APPROACHES TO IMPROVING EMBRYO IMPLANTATION

by

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Abstract

Embryo implantation represents a complex process vital in ensuring the normal development of pregnancy. Whether embryo implantation is the goal of natural conception or assisted reproductive treatment, the environment within the uterine cavity must be optimised in order to increase the chance of pregnancy.

This thesis uses a mixture of research methods to investigate potential approaches to improving embryo implantation. Below are the key findings from this thesis:

1. The vitamin D status in women undergoing assisted reproductive treatment is important. An interventional trial would prove or disprove the merits of vitamin D deficiency treatment in these women.
2. There is not enough evidence to suggest a clear association between vitamin D and recurrent miscarriage, however there is a strong argument for biological plausibility.
3. The use of endometrial fluid collected at the time of embryo transfer in women undergoing assisted reproductive treatments for metabolomics analysis is possible.
4. Women with hydrosalpinx associated tubal infertility should be offered salpingostomy as a treatment option as the natural conception rates are similar to that achieved in in vitro fertilisation treatment.

Dedication

I would like to dedicate this thesis to Anneke, Lily and Rolo – my wife, daughter and labradoodle who give me the greatest joy and happiness.

I also dedicate this thesis to my parents, Paul and Sylvia Denham, who have supported me throughout my career and have been there for me in good times and in bad.

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Lastly, I would like to thank all of the patients that consented to participation in my studies.

Abbreviations

$1,25[OH]_2D$	1,25-dihydroxyvitamin D / Calcitriol
25[OH]D	25-hydroxyvitamin D / Calcidiol
ART	Artificial Reproductive Treatment
BMI	Body Mass Index
CCG	Clinical Commissioning Group
ChRS	Centre of Human Reproductive Sciences
CI	Confidence Interval
CSF2	Colony Stimulating Factor-2
DIMS	Direct Infusion Mass Spectrometry
FET	Frozen Embryo Transfer
HFEA	Human Fertility and Embryology Authority
HOX10A	Homeobox-leucine zipper protein 10 A
ICSI	Intra-cytoplasmic Sperm Injection
IF-ϒ	Interferon gamma
IL	Interleukin
IQR	Interquartile Range
IVF	In Vitro Fertilisation
LC-MS/MS	Tandem Liquid Chromatography Mass Spectrometry
LIF	Leukaemia Inhibiting Factor
MeSH	Medical Subject Heading
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NRES	National Research Ethics Service
PCOS	Polycystic Ovarian Syndrome
PID	Pelvic Inflammatory Disease
SD	Standard Deviation
TNF-α	Tumour Necrosis Factor-alpha
TGF-β	Transforming Growth Factor Beta

UK	United Kingdom
VDBP	Vitamin D Binding Protein

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CHAPTER 1: THESIS INTRODUCTION

Embryo implantation

For many people, having children and a family is an ambition from a young age. However, many physiological processes must occur for pregnancy to be achieved. One of the most delicate and complex of these processes is embryo implantation. The complexities of the implantation process commence with fertilisation of an ovum and ends with embedding of the blastocyst into the endometrium. A series of precise steps ensures that placentation can occur to support the embryo throughout the gestation. Without the preceding step occurring normally, subsequent steps cannot follow. The steps involved for successful pregnancy to be initiated are divided into five stages[1]:

1. The fertilisation of the ovum
2. Division of the zygote
3. Entry of the morula into the endometrial cavity, with further development into a blastocyst
4. Blastocyst adherence and penetration into the endometrial stroma
5. Placentation

For the purposes of this introduction stages three and four will be considered in more detail.

Entry of the morula into the endometrial cavity

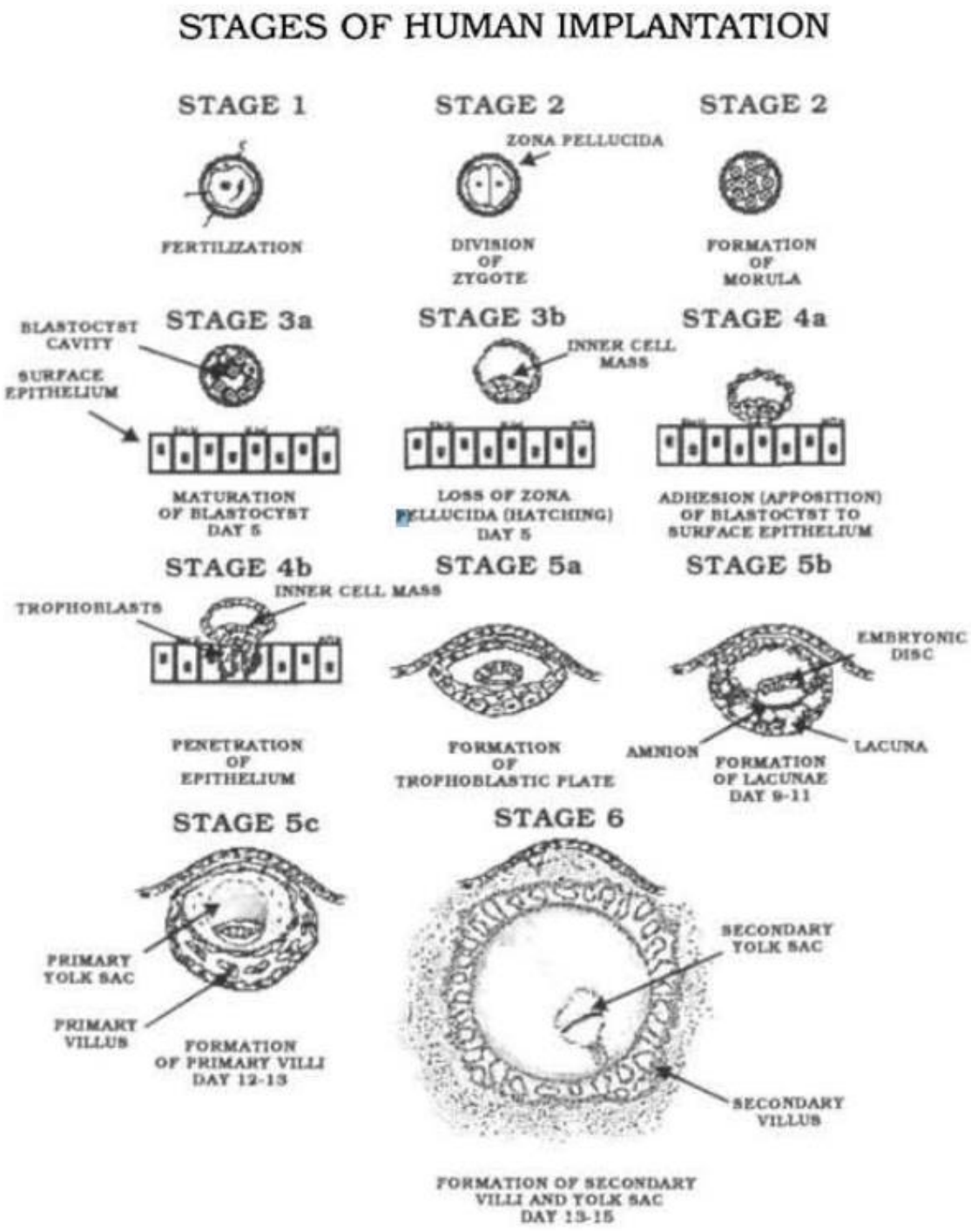
The pre-implantation blastocyst enters the endometrial cavity 72-96 hours after fertilisation of the ovum in the fallopian tube[2]. Cross talk between the blastocyst and the endometrium occurs immediately on entry in to the endometrial cavity. Further maturation of the

blastocysts occurs and it hatches from the zona pellucida[3] allowing contact between the trophoblast cells and the endometrium. It is important to note that at this stage, many blastocysts will never cause a clinically recognised pregnancy due to failure of the embryo to implant into the endometrium[4]. This is because successful embryo implantation can be prevented by a variety of factors making human reproduction relatively inefficient when compared with other mammalian species. These factors include karyotypical defects (which would not allow normal maturation of a zygote into a blastocyst), anatomical abnormalities within the female reproductive organs, and inflammation (which leads to an environment too hostile to allow implantation to take place)[5]. However, even in the absence of these factors, successful embryo implantation also depends on the synchrony in the development of both the embryo and the endometrium.

Blastocyst adherence and penetration

Transition of a free blastocyst within the endometrial cavity to implantation requires initial adherence of the blastocyst with the endometrium. This is initiated with apposition and then penetration of the endometrium[1] (see figure 1). Very little is known regarding this in humans due to the delicate nature of the processes. However, data from animal research suggests that apposition occurs five to six days after fertilisation, and is characterised by the close approximation of the plasma membranes of the trophoblast cells of the blastocyst with the plasma membranes of the surface epithelial cells of the endometrium[6]. A microfilament structure develops between the two cell surfaces allowing a cell to cell stable apposition.

Figure 1. The stages of human implantation[1]



The bonds formed in apposition are relatively weak, so implantation may still not occur[6]. It is only after penetration of the blastocyst that implantation is completed. Penetration in humans is thought to occur by outward processes of the syncitiotrophoblast invading in to

the spaces between the surface epithelial cells of the endometrium. Invasion causes the loss of inter-cellular junctions between the epithelial cells and the formation of new junctions between the syncitiotrophoblast cells and the epithelial cells. Invasion continues until the basement membrane of the endometrium is reached[7].

If pregnancy is to be achieved following blastocyst penetration, the placenta must form to provide an adequate blood supply to the blastocyst. Briefly, early placentation occurs between seven and 13 days after fertilisation[1]. Trophoblastic development results in the formation of cytotrophoblasts and syncytial trophoblasts. Further invasion occurs, with trophoblastic invasion into the maternal uterine blood vessels so that maternal blood floods spaces between the trophoblasts called lacunae. Placental villi develop and further anchor the pregnancy to the maternal endometrium[1]. For the purposes of this thesis introduction, the process of placentation will not be explained in any more detail.

The importance of the endometrium in embryo implantation

The complex processes of embryo implantation are described above. However, the importance of the endometrium in implantation cannot be understated. It must be receptive and prepared to allow effective cross talk with the blastocyst and for adherence and penetration to occur.

The endometrium is a dynamic structure, changing on a daily basis under the influence of a number of hormones. In a physiological menstrual cycle, these hormones cause recurring

proliferation and differentiation with the aim of supporting a potential pregnancy. The menstrual cycle consists of three phases; the menstrual phase, the proliferative phase and the secretory phase.

Menstruation begins on the first day of the menstrual cycles and is caused by progesterone withdrawal. The proliferative phase results from the growth of the endometrium from the endometrial basalis layer under the influence of oestrogen secreted by the growing cohort of ovarian follicles. Once a dominant follicle has emerged, ovulation is triggered by a surge in luteinising hormone from the anterior pituitary gland. The secondary oocyte is then released into the fallopian tube ready for fertilisation. The follicular remnant, or corpus luteum, produces high levels of progesterone and oestrogen, resulting in the differentiation of the endometrium to form secretory changes. Aside from the oestrogen and progesterone, endometrial changes are also caused by secondary autocrine and paracrine hormonal effects and by cell mediators such as cytokines[8,9]. At a histological level, the early secretory phase is characterised by the development of vacuoles within the endometrial cells. During the mid-secretory phase (six to ten days after ovulation), decidualisation occurs and the apical epithelium develops finger like protrusions called pinopodes[10,11]. It is during the mid-secretory phase of the menstrual cycle when embryo implantation is most likely to occur, and this is termed the "window of implantation"[12]. If implantation does not occur, no human chorionic gonadotrophin is produced by trophoblastic tissue; causing the endometrium to enter the late secretory phase, when preparations are made for menstruation. Progesterone production reduces from the corpus luteum leading to a breakdown in the endometrium causing menstruation and the cycle begins again.

Importantly, the mid-secretory phase and the "window of implantation" occur at the same time that a morula enters the endometrial cavity, if fertilisation of an ovum has occurred. The synchrony between the endometrium and the embryo is critical for implantation and pregnancy to occur. Asynchrony or abnormalities in the intrinsic factors outlined above manifests as infertility.

Infertility as a disease

Infertility is a cause of high anxiety and stress for one in seven couples in the United Kingdom (UK)[13]. Depending on the cause of infertility, women may suffer physical, psychological and emotional symptoms. Furthermore, the inability to have a family can cause breakdown of relationships leading to further negative health impact[14]. In women under the age of 40, having regular unprotected sexual intercourse, 80% of couples will have conceived at 12 months. After 24 months the cumulative pregnancy rate increases to 90%[13].

The National Institute for Health and Care Excellence (NICE) recommends that in couples not conceiving after 12 months, initial investigations and clinical assessments should be conducted. These investigations include a pituitary hormone profile, mid-luteal progesterone, rubella immunity testing, a fallopian tube assessment, pelvic ultrasonography, and a semen analysis. These investigations are performed to identify the cause of infertility.

Causes of infertility

The causes of infertility can be divided into male and female causes. Male infertility may be caused by primary testicular dysfunction, obstruction to the vas deferens, endocrine factors

(e.g. hypogonadotrophic hypogonadism and hyperprolactinaemia), autoimmune factors (e.g. anti-sperm antibodies), and drugs (e.g. tobacco, alcohol, steroid use and chemotherapy)[15].

Female infertility can be caused by anovulation (due to dysfunction of the hypothalamic-pituitary ovarian axis), endocrine factors (e.g. hyperprolactinaemia and thyroid dysfunction), tubal damage, and uterine factors[13].

In up to one third of couples no cause is found and this is termed unexplained infertility. Irrespective of the cause of infertility, the health impact is vast. Many couples have longstanding wishes to become parents and most couples would not expect difficulties in conceiving. This leads to detrimental effects on the psychological wellbeing of both the female and male partners. Furthermore, some causes of infertility, such as endometriosis, fibroids and endometrial polyps can also cause physical symptoms in the form of dysmenorrhoea, dyspareunia or abnormal menstruation[16,17].

Treatment of infertility

Once the cause of infertility is identified, treatment can commence. The nature of the treatment will depend on the type of infertility. This can range from lifestyle advice and mild medical treatments in the form of oral medications to more invasive treatments such as IVF and ICSI or even surgery to correct anatomical abnormalities.

Some couples may require very mild treatments to help them conceive. For example, in polycystic ovarian syndrome, all that may be required is advice regarding weight loss. In women where weight loss does not achieve regular menstruation, ovulation induction agents such as clomiphene can be used[18].

In couples where simple treatments have not been successful, more invasive medical treatments such as in vitro fertilisation (IVF) or intra-cytoplasmic sperm injection (ICSI) may be required. There are a number of indications for the use of IVF and ICSI treatment. These include; male factor infertility, tubal infertility unsuitable for surgical correction, anovulation refractory to ovulation induction agents, and unexplained infertility. The use of these high technology assisted reproductive treatments (ART) is increasing and the most recently published data from the Human Fertility and Embryology Authority (HFEA) states that in 2014, 52,288 women had a total of 67,708 cycles of IVF/ICSI in the UK. This represents a consistent five per cent rise in the number of IVF cycles performed each year[19].

Lastly, in some women with infertility, surgery may represent a treatment option. Before the availability of IVF and ICSI was widespread, fertility surgery was the only available option for many infertile couples. Uterine abnormalities such as fibroids or endometrial polyps were removed surgically. This was then followed by efforts at natural conception. Modern reproductive medicine now uses surgical correction of uterine abnormalities as a tool to optimise the uterus before IVF and ICSI treatment is performed. Tubal infertility can also be treated with fallopian tube conserving surgery. Surgery may offer some infertile couples the

chance of long lasting efforts at natural conception without depending on IVF or ICSI treatment once abnormal reproductive tract anatomy has been corrected[20].

Fertility treatment funding

Simple medical treatments and fertility surgery are funded by the National Health Service (NHS). However, if IVF or ICSI treatment is required, funding for these high technology treatments varies geographically according to local Clinical Commissioning Group (CCG) funding regulations[13]. This can mean that in some areas NHS funding is provided for up to three IVF cycles whilst in others there is no funding for fertility treatment at all. Once funding has been allocated and used up, subsequent attempts are self-funded by the infertile couple. Strict funding restrictions also apply; meaning that couples must fulfill certain criteria to be eligible to receive NHS funded IVF treatment. Examples of these criteria include; both partners being non-smokers, both partners having a body mass index of 30 or less, and neither partner having any living children[13].

IVF and embryo implantation

IVF was first performed successfully in 1978. Louise Brown was born after a natural treatment cycle with no exogenous pituitary hormones administered. Sir Robert Edwards, who later won the Nobel Prize for Physiology in 2010, pioneered IVF. IVF involves the fertilisation of an oocyte by a sperm in vitro. The ovaries are stimulated by exogenous follicular stimulating hormone and luteinising hormone to induce the growth of multiple ovarian follicles. Final maturation of the oocyte is then triggered using a human chorionic

gonadotrophin injection. This is followed by ultrasound guided oocyte retrieval. Sperm is then prepared and used to inseminate the oocytes. The fertilised oocytes are cultured, and the best embryo is transferred into the uterine cavity. The goal is to achieve embryo implantation and subsequently pregnancy by ART that substitute the physiological processes mentioned above.

Major advancements have occurred since the first IVF treatment cycles and success rates have improved steadily over the past four decades. Major advancements have included the development of ultrasonography, which is now used to monitor ovarian stimulation, as well as the increase in knowledge regarding embryology. Indeed, reproductive embryology is now a large branch of biology, which has benefitted from a great deal of research. This has led to a greater understanding of how normal embryos should develop allowing embryologists to select embryos with the best fertility potential with a high degree of confidence[21]. Most recently, time-lapse embryology has been developed further improving the ability of embryologists to choose the embryos with the greatest potential to cause pregnancy[22].

Despite these advancements, recent improvement in IVF treatment success has plateaued. Many patients fail to become pregnant with IVF treatments despite the transfer of high quality embryos[5]. Although our understanding of the developing embryo has gained clarity, knowledge concerning embryo implantation is not as clear. The implantation process is difficult to study as it is delicate and can easily be disrupted. It is now understood that for pregnancy to be achieved the endometrium must be at optimal conditions to allow for

implantation. As mentioned above, this is the case in natural conception when there is a putative 'window of implantation' in each physiological menstrual cycle. In ART, the goal is to mimic those intrauterine conditions found in the natural 'window of implantation'.

This thesis aims to highlight several approaches that may improve embryo implantation in both natural conception and in artificial reproductive treatments.

