias. Smoking status for this study was the only self-reported variable which may have been subject to reporting bias. This however was unlikely to affect the results as the distribution of smokers and non-smokers was not significantly different in the compared groups. For women with a double embryo transfer the values for serum AMH were duplicated during analysis. This may be considered a limitation. However, including only those women with a single embryo transfer would have considerably reduced the number of women in the dataset as only 25-30 % of all transfers are single embryo transfers in addition to introducing a selection bias.

For our systematic review we have used standard Cochrane methodology to ensure quality assurance. To minimise the impact of reporting bias, we have aimed to identify all eligible

studies for inclusion in the review by conducting a thorough search of the published literature. We evaluated studies for prospective trial registration and availability of a trial protocol to assess in-study reporting bias. We have included of all types of published literature rather than only randomised controlled trials for the review. Although it may be argued that inclusion of non-randomised studies has the potential to introduce bias and confounding, we have included these with the aim of increasing external validity and to provide a comprehensive review. Although RCTs may be generally accepted to safeguard against a biased estimate of the treatment effect, literature reports that the direction and magnitude of the effect may not differ due to inclusion of non-randomised studies in many cases (160). Where information was missing, we have attempted to contact the study authors to seek further information. Some information was received from authors but as we were unable to get information from others we were unable to include data from these studies.

Specific problems with study design, especially heterogeneity of populations and intervention were identified. The quality of evidence included in this review was assessed to be very low. This related to the risk of bias, imprecision, inconsistency, indirectness and publication bias. Major concerns regarding study design, prospective trial registration, sample sizes, sample size calculations and blinding were noted. All these may be considered major limitations of the evidence synthesized.

7 Future research

Our study on the per follicle AMH production supports the hypothesis for the mechanism of anovulation in PCOS. The correlation between the magnitude of higher per follicle AMH and the degree of increased FSH resistance remains unclear. Several other variables/factors in addition to an increased per follicle AMH production may impact the degree of FSH sensitivity and response to ovulation induction treatments. Cluster analysis of large cohorts utilising anthropometric, ultrasound and biochemical markers along with response to ovulation induction treatments will identify of specific subtypes allowing a more targeted approach to treatment.

Inositols are commonly prescribed by clinicians and patients themselves without good evidence about their benefit. Our review highlights the need for further studies with robust design and adequate sample size are needed to provide a definitive answer to the question. An

ideal study should aim to include an appropriate PCOS population with selection based on criteria such as BMI and insulin resistance. The dose and duration of effective treatment should be sought in appropriately designed dose-response studies. The two inositol isoforms need to be studied separately and in different dose combinations.

8 Conclusions

The results of our study show an association between AMH and ovulatory dysfunction, with a greater per follicle AMH production in anovulatory phenotypes as compared to isolated asymptomatic PCOM. This supports the hypothesis for a patho-physiological role for AMH in women with anovulatory PCOS where the physiological inhibitory role of AMH appears to be heightened. The absence of an association between androgens, LH and insulin to the per follicle AMH production, points to an intrinsic granulosa cell dysfunction.

Exposure to cigarette smoking in women ≤ 35 years seeking fertility treatment did not significantly change the antral follicle pool and no significant association was found between biomarkers of smoking or lifetime smoking exposure and biomarkers of ovarian reserve.

A systematic review of published literature did not provide adequate evidence for a change in ovarian reserve following the use of inositols in women with PCOS and to support the use of inositols as a pre-treatment in women with PCOS undergoing IVF/ICSI treatment to improve safety of the procedure or reproductive outcomes. The results of our study show no significant association between serum AMH and embryo quality in women undergoing IVF/ICSI treatment. Their positive association with improved pregnancy outcomes following IVF/ICSI is likely to be indirect through the increased numbers of oocytes retrieved.

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10 Appendix: Papers I-IV

Paper I

Phenotypic variation in anti-Mullerian hormone (AMH) production per follicle in women with polycystic ovary syndrome (PCOS) and isolated polycystic ovarian morphology (PCOM): an observational cross-sectional study.

Bhide P, Kulkarni A, Dilgil M, Dhir P, Shah A, Gudi A, Homburg R

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Paper II

Effect of cigarette smoking on serum anti-Mullerian hormone and antral follicle count in women seeking fertility treatment.

Bhide, P., Timlick, E., Kulkarni, A., Gudi, A., Shah, A., Homburg, R., Acharya, G.

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Title

Effect of cigarette smoking on serum anti-Mullerian hormone and antral follicle count in women seeking fertility treatment.

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Abstract page

Effect of cigarette smoking on serum anti-Mullerian hormone and antral follicle count in women seeking fertility treatment.

P Bhide, E Timlick, A Kulkarni, A Gudi, A Shah, R Homburg, G Acharya

Objective

The relationship between smoking and markers of ovarian reserve is inconclusive. The primary objective of our study was to assess the effect of cigarette smoking on the quantitative ovarian reserve parameters, serum anti-Mullerian hormone (AMH) and antral follicle count (AFC) in women seeking fertility treatment. Our secondary aims were to validate self-reported smoking behaviour using biomarkers and evaluate the association between biomarkers of ovarian reverse (serum AMH and AFC) with biomarkers of smoking exposure (i.e. breath carbon monoxide (CO) and urine cotinine levels).

Study Design

We conducted a single-centre, cross-sectional study in women ≤ 35 years and assessed the association between markers of cigarette smoking (self-reported smoking history, breath CO and urine cotinine) and serum AMH and AFC.

Results

Significant differences were found amongst current smokers, ex-smokers and never smokers for breath CO ($F(2,97)=33.32$, $p< 0.0001$) and urine cotinine levels ($p< 0.001$). However, no significant differences were found either for serum AMH ($F(2,91)=1.19$, $p=0.309$) or total AFC ($F(2,81)=0.403$, $p=0.670$) among these three groups. There was no significant correlation between the pack years of smoking and serum AMH ($r=- 0.212$, $n=23$, $p=0.166$)

or total AFC (r=-0.276, n=19, p=0.126). No significant correlation was demonstrated between breath CO and serum AMH (r=0.082, n=94, p=0.216) or total AFC (r=0.096, n=83, p=0.195). Similarly, no significant correlation was demonstrated between urine cotinine levels and serum AMH (r=0.146, n=83, p=0.095) or total AFC (r=-0.027, n=77, p=0.386).

Conclusion

We did not find a statistically significant difference in quantitative ovarian reserve markers between current, ex- and never smokers in our study population. We confirmed that self-reported smoking correlates well with quantitatively measured markers of smoking, validating the comparison groups based on self-reported smoking history to ensure a valid comparison of outcome measures. There was no significant association between biomarkers of smoking and biomarkers of ovarian reserve. We were also unable to demonstrate a correlation between the lifetime smoking exposure and ovarian reserve.

Keywords

Ovarian reserve, anti-Mullerian hormone, antral follicle count, smoking, cotinine, carbon monoxide

1 Introduction

Anti-Mullerian hormone (AMH) and antral follicle count (AFC) are well established biomarkers of ovarian reserve, commonly used in the context of fertility treatment(1, 2). Estimation of the size of the primordial follicle pool is difficult and impractical for routine clinical application as there is no known biochemical marker for estimating the number of primordial follicles, and their small size makes in-vivo imaging with sufficient resolution impossible using currently available technology. A subsection of the true ovarian reserve is the pool of pre-antral and antral follicles which are responsive to pituitary gonadotropins and are clinically relevant for menstruation, ovulation and fertility. The currently available biomarkers, AMH and AFC, measure the antral follicle pool. AMH is expressed exclusively by the granulosa cells of pre-antral and small antral follicles in the ovary and hence an excellent quantitative marker of the ovarian reserve(3). Antral follicle counts assessed by ultrasound scan measure the same biological entity and show a strong positive correlation with serum AMH levels(4).

Age remains one of the most important determinants of ovarian reserve and fertility (5), with a natural decline due to a decrease in the number of oocytes and a reduction in oocyte quality. Additionally, genetic, life-style and environmental factors are also recognised to affect variation in ovarian reserve(5, 6). The relationship between smoking and serum AMH and AFC reported in literature is inconsistent. Some studies suggest that smoking may negatively impact the ovarian reserve(7, 8), whereas the others have failed to corroborate this association(9).Differences in ascertainment of cigarette smoking exposure, potential inaccuracies in self-reported smoking history and selection biases in studies may have led to discrepancies in the results. The role of passive smoking has also not been well investigated.

Thus, the primary objective of our study was to assess the effect of cigarette smoking on the quantitative ovarian reserve parameters, AMH and AFC. Our secondary aims were to validate self-reported smoking behaviour using biomarkers and evaluate the association between biomarkers of ovarian reverse (serum AMH and AFC) with biomarkers of smoking exposure (i.e. breath carbon monoxide and urine cotinine levels).

2 Materials and methods

2.1 Study design, setting and population

We conducted a single-centre prospective cross-sectional study from July 2019 to February 2020. The study population comprised of couples referred to the fertility centre for investigations and treatment of subfertility. We compared the levels of serum AMH and AFC among current smokers, ex-smokers and never-smokers based on a self-reported smoking history and validated by the measurements of breath carbon monoxide (CO) and urine cotinine levels. We also explored the association between biomarkers of ovarian reserve (AMH and AFC) and biomarkers of smoking (breath CO and urine cotinine) and correlated the lifetime smoking exposure quantified as "pack years" with levels of serum AMH and AFC.

2.2 Inclusion and exclusion criteria

We included women aged ≤35 years attending the fertility unit for investigations and treatment. We excluded women on long term oral contraceptive pills or GnRH analogues, those not having both ovaries and with a history of previous chemotherapy, abdominal/pelvic radiotherapy or major ovarian surgery.

2.3 Study procedures, screening, consent, care pathway, study intervention, laboratory procedures

We screened and invited eligible participants to participate in the study. Following informed consent we assessed the participants for markers of smoking. This included a short self-reported questionnaire about the participant's current and past smoking history, a non-invasive breath test to detect the levels of carbon monoxide and a urine test to detect the levels of cotinine. Based on the smoking history we classified participants into one of three categories; current smokers, ex-smokers and never-smokers. The smoking history also accounted for passive smokers and smoking details aimed to quantify the smoking exposure in terms of "pack years". We measured serum AMH and AFC as a part of the standard fertility work up done for all fertility patients. We also collected baseline demographic and clinical data for confounding variables. We followed up all participants for the results of their tests.