Vitamin D and IVF treatment

The first two chapters of this thesis explore the association between vitamin D status in women undergoing IVF and ICSI treatment and the likelihood of treatment success. Vitamin D and health has recently been the subject of a great amount of research due to the discovery that vitamin D may have more health impact than its classical role in bone and calcium homeostasis[23]. In reproductive health, vitamin D is thought to have a role in obstetric medicine as well as in gynaecology. In particular, vitamin D receptors have been found in the endometrium leading to many in the field of reproductive medicine to consider vitamin D as an important regulator for implantation[24].

In the second thesis chapter, the importance of vitamin D in IVF treatment is explored by a systematic review and meta-analysis of existing literature. The methods, findings and inferences of a cohort study, performed to identify an association between vitamin D and IVF treatment outcome, are presented in the third thesis chapter.

Vitamin D and recurrent miscarriage

The high levels of interest in vitamin D and embryo implantation may also have implications for miscarriage. In some women, conceiving is not the primary problem that causes childlessness. Instead, it is the inability to maintain pregnancy. In patients with recurrent miscarriage, the main issue is not with embryo implantation. However, there has been a great deal of research that has suggested that the extravillous trophoblastic invasion that occurs after initial embryo implantation is affected by vitamin D. As a clinical population women with recurrent miscarriage lie adjacent to the population with infertility, being treated by the same reproductive medicine clinicians, therefore it was felt necessary to investigate the relationship between vitamin D and recurrent miscarriage further.

Recurrent miscarriage affects one per cent of couples and is diagnosed when three or more miscarriages have been suffered consecutively. In half of women with recurrent miscarriage a potential cause is identified. These causes include karyotypical abnormalities, thrombophilias and uterine anomalies. However, in the remaining half of women diagnosed with recurrent miscarriage a cause may not be found[25,26]. In order to improve understanding of the link between vitamin D and recurrent miscarriage, a systematic narrative review was undertaken. This is presented in the fourth thesis chapter.

The use of endometrial fluid in predicting IVF treatment outcome

The fifth and sixth thesis chapters explore the use of endometrial fluid in predicting IVF treatment outcome. As mentioned above, knowledge regarding the optimum environment

for embryo implantation lags behind advancements in embryology. Novel techniques to distinguish between the receptive versus the non-receptive endometrium are desperately required in order for continued improvement in IVF treatment success rates. One of these innovative techniques is to investigate the fluid within the endometrial cavity. This fluid represents the environment in which the embryo must implant and has been studied by many research groups who aim to uncover the optimum conditions for the implantation process to progress successfully in IVF treatment. A systematic narrative review was performed to identify the existing literature and this is presented in thesis chapter five. The narrative review presented in thesis chapter five demonstrates that endometrial fluid has been used to investigate the protein, genetic and immunological constituents of endometrial fluid sampled during IVF treatment[27]. However, there was no published evidence of endometrial fluid being used to explore metabolic profiling. A proof-of-principle cohort study is therefore presented in thesis chapter six. In this chapter, endometrial fluid from women undergoing IVF treatment is used for metabolomics profiling. This is a relatively new systems biology technique, which allows for the interrogation of the metabolic signature of bio-fluids. The aim of this cohort study was to confirm the feasibility of using endometrial fluid sampled in women undergoing IVF treatment for metabolomics analysis. Additionally, the study aimed to identify a panel of metabolites predictive of successful implantation.

Salpingostomy in the treatment of hydrosalpinx

Tubal infertility is caused by fallopian tube obstruction, which does not allow for in vivo fertilisation of the oocyte by sperm. Tubal obstruction is usually preceded by inflammation of the tubal mucosa. This inflammation of the fallopian tubes can be due to endometriosis or pelvic infection. With severe tubal obstruction, hydrosalpinx (fluid trapped in the tube causing swelling) can result. IVF treatment was initially developed for women with tubal infertility. In the case of tubal infertility caused by hydrosalpinx, it has been discovered that the fluid within the fallopian tube must be kept separated from the endometrial cavity so that the embryo fertilised in vitro has an increased likelihood of implanting[28,29]. The hydrosalpingeal fluid is thought to be toxic to the embryo and also cause a mechanical washout effect if allowed to communicate with the uterine cavity. Importantly, it is understood that the hydrosalpingeal fluid alters inflammatory cytokines, prostaglandin concentrations as well as endometrial natural killer activity, hindering embryo implantation. As a consequence it has become common practice to remove or disconnect the fallopian tubes affected with hydrosalpinx prior to beginning IVF treatment. Unfortunately this means that women with bilateral hydrosalpinges can be rendered sterile and reliant on IVF treatment for any further efforts at pregnancy. Before the widespread availability of IVF treatment, conservative tubal surgery was performed to correct hydrosalpinx[30]. The tube was drained by salpingostomy and couples were encouraged to continue efforts at natural conception. This treatment strategy is now rarely used but was effective. To investigate the effectiveness of salpingostomy in the treatment of hydrosalpinx a systematic review and meta-analysis was performed and this is presented in thesis chapter seven. Investigating the merits of tubal conservative surgery is an important approach to improving embryo

implantation in those with hydrosalpinx associated tubal infertility. This is the reason for investigating the efficacy of salpingostomy treatment for women with hydrosalpinx.

Conclusions drawn

This thesis seeks to explore the approaches that may be taken to improve the chances of embryo implantation in natural conception and in IVF treatment. The final chapter (chapter eight) summarises all of the findings of the thesis and highlights the potential directions that future research could take in this exciting and clinically important domain.

CHAPTER 2: VITAMIN D AND ASSISTED REPRODUCTIVE TREATMENTS – SYSTEMATIC REVIEW AND META-ANALYSIS

This work has been submitted to the British Medical Journal for publication. Reviewers' comments are awaited.

Introduction

In the UK, one in seven women have difficulties in conceiving pregnancy[31]. IVF treatment represents a viable treatment option in a significant proportion of these women. However, there are funding restrictions in place as well as strict criteria that couples must fulfill in order to access public funds. Furthermore, in many geographical regions in the UK, couples may only be entitled to funding for a single cycle of IVF treatment[32]. This has meant that it has become increasingly important to identify factors that can be improved in order to maximize the chances of IVF treatment success. Research into IVF outcome improvement has become an important research domain. One potential factor identified has been one of vitamin D status[23,24].

Many cohort studies have been published to substantiate an association between vitamin D status and IVF treatment success. Vitamin D deficiency is common in temperate countries such as the United Kingdom. Its prevalence has been reported as high as 49.5% in pregnant women[33] and has been found to be especially high in women of reproductive age with polycystic ovaries[34].

Vitamin D is a secosteroid hormone that exerts many biological effects[35]. Human intake is mainly through ultraviolet light skin exposure causing precursor conversion of 7-dehydrocholesterol into provitamin D3. In turn, provitamin D3 undergoes isomerization into cholecalciferol and is carried to the liver by vitamin D binding protein to the liver where it undergoes hydroxylation by 25-hydroxylase to 25-hydroxy vitamin D[36,37]. Further hydroxylation is carried out in the kidneys producing 1,25-dihydroxyvitamin D, which is the

biologically active form of vitamin D[35]. The biological actions are mediated by vitamin D receptors leading to activation of target genes through the retinoid receptor X[38,39]. An overview of vitamin D metabolism and its actions are provided in figure 2[40].

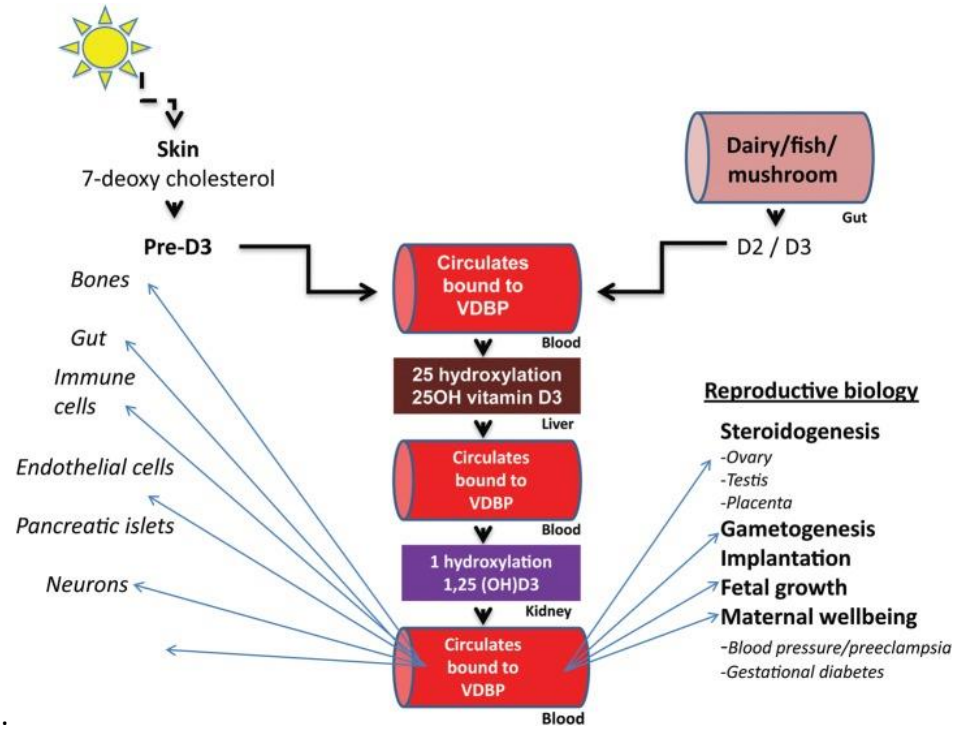


Figure 2. Metabolism of vitamin D and its physiological actions

Vitamin D status is ascertained by collecting blood serum and measuring 25-hydroxy vitamin D by a reliable assay. This is the most dependable method of investigating vitamin D status due to the long half-life of 25-hydroxy vitamin D (two to three weeks). The Endocrine Society and Institute of Medicine's classifies vitamin D deficiency as <50nmol/L, insufficiency is 50-75nmol/L and replete status is >75nmol/L[41].

The traditional role of vitamin D is in its importance in bone and calcium metabolism; however, more recently wider biological functions have been identified[42]. These include its roles in cancer, autoimmune disorders and cardiovascular disease[43]. In addition, vitamin D seems to be important in female reproductive physiology. This has been hypothesized as vitamin D receptors and the enzymes required for vitamin D metabolism have been found within the ovaries, placenta and endometrium[44,45]. Furthermore, vitamin D deficient mice are found to have reduced mating success with decreased litter sizes and uterine hypoplasia[46].

Vitamin D's immunomodulatory role is thought to be due to its effects on cytokine profiles within tissues. The presence of vitamin D allows for a preference to the T-helper 2 subset of cytokines including interleukin-4 (IL-4), IL-5 and IL-13 whilst also inhibiting the T-helper 1 cytokine response of IL-1, IL-2, interferon gamma (IF-Ɣ) and tumour necrosis factor alpha (TNF-α)[47,48]. It is postulated that this dampening of immune response within the endometrium allows for a more favourable environment for the embryo to implant and develop into a pregnancy[49]. Further immunomodulatory biological evidence is seen in decidual cells where vitamin D reduces natural killer cells release of pro-inflammatory cytokines such as Colony Stimulating Factor-2 (CSF2), IL-1, IL-6 and TNF-α[50].

Further studies have found that endometrial homeobox-leucine zipper protein 10 A (HOX10A) gene expression reflects vitamin D levels within the body and both have also been found to increase mid-cycle in naturally menstrual cycles[51]. In addition to HOX10A gene

expression, vitamin D also regulates gene expression for calbindin and osteopontin. Both of these genes are thought to be critical in embryo implantation and placentation[50].

This biological evidence above has prompted many research groups to investigate the actions of vitamin D on the endometrium, and specifically how vitamin D may influence the likelihood of IVF treatment success. The aim of this systematic review and meta-analysis was to compare the chances of clinical pregnancy in vitamin D deficient patients undergoing IVF treatment with the chances of clinical pregnancy in patients with a normal vitamin D status.

Methods

Literature Search

The population of interest was women undergoing any form of ART (IVF, ICSI and frozen embryo transfer [FET]) who had their vitamin D status checked. This could either be through serum blood testing or follicular fluid assay. The primary outcome was clinical pregnancy (the appearance of fetal heart activity at approximately seven weeks gestation after embryo transfer). Secondary outcomes included pregnancy test positive rates, miscarriage rates and live birth rates.

The following electronic databases were searched: MEDLINE, EMBASE, Cochrane Central Register of Controlled Trials and CINAHL (from inception to November 2015). A wide search strategy was developed based on the following key words and/or medical subject heading (MeSH) terms: pregnancy, in vitro fertilization, intracytoplasmic sperm injection, assisted

reproductive techniques and vitamin D (see appendix 1). The references of all included primary and review articles were examined to identify relevant articles not captured by the electronic searches. No language restrictions were applied in any of the searches or study selection.

Study Selection

Criteria for inclusion in the study were established a priori. Study selection was carried out by two independent reviewers. Firstly, the titles and abstracts identified by the electronic searches were examined by the reviewers. Examination of each title and abstract allowed the inclusion or exclusion of the article independently according to the predefined inclusion criteria; any disparities regarding inclusion between the two independent reviewers were resolved by a further independent reviewer. Full manuscripts of the titles and abstracts that were considered to be relevant for inclusion were obtained. When there was a duplicate publication, the most recent and complete version was selected and included. Studies that did not explicitly report results from assisted reproductive treatments in patients having their vitamin D status measured were excluded.

Further exclusion of full manuscripts was conducted if they did not meet inclusion criteria. Finally the relevant data from the final included full manuscripts were extracted by the two independent reviewers.

Validity Assessment

Two independent reviewers used the Newcastle-Ottawa Quality Assessment Scales for observational studies to complete a quality assessment of the included manuscripts. The Newcastle-Ottawa scale ranges from zero to nine, awarding one star for all categories (case-cohort representative, ascertainment of exposure, outcome negative at commencement of study, outcome assessment, duration of follow up and adequacy of follow up) except comparability by design or analysis where two stars can be awarded. An arbitrary score was allocated assuming that all items have equal weighting. Each study received a score from each of the reviewers.

Statistical Analysis

Clinical pregnancy, live birth, miscarriage and ectopic pregnancy rates were extracted from each study. The log of the ratio and its corresponding standard error for each study was computed. Meta-analysis using inverse-variance weighting was performed to calculate the random-effects summary estimates. The square root of this number is the estimated standard deviation of the underlying effects across studies. Because we had relative measures of effect, the confidence intervals were centered on the natural logarithm of the pooled estimate and the limits exponentiated to obtain an interval on the ratio scale. Forest plots were created for each outcome, showing individual study proportions with confidence intervals (CIs) and the overall DerSimmonian-Laird pooled estimate. Vitamin D insufficient and deficient data were combined in each study and compared against the data from participants replete in vitamin D. Heterogeneity of the treatment effects was assessed

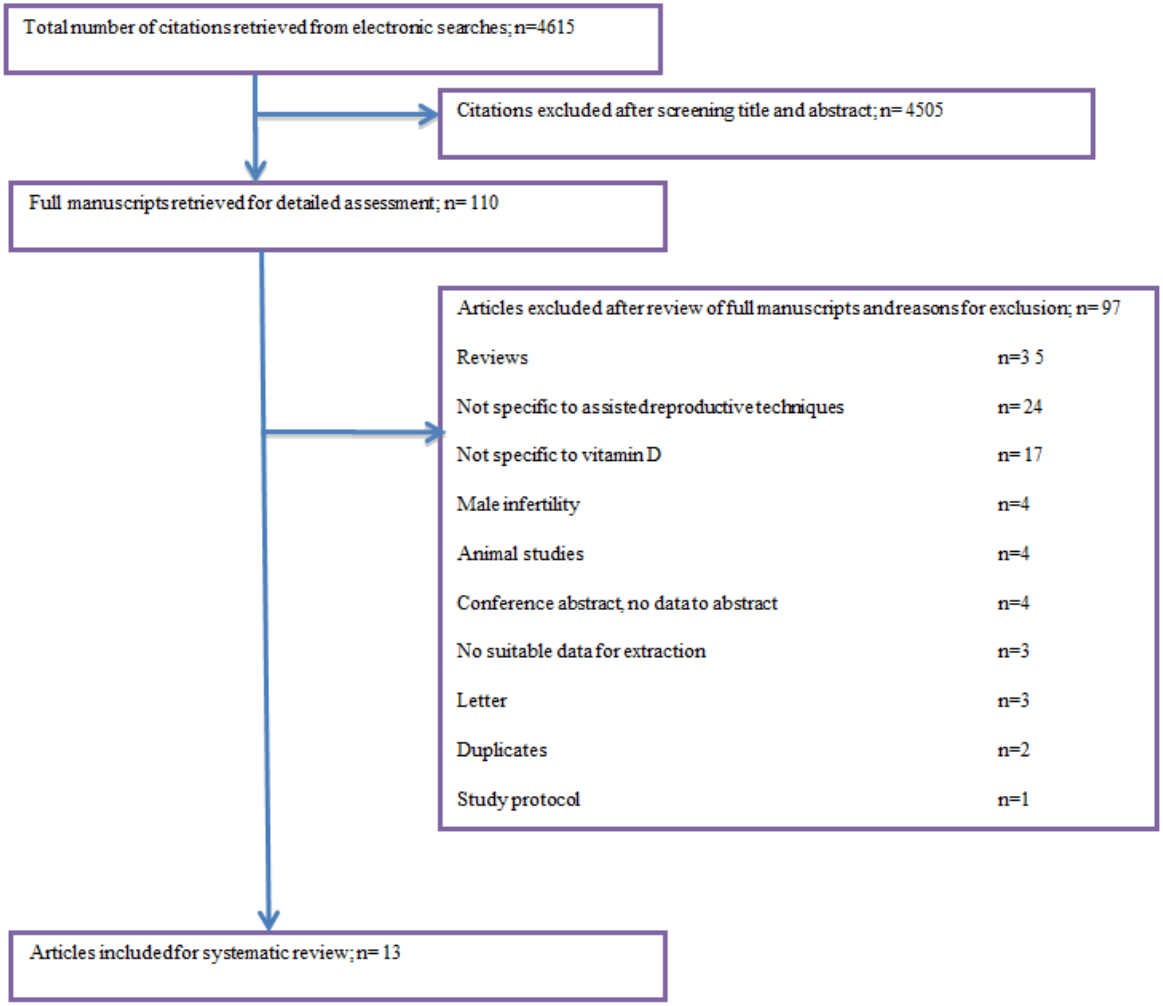
graphically with forest plots and statistically analyzed using the χ^2 test. Statistical analyses were performed using Stata 12.0 (StataCorp, College Station, TX).

Lastly, we performed a stratified analysis splitting studies that reported findings in women who had received a donor oocyte embryo and studies that reported findings in women using own oocyte embryo. This analysis was performed in an attempt to isolate the effect of vitamin D on endometrial implantation alone, thereby eliminating the effects of oocyte and embryo quality on the assisted reproductive treatment outcomes.

Results

The PRISMA flow diagram[52,53] of the review process is presented in figure 3. The search strategy yielded 4615 citations. Of these 4505 publications were excluded as it was clear from scrutinizing the title and abstract that they did not fulfil the selection criteria. This meant that 110 full manuscripts were obtained and examined. Of these, 110 full manuscripts 35 were review articles, 24 were publications not specific to assisted reproductive treatments, 17 were studies where vitamin D was not measured, four studies investigated the impact of vitamin D deficiency on sperm and male infertility, four were animal studies, four were conference abstracts and had no extractable data, three studies had data unsuitable for extraction, three were letters, two were duplicate publications, and one was a study protocol with no results. This left a total of 13 citations for final inclusion in this review[54–66]. Three interventional studies[67–69] were also identified between the time of the literature search and preparation of the thesis.

Figure 3. PRISMA flow diagram for study selection. Vitamin D and assisted reproductive techniques systematic review and meta-analysis



Study characteristics

The study characteristics of the 13 included manuscripts are presented in Table 1. The studies varied in publication date between 2010 and 2014. All 13 included studies were cohort studies; seven were retrospective and six were prospective in design. Sample sizes varied between 64 women to 517 women. Ten of the 13 included studies reported the ages of their study population. Eight studies had a mean age of below 37 years and two had a higher mean age of 40.5 and 40.9 years. The included studies varied in their method of investigating vitamin D status. Nine included studies used serum measurement of vitamin D, three used both follicular fluid and serum vitamin D (finding that there was high correlation between the follicular fluid vitamin D and serum vitamin D in their participants) and one study only used follicular fluid for vitamin D measurement. Of the 13 included studies, ten studies reported ART where women had used autologous oocytes. Three reported results from women who were donor egg recipients. One study used pre-implantation genetic diagnosis to ensure that patients had karyotypically normal embryos transferred into their endometrial cavities. One study chose to only study women that underwent a single blastocyst transfer.

Table 2 shows the results of the Newcastle-Ottawa Quality Assessment. All studies scored well achieving a score between 7 and 9.

Table 1. Characteristics of included studies. Vitamin D and IVF treatment systematic review and meta-analysis

Author (year)	Study design	Study population	Age of study population	Method of vitamin D investigation	Autologous or donated oocyte	Summary of results		Conclusions
Aleyasin et al., (2011)	Prospective Cohort	82 women undergoing IVF in Iran	Mean age 29.8 +/-4.3 years	Vitamin D in follicular fluid and serum	Autologous	<u>Clinical pregnancy</u> 24/82 deficient group 0/0 insufficient group 0/0 replete group <u>Pregnancy test positive</u> 29/82 deficient group 0/0 insufficient group 0/0 replete group	<u>Miscarriage</u> Data not provided <u>Live birth</u> 19/82 deficient group 0/0 insufficient group 0/0 replete group	Significant correlation between serum and follicular fluid and vitamin D. No significant differences in IVF outcome when participants were divided into tertiles by vitamin D
Anifandis et al., (2010)	Prospective Cohort	101 women undergoing IVF in Greece	Not reported	Vitamin D in follicular fluid	Autologous	<u>Clinical pregnancy</u> 10/31 deficient group 16/49 insufficient group 3/21 replete group <u>Pregnancy test positive</u> Data not provided	<u>Miscarriage</u> Data not provided <u>Live birth</u> Data not provided	Follicular fluid vitamin D levels significantly correlated to the quality of the embryos. Data suggested that high levels of vitamin D led to a decreased chance of clinical pregnancy

Fabris et al., (2014)	Retrospective Cohort	267 women undergoing donor oocyte IVF in Spain	Mean age 40.5 years	Vitamin D in serum	Donated	Clinical pregnancy 68/92 deficient group 94/134 insufficient group 29/41 replete group Pregnancy test positive 60/92 deficient group 85/134 insufficient group 25/41 replete group	Miscarriage 8/92 deficient group 9/134 insufficient group 4/41 replete group Live birth 56/92 deficient group 71/134 insufficient group 23/41 replete group	No significant difference in implantation or clinical pregnancy rates between deficient, insufficient and replete vitamin D groups
Firouzabadi et al., (2014)	Prospective Cohort	221 women undergoing IVF in Iran	Mean age 29.2 years	Vitamin D in follicular fluid and serum	Autologous	Clinical pregnancy 23/50 deficient group 47/155 insufficient group 4/16 replete group Pregnancy test positive Data not provided	Miscarriage Data not provided Live birth Data not provided	No significant correlation between follicular fluid or serum vitamin D and clinical pregnancy rate. Significant correlation between follicular fluid vitamin D levels and serum vitamin D levels (NOTE***USED <25NMOL = DEF, 25-75NMOL = INSUFF, >75NMOL = REPLETE)
Franasiak et al., (2014)	Retrospective cohort	517 women undergoing IVF with euploid blastocyst	Mean age 35.0 years	Vitamin D in serum	Autologous	Clinical pregnancy 144/206	Miscarriage 32/206 deficient group	Vitamin D status unrelated to pregnancy rates in women undergoing euploid blastocyst transfers

		transfer in USA				deficient group 151/215 insufficient group 64/96 replete group <u>Pregnancy test positive</u> 163/206 deficient group 162/215 insufficient group 74/96 replete group	29/215 insufficient group 14/96 replete group <u>Live birth</u> 131/206 deficient group 133/215 insufficient group 60/96 replete group	
Fru et al., (2014)	Retrospective Cohort	102 women undergoing IVF in USA	Not reported	Vitamin D in serum	Autologous	<u>Clinical pregnancy</u> 6/18 deficient group 24/47 insufficient group 37/58 replete group <u>Pregnancy test positive</u> Data not provided	<u>Miscarriage</u> 1/6 deficient group 9/24 insufficient group 12/37 replete group <u>Live birth</u> 5/18 deficient group 15/47 insufficient group 25/58 replete group	Higher vitamin D levels correlated with increased likelihood of positive pregnancy test. Overall live birth rates highest in vitamin D replete group.

Garbedian et al., (2013)	Prospective Cohort	173 women undergoing IVF in Canada	Mean age 34.5 years	Vitamin D in serum	Autologous	<u>Clinical pregnancy</u> 33/95 deficient and insufficient groups combined 41/78 replete group <u>Pregnancy test positive</u> Data not provided	<u>Miscarriage</u> Data not provided <u>Live birth</u> Data not provided	Implantation and clinical pregnancy rates are higher in the vitamin D sufficient group (>75nmol/L). Statistical significant difference in clinical pregnancy rate, no statistical difference in pregnancy positive rate. NOTE*** DEF AND INSUFF (<75NMOL) PUT TOGETHER VERSUS REPLETE (>75NMOL)
Ozkan et al., (2010)	Prospective Cohort	84 women undergoing IVF in Turkey	Mean age 34.4 years	Vitamin D in follicular fluid and serum	Autologous	<u>Clinical pregnancy</u> 5/23 deficient group 6/30 insufficient group 15/31 replete group <u>Pregnancy test positive</u> 3/23 deficient group 4/30 insufficient group 8/31 replete group	<u>Miscarriage</u> Data not provided <u>Live birth</u> Data not provided	Serum and follicular fluid strong correlated. Higher implantation and clinical pregnancy rates in insufficient (20-30ng/ml) and replete levels (>30ng/ml) when compared to deficient group - highest in replete group
Paffoni et al., (2014)	Prospective cohort	335 women undergoing IVF in Italy	Mean age 36.9 years	Vitamin D in serum	Autologous	<u>Clinical pregnancy</u> 30/154 deficient group 33/117 insufficient	<u>Miscarriage</u> Data not provided <u>Live birth</u>	Analysis suggested those with a vit D >75nmol/L had the highest chance of clinical pregnancy when compared with those with vitamin D deficiency or insufficiency.

						group 23/64 replete group <u>Pregnancy test positive</u> 34/154 deficient group 36/117 insufficient group 25/64 replete group	29/154 deficient group 28/117 insufficient group 19/64 replete group	
Polyzos et al., (2014)	Retrospective cohort	368 women undergoing IVF resulting in single blastocyst embryo transfer in Belgium	Mean age 30.6 years	Vitamin D in serum	Autologous	<u>Clinical pregnancy</u> 98/239 deficient group 70/129 insufficient and replete group combined <u>Pregnancy test positive</u> 124/239 deficient group 86/129 insufficient and replete groups combined	<u>Miscarriage</u> 44/239 deficient group 25/129 insufficient and replete groups combined <u>Live birth</u> 78/239 deficient group 61/129 insufficient and replete groups combined	Clinical pregnancy rate significantly lower in vitamin D deficient group p=0.015. Controlled for 16 confounding factors. NOTE*** INSUFF (50-75NMOL) AND REPLETE (>75NMOL) PUT TOGETHER VERSUS DEFICIENT (<50NMOL)

Rudick et al., (2011)	Retrospective cohort	64 women undergoing donor oocyte IVF in USA	Not reported	Vitamin D in serum	Donated	<u>Clinical pregnancy</u> 7/18 deficient group 10/26 insufficient group 14/20 replete group <u>Pregnancy test positive</u> Data not provided	<u>Miscarriage</u> Data not provided <u>Live birth</u> Data not provided	Lower CPRs in those with vitamin D deficiency suggesting that the effects are localised within the endometrium. NOTE*** CONFERENCE ABSTRACT SO LIMITED RESULTS
Rudick et al., (2012)	Retrospective cohort	188 women undergoing IVF in USA	Mean age 36.0 years	Vitamin D in serum	Autologous	<u>Clinical pregnancy</u> 14/39 deficient group 29/70 insufficient group 34/79 replete group <u>Pregnancy test positive</u> Data not provided	<u>Miscarriage</u> 3/39 deficient group 7/70 insufficient group 8/79 replete group <u>Live birth</u> 11/39 deficient group 22/70 insufficient group 26/79 replete group	Vit D deficiency associated with lower CPR in non-hispanic whites but not in Asians

Rudick et al., (2014)		99 women undergoing donor oocyte IVF in USA	Mean age 40.9 years Range 21-39	Vitamin D in serum	Donated	<u>Clinical pregnancy</u> 9/26 deficient group 16/38 insufficient group 26/35 replete group <u>Pregnancy test positive</u> Data not provided	<u>Miscarriage</u> 1/26 deficient group 3/38 insufficient group 6/35 replete group <u>Live birth</u> 8/26 deficient group 13/38 insufficient group 20/35 replete group	Lower CPRs in those with vitamin D deficiency suggesting that the effects are localised within the endometrium

Table 2. Appraisal of methodological quality (Newcastle-Ottawa Scale) of included studies. Vitamin D and IVF treatment systematic review and meta-analysis

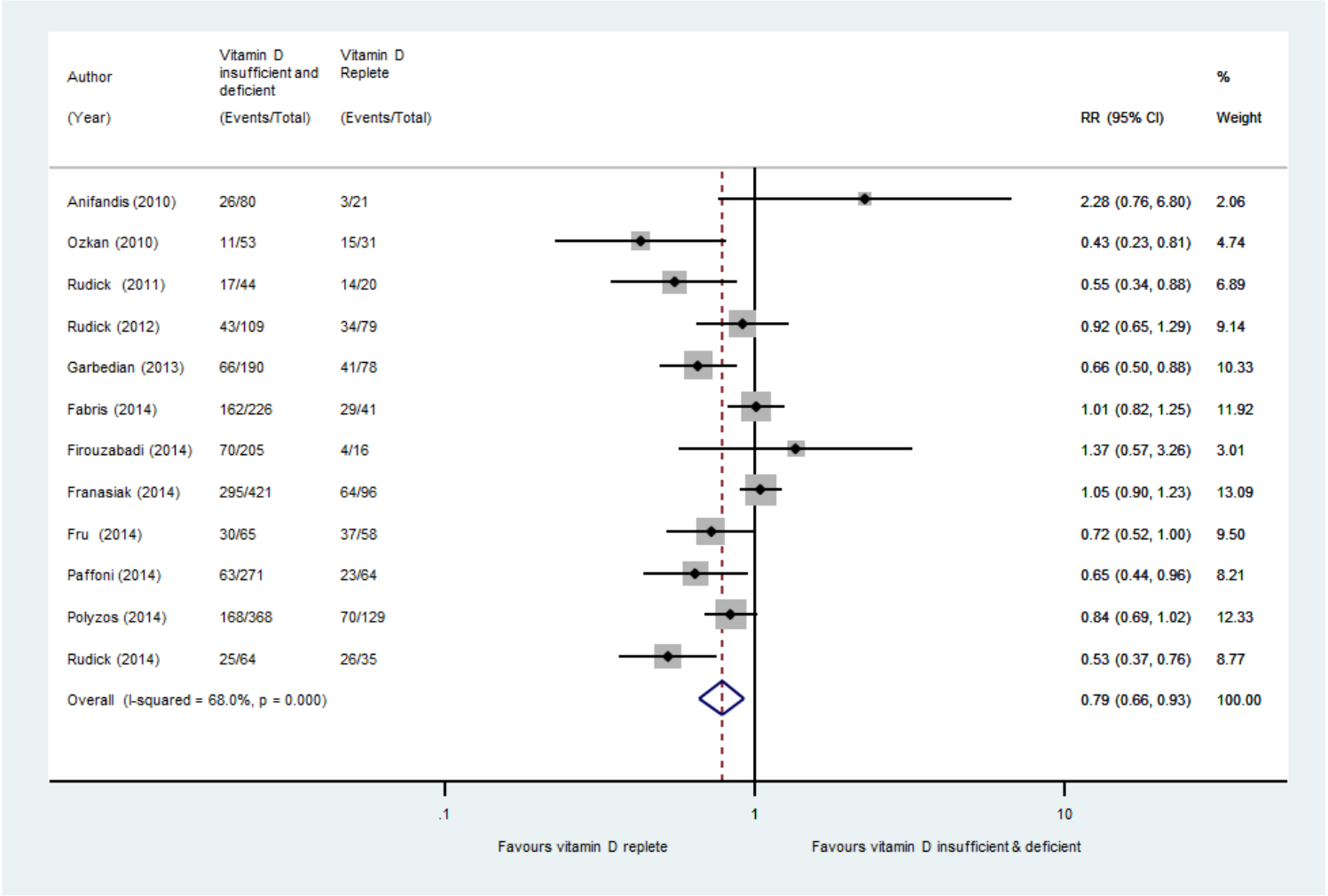
Study	Case representative	Control representative	Ascertainment of exposure	Outcome negative at start	Comparability by design or analysis	Outcome assessment	Duration of follow up	Adequacy of follow up	Score
Aleyasin et al., (2011)	*	*	*	*	**	*	*	*	9
Anifandis et al., (2010)	*	*	*	*	**	*	*	x	8
Fabris et al., (2014)	*	*	*	*	*	*	*	*	8
Firouzabadi et al., (2014)	*	*	*	*	x	*	*	*	7
Franasiak et al., (2014)	*	*	*	*	*	*	*	*	8
Fru et al., (2014)	*	*	*	*	x	*	*	*	7
Garbedian et al., (2013)	*	*	*	*	*	*	*	*	8
Ozkan et al., (2010)	*	*	*	*	**	*	*	*	9
Paffoni et al., (2014)	*	*	*	*	*	*	*	*	8
Polyzos et al., (2014)	*	*	*	*	**	*	*	*	9
Rudick et al., (2011)	*	*	*	*	x	*	*	*	7
Rudick et al., (2012)	*	*	*	*	x	*	*	*	7
Rudick et al., (2014)	*	*	*	*	**	*	*	*	9

*Indicates that a feature is present. X that a feature is absent. For comparability by design or analysis this checklist awards the maximum of two stars (**), one (*) or none if the feature is completely absent (x).

Clinical pregnancy

All 13 studies (n=2601) reported on clinical pregnancy rate (the presence of fetal heart activity at seven weeks gestation) as an outcome (figure 4). As the study by Aleyasin et al., did not include any women with a serum vitamin D level >50nmol/L, their data could not be used in this meta-analysis. However, pooling of the clinical pregnancy outcomes from the remaining 12 studies showed an improved chance of clinical pregnancy in the vitamin D replete population when compared to the vitamin D deficient and insufficient population. The relative risk of achieving a clinical pregnancy in the vitamin D deficient and insufficient population was 0.78 (95% CI 0.66 to 0.91) when compared to the vitamin D replete population. There was a moderate level of statistical heterogeneity in these studies indicated by an I^2 value of 67.1%, p=<0.001.

Figure 4. Clinical pregnancy outcomes forest plot. Vitamin D and assisted reproductive techniques systematic review and meta-analysis



Clinical pregnancy rates according to source of oocyte used

The 13 included studies were divided into two groups according to the source of the oocyte used to form the embryo for transfer (figure 5). The population of women in the first group of studies used autologous oocyte embryos. The second group of studies consisted of women who received a donor oocyte embryo. Meta-analysis of the studies in two groups was performed to try and elucidate whether there was a different association between vitamin D and clinical pregnancy rate achieved in women using their own oocytes compared to women using donor oocytes. In theory women using donor oocytes would use higher quality oocytes from young healthy donors. This would therefore eliminate poor fertility treatment outcomes due to poor oocyte quality; placing more emphasis on the effect that vitamin D has on implantation. Ten studies reported fertility outcomes in infertile women receiving an autologous oocyte embryo. As mentioned above, because the study by Aleyasin et al., did not include any women with insufficient or replete levels of vitamin D, their data could not be used in the meta-analysis. In the remaining nine studies (n=2171) where infertile women received an autologous oocyte embryo, the chances of achieving a clinical pregnancy was higher in the vitamin D replete population when compared to the vitamin D deficient and insufficient population. The relative risk of clinical pregnancy was 0.82 (95% CI 0.68 to 0.99 in the vitamin D deficient women compared to vitamin D replete women. Again, there was moderate heterogeneity in these studies with an I^2 value of 62.9%, p=0.006.