2.4 Products, devices, techniques and tools

A bespoke questionnaire was used to obtain self-reported smoking history. This was designed with the input of clinical and research members of the team to ensure content validity and reliability. The questionnaire was tested on a pilot sample of the target population. This highlighted deficiencies and allowed improvements in the final questionnaire used. The questionnaire details are provided in Appendix S1.

The device used to measure the breath CO (Smokelyser) is a CE marked, commercially available, non-invasive CO breath test that uses an electrochemical sensor to measure the breath concentration of CO with a concentration range of 0-150 ppm with a sensor sensitivity of 1 ppm and an accuracy of $\pm$2 ppm. The instrument was used within the specified warranty period and used and serviced according to manufacturer's specifications.

The urine cotinine was measured using the DRI®Cotinine assay (Thermo Fisher Scientific). The DRI® Cotinine Assay is an in vitro diagnostic medical device intended for the qualitative and semi-quantitative determination of cotinine in human urine at a cut off level of 500 ng/mL. The accuracy of the assay has been confirmed by gas chromatography /mass spectrometry. According to manufacturer, the sensitivity, defined as the lowest concentration that can be differentiated from the negative urine calibrator with 95% confidence, is 34 ng/mL.

All serum AMH assays were performed in an on-site clinical laboratory using the bench-top fully automated assay Access 2 immunoassay system (Beckman-Coulter) and values were expressed as pmol/l. Inter-assay coefficients of variation for a low and high control were 0.056 and 0.44, respectively. Venous blood samples were obtained and delivered to the laboratory immediately, centrifuged, and stored at 2-8°C, and analysed every day.

Ultrasound imaging of ovaries was performed using a Voluson S10 diagnostic ultrasound system (GE Healthcare) equipped with a multi-frequency transvaginal probe (RIC5-9W-RS: 9-5MHz) to visualize antral follicles systematically. AFC was obtained automatically using the sono-AVC™ software. Manual image post-processing was done if required. A total AFC was calculated as the sum of total number of follicles between 2-9 mm on each ovary. This measurement was not restricted to a particular time of the cycle.

2.5 Outcome measures

The primary outcome measures were serum AMH and total AFC.

2.6 Data collection

Data were recorded onto study specific paper Case Report Forms (CRFs) and subsequently transferred to a study database. We collected baseline demographic characteristics of the study population (age, ethnicity), baseline clinical data (BMI, presence of PCO/PCOS, history of ovarian surgery), data for on smoking parameters (type of smoker, passive smoking, smoking in pack years, breath CO and urine cotinine levels) and data for primary outcomes (serum AMH, AFC).

Data for smoking parameters were collected by members of the research team directly from the participant. All other data were collected from the participants' medical records and electronic hospital records.

2.7 Statistical considerations, sample size, analysis

The sample size calculation was based upon the primary outcome of serum AMH. Approximately 13% of women in the UK are current smokers (10) and the number of ex-smokers exceeds that of smokers. The proportion of never smokers in the UK population is increasing and reported at 59% in 2014 (11). Hence we estimated that at the fertility clinic approximately one third of our population would be either smokers or ex-smokers. We have previously found the mean serum AMH to be 28.28 pmol/l and a significantly lower pregnancy rates among women in the lowest quartile of AMH, i.e. below 10.28 pmol/l.(12) To detect an absolute decrease in AMH from 28.28 to 10.28 pmol/l with 80% power at a 5% significance level with an enrolment ratio of 0.5, we would require 96 participants (32 smokers/ex-smokers and 64 non-smokers). We planned to recruit approximately 100 participants to compensate for dropout and loss to follow up. Appropriate descriptive statistics were used to describe the baseline variables in the dataset. Normality of data was checked using Shapiro-Wilk test and skewed data were log transformed to achieve normal distribution before using parametric test. Nonparametric tests were used for data analysis if normal distribution was not achieved. An one-way between-groups analysis of variance (ANOVA), a Chi-squared test or a Kruskal-Wallis test were used to assess differences between baseline variables and smoking markers between current smokers, ex -smokers

and never smokers. An ANOVA was used to assess differences in outcome variables between the three study groups. When the P-value was <.05, the difference was considered statistically significant. When a difference was found to be significant, a post-hoc Tukey multiple comparison test was performed. A one-way between-groups analysis of co-variance (ANCOVA) was performed to assess the differences between groups taking into account the variability of other confounding variables. Differences in breath CO concentrations and urine cotinine levels in the three comparison groups were used to validate group stratification and the results for the primary outcome variables. Pearsons correlation test was used to explore the relationship between lifetime exposure to smoking (pack years), breath CO or urine cotinine and outcome variables. Statistical analysis was done using the Statistical Package for Social Sciences (SPSS version 26).

3 Results

101 women were recruited to the study over a period of nine months. Based on a self-reported smoking history women were classified into three comparison groups: current smokers, ex-smokers and never smokers. The baseline clinical characteristics of the participants are summarised in Table 1. There were no significant differences in the baseline variables amongst the three groups.

The smoking markers for the three groups are detailed in table 2. The pack years of smoking, quantifying exposure to cigarette smoking, were not significantly different between current and ex-smokers ($F(1,25) = 0.547$, $p=0.467$). The breath CO levels were significantly different amongst current, ex- and never smokers ($F(2,97) = 33.32$, $p< 0.0001$). Urine cotinine levels were also significantly higher in current smokers as compared to ex-smokers and never smokers. ($p< 0.001$). Current smokers reported to be more exposed to passive smoking (75%, 9/12) as compared to ex-smokers (20%, 5/25) and never smokers (25%, 16/64) ($p=0.001$).

No significant difference was observed amongst current, ex- and never smokers either for serum AMH ($F(2,91) = 1.19$, $p=0.309$) or total AFC ($F(2,81) = 0.403$, $p= 0.670$). When comparing baseline variables, age showed borderline non-significance between the groups ($p=0.057$). Hence, we performed an analysis of covariance (ANCOVA) to explore the impact

of smoking status on serum AMH using age as a covariate. No significant difference was demonstrated among the three groups (F(2,90) = 0.398, p = 0.673).

No significant correlation was demonstrated between the pack years of smoking and serum AMH (r= -0.212, n=23, p=0.166) or total AFC (r= -0.276, n=19, p=0.126). No significant correlation was found between breath CO and serum AMH (r= 0.082, n=94, p=0.216) or total AFC (r= 0.096, n=83, p=0.195). Similarly, no significant correlation was found between urine cotinine levels and serum AMH (r= 0.146, n=83, p=0.095) or total AFC (r= -0.027, n=77, p=0.386).

4 Discussion

4.1 Main results

We did not find a statistically significant difference in quantitative ovarian reserve markers serum AMH and AFC between current, ex and never smokers in our study population. By demonstrating significant differences in breath CO and urine cotinine levels among the groups, we confirmed that self-reported smoking correlates well with quantitatively measured markers of smoking. We were hence able to validate the comparison groups created by a self-reported history to ensure a valid comparison of outcome measures. We were unable to demonstrate a significant correlation between the pack years smoked and serum AMH and AFC. We did not find a significant association between biomarkers of smoking and biomarkers of ovarian reserve.

4.2 Interpretation of results

Biological plausibility exists for the effect of smoking on ovarian reserve and ovarian ageing. Animal studies have suggested adverse effects of cigarette smoking on ovarian reserve (13, 14). Several mechanisms have been postulated, which may affect quality, quantity or both. Gannon et al in 2012 (15) hypothesised a mechanism of direct toxicity to ovarian follicles resulting in an accelerated follicle loss. An indirect effect on ovarian follicle numbers has been suggested through an action on the hypothalamic pituitary axis (16) . These effects are however not evident in our study population of younger women based on serum AMH and AFC.

This may be because the natural decline of ovarian reserve with age does not follow a linear function but shows a rapid decline with increasing age(5).It has also been suggested that ovarian follicles may differ in susceptibility to the effects of smoking at different ages with older oocytes being more susceptible to negative effects of smoking.

The effect of smoking may be dose related. The pack years of smoking in our study population was relatively low at 2.13 pack years. It is possible that the deleterious effects are evident only at higher levels of smoking exposure or smoking is associated with smaller magnitude of reduction in ovarian reserve markers. Although it may be possible to demonstrate such small differences with a larger sample size, the clinical implications of such findings would be questionable. Serum AMH and AFC are largely used in young women in the context of fertility treatment, to predict ovarian response to treatment and pregnancy rates. Hence in younger women seeking fertility treatment , a clinically relevant decrease in ovarian reserve may be considered one which significantly reduces the probability of the most important outcome for this group of women; the pregnancy rate. Significantly lower pregnancy rates have been reported in the lowest quartile of AMH below 10.28 pmol/l(12). Pregnancy rates in women with serum AMH in the upper three quartiles are not statistically different from each other.(12). The absence of an association between smoking and serum AMH and AFC also argues for a mechanism against follicular atresia. This is strengthened by the finding of no association between ex-smokers and lower AMH values in our study and also in other studies such as Dolleman et al(7).

Our results are in agreement with those of Bressler et al, 2016 (9). They were unable to demonstrate an association between smoking exposure and serum AMH in a population based cross-sectional analysis. The age of their study population was women aged 23-35 years which is similar to that of our study. However, exposure ascertainment was done using only a self-reported questionnaire. Similarly, Kline et al in 2016 reported no association between AMH and smoking in a cross-sectional study using self-reported smoking to ascertain exposure. Dolleman et al in 2013 in a large population based study reported lower serum AMH in current smokers but not in ex-smokers as compared to never smokers. The study population was however significantly older (mean 37.3, SD 9.2) than our study population, which may explain a difference in the results. It has been suggested that the increase in follicular decline may be accelerated and more evident with advancing

age(16). Also, the smoking exposure in pack years was higher in this population (mean 10.2, SD 9.1) as compared to our study (median 2.13 (IQR 0.59-3.48)) which could account for the differences. Dolleman also reported a threshold after which the linear association of pack years and serum AMH was significant. They reported this at 10 pack years of smoking below which there was no significant association with serum AMH. Hence, these results could be considered to be in agreement with our study.