In the three studies (n=430) where women received a donor oocyte embryo, no significant difference was found when comparing the clinical pregnancy in women receiving a donor oocyte embryo who were vitamin D deficient or insufficient when compared to women

Figure 5. Clinical pregnancy outcomes by source of oocyte forest plot. Vitamin D and assisted reproductive techniques systematic review and meta-analysis

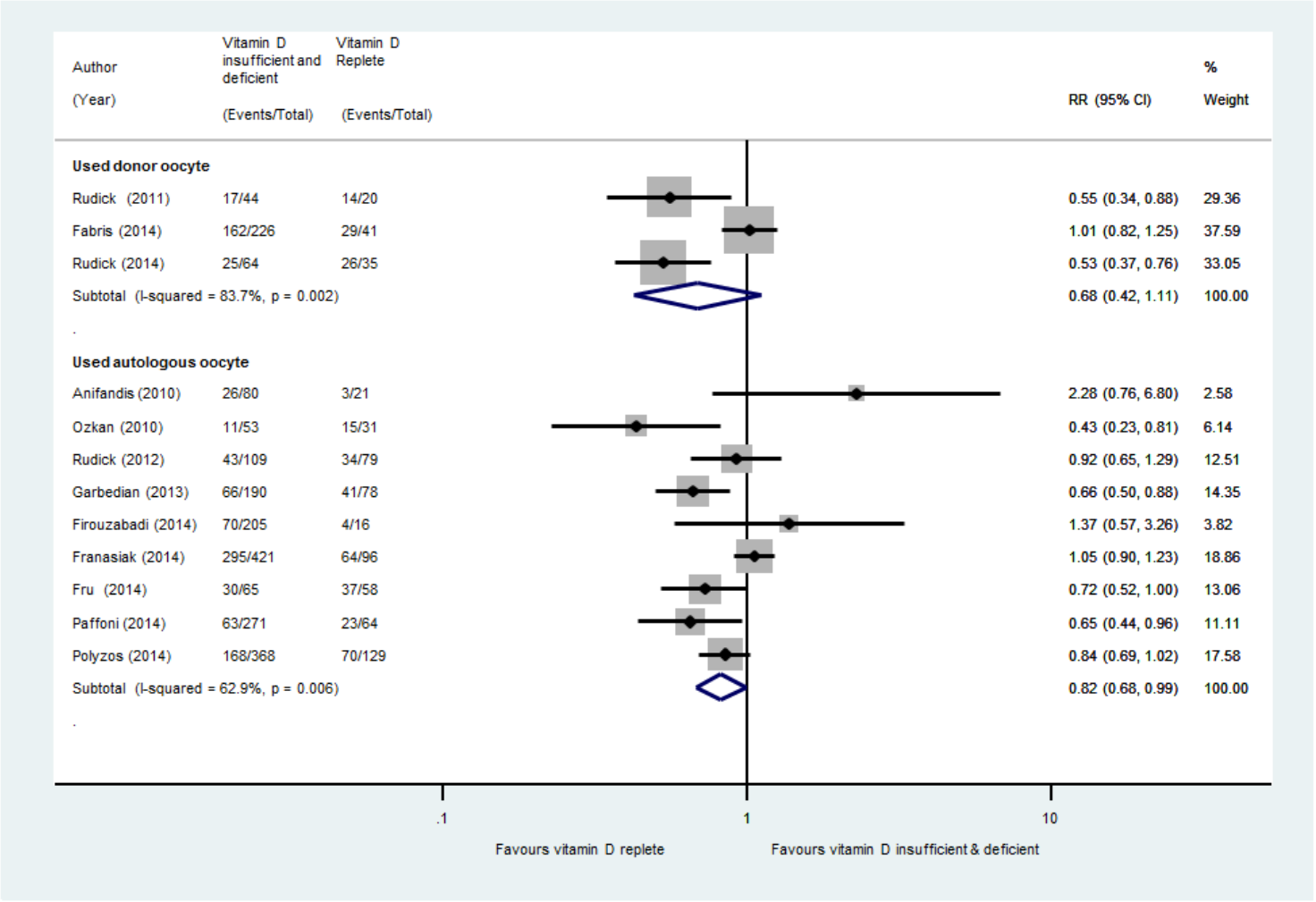
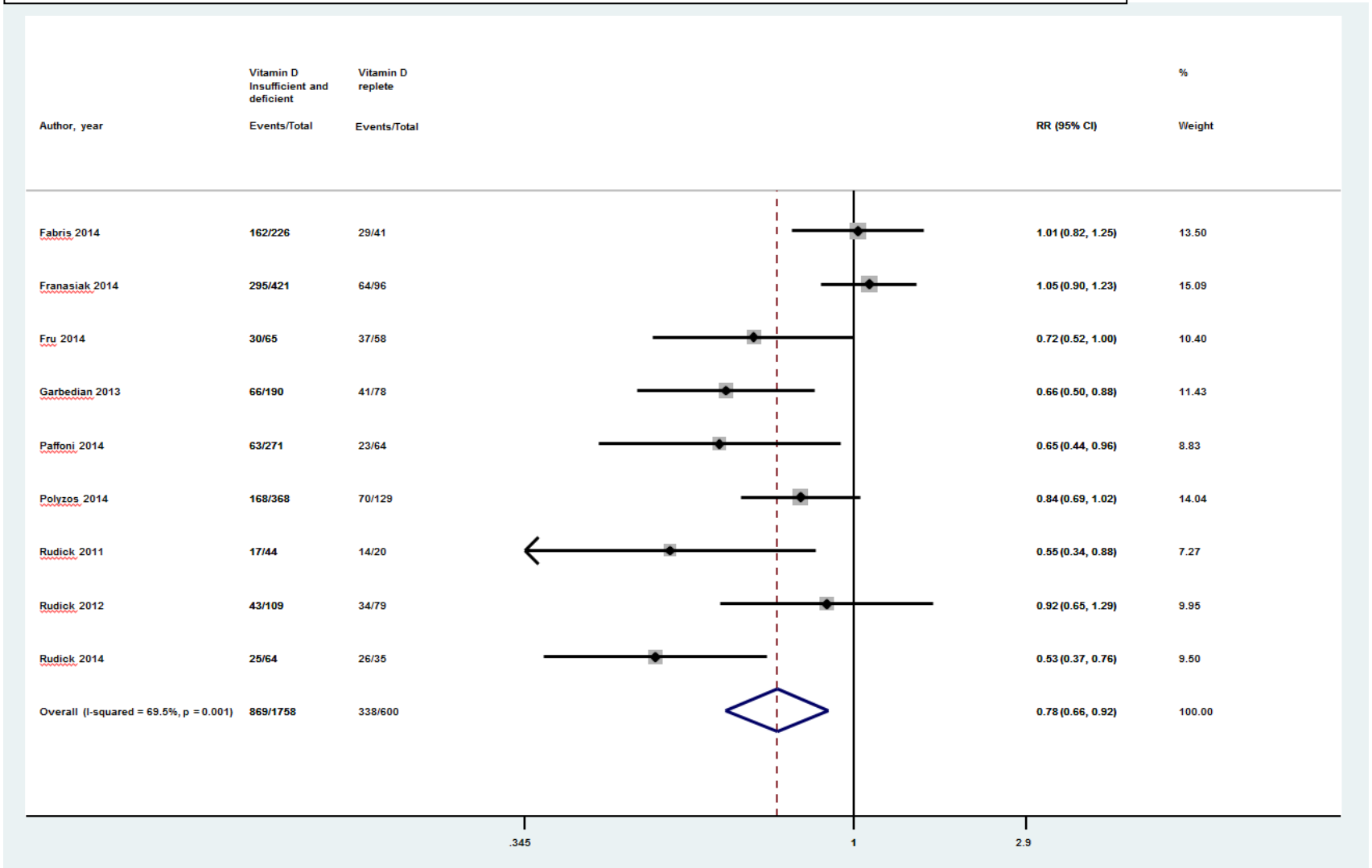


Figure 6 Clinical pregnancy outcomes from studies that have only used serum for vitamin D assessment. Vitamin D and assisted reproductive techniques systematic review and meta-analysis



receiving a donor oocyte embryo who were also replete in vitamin D. The relative risk was 0.68 (95% CI 0.42 to 1.11). The I^2 value was 83.7%, p=0.002, indicating a high level of heterogeneity in these three studies.

Clinical pregnancy rates according to biofluid used to assess vitamin D status

Nine studies used (n=2358) used blood serum as the biofluid that was examined to ascertain vitamin D status. These studies showed that women who are vitamin D replete are more likely to achieve a clinical pregnancy when compared with women that are vitamin D deficient or vitamin D insufficient. The relative risk of achieving a clinical pregnancy was 0.78 (95% CI 0.66 to 0.92) in the vitamin D deficient and insufficient women when compared to the vitamin D replete population. The nine studies showed a moderate level of statistical heterogeneity with an I^2 value of 69.5%, p=0.001.

Live birth

Eight studies (n=1958) reported the live births achieved by women when categorized by vitamin D (figure 6). These studies showed that women who are vitamin D replete have a higher chance of achieving a live birth from ART when compared with women with vitamin D deficiency or insufficiency. The relative risk of achieving a live birth was 0.85 (95% CI 0.74 to 0.99) in the vitamin D deficient and insufficient population when compared to the vitamin D replete population. These studies showed a low level of heterogeneity indicated by an I^2 value of 32.2%, p=0.183.

Implantation

Five studies (n=1700) reported number of women that achieved a positive pregnancy test approximately two weeks after embryo transfer for the three vitamin D categories. The relative risks of embryo implantation in the vitamin D deficient and insufficient population versus the vitamin D replete population are presented in figure 7. When the data from the five studies are pooled, the relative risk of achieving an implanted embryo in the vitamin D deficient and insufficient population is 0.90 (95% CI 0.77 to 1.05) versus the vitamin D replete population. There was a moderate heterogeneity between these studies, the I^2 value was 52.5%, p=0.077.

Figure 7 Live birth outcomes forest plot. Vitamin D and assisted reproductive techniques systematic review and meta-analysis

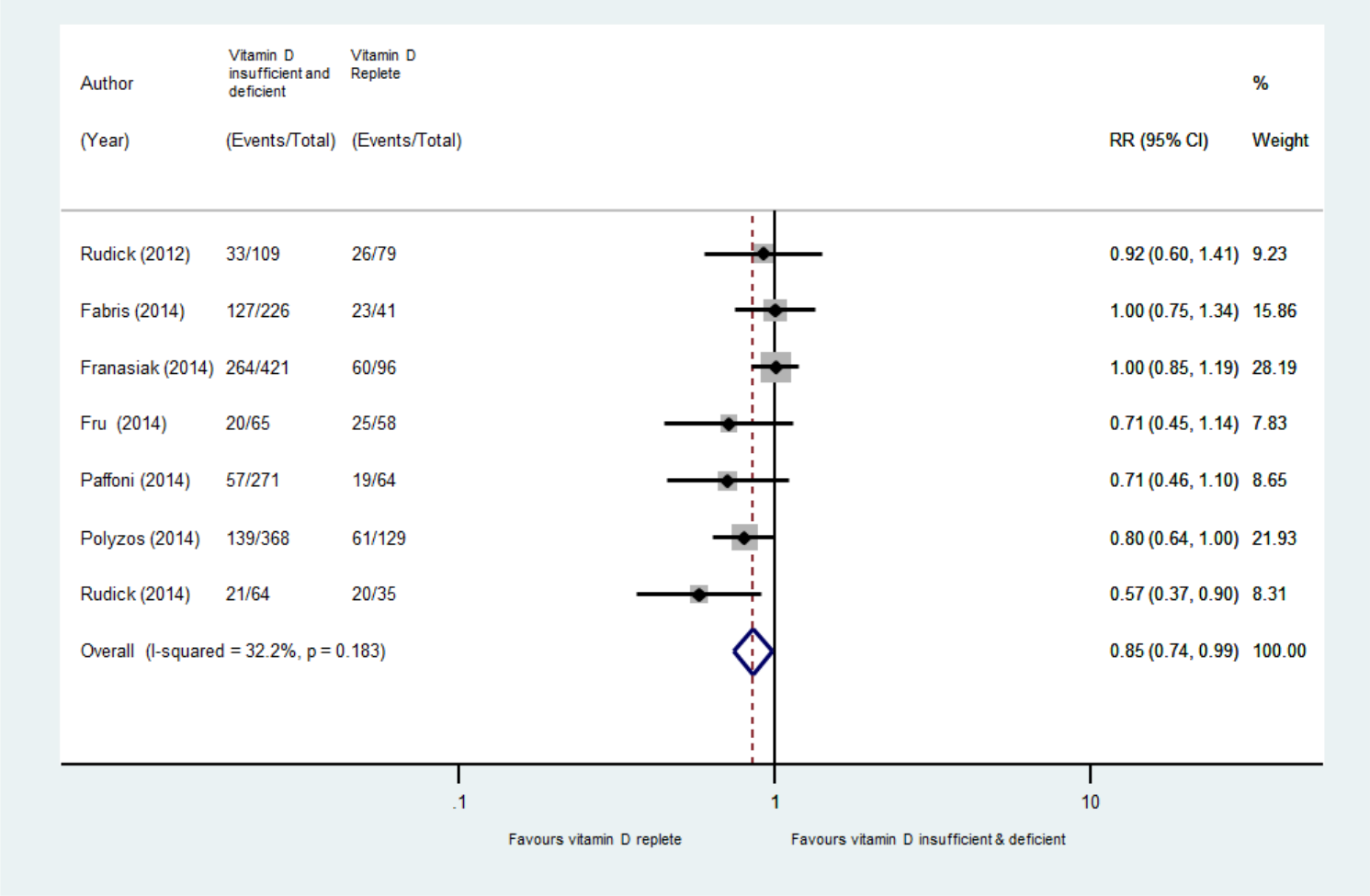
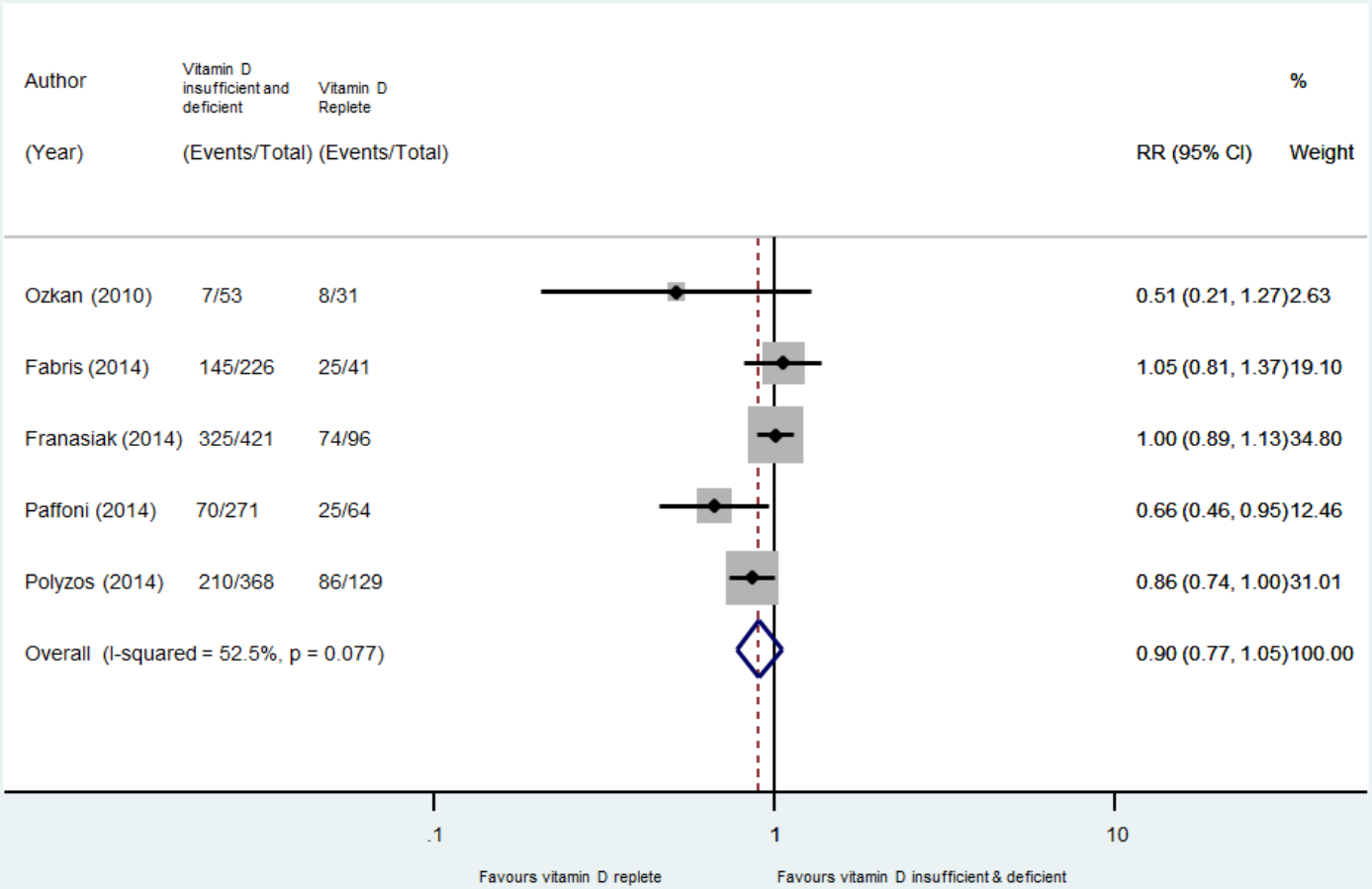