We have used breath CO and urine cotinine as biomarkers of smoking to validate self - reported smoking history. This is in agreement with previously reported studies. Marrone et al report significantly higher breath CO and cotinine levels in smokers compared with non-smokers ($P<0.001$), with 100% specificity and sensitivity at a concentration of 5ppm(17). Similarly, MacLaren et al reported a strong agreement between self-reported smoking and breath CO levels with a sensitivity of 96% and specificity of 93.3% using a cut off of 7ppm(18).

4.3 Strengths and limitations

A major strength of our study is that we used a comprehensive and detailed self-reported questionnaire to assess smoking exposure, which allowed estimation of lifetime smoking exposure in terms of pack years and also accounted for passive smoking. Furthermore, we also used breath CO and urine cotinine concentrations to validate our study groups. The CO breath test shows the amount of CO in the breath (ppm), as an indirect, non-invasive measure of blood carboxyhemoglobin (%COHb). CO leaves the body rapidly and the half-life is about 5 hours. Within 24 to 48 hours of not smoking, smokers will be at non-smoker levels. Cotinine is the predominant metabolite of nicotine. It has a half-life of 20 hours and is detectable for up to one week after the use of tobacco. This is useful to identify smokers who have abstained from smoking for several hours.

The participants included an unselected population of women attending the clinic for various investigations and treatments. There were wide variations in the baseline characteristics of participants such as ethnicity, cause of infertility and diagnosis. By using a wide-ranging unselected population of women we have attempted to improve the generalisability of the results.

Age remains a major determinant of ovarian reserve. We have included only women 35 years and younger to reduce bias due to the impact of advancing age. The participants included only sub-fertile women with a limited range of BMI and age. This is because fertility treatment within the UK and funded by the National Health Service is restricted by limits on age and BMI. Therefore, caution should be exercised when extrapolating these results to other populations. Our study was powered only to detect differences in ovarian markers of relatively large magnitude that we considered to have a clinical significance in the management of young women seeking fertility treatment However, a much larger sample size would be required to detect statistically significant differences of smaller magnitude which may be relevant to different study populations and research questions.

5 Conclusion

We did not find a quantitative change in the antral follicle pool following exposure to cigarette smoking in women ≤35 years seeking fertility treatment. We confirmed that self-reported smoking correlates well with quantitatively measured biomarkers of smoking. There was no significant association between biomarkers of smoking and biomarkers of ovarian reserve. We were also unable to demonstrate a correlation between the lifetime smoking exposure and ovarian reserve parameters

6 Disclosure of interests

The authors have no conflicts of interest to declare.

7 Contribution to authorship

PB: Study concept and design, participant recruitment, data collection, data analysis and interpretation, drafting the article, critical review and final approval.

ET: participant recruitment, data collection, critical review and final approval.

AK: participant recruitment, data collection, critical review and final approval.

AG: critical review and final approval

AS: critical review and final approval

RH: critical review and final approval

GA: Study concept and design, critical review and final approval.

All authors read the manuscript critically, commented on the draft and approved the final version before submission.

8 Details of ethics approval

The study was approved by Health Research Authority and Health and Research Care Wales-Central Research Ethics Committee on 10/Apr/2019. (REC reference: 19/WA/0089)

9 Funding

The authors have no funding to declare.

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Table 1: Baseline variables

	Current smokers (n=12)	Ex-smokers (n=25)	Never smokers (n=64)	p
Age (years)	30 (25.5-33.0)	32.5 (31.0-33.5)	31.0 (28.0-33.0)	0.057
BMI	23.2 (21.8-26.2)	25.3 (20.8-28.3)	25.1 (22.1-27.8)	0.632
Ethnicity				0.208
White European	8	21	35	
Asian	2	4	16	
Afro-Caribbean	1	0	8	
Others	1	0	5	
Category of infertility				0.077
Anovulatory	4	1	11	
Male	4	5	14	
Tubal	2	0	9	
Unexplained	1	14	20	
Other	0	4	4	
Ovarian surgery				0.659
No	12	23	60	
Yes	0	1	1	
PCOS/PCOM				0.351
N	7	17	42	
Y	5	4	20	

Values expressed as median (IQR) or n

Table 2: Smoking markers

	Current smokers (n=12)	Ex-smokers (n=25)	Never smokers (n=64)	p
Pack years of smoking	2.13 (0.59-3.48)	2.13 (0.05-5.40)	0.00 (0.00-0.00)	0.467*
Breath CO (ppm)	9 (3.5-21)	2 (2-3)	1 (1-2)	<0.001
Urine Cotinine (ng/ml)	837 (22.42 – 1571.8)	22.42 (22.42-22.42)	22.42 (22.42-22.42)	<0.001

*comparison between current and ex-smokers only
Values presented as median (IQR)

Table 3: Outcomes

	Current smokers (n=12)	Ex-smokers (n=25)	Never smokers (n=64)	p
Serum AMH (pmol/l)	38.9 (20.4-66.2)	26.0 (14.7-32.2)	27.6 (16.4-39.7)	0.309
Total AFC (n)	30.5 (16-41.5)	22.5 (13-30)	21.5 (15-35.5)	0.670

Paper III

Bhide P, Pundir J, Gudi A, Shah A, Homburg R, Acharya G.

The effect of myo-inositol/di-chiro-inositol on markers of ovarian reserve in women with PCOS undergoing IVF/ICSI: A systematic review and meta-analysis.

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SYSTEMATIC REVIEW

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The effect of myo-inositol/di-chiro-inositol on markers of ovarian reserve in women with PCOS undergoing IVF/ICSI: A systematic review and meta-analysis

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Abstract

Introduction: High levels of anti-Mullerian hormone and a high antral follicle count in women with polycystic ovary syndrome, reflecting increased ovarian antral follicles, predisposes them to have a high number of retrieved oocytes with in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) and an increased risk of ovarian hyperstimulation syndrome. Inositols, which act as insulin sensitizers, have the potential to alter folliculogenesis and the functional ovarian reserve, with subsequent benefits to reproductive outcomes following IVF/ICSI treatment. Published literature is, however, unable to provide definitive evidence of its efficacy. The objective of our review was to evaluate the effect of inositols on anti-Mullerian hormone, antral follicle count and reproductive outcomes in women with polycystic ovary syndrome undergoing IVF/ICSI.

Material and methods: We performed a literature search using standard methodology recommended by Cochrane. Randomized controlled trials and non-randomized studies comparing inositols with no treatment, placebo or other treatment were included in the review. Using standard methodology recommended by Cochrane we pooled results using the random effects model; our findings were reported as relative risk or mean differences. PROSPERO registration: CRD42017082275.

Results: We included 18 trials. The primary outcome was a change in anti-Mullerian hormone and antral follicle count before and after treatment, for which data were unsuitable for meta-analysis. A narrative review showed no consistent direction or size of effect. A meta-analysis for the secondary outcomes showed no evidence of a significant difference between inositol and control groups for any outcome: number of oocytes (mean difference −0.39, 95% confidence interval [CI] −1.11 to 0.33), number of metaphase II oocytes (mean difference 0.29, 95% CI −0.83 to 1.40), number of top grade embryos (risk ratio [RR] 1.02, 95% CI 0.93-1.12), clinical pregnancy rate (RR 1.16, 95% CI 0.87-1.53), and risk of ovarian hyperstimulation syndrome (RR 0.73, 95% CI 0.39-1.37). The quality of evidence was assessed as very low.

Abbreviations: AFC, antral follicle count; AMH, anti-Mullerian hormone; CI, confidence interval; DCI, di-chiro inositol; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; MD, mean difference; MI, myo-inositol; OHSS, ovarian hyperstimulation syndrome; PCOS, polycystic ovary syndrome; RCT, randomized controlled trial; RR, risk ratios; SMD, standardized mean difference.

Conclusions: There is insufficient evidence for an effect of inositols on ovarian reserve markers and to support their use as pretreatment before IVF/ICSI in women with polycystic ovary syndrome.

KEYWORDS
anti-Mullerian hormone, antral follicle count, in vitro fertilization, inositol, intracytoplasmic sperm injection, polycystic ovary syndrome

1 | INTRODUCTION

Ovarian reserve markers, anti-Mullerian hormone (AMH) and antral follicle count (AFC), are increased in women with polycystic ovary syndrome (PCOS),[1,2] reflecting the increased density of ovarian antral follicles in these women.[3] This predisposes them to hyper-respond to controlled ovarian stimulation following in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatment and also puts them at a higher risk of developing ovarian hyperstimulation syndrome (OHSS).

Hyperinsulinemia secondary to insulin resistance is an important contributor to hyperandrogenism and excessive follicular growth and is thought to play a central role in the pathophysiology of PCOS.[4] Hyperinsulinemia directly and indirectly stimulates increased androgen production, resulting in increased ovarian folliculogenesis. A reduced glucose uptake by oocytes secondary to insulin resistance is postulated to result in poor quality oocytes and subsequently embryos.[5]

Inositol is a sugar alcohol, belonging to the vitamin B complex family. Inositol and its metabolites, among others, function as secondary messengers in the insulin signaling pathway.[6] Deficiency of inositols[7] and an altered inositol metabolism are hypothesized to play a role in the pathogenesis of insulin resistance in PCOS.[8] Inositols also have a role in oocyte maturation and fertilization through their actions in the calcium signaling pathway.

Exogenously administered inositols act as insulin sensitizers to reduce insulin resistance and insulin levels with a subsequent reduction in downstream androgen production.[9,10] Thus inositols have the potential biological plausibility to alter ovarian folliculogenesis and hence the functional ovarian reserve in women with PCOS. AMH and AFC, which are direct markers of the antral follicle pool, serve as the most appropriate markers to reflect this effect. Through their action on the antral follicle pool, inositols have the potential to alter the number and quality of oocytes and embryos following IVF/ICSI and reduce the risk of OHSS, making IVF/ICSI a more effective and safe treatment. They are also reported to improve quality of oocytes and subsequently embryos.[11,12]

In view of their hypothesized benefits, inositols are commonly prescribed as a pretreatment to women with PCOS undergoing IVF/ICSI to reduce the number of retrieved oocytes and the risk of OHSS. They are also given with the aim of improving the quality of oocytes and embryos in order to improve pregnancy rates after IVF/ICSI treatment. Although inositols have been reported to improve menstrual regularity and ovulation rates,[13] most studies assessing the effects of inositol on ovarian function markers/IVF outcomes are small randomized controlled trials (RCTs) or non-randomized studies unable to provide definitive evidence of its efficacy. Reviews summarizing the effects of inositols on reproductive outcomes have been published. However, two of these reviews are narrative rather than systematic reviews[5,14] whereas the Cochrane review by Morley et al[15] may be considered non-comprehensive, as due to stringent selection criteria it presents only two randomized controlled trials and none of the outcomes presented in this review.