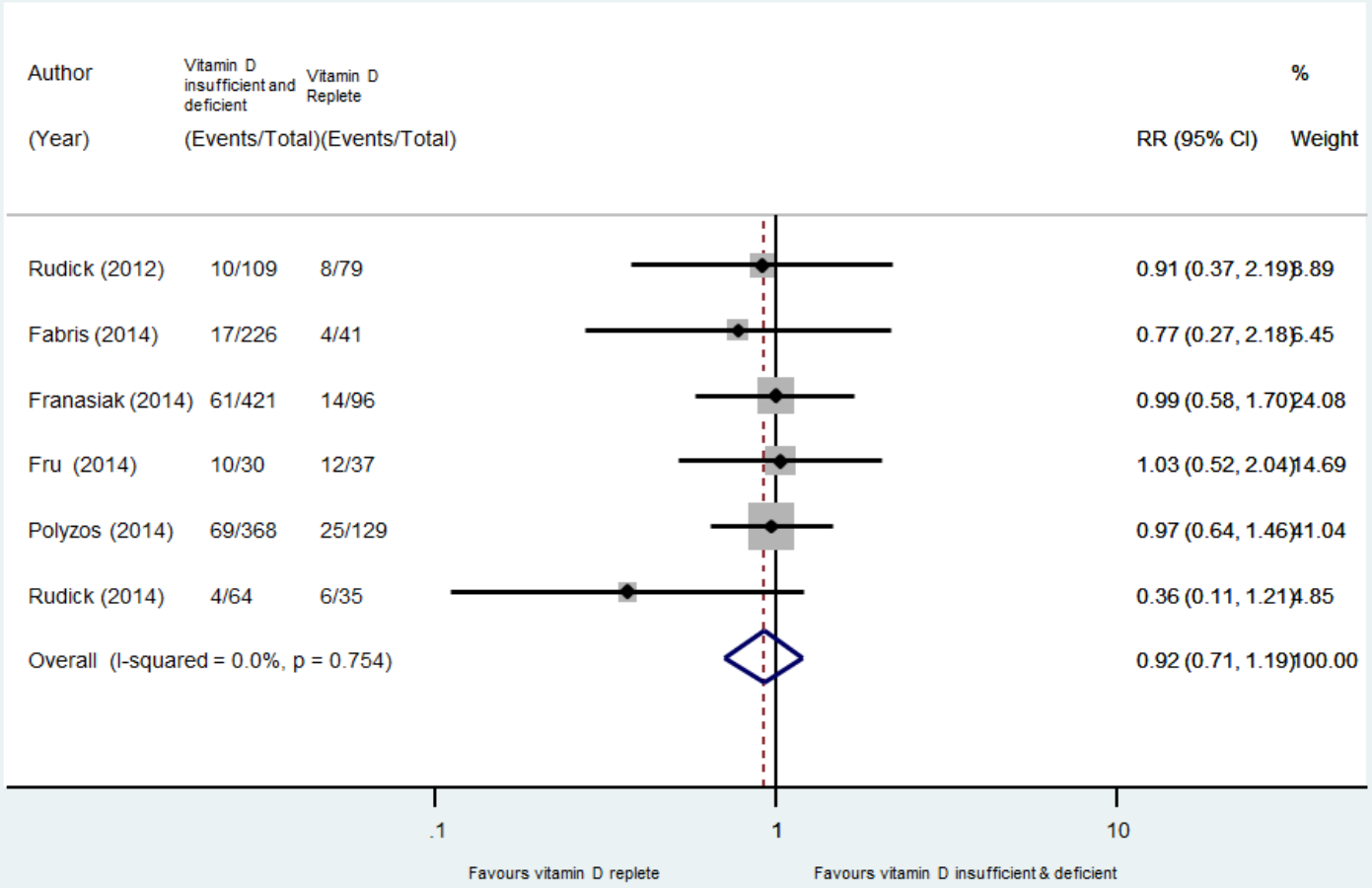
Figure 8 Embryo implantation rate forest plot. Vitamin D and assisted reproductive techniques systematic review and meta-analysis



Miscarriage

Six studies (n=1635 women) reported on the outcome of miscarriage (figure 8). When the data from these six studies are pooled, the chance of miscarriage in the vitamin D replete women is similar to that of vitamin D deficient and insufficient women. The relative risk of miscarriage in vitamin D deficient and insufficient population is 0.92 (95% CI 0.71 to 1.19) compared to miscarriage in vitamin D replete women. These six studies showed a low level of heterogeneity with an I^2 value of 0.00% p=0.754.

Figure 9 Miscarriage rate forest plot. Vitamin D and assisted reproductive techniques systematic review and meta-analysis



Discussion

This systematic literature search identified 13 relevant studies. Meta-analysis of the data from these studies suggests that the chances of achieving a clinical pregnancy in vitamin D are higher in vitamin replete women compared to women with vitamin D deficiency or insufficiency. Furthermore, the chance of live birth is higher in the vitamin D replete women when compared to vitamin D deficient and insufficient women. The effect of vitamin on embryo implantation is not proven with a relative risk which does not reach statistical significance. Lastly, the meta-analysis shows that vitamin D does not have an effect on miscarriage.

This review has shown that many research groups share the same hypothesis; that vitamin D status is important in patients undergoing IVF treatment. All of the cohorts identified have been published since 2010, and the results have been encouraging, suggesting that the association between vitamin D and IVF treatment outcome exists. With the recent rapid increase in publications linking vitamin D to various disease states[70], the association between vitamin D and IVF treatment success is reinforced by sound biological plausibility. In its immunomodulatory role as a suppressor of adverse inflammatory adaptive immunity, it is reasonable to consider that vitamin D has an important function specific to implantation[47,71]. Three of the studies identified by this review have specifically tested this hypothesis by presenting data from oocyte recipient IVF treatment cycles.

This systematic review and meta-analysis has been strengthened by a number of factors. A comprehensive search strategy was used, employing relevant research databases. Additionally, a valid data synthesis method was implemented. No language barriers were applied to ensure further comprehensiveness of the literature search and therefore there can be maximum confidence that all relevant studies have been captured. Furthermore, assessment of the quality of the included cohort studies using the Newcastle-Ottawa Quality Assessment Scale showed high scores, suggestive of a low risk of bias in our analysis.

There are also weaknesses to the present study, which mainly stem from the clinical heterogeneity of the publications that were used in the meta-analysis. As the enquiry into the association between vitamin D and IVF treatment success is an area of research that is relatively new, some degree of heterogeneity is to be expected as no standard methodology has been set. The clinical and statistical heterogeneity between the included studies does increase the risk that the findings could be due to chance. Ideally one would want all studies to be homogeneous from a clinical and statistical perspective to increase the strength of the overall findings of this systematic review and meta-analysis. However, this is not necessarily a disadvantage as some degree of clinical heterogeneity can increase the generalizability and applicability of the findings to wider infertility populations.

The cohort studies included in the analysis varied in the timing of vitamin D assessment. It is well documented that vitamin D status does not fluctuate a high degree over time unless vitamin D deficiency or insufficiency is actually treated[24]. Therefore, the importance of the timing of the assay reduces. Additionally, the studies varied according to the methods used

to ascertain vitamin D status. Some studies measured vitamin D in the follicular fluid aspirated at the time of oocyte retrieval whereas others used blood serum for vitamin D measurement. However, a number of previously published studies have found that assays of vitamin D in follicular fluid or blood serum produce results that are highly correlative[54,55,57 61]. This means that, although four of our included studies used follicular fluid vitamin D assay, our results remain reliable. Furthermore, when we analysed the data from studies where serum vitamin D was used as the biofluid, and excluded studies where follicular fluid was used, our meta-analysed results show that women who were vitamin D replete are more likely to achieve a clinical pregnancy. From a practical and clinical perspective, this is an important finding, as measuring serum vitamin D would be more convenient for the patient and clinician. This is because correction of vitamin D deficiency is feasible when vitamin D status is checked in peripheral blood. In contrast, vitamin D deficiency treatment when the follicular fluid is collected at transvaginal oocyte retrieval will be too late to illicit any clinical benefit in reproductive outcomes.

The definition of vitamin D deficiency, insufficiency and normal status is still debated. All of our studies used the Endocrine Society's definitions[41], however, in reporting their results, some authors chose to group women according to vitamin D status in two groups instead of three. This occurred in two studies. In the study by Garbedian et al.,[60] the vitamin D deficient and insufficient groups were combined and compared to the IVF treatment results in the vitamin D replete group. The study by Polyzos et al.,[63] compared the IVF treatment results of the vitamin D deficient group versus that of the insufficient and replete groups. Although the authors were contacted to retrieve data for the three vitamin D categories, no

response was received. It would be advisable that further studies aiming to identify the association between vitamin D and IVF treatment outcome report results using the Endocrine Society's definition of the three vitamin D categories.

Although the total number of women from the meta-analysis totaled 2601 patients undergoing ART, the sample sizes in each cohort varied leading to further clinical heterogeneity. In addition, there was also variability in the sample population with regards to age, body mass index and also the type of IVF treatment performed. However, this should aid the generalisability of the findings from our final meta-analysis.

Four studies[58,63,64,66] conducted logistical regression to adjust for potential confounding factors that could affect vitamin D status and pregnancy rate after IVF. The cohort studies performed by Rudick et al.,[64,66] adjusted for race and body mass index (BMI). Results indicated that when race and BMI were adjusted, the association between vitamin D and IVF outcome persisted. Franasiak et al.,[58] adjusted for a range of potential confounders including; BMI, number of previous IVF cycles, timing of embryo transfer, ethnicity, age and number of embryos transferred. They found that there was no difference in ongoing pregnancy rates among strata of vitamin D level. In contrast, Polyzos et al.,[63] performed a logistic regression analysis to identify whether vitamin D deficiency was independently associated with clinical pregnancy rates controlling for 16 potential confounders in their cohort of 368 women. The results showed that vitamin D deficiency was independently associated with lower clinical pregnancy rates with an odds ratio of 0.61 (95% CI 0.39-0.95).

Of particular interest, three included studies investigated the association between vitamin D status and IVF treatment outcome amongst women undergoing oocyte recipient treatment cycles[56,64,66]. Although meta-analysis of the data from these three studies did not show a statistically significant difference in chance of clinical pregnancy between the vitamin D replete and vitamin D deficient and insufficient populations, the data does show a trend towards a higher chance of clinical pregnancy in the vitamin D group. It is likely that the failure to reach statistical significance is due to the low number of studies and therefore participants (n=430). Further studies into the reproductive outcomes of oocyte recipient IVF patients would be helpful to clarify whether there is a clear association between vitamin D and pregnancy outcome in this sub-population. If this were the case then it would support the hypothesis that vitamin D status exerts its effects mainly on the endometrium, increasing the chance of implantation rather than any other prognostic factors, such as oocyte retrieval number or oocyte quality.

To a certain degree, the embryo implantation rates seen in the forest plot presented in figure 7 supports the hypothesis that vitamin D is key in initial embryo and endometrial interaction. Although the pooled risk ratio for embryo implantation does not reach statistical significance, the data suggests that implantation is more likely in a woman that is vitamin D replete when compared to a woman that has vitamin D deficiency or insufficiency. However, firm assumptions cannot be made from the pooled implantation risk ratio as the 95% confidence interval does cross the line of unity. This lack of statistical significance is likely to be due to there being an insufficient number of women in the six studies where implantation is reported.

Apart from the 13 cohort studies identified by this systematic review, three interventional studies were also found after the literature search was performed. The first trial, by Choi et al.,[67] was a conference abstract describing a trial aimed at elucidating whether treatment of vitamin D deficiency aids clinical outcome in IVF treatment. In this trial of 100 patients undergoing treatment, women were divided into two groups; one group received no vitamin D supplementation and the other received vitamin D treatment without assessment of vitamin D status. Unsurprisingly, the study found no statistical differences in implantation, clinical pregnancy or miscarriage rates between the treatment and control groups. Similarly, a trial by Polak di Fried et al.,[68] of 52 women undergoing IVF, administered vitamin D treatment in half of their population and placebo in the other half without measuring vitamin D status. Again no significant differences were found in endometrial thickness, number of oocytes retrieved, implantation and clinical pregnancy rates between the treatment and placebo group. Lastly, Aflatoonian et al.,[69] performed a randomized trial in 106 women undergoing frozen embryo transfer. All women included in the trial had a serum vitamin D of less than 75nmol/L. Half the population received 50,000 units of vitamin D on a weekly basis, with the other half receiving placebo. This study found no improvement in pregnancy rate in the treatment group. However, the authors conceded that this study was underpowered and that larger trials should be conducted to further investigate whether treatment of vitamin D deficiency and insufficiency could improve IVF treatment outcomes. As all three of these trials were yet to be published at the time of the systematic review, the data from these studies have not been included in the meta-analysis presented in this thesis chapter.

The findings of this review suggest that a large robustly performed randomised clinical trial should be performed to investigate the merits of vitamin D insufficiency treatment in the IVF population. Assessment of vitamin D is cheap and widely available[72]. If the results of a trial confirmed that correction of vitamin D insufficiency led to more favorable ART outcomes, vitamin D assessment could become a standard baseline investigation of all women suffering with infertility. Furthermore, treatment regimens such as that from the Institute of Medicine or Endocrine Society could be implemented (which are safe and carry low cost) before IVF treatment is commenced. However, the design of such a trial is complex. As the risks of vitamin D deficiency are well documented and wide ranging (e.g. osteoporosis, diabetes and certain cancers)[73], it would be difficult to justify not treating women identified as being deficient. One trial methodology that could be used to bypass this ethical dilemma would be to perform a cluster stepped wedge randomised controlled trial[74]. This would involve commencing vitamin D 'test and treat' policies in similar IVF units at a randomly assigned time. In this way unit wide clinical pregnancy rates could be collected, with a comparison of success rates before and after the implementation of the vitamin D 'test and treat' policy (i.e. with each unit acting as its own control).

Summary

Vitamin D deficiency is common and the health implications are gaining increasing clarity. Knowledge regarding the importance of vitamin D in reproductive processes such as ovarian steroidogenesis, polycystic ovarian syndrome, endometriosis and implantation is growing at

a rapid pace[75]. For infertile women, desperate to start a family, any effort to improve their treatment prognosis is welcomed. A simple, safe, and cost effective method in achieving this could be in the treatment of vitamin D deficiency prior to starting IVF treatment. This review has shown an association between vitamin D status and IVF outcome. Further investigation of the benefits of vitamin D deficiency treatment is justified and should be achieved by performing a robustly designed trial.

CHAPTER 3: VITAMIN D AND IVF TREATMENT OUTCOMES – A PROSPECTIVE COHORT STUDY

This work has been submitted to the British Medical Journal for publication. Reviewers' comments are awaited.

Introduction

There has been recent interest in the role of vitamin D in reproductive physiology due to findings that illustrate the high prevalence of vitamin D deficiency[42]. Studies have shown that as much as 20 to 52% of women of reproductive age are deficient in vitamin D[76–78].

In human beings, the main source of vitamin D, a fat soluble steroid hormone, is from sunlight. Only a small amount is obtained from our diet. The majority of the body's vitamin D is in the form of vitamin D3 (cholecalciferol). This is photo-chemically synthesized in the skin. Cholecalciferol is bound to serum vitamin D binding protein (VDBP). The cholecalciferol is hydroxylated by the liver to 25-hydroxyvitamin D (25[OH]D), also known as calcidiol. The second hydroxylation occurs primarily in the kidney and forms the biologically active 1,25-dihydroxyvitamin D ($1,25[OH]_2D$), also known as calcitriol ($1\alpha,25(OH)_2D_3$)[79].

Although $1\alpha,25(OH)_2D_3$ is the biologically active form of the hormone, vitamin D levels are usually measured according to the serum 25 (OH) D_3 concentration. Serum concentration of $25(OH)D_3$ is the best indicator of vitamin D status as it best reflects vitamin D produced in the skin and the smaller amount obtained from food and supplements[41]. $25(OH)D_3$ also has a relatively long circulating half-life of 15 days.

There is still great debate over the definition of vitamin D deficiency and the levels required for adequacy for bone health, and optimal overall health. However, most nutritional bodies of expertise have concluded that people are at risk of the detrimental effects of vitamin D deficiency at serum $25(OH)D_3$ concentrations of less than 50 nmol/L (less than 20ng/mL). A level of 50 to 75 nmol/L (21 to 29 ng/mL) is considered insufficient and greater than

75nmol/L (greater than 30 ng/ml) is considered vitamin D replete[78,80–82]. Serum concentrations greater than 150 ng/mL (greater than 374 nmol/L) are associated with toxicity and adverse effects.

The established role of Vitamin D is to promote calcium absorption in the gut and maintain adequate serum calcium and phosphate concentrations and promote bone mineralisation[41] . Without sufficient vitamin D, bones can become thin, brittle, or misshapen. Vitamin D at sufficient levels prevents rickets in children and osteomalacia in adults. Together with calcium, vitamin D also helps protect older adults from osteoporosis. However, the discovery that most tissues and cells in the body possess a vitamin D receptor[42] suggests this hormone has more widespread function.

In reproductive physiology, vitamin D deficient animals have decreased fertility capacity, impaired neonatal growth, hypogonadism, uterine hypoplasia, impaired folliculogenesis and pregnancy complications, implying that vitamin D may have a crucial role in conception and in pregnancy[46,83–85]. Vitamin D may also have an effect on endometrial decidualisation and placental function[80–82]. This function is the most studied aspect of vitamin D in reproduction, with vitamin D deficiency linked to poor placentation (due to abnormal inflammatory responses and endothelial dysfunction) causing hypertensive disorders of pregnancy and fetal growth restriction. More recently, it has been proposed that vitamin D may be a regulator of target genes associated with implantation and trophoblast invasion[86–88]. Studies have been conducted to investigate the importance of vitamin D and subfertility. Human studies in women with polycystic ovarian syndrome (PCOS) have shown patients

deficient in vitamin D have a higher incidence of metabolic risk factors such as insulin resistance and obesity[34,89,90]. Clinical trials with vitamin D supplementation in patients with PCOS have used small sample sizes but have shown positive effects on the metabolic profile and menstrual cycles of these patients[91–95].

The biological plausibility for vitamin D playing an important role in implantation and trophoblastic invasion has led many research groups to investigate the importance of vitamin D in patients undergoing IVF treatment. The findings of these studies have been meta-analysed in chapter two of this thesis. Some of these studies have found that a replete level of vitamin D leads to an increase in clinical pregnancy and live birth rates[60–62,64,65]. However, others have found conflicting evidence suggesting that vitamin D has no bearing on the outcome of IVF treatment[54–58]. None of these studies have been conducted in the UK. Some have also been of small sample size and been retrospective in their design and therefore have not offered a strong enough evidence base to confirm the benefit of measuring vitamin D in the IVF population in clinical practice. In this present chapter we prospectively examined the vitamin D levels of a diverse population of women undergoing ART at an assisted conception unit in Birmingham, UK.

Specific human data on the role of vitamin D in reproduction is lacking. However, considering the expected high levels of vitamin D deficiency in Birmingham (due to the multiethnic population), it was felt that it would be important to evaluate the role of vitamin D in achieving pregnancy. The aim of the cohort study was to identify whether there is an

association between serum blood levels of 25-hydroxy vitamin D and fertility treatment outcomes.

Methods

Study Design

Before beginning this cohort study, the aim was to recruit 490 patients undergoing IVF, ICSI or FET treatment. A clinical pregnancy rate of 35% for patients was hypothesized for women with replete serum vitamin D and 25% for patients with insufficient or deficient serum vitamin D levels. This gives an absolute risk difference of 10%. A 10% attrition rate was anticipated. Aiming for 90% power with a 5% type 2 error rate, the sample size required was calculated as 490.

The study was approved by the National Research Ethics Service (NRES) Committee West Midlands – Black Country (REC 13/WM/0258) (see appendix 2). A total of 504 patients who underwent ART at the Birmingham Women's Fertility Centre from September 2013 to December 2014 were recruited.

All patients that were referred for IVF, ICSI and FET treatment met the inclusion criteria and were approached to participate. Those that consented to participation in this study were included. Prospective participants were approached at their IVF consent signing appointment. A participant information sheet was provided and written consent obtained (see appendix 3 and 4). Vitamin D assay for total 25-hydroxy vitamin D, 25-hydroxy vitamin

D2 and 25-hydroxy vitamin D3 were measured using blood serum samples taken at the time of consent. Assays used a liquid-liquid extraction method using highly sensitive liquid chromatography mass spectrometry (Waters Premier XE MS detector with ACQUITY Ultra Performance LC). Patients were allocated into three groups according to their total 25-hydroxy vitamin D levels; vitamin D deficient group (25-hydroxy vitamin D less than 50nmol/L), vitamin D insufficient group (25-hydroxy vitamin D 50-75nmol/L) and vitamin D replete group (25-hydroxy greater than 75nmol/L) according to the Endocrine Society definitions[41].

After embryo transfer the result of the vitamin D assay was communicated with the participant and the General Practitioner to initiate treatment and supplementation of vitamin D if required (see appendix 4).

The primary outcome was clinical pregnancy (presence of a fetal heartbeat on ultrasound scan at seven weeks gestation). Secondary reproductive treatment outcomes included implantation rates (positive pregnancy test rates prior to ultrasound), live birth rates and miscarriage rates. Secondary fertility treatment outcomes included FSH dose requirements, endometrial thickness at last monitoring pelvic ultrasound, number of oocytes retrieved, and quality of embryo transferred.

IVF, ICSI and FET treatments

All participants undergoing fresh cycle IVF or ICSI received either a short antagonist protocol or a long down regulation protocol using Cetrotide (Merck Serono, France) or Buserelin

(Sanofi-Aventis, France) respectively. After complete down regulation (checked by transvaginal ultrasonography to ensure endometrial thickness less than 4mm and inactive ovaries), participants underwent ovarian stimulation with recombinant follicular stimulating hormone (Gonal-F [Merck Serono, France] or Menopur [Ferring, France]). Starting doses depended on the patient's age. The starting doses were 150iU of menopur if women were less than 35 years, 225iU is women were 36-38 years, 300iU if women were 39-42 years and 300 to 450iU in the woman was over the age of 42. Additionally, if baseline FSH was greater than 10iU/ml in women less than 31 years, the start dose of menopur would be increased to 225iU. In women with a baseline FSH greater than 10iU/ml over 31 years, the start dose of menopur would be increased to 300iU. In women with anovulatory cycles with polycystic ovarian syndrome, an individualized stimulation protocol was planned. Previous poor responders would also have a higher starting menopur dose.

Doses of follicular stimulating hormone were then modified according to the patient's ovarian response tracked by transvaginal ultrasonography. The endometrial thickness was also measured using transvaginal ultrasonography at each ovarian response monitoring visit. When at least three follicles reached a diameter of at least 17 mm, 250 micrograms/0.5ml choriogonadotropin alfa Ovitrelle (Merck Serono, France) was used as a trigger for ovulation. In the event of ovarian hyperstimulation or inadequate ovarian stimulation, the treatment was abandoned if clinically required. In women with a suitable ovarian response to follicular stimulating hormone, transvaginal oocyte retrieval was performed 36 hours after trigger injection. Morphological grade of all embryos was assessed on days two, three and five post oocyte retrieval. Embryos were graded according to the degree of fragmentation and the regularity of blastomeres. Day five blastocyst grading was according to the development

stage, inner cell mass quality and trophectoderm quality. Fresh embryo transfer was conducted in line with the Birmingham Women's Fertility Centre single embryo transfer policy.

All participants having frozen embryo transfer treatment underwent down-regulation using Buserelin (Sanofi-Aventis, France) followed by oestrogen (Progynova [Bayer, UK] and progesterone administration in the form of Gestone injections (Nordic Pharma, France) or Cyclogest pessaries (Actavis, UK) to prepare the endometrium prior to embryo transfer.

Statistical analysis

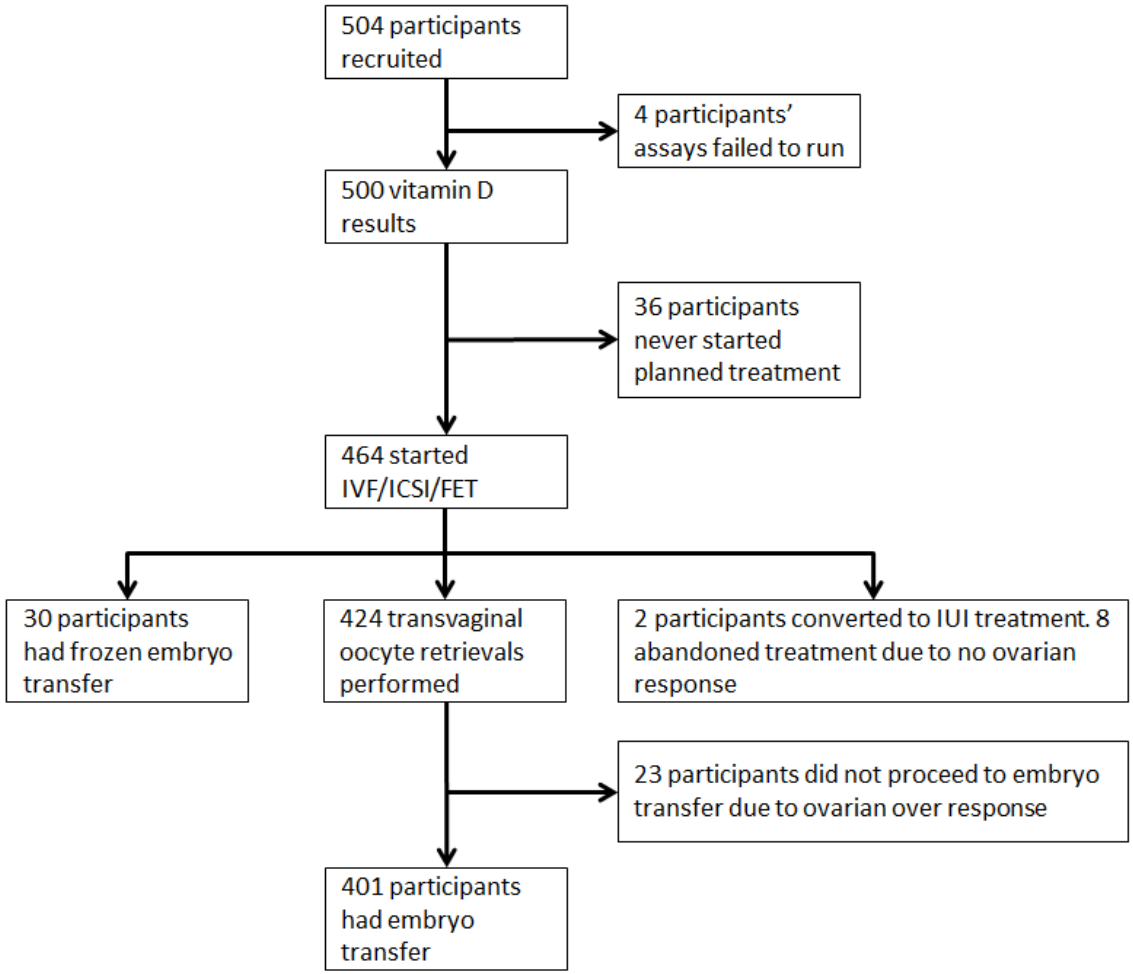
The baseline characteristics and outcomes for the vitamin D deficient, insufficient and replete groups were analyzed using Mann–Whitney U-tests for non-parametric data and Pearson x2 tests for categorical data. Analysis of outcomes between the groups was performed by logistic regression to compute odds ratios with their 95% confidence intervals (CIs) adjusting for potential confounding factors. Missing data were handled by complete case analysis for vitamin D levels and fertility outcomes and by multiple imputation for confounding variables. All analyses were performed using STATA Version 12.1 (Stata Corp, College station, TX, USA).

Results

Figure 9 shows the flow of participants through the study. A total of 504 women were recruited to the study and had serum collected for vitamin D assay. Four blood samples

could not be processed for serum vitamin D status as the clinical chemistry laboratory reported that the blood sample was inadequate or the sample was misplaced. Thirty-six recruited participants never commenced their planned treatment. The remaining 464 participants commenced their treatment and had their reproductive treatment outcomes collected. Of these 30 underwent a planned frozen embryo transfer, 424 underwent a transvaginal oocyte retrieval, two participants had their treatment converted to intrauterine insemination treatment (as only one follicle grew in the upregulation phase of IVF treatment), and eight participants had their treatment cycles cancelled as there was no ovarian response to FSH. Of the 424 women that had oocyte retrieval, 23 had cryopreservation of embryos as there was a risk of ovarian hyperstimulation syndrome.

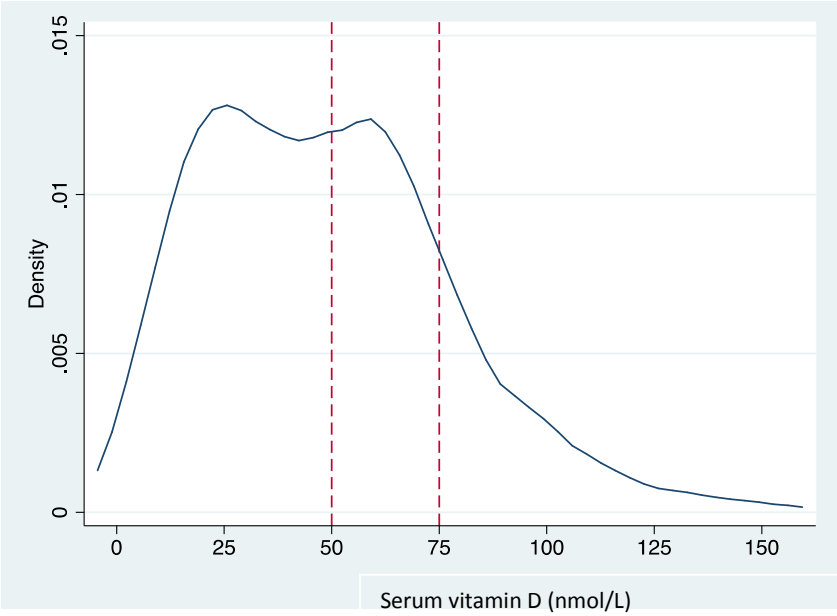
Figure 10. Vitamin D and IVF cohort study participant flow chart



Vitamin D status

The vitamin D status of the 500 recruited participants is shown in figure 10. Vitamin D deficiency (total vitamin D 0-49.9nmol/L) affected 266 out of 499 (53.3%) participants. Vitamin D insufficiency (total vitamin D 50-75nmol/L) affected 154 of 499 participants (30.9%). Eighty out of the 499 participants (16.0%) were replete in vitamin D (>75nmol/L).

Figure 11. Vitamin D and IVF cohort study. Vitamin D results distribution



Baseline characteristics

The baseline characteristics of the participants, the season at which the IVF treatment was performed, and the smoking status of participants are shown in table 3. The three vitamin D groups were similar in mean age (p=0.196). The mean age of the deficient group was 33.2 years (SD 4.9 years). The mean age of the insufficient group was 33.7 years (SD 4.4 years) and the mean age of the replete group was 34.2 years (SD 4.6 years).

The mean BMI for the three vitamin D groups were also comparable (p=0.182). The mean BMI for the deficient, insufficient and replete groups were 24.7 (SD4.0), 25.1 (SD 3.6) and 24.1 (SD 3.1) respectively.

The serum vitamin D levels varied according to race. More South Asian and Black women were vitamin D deficient, 71.8% and 66.7% respectively). Vitamin D status was more evenly distributed amongst White participants (36.0% deficient, 39.2% insufficient and 24.8% replete).

Table 3. Vitamin D and IVF cohort study. Baseline characteristics of participants

	Vitamin D Category			
	Deficient (<50nmol/L) N=266	**Insufficient (50-75nmol/L) N=154**	**Replete (>75nmol/L) N=80**	**p-value**
Age - years(SD)	33.2 (4.9)	33.7 (4.4)	34.2 (4.6)	0.196
BMI (SD)	24.7 (4.0)	25.1 (3.6)	24.1 (3.1)	0.182
Race (%)				
*White**	93 (36.0)	101 (39.2)	64 (24.8)	<0.001
South Asian	125 (71.8)	37 (21.3)	12 (6.9)	
Black	20 (66.7)	8 (26.7)	2 (6.6)	
Chinese	5 (62.5)	2 (25.0)	1 (12.5)	
Other	23 (76.7)	6 (20.0)	1 (3.3)	
Season (%)				
Winter	76 (59.4)	41 (32.0)	11 (8.6)	<0.001
Spring	77 (61.6)	39 (31.2)	9 (7.2)	
Summer	31 (31.0)	40 (40.0)	29 (29.0)	
Autumn	82 (55.8)	34 (23.1)	31 (21.1)	
Smoking (%)				
Non-smokers	250 (52.7)	148 (31.2)	76 (16.1)	0.639
Smokers	16 (61.5)	6 (23.1)	4 (15.4)	

Vitamin D status was unevenly distributed when comparing differing ethnicities and this reached statistical significance ($p<0.001$).

Seasonal vitamin D changes

A statistically significant difference was found when comparing vitamin D status with the season that the IVF treatment was performed. Vitamin D was more likely to be deficient in the participants in the Winter (59.4%) and Spring (61.6%) when compared to the Summer (31.0%) ($p<0.001$).

Smoking status

Only 26 of the participants smoked. The proportion of smokers in the vitamin D deficient, insufficient and replete groups were comparable ($p=0.639$).

Fertility History

The fertility history variables are displayed in table 4. Amongst the participants, the duration of infertility was found to be longer in the vitamin D deficient group when compared to the vitamin D insufficient and replete groups. The median durations of infertility for the deficient, insufficient and replete groups were 48 months (IQR 36-72 months), 36 months (IQR 24-60 months), and 36 months (IQR 24-60 months). The differences in the durations of infertility were statistically significant ($p=0.044$). The type of infertility (primary or secondary) was comparable between the three groups ($p=0.627$). In the deficient group, 55.3% of participants had primary infertility, the insufficient group had 57.5% of participants with primary infertility and 50.7% of the replete group had primary infertility.

Table 4. IVF treatment cycle variables by vitamin D categories

	Vitamin D Category			
	Deficient (<50nmol/L) N=266	**Insufficient (50-75nmol/L) N=154**	**Replete (>75nmol/L) N=80**	**p-value**
Duration of infertility in months (Median-IQR)	48 (36-72)	36 (24-60)	36 (24-60)	0.044
Type of infertility (%)				
Primary	136 (55.3)	81 (57.5)	39 (50.7)	0.627
Secondary	110 (44.7)	60 (42.5)	38 (49.3)	
Cause of infertility (%)				
Unexplained	47 (19.0)	37 (26.2)	15 (19.5)	0.771
An ovulatory	24 (9.7)	13 (9.2)	9 (11.7)	
Tubal disease	39 (15.7)	16 (11.4)	15 (19.5)	
Uterine or peritoneal	15 (6.1)	6 (4.3)	5 (6.5)	
Male factor	91 (36.7)	51 (36.2)	21 (27.3)	
Mixed aetiology	24 (9.8)	15 (10.6)	9 (11.7)	
Other cause	6 (2.4)	3 (2.1)	3 (3.9)	
Mean FSH (iU/ml) (SD)	7.9 (2.9)	7.6 (2.9)	7.9 (4.0)	0.725
Mean AMH (SD)	18.7 (21.7)	22.6 (27.4)	14.3 (14.1)	0.341
Parity – Nulliparous (%)				
No	48 (19.5)	30 (21.3)	15 (19.5)	0.908
Yes	198 (80.5)	11 (78.7)	62 (80.5)	
Treatment type (%)				
IVF	99 (40.2)	54 (38.3)	40 (52.0)	0.367
ICSI	130 (52.9)	78 (55.3)	33 (42.9)	
FET	17 (6.9)	9 (6.4)	4 (5.1)	
Treatment protocol (%)				
Long	175 (76.4)	83 (62.9)	47 (64.4)	0.036
Flare	12 (5.3)	13 (9.9)	9 (12.3)	
Short	42 (18.3)	36 (27.3)	17 (23.3)	
Starting FSH dose (%)				
150 or less	97 (42.4)	55 (41.7)	28 (38.4)	0.402
225-300	78 (34.1)	51 (38.6)	22 (30.1)	
300 or more	54 (23.6)	26 (19.7)	23 (31.5)	
Mean number of FSH	40.7 (18.7)	37.4 (19.6)	42.0 (19.0)	0.171

ampoules (SD)				
Mean endometrial thickness (mm) (SD)	10.9 (2.3)	10.6 (2.6)	10.4 (1.8)	0.242
Mean number of oocytes retrieved (SD)	10.3 (6.6)	10.4 (5.6)	9.7 (5.8)	0.744
Abandoned cycles (%)				
No	209 (85.0)	123 (87.2)	71 (92.2)	0.256
Yes	37 (15.0)	18 (12.8)	6 (7.8)	
Number of embryos transferred at day 5 post oocyte retrieval (%)				
No	111 (53.4)	57 (46.7)	31 (43.7)	0.275
Yes	97 (46.6)	65 (53.3)	40 (56.3)	
Number of embryos transferred (%)				
Single	153 (73.6)	79 (64.8)	51 (71.8)	0.230
Double	55 (26.4)	43 (35.2)	20 (28.2)	
Top grade embryo transfer				
No	39 (18.7)	29 (23.8)	15 (21.1)	0.551
Yes	169 (81.2)	93 (76.2)	56 (78.9)	

The causes of infertility were also similar between the three groups (p=0.771). Male factor infertility was found to be the most common single cause of infertility in all three groups. Ovarian reserve using day two to five follicular stimulating hormone as a marker was similar in the three vitamin D categories (p=0.725). The mean follicular stimulating hormone levels in the deficient, insufficient and replete groups were 7.9 (SD 2.92), 7.6 (SD2.88) and 7.9 (SD3.97) respectively.