Key message

There is insufficient evidence that inositols change ovarian reserve markers and improve outcomes in women with polycystic ovary syndrome having in vitro fertilization/intracytoplasmic sperm injection

Thus our primary objective was to assess the effect of treatment with myo-inositol (MI)/di-chiro inositol (DCI) compared with no treatment, placebo or other treatment on markers of ovarian reserve in women with PCOS combining data from all published literature. Our secondary aim was to assess the effect of these treatments on reproductive outcomes in women with PCOS undergoing an IVF/ICSI procedure.

2 | MATERIAL AND METHODS

2.1 | Eligibility criteria

All published studies, randomized controlled trials (including crossover trials) supplemented by non-randomized studies such as quasi-randomized studies, controlled clinical trials, cohort studies and observational studies were included. We included only those studies that were published as full-length manuscripts (not just abstracts) and only those published in the English language. We included studies with a study population of women with PCOS. The intervention was MI, DCI or a combination of the two compared with no treatment, folic acid, placebo or other treatment. Studies with any dosage, frequency and duration of the intervention/control were included. Any other treatment given in addition to MI/DCI and appearing in both the intervention and comparator arms was analyzed as MI/DCI vs no treatment, for example, MI+metformin vs metformin = MI vs no treatment. PROSPERO registration: CRD42017082275.

2.2 | Assessment of study quality and the risk of bias

Randomized studies were evaluated for the risk of bias independently by two authors (P.B. and J.P.) using the Cochrane risk of bias assessment tool. Studies were evaluated for bias in sequence generation and allocation concealment in RCTs, adequacy of blinding of participants, assessors and outcome assessors, completeness of outcome data, risk of selective reporting of outcomes and other potential sources of bias. Non-randomized studies were evaluated for the risk of bias using The Risk Of Bias In Non-randomized Studies-of Interventions (ROBINS-I) assessment tool. Disagreements were resolved by consensus.

We conducted a comprehensive search for eligible studies to minimize the impact of reporting bias. Within-study reporting bias was assessed by evaluating studies for prospective trial registration and availability of a trial protocol. The study was considered to have a low risk of bias if all of the prespecified outcomes were reported as outlined in the trial protocol. Funnel plots were produced for the primary outcome measures to assess publication bias.

We prepared a summary of findings table using GUIDELINE DEVELOPMENT TOOL software. This was used to evaluate the overall quality of evidence for all outcomes using GRADE criteria (risk of bias, consistency of effect, imprecision, indirectness and publication bias). Based on this, the judgements about strength of recommendation (high, moderate, low or very low) were made and reported in the Results section for each outcome and in the summary of findings table.

2.3 | Main outcome measures

The primary outcomes were serum AMH level and AFC. The secondary outcomes were number of retrieved oocytes, number of mature (metaphase II) oocytes, number of top-grade embryos, pregnancy rate, live birth rate and risk of OHSS.

2.4 | Data sources

The authors P.B. and J.P. independently screened and identified relevant studies for the review. We used both electronic searches of bibliographic databases and hand-searching as described in the Cochrane Handbook for Systematic Reviews of Interventions. An up-to-date search for any recent data was conducted 1 month prior to submission of this systematic review. We searched the electronic databases MEDLINE, Embase, CENTRAL and CINAHL. The study period was from inception till 31 December 2017. The search strategy included a combination of subject headings (MeSH/Emtree) and text words relating to or describing the participants (polycystic ovary syndrome, PCOS, PCO polycystic ovar*) and intervention (inositol, myo-inositol, di-chiro inositol). The draft search strategy for CENTRAL can be seen in Supporting Information Appendix S1. A similar strategy was used for the other databases searched. We also searched other published reviews and guidelines. The reference lists of all known primary and review articles were searched for relevant citations not captured by the electronic searches. We also searched trial registers, ClinicalTrials.gov and World Health Organization (WHO) International Clinical Trials Registry Platform (ICTRP) for ongoing studies and completed or ongoing studies which are unpublished.

2.5 | Data collection and analysis:

2.5.1 | Data collection

The authors P.B. and J.P. independently screened the titles and abstracts of articles retrieved from the search. The full texts of potentially suitable articles were obtained and assessed for suitability for the review. In the case of disagreement, a third author (G.A.) was consulted and consensus reached for inclusion/exclusion of the article in question.

Data were extracted independently by two authors (P.B. and J.P.). In case of disagreement a third author (G.A.), was consulted to achieve consensus. Data were collected on a bespoke data collection EXCEL spreadsheet. If a study was reported in multiple publications, these were pooled together under a single study ID. Data were collected for participants, intervention and outcomes. Data were tabulated in 2 × 2 tables for dichotomous outcomes and 1 × 2 tables for continuous outcomes.

2.5.2 | Data analysis

The pooled estimates for outcomes were presented as risk ratios (RR) for dichotomous variables and mean difference (MD)/standardized mean difference (SMD) for continuous variables with 95% confidence intervals using the random effects model and inverse variance method. Statistical significance was assumed when $P < .05$. When published articles were unclear about the presence and amount of missing data, the original investigators[16-25] of the trial were contacted to request this information. We were able to get this information from Colazingari et al,[18] Ozay et al[21] and Pkhaladze et al[23] and have updated the risk of bias accordingly. Although we were not able to get information from some authors, the required data were available in a previously published systematic review[26] from which it was extracted.

Included studies were assessed for clinical and methodological characteristics to determine whether they were sufficiently similar to allow meta-analysis and a pooled estimate of the outcomes to be studied. The data were analyzed per participant except for the number of top-grade embryos where the unit of analysis was embryos. Statistical heterogeneity was assessed by measuring the I^2 statistic. Substantial heterogeneity was assumed when I^2 was calculated to be greater than 50%.[27]

2.5.3 | Data synthesis

Meta-analysis was planned for primary and secondary outcomes where adequate and appropriate data were available to produce pooled estimates of effect. Where sufficient data were available, subgroup analyses was planned for the two different types of inositols: MI and DCI.

3 | RESULTS

3.1 | General characteristics of the studies

From the originally retrieved 263 search results, 18 studies were selected for the review. The search and selection process is documented with a PRISMA flow chart in Supporting Information Appendix S2 and the list of included studies, excluded studies with reasons for exclusion and ongoing studies is also provided (Supporting Information Table S1). Seven studies assessed changes in ovarian reserve markers. Twelve studies assessed treatment outcomes following IVF/ICSI treatment. One study assessed both categories of outcomes. The characteristics of the included studies are detailed in in Supporting Information Table S2.

3.2 | Included studies

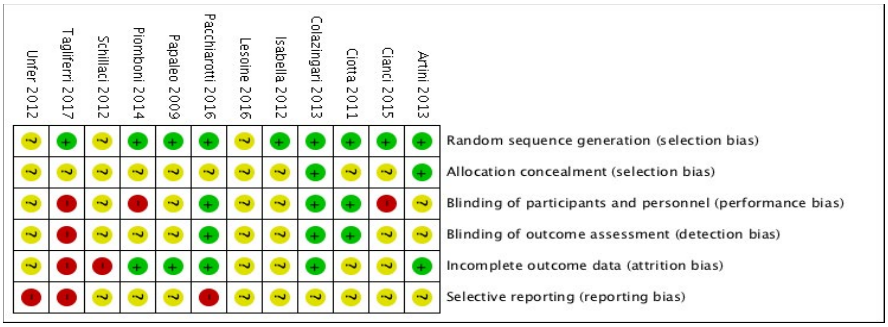
3.2.1 | Studies assessing changes in ovarian reserve markers

Two of the seven included studies were RCTs[16,24] and five were non-randomized studies.[21,23,28-30] All the studies were single-center studies with five of the seven studies conducted in Italy, one in Turkey and one in Georgia. In total, 415 women were recruited to these seven studies. All women were diagnosed with PCOS. Some studies restricted inclusion based on age, body mass index and insulin resistance, the details of which are included in Table S2. The study by Pkhaladze et al[23] included participants between 13 and 19 years of age. The diagnostic criteria for PCOS in teenagers have been debated. We have, however, included the study in our review as a consensus by international pediatric subspecialty societies states that a persistence of hyperandrogenic anovulation for ≥2 years distinguishes PCOS from physiologic anovulation.[31] Five of the included studies[21,23,24,28,29] assessed MI in varying doses ranging from 1 to 4 g daily and for varying durations between 12 weeks and 6 months with no explanations for variations in dose or duration of given treatment. Four of these looked at its effect on serum AMH[21,23,24,29] and three looked at its effect on AFC.[21,28,29] Two of the studies[16,30] assessed the effects of DCI in doses ranging from 1000 to 1500 mg daily for durations from 180 days to 13 months. One of these assessed the effect of DCI on serum AMH[30] and the other assessed its effect on AFC.[16]

3.2.2 | Studies assessing reproductive outcomes after IVF/ICSI

Ten of the 12 included studies were RCTs[11,12,17-20,22,32-34] and two were non-randomized studies.[25,28] All the studies were single-center studies with 10 of the 12 studies conducted in Italy, one in Poland and one in Germany. In all, 1225 women, all diagnosed with PCOS were recruited to the 12 trials included. Some studies restricted inclusion based on age, body mass index and insulin resistance, the details of which are included in Table S2. Nine of the 12 included studies[11,17,18,20,22,25,28,32,34] assessed the effects of MI in doses varying from 1 to 4 g daily and for varying durations of from 2 weeks to continuous ongoing treatment. Two studies[19,33] assessed the effects of DCI in doses ranging from 300 to 1200 mg daily in durations ranging between 8 weeks and 3 months. One study compared MI with DCI.[12]

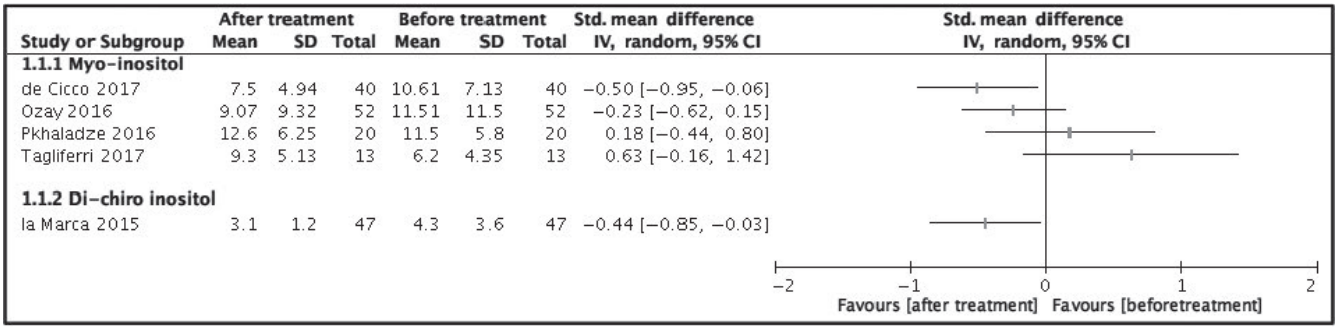
Risk of bias summary for randomised studies (Cochrane RoB tool):



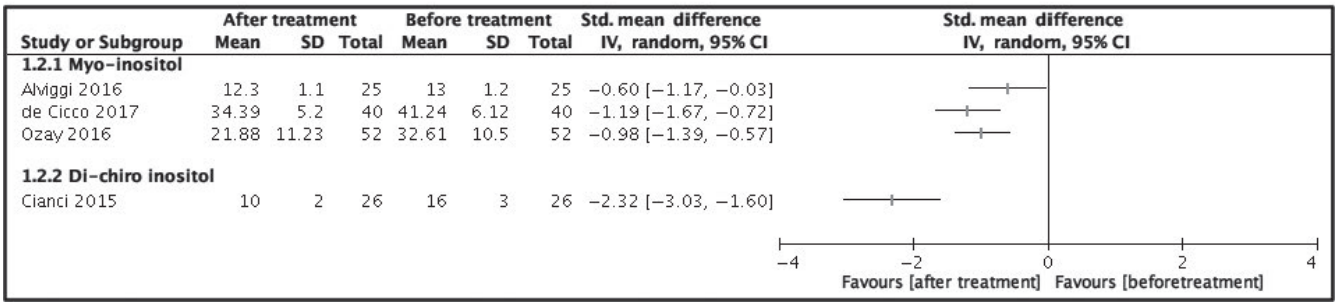
Risk of bias for non-randomised studies (ROBINS-I tool):

No	Study	Study design	Bias due to confounding	Bias in selection of participants into the study	Bias in classification of interventions	Bias due to deviations from intended interventions	Bias due to missing data	Bias in measurement of outcomes	Bias in selection of the reported result	Overall Risk of Bias
1	Pkhaladze, 2016[23]	Quasi randomised	Moderate risk	Moderate risk	Low risk	No information	Low risk	Low risk	Moderate risk	Moderate risk
2	Alviggi, 2016[28]	Retrospective controlled before and after study	Moderate risk	No information	Moderate risk	No information	Low risk	Low risk	Moderate risk	Moderate risk
3	De Cicco, 2017[29]	Before and after study	Serious risk	No information	Low risk	Low risk	Low risk	Low risk	Moderate risk	Serious risk
4	Ozay, 2017[21]	Quasi randomised	Moderate risk	Moderate risk	Low risk	Serious risk	Serious risk	Low risk	Moderate risk	Serious risk
5	La Marca, 2015[30]	Retrospective before and after study	Critical risk	Critical risk	Low risk	Moderate risk	Low risk	Low risk	Moderate risk	Critical risk
6	Wdowiak, 2016[25]	Retrospective cohort study	No information	Moderate risk	Low risk	No information	No information	Low risk	Moderate risk	Moderate risk

FIGURE 1 Risk of bias in included studies. [Colour figure can be viewed at wileyonlinelibrary.com]



Analysis 1: Forest plot of comparison: Ovarian reserve markers before and after treatment with inositol, Antimullerian hormone.



Analysis 2: Forest plot of comparison: Ovarian reserve markers before and after treatment with inositol, Antral follicle count.

FIGURE 2 Forest plot of comparison: Ovarian reserve markers before and after treatment with inositol

3.2.3 | Risk of bias and quality of evidence in included studies

Of the randomized trials, 75% of trials were at low risk for selection bias for random sequence generation, but only 17% were considered low risk for allocation concealment. Similarly, only 25% of studies were considered at low risk of performance bias due to blinding. The risk of attrition bias due to incomplete outcome data was low in 42% of studies. None of the trials was considered at low risk of reporting bias. The details of the assessment of risk of bias are presented in the Supporting Information Table S3 and Figure 1. None of the non-randomized studies was considered to be at a low risk of bias due to confounding or selection of participants into the study, but 83% were at low risk for bias in classification of interventions and 67% were at low risk of bias due to missing data. In all, 50% of studies provided no information on deviations from intended interventions and none of the studies was at low risk of reporting bias. The quality of evidence contributing to the review was assessed as very low across most domains of inconsistency, indirectness, imprecision, and a high risk of bias. This is detailed in the summary of findings in Supporting Information Table S4.

3.2.4 | Synthesis of the results

Primary outcomes

Anti-Mullerian Hormone. Due to clinical and methodological heterogeneity, none of the studies using MI/DCI were suitable for inclusion in a meta-analysis for this outcome. The SMDs in the levels of serum AMH before and after treatment with inositols for the five individual studies with 172 participants are shown in Figure 2. There was no consistent direction or size of effect.

Antral follicle count. Due to clinical and methodological heterogeneity, none of the four studies using MI/DCI was suitable for inclusion in a meta-analysis. The SMDs in the AFC before and after treatment for the individual studies with 143 participants are shown in Figure 2. The results show a high heterogeneity in effect size for AFC. The results do not suggest any clear change in AMH or AFC values following treatment with inositol.

Secondary outcomes

Number of retrieved oocytes. Of the 11 studies reporting the number of oocytes retrieved, 7 RCTs with 722 participants were included in the meta-analysis. No statistically significant difference was found between the intervention and control arms (MD −.39, 95% confidence interval [CI] −1.11 to .33). The results were similar for a subgroup analysis for MI (MD −.76, 95% CI −2.04 to .52) and DCI (MD −.18, 95% CI −1.11 to .74). These results are shown in Figure 3. Studies by Ciotta[17] and Lesoine & Regidor[20] were not included in the meta-analysis as trial data were not available in a format suitable for meta-analysis. Ciotta[17] reported a significantly higher number of oocytes in the MI treated group as compared with control ($P < .05$), whereas Lesoine & Regidor[20] reported a higher number of oocytes in the control group than in the MI group. Alviggi et al[28] in a non-randomized study reported no significant difference between the MI and control groups ($P = .23$). Unfer[12] compared treatment with MI with treatment with DCI and reported no significant difference between the number of retrieved oocytes.

Number of metaphase II oocytes. Of the 10 studies reporting on the number of metaphase II oocytes retrieved, only 3 RCTs with 207 participants were included in the meta-analysis. No statistically significant difference was observed between the intervention

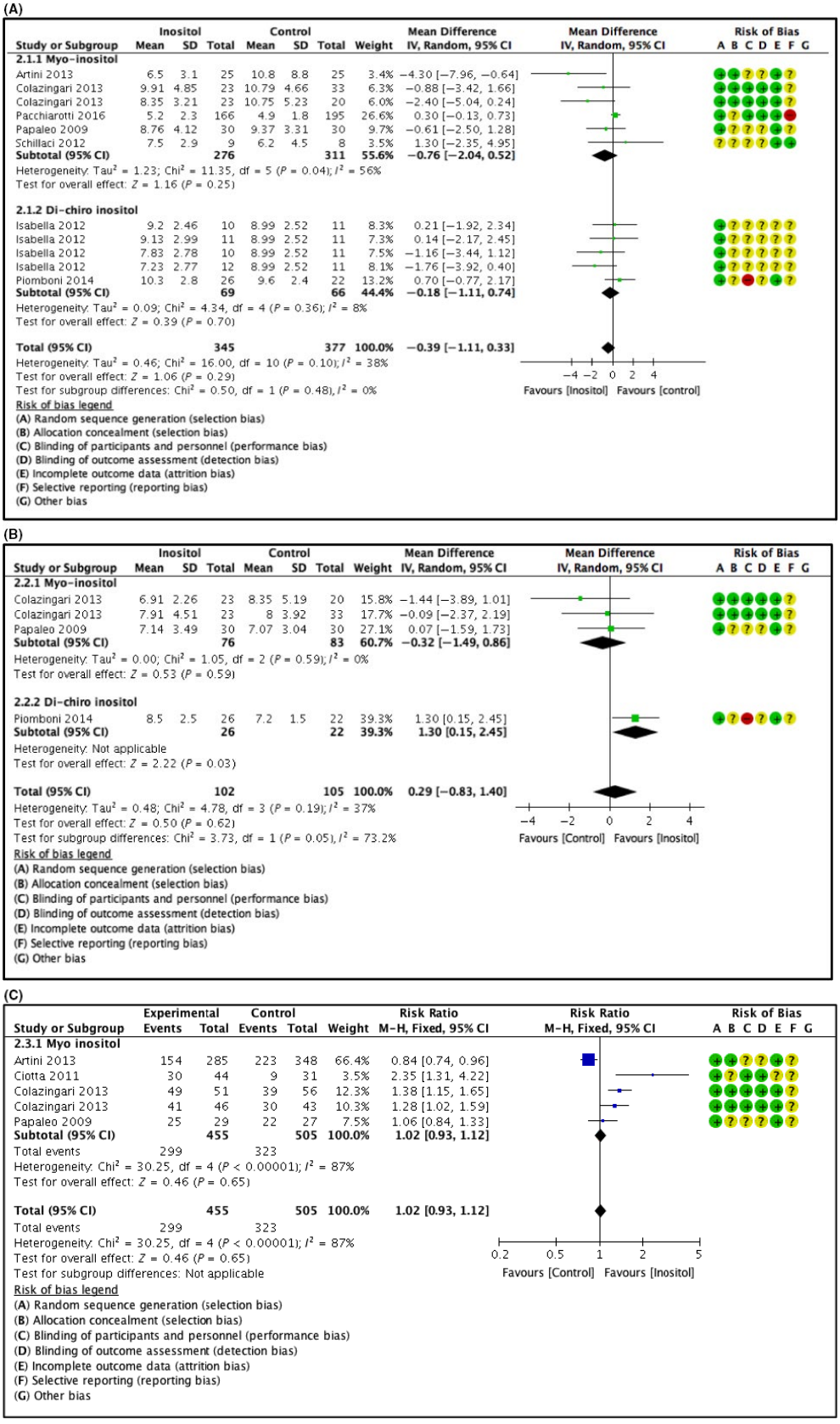


FIGURE 3 Forest plot of comparison: Inositol vs control, reproductive outcomes for IVF/ICSI. (A) Number of retrieved oocytes; (B) number of MII oocytes; (C) number of top-grade embryos. [Colour figure can be viewed at wileyonlinelibrary.com]