The three groups were comparable with regards to the index treatment (IVF, ICSI or FET) (p=0.367). However, there was a statistical difference between the treatment protocol (long down regulation, short antagonist and flare) used (p=0.036). Women in the vitamin D

deficient group were more likely to receive the long down regulation protocol than the vitamin D insufficient and replete groups (p=0.036).

Treatment Cycle Variables

The IVF treatment cycle variables are also displayed in table 4. The starting follicular stimulating hormone doses used for ovarian stimulation were similar between the three vitamin D groups (p=0.402). Furthermore, the mean number of follicular stimulating hormone ampoules used for ovarian stimulation was 40.7 ampoules (SD18.7) in the deficient group, 37.4 ampoules (SD19.6) in the insufficient group and 42.0 ampoules (SD19.0) in the replete group. Therefore the total FSH required for stimulation in all vitamin D groups were comparable (p=0.171).

During the last monitoring scan in the participants' index treatments, the endometrial thickness was similar in all three of the vitamin D groups (p=0.242). The endometrial thicknesses in the vitamin D deficient, insufficient and replete groups were 10.9mm (SD2.33mm), 10.6mm (SD2.60mm) and 10.4mm (SD1.77mm) respectively.

The numbers of abandoned cycles were similar for all three vitamin D categories (p=0.256). Thirty-seven of 246 participants (15.0%) with vitamin D deficiency had their treatment cycle cancelled due to under or over ovarian stimulation. Eighteen out of 141 vitamin D insufficient participants (12.8%) had their treatment cycle cancelled. Lastly, six out of 77 vitamin D replete participants (7.8%) had to discontinue their cycle.

Oocyte retrieval yielded comparable number of oocytes in all three vitamin D groups (p=0.744). The mean number of oocytes collected in the deficient, insufficient and replete vitamin D groups were 10.3 (SD6.60), 10.4 (SD5.56) and 9.7 (SD5.77) respectively.
The quality of embryos cultured was largely similar in the three vitamin D groups (p=0.275). Comparable proportions of participants in the deficient, insufficient and replete groups underwent a day five blastocyst transfer, 46.6%, 53.3% and 56.3% respectively. Similar proportions underwent a single embryo transfer, 73.6% in the deficient group, 64.8% in the insufficient group and 71.8% in the replete group (p=0.230). Most participants received a top grade embryo and transfer. Of the participants with deficient vitamin D, 81.2% received a top grade embryo, 76.2% of vitamin D insufficient women underwent a top grade embryo transfer, and 78.9% of vitamin D replete women had a top grade embryo transfer (p=0.551).

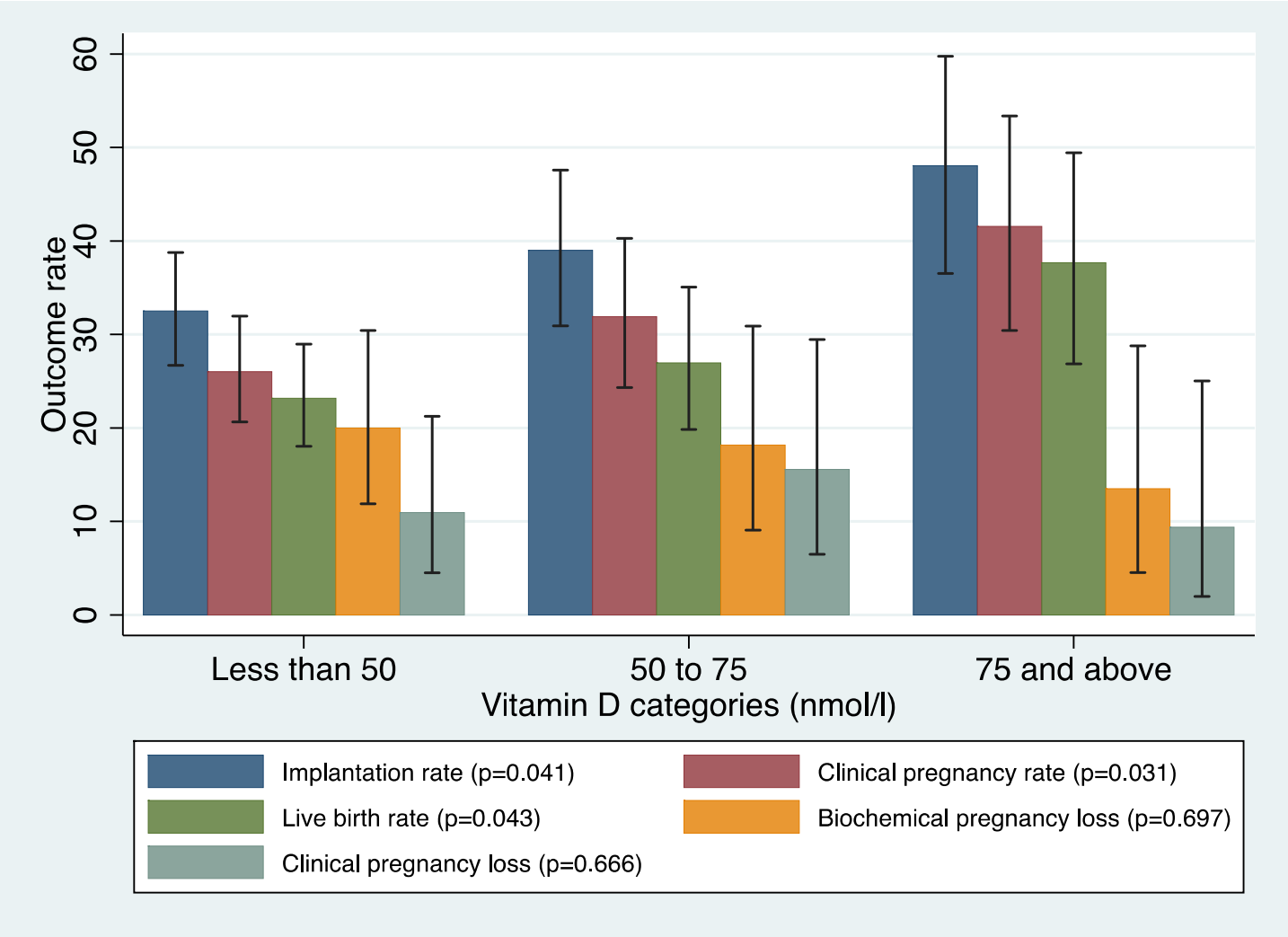
IVF treatment outcomes

The IVF treatment outcomes are shown in table 5 and figure 11. In the vitamin D deficiency group the clinical pregnancy rate was 26.0% (95% CI 20.6-32.0). In the vitamin D insufficient group the clinical pregnancy rate achieved was 31.9% (95% CI 24.3-40.3). In women with a replete vitamin D result, the clinical pregnancy rate was 41.6% (95% CI 30.4-53.4). This difference in clinical pregnancy between the three groups is statistically significant (p=0.031). It should be noted that any subsequent reproductive outcomes resulting from frozen embryos that resulted from the index treatment each participant received were not recorded.

Table 5: Reproductive treatment outcomes by vitamin D category

Reproductive treatment outcome	Vitamin D category (nmol/L)	Point estimate % (95% CI)	P-value
Clinical pregnancy (Primary outcome)	<50	26.0 (20.6-32.0)	0.031
	50-75	31.9 (24.3-40.3)	
	>75	41.6 (30.4-53.4)	
Biochemical pregnancy	<50	32.5 (26.7-38.8)	0.041
	50-75	39.0 (30.9-47.6)	
	>75	48.1 (36.5-59.7)	
Live birth	<50	23.2 (18.0-29.0)	0.043
	50-75	27.0 (19.8-35.1)	
	>75	37.7 (26.9-49.4)	
Biochemical pregnancy loss	<50	20.0 (11.9-30.4)	0.697
	50-75	18.2 (9.1-30.9)	
	>75	13.5 (4.5-28.8)	
Clinical pregnancy loss	<50	10.9 (4.5-21.2)	0.666
	50-75	15.6 (6.5-29.5)	
	>75	9.4 (2.0-25.0)	

Figure 12: Reproductive treatment outcomes by vitamin D category

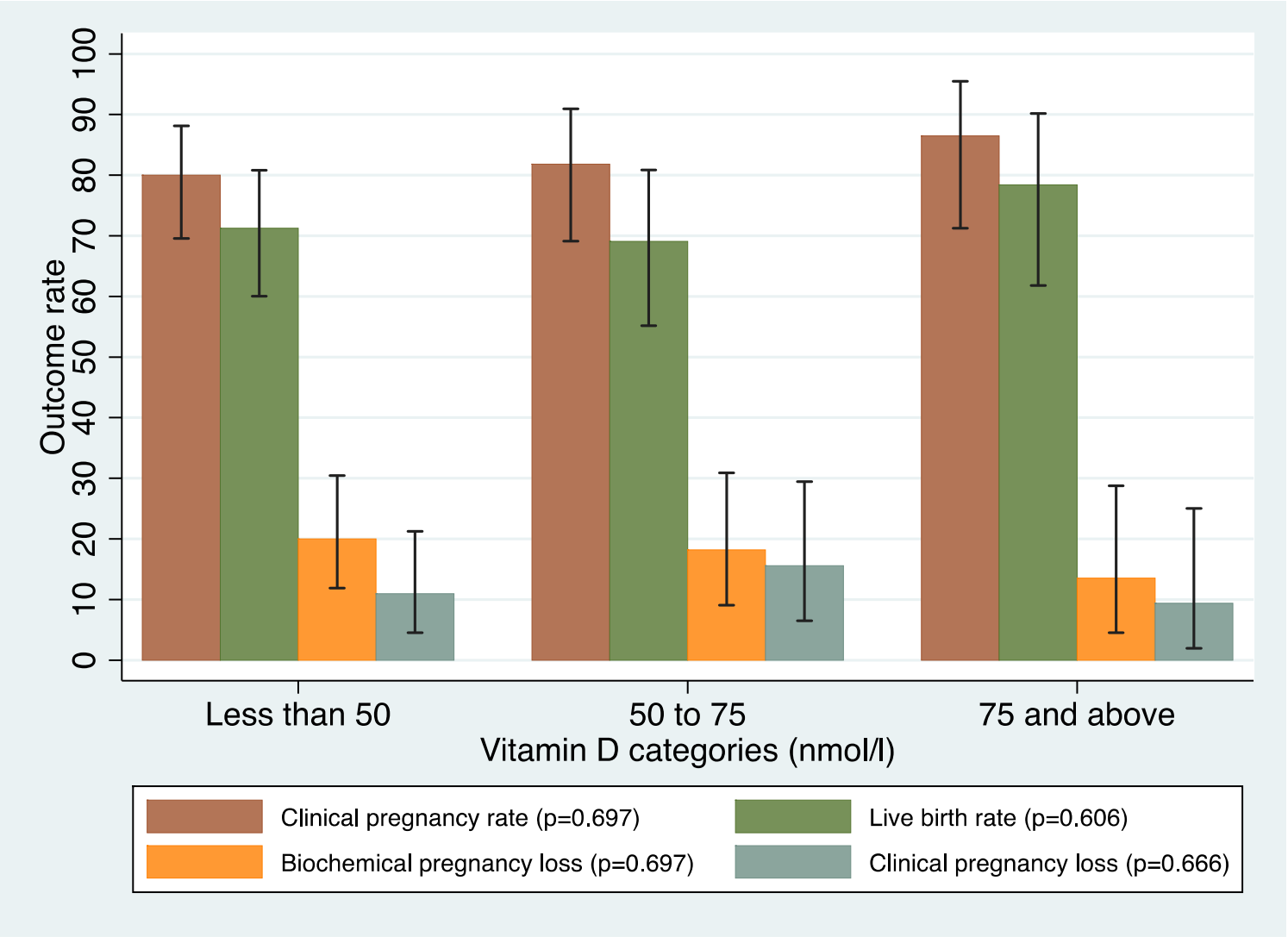


Implantation rates (the percentage of women in each vitamin D group achieving a positive pregnancy test) in deficient, insufficient and replete groups were 32.5% (95% CI 26.7-38.8), 39.0% (95% CI 30.9-47.6) and 48.1% (95% CI 36.5-59.7) respectively (p=0.041). The implantation rates show a statistically significant difference between the three groups. Of the women achieving a positive pregnancy test, the biochemical pregnancy loss rates for vitamin D deficient, insufficient and replete vitamin groups were 20.0% (95% CI 11.9-30.4), 18.2% (95% CI 9.1-30.9) and 13.5% (95% CI 4.5-28.8) respectively (p=0.697).
Women achieving clinical pregnancy had comparable miscarriage rates in the vitamin D deficient, insufficient and replete groups; 10.9% (95% CI 4.5-21.2), 15.6% (6.5-29.5) and 9.4% (95% CI 2.0-25.0) respectively (p=0.666). Finally, live birth rates in the vitamin D groups were significantly different. The live birth rates in the deficient, insufficient and replete groups were 23.2% (95% CI 18.0-29.0), 27.0% (95% CI 19.8-35.1) and 37.7% (95% CI 26.9-49.4) respectively (p=0.043).

Reproductive outcomes if implantation achieved

In participants who achieved a biochemical pregnancy, the chance of progressing to a clinical pregnancy and live birth were comparable between the three vitamin D groups (figure 12). The clinical pregnancy rate in women achieving biochemical pregnancies in the vitamin D deficient, insufficient and replete groups were 80.0% (95% CI 69.6-88.1), 81.8% (95% CI 69.1-90.9) and 86.5% (95% CI 71.2-95.5) respectively (p=0.697). The proportion of women progressing to a live birth who achieved a biochemical pregnancy in the vitamin D deficient, insufficient and replete groups were 71.3% (95% CI 60.0-80.8), 69.1% (95% CI 55.2-80.9) and 78.4% (95% CI 61.8-90.2) respectively (p=0.606).

Figure 13: Reproductive outcomes of participants that achieve a positive pregnancy test by vitamin D category



IVF treatment outcomes adjusted by ethnicity

The IVF treatment outcomes were adjusted according to ethnicity, as vitamin D deficiency was more prevalent in the non-White population. The statistically significant trends seen in clinical pregnancy and live birth rates between the three vitamin D groups was lost after adjustment analysis was performed. The adjusted clinical pregnancy rates in the vitamin D deficiency, insufficiency and replete groups were 26.0%, 31.2% and 41.5% respectively (p=0.227). The adjusted live birth rates for the vitamin D deficiency, insufficiency and replete groups were 21.5%, 24.7% and 36.3% respectively (p=0.212).

The White population

We performed a post-hoc analysis of the IVF treatment outcomes in the White population alone. This was performed to ascertain whether vitamin D is an independent variable that affects IVF treatment outcome in our largest ethnic group. When comparing vitamin D deficient and insufficient White women with vitamin D replete White women, the clinical pregnancy rates and live birth rates showed clear trends, however, the trend did not reach statistical significance. The clinical pregnancy rates in vitamin D deficient, insufficient and replete White women were 31.4%, 35.2% and 46.8% respectively (p=0.147). The live birth rates in vitamin D deficient, insufficient and replete White women were 29.1%, 30.8% and 43.6% respectively (p=0.144).

Discussion

This prospective cohort study indicates that serum vitamin D status is associated with IVF treatment outcomes. This finding confirms what other research groups have found[60–65,96,97], that women with vitamin D replete status are more likely to become pregnant through their IVF treatment than those who are vitamin D deficient or insufficient. Moreover, these findings suggest an association between vitamin D status and IVF treatment outcomes in the White population, although in the Asian and Black population this association was lost. The loss of a the association between vitamin D and IVF treatment outcome in the Asian and Black population is likely to be due to the low numbers of women of dark pigmented skin (Asian and Black women) that had a normal serum vitamin D.

Study strengths

There were several strengths to this present study. Compared to other similar cohort studies, this is large in number and a sample size calculation was conducted prior to the commencement of the study. This reduces the likelihood for our association to be due to chance. Additionally, we conducted this study with inclusivity in mind meaning that all patients that underwent IVF treatment were approached for recruitment. This enables our findings to have greater generalisability and widens applicability. Furthermore, the population that we recruited was ethnically diverse, increasing representativeness for the rest of the UK population and specifically all other UK IVF care providers.

Study weaknesses

This study also had weaknesses. As mentioned above, the biological plausibility that vitamin D affects IVF treatment outcome stems from its effects on the endometrium. Ideally, in order to prove the hypothesis that vitamin D exerts its effects on treatment outcome via the endometrium; serum for vitamin D assay should have been obtained on the day of embryo transfer. This ensures that vitamin D status is as close temporally to the time of embryo transfer as possible. In our study, women were approached at the start of their IVF treatment. Therefore there may have been changes and fluctuations to the serum vitamin D level during IVF treatment after the assay was performed. However, this is unlikely as changes in vitamin D levels only occur when vitamin D deficiency or insufficiency is treated. All of the participants would have been taking preconception vitamins at the time the blood test was obtained and would have continued this supplementation throughout their IVF treatment. However, this is not at a dose high enough to correct vitamin D deficiency or insufficiency.

Another weakness in the study is the fact that it is observational in nature. This means that, although an association between vitamin D and IVF treatment outcome is demonstrated, no clear causal pathway can be elucidated.

Seasonal variation in conception

Seasonal variations in conception rates have already been established[98] with higher conception rates found in the Summer and Autumn. However, although many hypotheses have been postulated to explain this phenomenon (e.g. reduced ovulation rates and sperm

quality in darker months) the exact mechanism behind this has not been explained. It is possible that an increase in sun exposure and greater sunlight luminosity increases the body's store of vitamin D, thereby yielding higher conception rates in Summer and Autumn.