and control arms (MD .29, 95% CI −.83 to 1.40). The results were similar for a subgroup analysis for MI (MD −.32, 95% CI −1.49 to .86). A single trial, Piomboni et al[33] reported significantly higher number of MII oocytes, with a moderate effect size, following treatment with DCI as compared with control (MD 1.30, 95% CI .15-2.45). The results are shown in Figure 3. Data from other studies were not available in a format suitable for meta-analysis. Artini et al[32] reported a significant increase in the percentage of top-quality oocytes in the group treated with MI as compared with control ($P < .05$). Pacchiarotti et al[22] and Ciotta et al[17] reported no significant differences between the groups. Isabella et al[19] reported a significant decrease in the number of MII oocytes following treatment with DCI as compared with control. The results from Lesoine & Regidor[20] are unclear. Alviggi et al[28] in a non-randomized study reported a significant increase in the number of MII oocytes following treatment with MI as compared with control ($P = .03$). Unfer et al[12] compared treatment with MI with treatment with DCI and reported a significant increase in the number of MII oocytes following treatment with MI as compared with DCI ($P < .05$).

Number of top-grade embryos. Four of the eight RCTs including 957 participants and reporting the number of top-grade embryos were included in the meta-analysis. No significant difference was seen between the groups (RR 1.02, 95% CI .93-1.12). The other four studies reporting this outcome did not contain data in a suitable format and hence could not be included in the meta-analysis. The results are shown in Figure 3. Pacchiarotti et al[22] reported no significant difference in the number of top grade embryos between the MI and control groups. Isabella et al[19] reported a significant decrease in the number of top-grade embryos following treatment with DCI ($P < .01$) whereas Lesoine & Regidor[20] reported a significantly higher number of top-grade embryos following treatment with MI as compared with control ($P < .05$). Unfer et al[12] reported a significantly higher number of top-grade embryos following treatment with MI than treatment with DCI ($P < .01$).

Clinical pregnancy rate. Three RCTs with 488 participants reporting clinical pregnancy rates were included in the meta-analysis. No significant difference was seen between the groups (RR 1.16, 95% CI .87-1.53). The results are shown in Figure 4. Schillaci et al,[34] Alviggi et al[28] and Wdowiak et al[25] did not indicate whether the pregnancies were biochemical or clinical. No significant differences were reported between the groups in these studies. Unfer & Regidor[12] compared treatment with MI with treatment with DCI and reported no significant difference in clinical pregnancy rates between the groups.

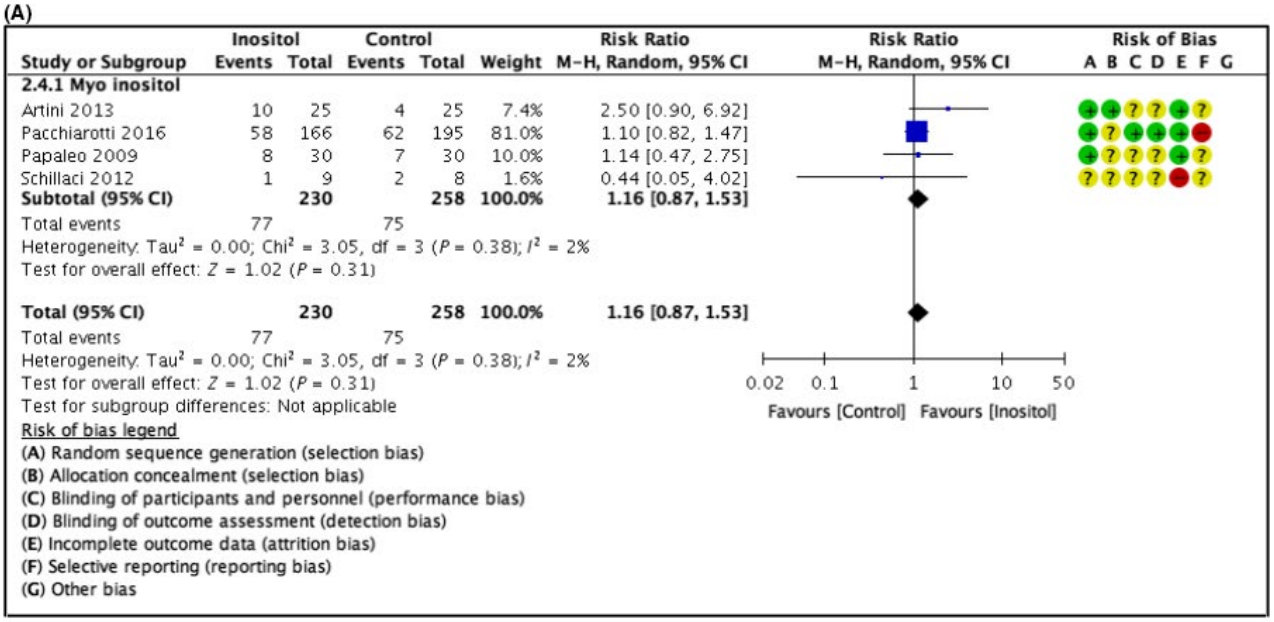
Live birth rate. Only one trial, Artini et al,[32] reported live birth rate. This reported a significant improvement in live birth rate following treatment with MI compared with placebo ($P < .05$).

Risk of cycle cancellation due to the risk of OHSS. Six of the seven studies reporting cycle cancellations due to the risk of OHSS were included in the meta-analysis. No significant difference was seen between groups (RR .73, 95% CI .39-1.37). The results were similar for a subgroup analysis for MI (RR .70, 95% CI .34-1.42) and DCI (RR .85, 95% CI .22-3.29). The results are shown in Figure 4.

4 | DISCUSSION

A systematic review of the available data showed no consistent direction or size of effect for a change in AMH or AFC after treatment with MI/DCI. The review is unable to provide adequate evidence for a quantitative change in the antral follicle pool following treatment with inositols. Due to clinical and methodological heterogeneity, the data were unsuitable for meta-analysis regarding the primary outcome. A meta-analysis for secondary outcomes showed no significant difference between the inositol and control groups for the number of retrieved oocytes, number of metaphase II oocytes, number of top-grade embryos and pregnancy rates. There was no significant difference between the rates of cycle cancellation due to the risk of OHSS between the inositol and control groups.

To our knowledge, our review is the first to assess the impact of treatment with inositols on ovarian reserve markers. Although the review does not provide definitive evidence of effect, it very clearly highlights the absence of good-quality evidence and heterogeneity of existing literature, which we consider useful new information to guide clinical practice and direct future research. Other systematic reviews reported the effects of inositol on outcomes following IVF/ICSI treatment.[5,14,15,26] The first systematic review by Unfer et al[5] reported an improvement in oocyte and embryo quality based on five studies. The findings of that review disagree with those of our review. The conclusions of that review were, however, based on a narrative systematic review rather than a meta-analysis. It is possible that the discordant results may be due to positive outcomes in earlier studies included for analysis by Unfer et al, whereas some later studies (after 2012) included in our review have contradictory findings, resulting in a pooled estimate showing no significant difference in outcomes. Unfer et al discuss neither the quality of evidence presented nor the risk of bias but there may be a concerns about publication bias. Similar positive outcomes were reported by Gateva et al in 2018.[14] That was a narrative review rather than a systematic review and meta-analysis published by the same group of authors and hence subject to similar criticisms.[14] Findings of a more recent review by Mendoza et al[26] which added more studies, are broadly in agreement with ours. However, our review has included 12 studies, adding four more studies with 206 participants than Mendoza et al, thus making our findings more robust. We have added information on ovarian reserve markers, AMH and AFC which was not investigated and discussed by Mendoza et al. Our review is also the first review to assess the effect of inositols on the risk of OHSS following IVF/ICSI treatment and provide a pooled effect estimate. Women with PCOS are at an inherently greater risk of OHSS following IVF/ICSI treatment and this remains an important safety outcome for



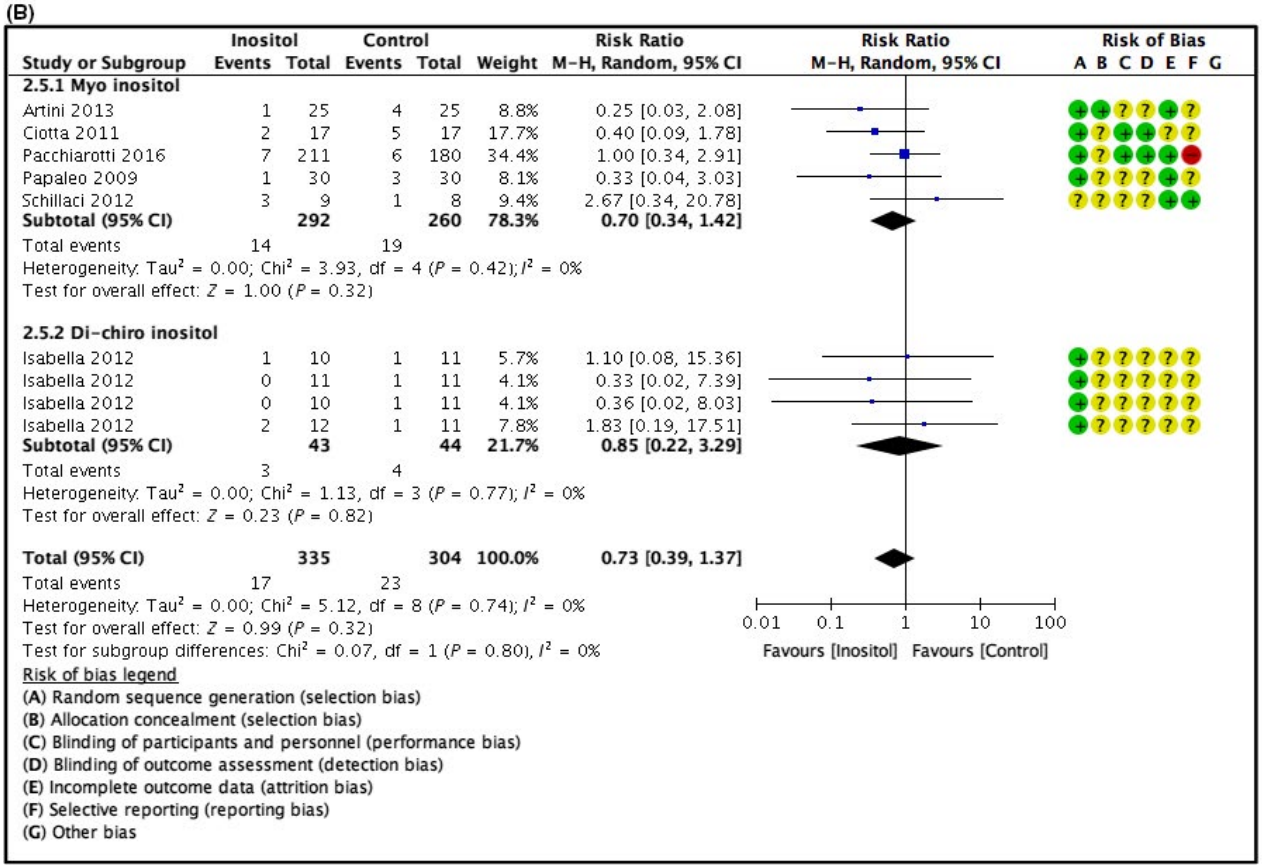


FIGURE 4 Forest plot of comparison: Inositol vs control, reproductive outcomes for IVF/ICSI. (A) Clinical pregnancy rate; (B) cycle cancelation due to the risk of ovarian hyperstimulation. [Colour figure can be viewed at wileyonlinelibrary.com]

any trial intervention. Although Mendoza et al have discussed the limitations of the included literature in terms of study design and methodology, we have provided a formal and hence more objective assessment of the quality of evidence included and certainty of the evidence presented. A Cochrane review by Morley et al[15] on the effects of insulin sensitizing drugs in women with PCOS includes a section on inositols.[15] However, due to very stringent inclusion criteria, only two studies and none of the outcomes of this review are included.[15] A very recent Cochrane review discusses the effects of inositols on outcomes following IVF/ICSI treatment.[35] They report

on only two outcomes investigated by this review (live birth and clinical pregnancy). The results are in broad agreement with those of our review.

The quality of evidence contributing to this review assessed by the GRADE criteria ranged from low to very low. This was across all domains assessed, that is, the risk of bias, imprecision, inconsistency, indirectness and publication bias. This is detailed for individual outcomes in the results section. There were major concerns regarding study design, prospective trial registration, sample sizes, sample size calculations and blinding. These along with significant heterogeneity in the patient population as well as dose and duration of the inositols used may be considered the major limitation of the evidence synthesized.

We have conducted a thorough search of the published literature and thus aimed to identify all eligible studies for inclusion in this review. Inclusion of all types of published literature rather than only randomized controlled trials has allowed us to gauge the direction and size of a potential effect to direct future research. Where information was missing, we attempted to contact the study authors on multiple occasions to seek as much information for inclusion as possible. We have received information from some authors but were unable to get information from several others. We were hence unable to include data from these studies and remain uncertain about some aspects of the study design and methodology.

Insulin resistance and hyperinsulinemia are central to the pathophysiology of PCOS, observed in a significant proportion of lean and obese PCOS.[4] Although the exact cause of insulin resistance is unknown, the resultant high insulin levels directly stimulate the ovarian theca cells to produce androgens and these may also affect luteinizing hormone secretion centrally.[36] High blood glucose levels due to insulin resistance, reduce synthesis of sex hormone binding globulin (SHBG) by the liver, resulting in even higher levels of circulating free androgens. This can be used to modify treatment protocols to reduce the risks.

Inositols are available as over-the-counter nutritional supplements with a minimal burden of adverse effects, though their actions on the insulin pathway have the potential to reduce these risks, making IVF/ICSI a more effective and safe treatment for women with PCOS. However, our review reported no consistent direction or size of effect and no robust evidence of benefit in this respect. This may be due to heterogeneity in the study design and the patient population included. Inositols exist in different tissue isoforms. MI and DCI are the predominant isomers involved in the insulin second messenger pathways and are present in variable proportions. The effective dose and duration of exogenously administered inositols still remains unclear. Likewise, PCOS is a heterogeneous clinical condition exhibiting several phenotypes reflecting differences in pathophysiological processes. Inositols may be effective only in a subset of women with PCOS demonstrating insulin resistance. Hence, the inclusion of all women with PCOS, irrespective of their insulin sensitivity, may lead to variable and inconsistent results after treatment with inositols.

5 | CONCLUSION

In conclusion, the findings from this systematic review do not provide adequate evidence for changes to the antral follicle pool following the use of inositols. Also, the review does not provide adequate high-quality evidence to support the use of inositols as pretreatment in women with PCOS undergoing IVF/ICSI treatment with the aim of improving reproductive outcomes or safety of the procedure.

Inositols are commonly prescribed by clinicians and patients themselves without good evidence for their benefit. This review highlights the need for further studies with robust design and adequate sample size to provide a definitive answer to the question of benefit. An ideal study should aim to include an appropriate PCOS population with selection based on criteria such as body mass index and insulin resistance. The dose and duration of effective treatment should be sought in appropriately designed dose-response studies. The two inositol isoforms need to be studied separately and in different dose combinations.

CONFLICT OF INTEREST

None.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Paper IV

Anti-Mullerian hormone (AMH) and embryo quality assessed by time-lapse imaging (TLI): a cross-sectional observational study.

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Anti-Mullerian hormone (AMH) and embryo quality assessed by time-lapse imaging (TLI): a cross-sectional observational study

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Abstract
Purpose Serum anti-Mullerian hormone shows a strong positive correlation to the quantitative ovarian reserve but its correlation to embryo quality is unclear. This study assessed the association between serum AMH as a marker of ovarian reserve and embryo quality, using the technology of time-lapse imaging of the embryos in women undergoing in vitro fertilisation (IVF) treatment.
Methods 304 embryos from 198 women undergoing IVF were included in the study. Serum AMH was assessed for all women. Embryo quality was assessed with the known implantation data (KID) score generated by the time-lapse imaging system.
Results There was no statistically significant difference in mean serum AMH among different KID score categories ($p = 0.135$). This remained non-significant after controlling for confounding variables ($p = 0.305$).
Conclusions The results of our study show no significant association between serum AMH and embryo quality in women undergoing IVF treatment when embryo quality was assessed using the KID scores generated by time-lapse imaging which is a better method of embryo assessment rather than conventional morphological assessment.

Keywords Anti-Mullerian hormone · Embryoscope · Time-lapse imaging · Ovarian reserve

Introduction

Anti-Mullerian hormone (AMH) produced by the granulosa cells of the ovary is an indicator of ovarian reserve. It is an excellent predictor of response to controlled ovarian stimulation in in vitro fertilisation (IVF)/intracytoplasmic sperm injection (ICSI) treatment [1]. Although a strong positive correlation between serum AMH levels and number of oocytes retrieved during IVF is reported [2–4], previous studies have not shown a clear correlation between AMH and embryo quality [5–7]. Embryo quality in these studies is evaluated using standard morphological assessment of developing embryos [5–7]. The predictive ability of standard morphological assessment for successful pregnancy remains poor [8] and methods for improved embryo selection are constantly being sought. Time-lapse imaging systems provide more detailed, objective and reproducible assessment of embryo development and quality compared to standard morphological assessment [9].

This study aimed to assess the association between ovarian reserve and embryo quality using the newer technology of time-lapse imaging. This was done by examining the correlation between serum AMH levels as a marker of ovarian reserve and 'KID' (Known Implantation Data) scores generated by time-lapse imaging of the developing embryos as an indicator of embryo quality.

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Materials and methods

A single centre, cross-sectional observational study was conducted at a university IVF clinic in the UK from June 2014 to December 2016. Eligible participants included couples having IVF/ICSI treatment during the recruitment period with embryos cultured and assessed in time-lapse incubators and with known implantation data. Thus, the study included all women with a single embryo transfer and those women with two embryos transferred who either had a negative pregnancy test or a dichorionic twin pregnancy. The participants were followed from the start of their IVF/ICSI treatment up to a time when either a clinical pregnancy or a negative pregnancy test was established. Data were collected from medical notes and electronic patient records. Collected data included serum AMH level and the KID score generated by the time-lapse system. Serum AMH is measured as a part of the routine baseline investigations for all women attending the fertility clinic and proceeding to IVF/ICSI treatment. Measurement of AMH was not restricted to a particular time of the menstrual cycle. All AMH assays were performed using the Beckman Coulter Generation II assay, and values were expressed as pmol/l. Inter-assay coefficients of variation for a low and high control were 10.3 and 10.0%, respectively. The time-lapse system used was the Embryoscope (Vitrolife, Sweden). Embryos were placed in the time-lapse incubators following IVF fertilisation check/ICSI and assessed on day 3 of embryo culture. Each embryo was annotated for five variables related to timing and synchronicity of cell divisions (morphokinetic parameters) which were combined to generate a composite score—KID score. The variable annotations are, time of pronuclear fading-tPNf, time of first cell division to two cells—t2, time of 3 cells—t3, time of 4 cells—t4 and time of 5 cells—t5. The models are based on deselection of embryos showing erratic morphokinetic patterns rather than selection intervals. The range of available scores was 1–5 with five denoting the best embryo quality and one a poor embryo quality. One or two embryos were replaced either on day 3 or day 5 of culture depending on the number and quality of available embryos. A pregnancy test, serum beta HCG, was done 14 days after embryo transfer. The primary outcome was the correlation between serum AMH and KID score. Secondary outcome measures were pregnancy rate (positive beta HCG) and clinical pregnancy rate. A positive pregnancy test was defined as levels of serum beta HCG greater than 30 IU/ml. Clinical pregnancy was defined as the presence of at least one fetal heartbeat seen on ultrasound scan. Data was also collected for confounding variables such as age, BMI, smoking status (current smokers and self-reported) and method of insemination (IVF/ICSI).

Statistical methods

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS Version 20). Basic descriptive statistics were used to describe all the variables in the dataset. The Kolmogorov–Smirnov test of normality was performed to assess distribution of the data. Skewed data was transformed prior to analysis using log transformation. A one-way between group's analysis of variance (ANOVA) or a Chi-squared test was performed depending on the type of data for all univariate analysis. When $p < 0.05$, the difference was considered to be statistically significant in all tests. When a difference was found to be significant, a post hoc Tukey's multiple comparison test was performed. Analysis of covariance (ANCOVA) was used to test for differences of AMH levels between KID scores while controlling for confounding factors. To explore the relative impact of serum AMH and the number of retrieved oocytes on the pregnancy rate, we performed a logistic regression analysis with pregnancy as a binary outcome variable and serum AMH and number of oocytes as explanatory variables.

Local Institutional Review Board approval was obtained (Ref No. 1675) and all data collection was done in accordance with data protection rules.

Results

A total of 198 women and 304 embryos were included in the study, as some women had more than one embryo transferred to the uterine cavity. The baseline characteristics of the participants are summarised in Table 1.

Participants were grouped into five categories based on their KID score. A univariate analysis (ANOVA) for each of the variables was done for the different KID score categories. The results are summarised in Table 2.

Table 1 Baseline characteristics of the study polulation

Age (years)	36 (33–39)
Body mass index	24 (22–27)
Current smokers	22 (7.2)
IVF/ICSI	88/216 (29/71)
Serum AMH (pmol/l)	12.95 (8.57–18.33)
KID scores	4 (2–5)
Oocytes retrieved	10 (6–13)

Values expressed as median (IQR) or number (%)

Table 2 Baseline and outcome variables in KID score categories

No.		KID 5	KID 4	KID 3	KID 2	KID 1	*p*
1	Serum AMH (pmol/l)	16.36	15.05	15.38	16.77	12.76	0.135
2	Age (years)	35.07	35.16	37.31	36.14	36.81	0.049*
3	Body mass index	24.73	24.66	25.72	23.91	23.34	0.411
4	Current smokers (as a % of smokers)	31.8%	40.9%	4.5%	4.5%	18.2%	0.660
5	Method of insemination: IVF/ICSI(%)	20.3/79.7	38.4/61.6	15.5/84.6	39.3/60.7	36.5/63.5	0.033*
6	Pregnancy rate (positive serum beta HCG, IU/L)(%)	37	15.1	0	21.7	11.5	<0.001*

There was no statistically significant difference in mean serum AMH among different KID score categories as determined by one-way ANOVA ($F(4, 293) = 1.769$, $p = 0.135$).

An ANCOVA to test for differences in AMH levels among KID scores while controlling for confounding variables, such as age and method of insemination (IVF/ICSI), showed no significant differences ($p = 0.305$). Pregnancy rates were significantly different among different KID score categories ($p < 0.001$) but there was no significant impact of serum AMH on the chance of pregnancy as shown in Table 3.

Discussion

Main results

The results of our study show no significant differences in mean serum AMH levels among different KID score categories (1–5) generated by the time-lapse imaging system. This indicates that serum AMH is not significantly associated with embryo quality as determined by time-lapse imaging. Our findings are in agreement with previous studies assessing the relationship between AMH and embryo quality assessed using other methods. Smeenk et al. [7], in an observational study, correlated basal serum AMH with embryo quality in 112 women undergoing controlled ovarian stimulation and IVF/ICSI. Serum AMH failed to show a predictive capacity with respect to embryo quality. Fong et al. [5] reported on correlation between basal serum AMH and embryo quality in 125 women undergoing IVF. Women were randomly assigned to either mild stimulation or conventional stimulation and parameters were assessed in both groups. Although a positive correlation was seen between serum AMH and embryo quality in the mild stimulation group, no significant correlation was seen in the conventional stimulation group. The latter group of women is a similar population to our dataset, and hence the results may be considered to be in agreement. Results from the study by Silberstein et al. [6] are not in agreement with our findings. This study compares serum AMH levels measured on the day of hCG trigger with embryo quality. Therefore, the discrepancy could be attributed to a different time frame used for the measurement of AMH. None of the previous studies employed the KID scores for a qualitative assessment of embryo quality.

Strengths and limitations

Embryo quality in previous studies was mostly evaluated using standard morphological assessment. This involves a single snapshot assessment of embryos at pre-specified times following IVF. The number of cells, degree of fragmentation and uniformity of the blastomeres are incorporated into this embryo scoring system. Standard morphological assessment has shown poor predictive ability for optimal embryo selection and subsequent correlation to pregnancy rates [8]. The time-lapse systems and KID scores used in our study provide an objective and reproducible assessment of embryo quality. The KID

Table 3 Results of logistic regression analysis

	95% CI for odds ratio				Sig.
	B (SE)	Lower	Odds ratio	Upper	
Constant	−3.098 (0.749)				0.000
Serum AMH*	0.651 (0.570)	0.627	1.918	5.865	0.253
Number of oocytes*	1.243 (0.730)	0.829	3.468	14.501	0.088

* Data log transformed to fit normal distribution

scores are generated by the manufacturers algorithm using 'morphokinetic' parameters. These parameters are generated based on the timing and synchronicity of early mitotic divisions and abnormal cleavage patterns of embryos. KID scores in our study show a strong correlation to pregnancy rate, which is in agreement with previous studies [10]. These scores may be considered a more robust indicator of embryo development and quality than previously used standard assessment, and hence strengthen the validity of our results as compared to previous studies. Embryo quality is a reflection of both oocyte and sperm quality. Oocyte and subsequently embryo quality is further dependent on a number of variables such as age and lifestyle factors such as smoking. Our study controls for these confounding variables when assessing the relationship between ovarian reserve and embryo quality.

The possibility of some selection bias cannot be excluded due to the observational design of the study. However, we feel that this would not impact on the results, as the study variables are objective and a wide range of serum AMH and KID score values are present in the dataset. There has been some recent concern about measurement and reported values of serum AMH. This is related to the instability of AMH and mainly caused by delays in processing and long storage times for samples. As the samples were delivered, spun, and stored at −20 °C, and analysed within 14 days in an onsite laboratory, we remain confident about the accuracy of our measurements. The study was conducted in the UK in a NHS funded IVF unit which places age restrictions on the women being treated. We hence have data only between 23 and 42 years of age and the results only applicable to this age group. It is, however, uncommon to have many women outside these age ranges seeking fertility treatment. We recruited 198 women but analysed the data for 304 embryos as some women had two embryos replaced. Hence, some AMH values have been duplicated in the analysis. Including only those women with a single embryo transfer would have considerably reduced the number of women in the dataset as only 25–30% of all transfers are single embryo transfers. The smoking status of women in the study is self-reported and we did not have confirmatory cotinine testing for participants. NHS funding for IVF/ICSI treatment is not available for smokers, and this may have led to a reduced self-reporting of the condition. This is, however, unlikely to affect the final outcomes as these women would be equally distributed across the dataset.

Interpretation

Pregnancy rates following fertility treatment have been positively correlated with serum AMH levels [1, 4, 11, 12]. This positive correlation has thought to be mainly due to the increased number of oocytes retrieved during the treatment rather than oocyte/embryo quality. Our study confirms these findings. Others have been unable to find a significant relationship between these two variables [13, 14]. The results of our study confirm that there is no significant relationship between serum AMH and embryo quality. These results are important for counselling women prior to the start of IVF treatment. Embryo quality may be dependent on several other known and unknown oocyte and sperm factors. However, serum AMH, a quantitative rather than qualitative indicator of ovarian reserve, does not appear to be associated with embryo quality, although it does help to decide the dose of gonadotropins to be used for controlled ovarian stimulation and estimate the number of oocytes that may be retrieved.

Conclusions

The results of our study show no significant association between serum AMH and embryo quality in women undergoing IVF/ICSI treatment when embryo quality was assessed using the KID scores generated by time-lapse imaging which is a better method of embryo assessment rather than conventional morphological assessment.

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Author contribution statement PB: Study idea and design, protocol development, data collection, analysis and interpretation, manuscript writing, editing and critical review. ME: Collection and analysis of data, editing of the manuscript and critical review. AS: Collection and analysis of data, editing of the manuscript and critical review. HJ: Collection and analysis of data, editing of the manuscript and critical review. AG: Data analysis and interpretation, editing of the manuscript and critical review. AS: Data analysis and interpretation, editing of the manuscript and critical review. GA: Study idea and design, protocol development, data analysis and interpretation, editing of the manuscript and critical review. RH: Study idea and design, protocol development, editing of the manuscript and critical review. All authors read the manuscript critically, commented on the draft and approved the final version before submission.

Compliance with ethical standards

Conflict of interest PB, ME, AS, HJ, AG, AS, GA, and RH have no conflict of interest to declare.

Ethical approval Local Institutional Review Board approval was obtained (Ref No. 1675) and all data collection was done in accordance with data protection rules. This article does not contain any studies with animals performed by any of the authors. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Informed consent Informed consent was not required from individual participants included in the study and all data collection was done in accordance with data protection rules.

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