The debate regarding the importance of vitamin D in human beings continue, however, its impact on immunomodulation with a resultant dampening of active inflammatory cytokines is now well understood[42]. Additionally, the expression of vitamin D receptors at the level of the endometrium and the role of vitamin D in the transcription of HOX10A gene, which has been found to be of key importance in implantation, suggest that the immunomodulatory effects of vitamin D may have a direct impact on implantation and therefore the likelihood of reproductive treatment success.

These theories would explain why in the population studied, vitamin D levels were strongly associated with the season at which IVF treatment was performed and why those with a normal serum vitamin D level were more likely to achieve a clinical pregnancy.

Reproductive outcomes

Interestingly, vitamin D status did not have an impact on IVF treatment itself, only on its outcome. In our cohort of infertile women, patient demographic was comparable when the population was divided into the three vitamin D categories (deficient, insufficient and replete). The cohort was also homogeneous with regards to the types of infertility (primary or secondary) suffered as well as the cause of infertility when participants were divided into the three vitamin D groups. The only statistically significant difference between the three

vitamin D groups in our cohort was the duration of infertility. This is one of many important prognostic markers for the success of IVF treatment. The duration was significantly increased in the vitamin D deficient group, further supporting the theory that vitamin D deficiency could reduce endometrial receptivity. Most importantly, when divided into vitamin D deficient, insufficient and replete categories, the women in our cohort faired similarly during the IVF treatment, with important prognostic factors such as requirement for follicular stimulating hormone, endometrial thickness and availability of top grade embryo for transfer being similar.

In the present cohort study, vitamin D seemed to have the most profound effects on the treatment outcomes themselves. Furthermore, the data shows that if participants are able to achieve implantation (by achieving a biochemical pregnancy) the chances of the implanted embryo progressing to become a clinical pregnancy and a live birth are comparable between the three groups. This substantiates the belief that vitamin D's function in fertility is mainly in initial implantation of the embryo.

Other studies have investigated the link between vitamin D status and implantation further. Some research groups have attempted to isolate the effects of vitamin D on the endometrium and implantation by studying women undergoing donor oocyte IVF treatment cycles. In such cycles, one would expect all oocyte prognostic determinants to be nullified, as only good quality oocytes are used. In effect endometrial receptivity and implantation alone are tested. Examples of such studies include the study by Fabris et al.,[56] (who did not find an association between vitamin D and implantation in women undergoing IVF treatment using

donor oocytes), and the study by Rudick et al.,[64] who found a strong association between the vitamin D status of donor oocyte recipients and the IVF treatment success.

When reproductive outcomes were adjusted for ethnicity, the associations between vitamin D and clinical pregnancy, live birth, and miscarriage rates were lost. The results do demonstrate that women of Asian or Black origin were far less likely to be replete in vitamin D status when compared to White women. In this study, there are low sample sizes in each ethnic group. This would have caused under-powering leading to the loss of association after adjustment for ethnicity. However, in other published studies, ethnicity has been found to be a prognostic marker for IVF treatment success on its own[99]. Perhaps the reason for this could be due to higher prevalence of vitamin D deficiency in these ethnic groups due to darker pigmented skin, absorbing a lower level of ultraviolet light in the UK. Consequently, this would reduce the stores of vitamin D in Asian and Black women. Vitamin D receptor gene polymorphisms have already been identified in the Asian population which may act as a confounding or modifying factor[100,101].

Interestingly, when the IVF treatment outcomes were analysed in isolation in the White population, women with replete vitamin D levels achieved an increased rate of clinical pregnancy and live birth when compared to those with vitamin D deficiency/insufficiency. Although strong conclusions cannot be drawn from observational data, this would suggest that in White women, vitamin D does play an important independent role in predicting IVF treatment success. This could be via its actions within the endometrium promoting implantation or as a surrogate marker for another variable.

Summary

In many countries like the UK, strict government funding restrictions apply to the provision of IVF treatment to infertile couples. The identification of treatable factors that can improve the chances of each IVF treatment success is therefore crucial. Vitamin D serum testing is relatively cheap and widely available. Furthermore, treatment of deficiency or insufficiency with subsequent maintenance therapy is not costly. Additionally, it is now well understood that vitamin D deficiency during pregnancy may also increase the risk of obstetric complications such as gestational diabetes[102,103], pre-eclampsia[104–106], and fetal growth restriction[107,108]. Treatment of vitamin D deficiency pre-conceptually could be the goal in the future, extending to pregnancies achieved through natural conception as well and therefore having far reaching public health implications. However, before population based vitamin D screening and treatment of deficiency can be implemented, a randomized controlled trial is required to ensure that treatment of vitamin D deficiency does improve reproductive treatment outcomes. Moreover, more understanding is needed regarding the different vitamin D requirements in the Asian and Black populations, as vitamin D deficiency treatment in these sub-populations may be different to that of the White population.

CHAPTER 4: VITAMIN D AND RECURRENT MISCARRIAGE – SYSTEMATIC NARRATIVE REVIEW

Introduction

Vitamin D is a fat soluble secosteroid that human beings mainly source from skin production as a result of sunlight exposure[37,109]. The precursor 7-dehydrocholesterol is converted to provitamin D3 by UVb light[42]. Spontaneous isomerisation produces cholecalciferol or vitamin D3. This provides 80-90% of total body vitamin D[45]. Diet in the form of plants, fungi and fatty fish provides the remaining 10-20%[42]. Vitamin D3 is transported bound to VDBP to the liver where it is metabolised into 25-hydroxy vitamin D by 25-hydroxylase. Further metabolism then converts 25-hydroxy vitamin D into 1-25-dihydroxyvitamin D3 in the kidneys[47]. This is the biologically active form of vitamin D and exerts its biological actions by activation of target genes through the retinoid X receptor[110].

Vitamin D deficiency has been found to be highly prevalent in areas of low sunlight and has been found to be especially prevalent in women at reproductive age[42]. This is postulated to be due to a reduction in sun exposure due to lifestyle changes and also because of rising prevalence of obesity (as vitamin D is sequestered in adipose tissue)[111]. The importance of this epidemiological problem is far reaching. It is well recognised that vitamin D is essential in calcium and phosphorous homeostasis as well as bone metabolism. Severe vitamin D deficiency can lead to rickets in childhood and osteomalacia in adulthood[112]. However, more recently the role of vitamin D in other non-skeletal pathological processes such as cancer, infections, autoimmune disorders and cardiovascular disease has been called into question[113,114]. It is thought that vitamin D has a role in immunomodulation, reducing T-helper 1 cells and their production of inflammatory cytokines such as IL-2, TNF-α and IF-ϒ.

Additionally, vitamin D is also thought to increase the action of T-helper 2 cells leading to further suppression of inflammatory responses[115].

Vitamin D receptors and enzymes required for its metabolism have been found to be widespread in female reproductive organs. The ovaries and uterus have been found to contain these enzymes and receptors[23,116,117]. Due to the immunomodulatory role of vitamin D it has been suggested that vitamin D has a key role in implantation, placental formation and progression to a health pregnancy[40,44,118]. Published research has investigated vitamin D's role in ART[58,69,96], PCOS[119,120], endometriosis[121,122], uterine fibroids[123,124] as well pre-eclampsia, gestational diabetes mellitus and intrauterine growth restriction[125,126]. Interest in the areas of ART, pre-eclampsia and fetal growth restriction stem from the belief that vitamin D has a crucial role in ensuring the dampening of inflammation required to ensure that normal and effective fetal-maternal interface is formed at the time of implantation. It is thought that deficiency in vitamin D may lead to poor endometrial decidualisation leading to poor trophoblastic invasion[47] poor IVF treatment outcomes and pregnancy complications.

Recurrent miscarriage is the loss of three or more consecutive pregnancies before 20 weeks gestation. It affects 1% of women in their reproductive age and can have severe physical and emotional effects[127]. In approximately 50% of patients with recurrent miscarriage the cause of the condition is known. Recurrent miscarriage is associated with parental karyotypical abnormalities, uterine anomalies, thrombophilia, and dysfunction of the immune system as well as endocrine diseases such as thyroid dysfunction or diabetes mellitus. However, in the 50% of patients where a cause is not found there may be a

combination of unidentifiable risk factors[128,129]. One such risk factor may be deficiency in vitamin D. Improper implantation may lead to increased risk of recurrent miscarriage.

The purpose of this systematic review was to identify and examine all published literature that has investigated the association between vitamin D and recurrent miscarriage.

Methods

Literature Search

Literature of interest consisted of journal publications reporting on the association between vitamin D and recurrent miscarriage.

The following electronic databases were searched: MEDLINE, EMBASE, Cochrane Central Register of Controlled Trials and CINAHL. Databases were searched from inception to November 2015. A search strategy was developed based on the following key words and or MeSH terms: vitamin D, cholecalciferol, ergocalciferol, vitamin D deficiency, recurrent miscarriage and pregnancy. The reference lists of all primary and review articles were examined to identify relevant articles that had not been identified by the electronic searches. No language restrictions were applied to the searches or study selection.

Study selection

Prior to the literature searches criteria for inclusion into the review were established. Study selection was performed by two independent reviewers. The titles and the abstracts were scrutinised by the independent reviewers and included and excluded independently

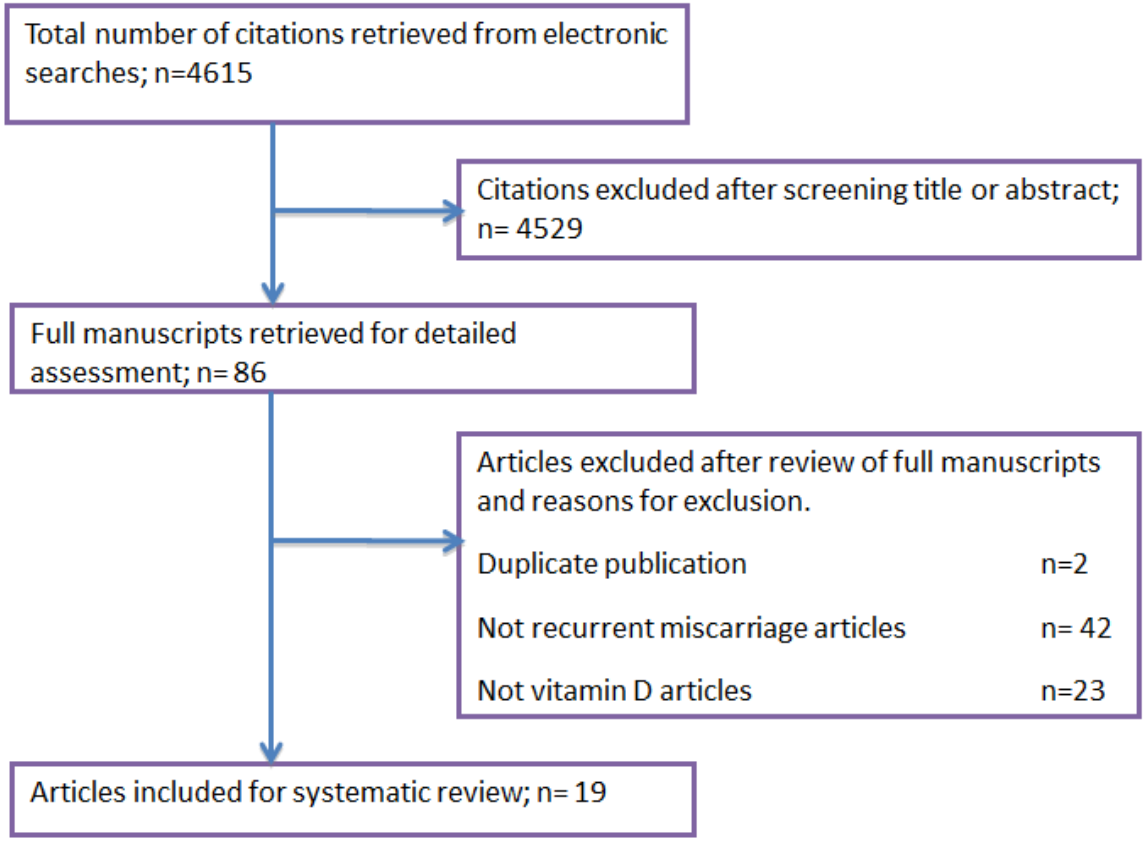
according to the preset criteria. Any disagreements regarding inclusion were resolved by a third reviewer. The full manuscripts of the abstracts identified as relevant for inclusion were retrieved and examined. In the scenario where duplication publications were found then the most recent and complete versions were selected. Studies that did not explicitly report findings for recurrent miscarriages were excluded. Similarly, studies that did not explicitly report findings for vitamin D were also excluded.

Studies were divided into two groups. The first group included studies that reported on findings from clinical research. The second group reported findings from basic science research.

Results

The PRISMA flow diagram[52,53] of the review process is presented in figure 13. The search strategy generated 4615 citations. Of these, 4529 of these citations were excluded because it was clear from the title and abstract that they did not fulfill the search criteria. A total of 86 full manuscripts were retrieved for further examination. A further 67 of these publications were excluded because 42 were not specifically concerning recurrent miscarriage and 23 were not specifically concerning vitamin D. Two publications were duplicate articles. Therefore the total number of studies included in the review was 19.

Figure 14. PRISMA flow diagram for study selection. Vitamin D and recurrent miscarriage



Study characteristics

Of the 19 studies that are included in the review, eight are clinical studies and 11 are basic science studies. As the studies that were found to satisfy inclusion criteria were low, conference abstracts were also included.

Clinical Studies

Eight clinical studies fulfilled the criteria of the search.

One small randomised controlled trial published by Ibrahim et al., in 2013 investigated the benefits of using vitamin D supplementation in women with a history of unexplained recurrent miscarriage[130]. In this trial, 40 women with a history of recurrent miscarriage were recruited at less than six weeks gestation to either receive vitamin D3 supplementation at a dose of 0.25 micrograms twice daily or no treatment. This group of patients did not have their serum vitamin D measured. The primary outcome was the outcome of the index pregnancy. The authors did not find a statistically significant difference between the treatment and control groups but did find the risk of miscarriage was 15% lower in the treatment group than the control group. As a surrogate marker of immunomodulation, this study found that the serum level of IF-ϒ was significantly higher in the control group. The group also concluded that their findings warranted further investigation by way of a larger trial.

Several observational cohort studies have investigated the effects of plasma vitamin D on early pregnancy outcomes in women with a history of recurrent miscarriage. A study by Moller et al., in 2012 on women with recurrent miscarriage, did not find an association between pre conception plasma vitamin D levels and miscarriage in an index pregnancy, although they did find a statistically significant association between low plasma vitamin D and second trimester miscarriage[131].

In 2013, Charatcharoenwitthaya et al., published a cohort of 120 women who had been previously diagnosed with recurrent miscarriage. All 120 women were followed up during a subsequent pregnancy. Only 10 women had a miscarriage. [132]. Out of the ten women who had miscarriages, nine had low vitamin D (four had vitamin D deficiency and five had vitamin D insufficiency). Only one out of the ten women who had a miscarriage was vitamin D replete. Other studies have shown no association between vitamin D status and subsequent miscarriage in recurrent miscarriage patients. Most recently, Schneuer et al., found that there was no relationship between vitamin D and miscarriage in an index pregnancy in 2014[133]. This was a large study of 5109 women where serum vitamin D was measured in the first trimester of pregnancy. Although this study found that vitamin D deficiency was highly prevalent in the recurrent miscarriage (being present in 80.4% of their population), index pregnancy miscarriage was seen in equal proportions in women with varying ranges of serum vitamin D. Flood Nichols et al., also found no relationship between vitamin D deficiency and adverse pregnancy outcomes including miscarriage in a small retrospective cohort study of 108 women diagnosed with recurrent miscarriage who had plasma collected and stored in the first trimester of pregnancy[134].

Ota et al., performed a retrospective cross-sectional study of 133 women with a history of recurrent miscarriage in 2014[135]. They investigated the prevalence of auto and cellular immune abnormalities in women with low vitamin D when compared with those that have a normal vitamin D level. The authors found a significantly higher prevalence of antiphospholipid antibody, antinuclear antibody, anti-single-strand DNA antibody and thyroperoxidase antibody in women with vitamin D deficiency. Interestingly, natural killer cytotoxicity was also found to be higher in the vitamin D deficient group when compared to the vitamin D replete group. High natural killer cytotoxicity has been implicated in the aetiology of unexplained recurrent miscarriage[136] and is offered as a test in some recurrent miscarriage clinics. In summary, this study found that a high proportion of women with recurrent miscarriage have vitamin D deficiency and that vitamin D deficiency has immunological implications.

Natural killer cytotoxicity was also found to be higher in vitamin D deficient women with recurrent miscarriage by Kim et al.,[137]. This article reported that there was increased vitamin D receptor expression in peripheral blood natural killer cells in women with vitamin D deficiency when compared to those who were vitamin D replete. This infers that natural killer cell activity is dependent on vitamin D status of women with recurrent miscarriage.

A case-control study by Kashani et al., suggested that vitamin D was higher in patients with recurrent miscarriage when compared to healthy controls without a history of recurrent

miscarriage[138]. This author group found that there was a higher proportion of vitamin D replete women in the recurrent miscarriage group when compared to healthy cohorts. The last clinical article was a review of all immunomodulatory therapies that are used in the treatment of recurrent miscarriage. Published in 2012, Bansal et al., discuss the value and basis of potential immune therapies in this patient group[139]. Along with glucocorticoid therapy, immunoglobulin therapy, anti-TNF-α therapy and intralipid therapy, Vitamin D is one of the therapies discussed. Its value in reducing adverse decidual inflammatory cytokines and increase the number of regulatory T-cells is recognised.

Basic Science Studies

Of the 19 included studies in the review, 11 were basic science studies.

Most recently Gysler et al., investigated the trophoblastic cell line (HTR8) secretion of inflammatory cytokines (IL-8 and IL-1β) when they were treated with vitamin D[140–142]. This study found that when trophoblastic cells are treated with vitamin D alone, or in combination with low molecular weight heparin, the antiphospholipid antibody induced trophoblast inflammatory response in the HTR8 cells was attenuated. The study called for further research into the benefits that may be gained by using vitamin D to prevent faulty antiphospholipid antibody induced trophoblast invasion and recurrent miscarriage.

Tavakoli et al., investigated the immunomodulatory effect of 1,25(OH)2 vitamin D3 on cytokine production by the endometrial cells of women with unexplained recurrent miscarriage[143]. In this study endometrial samples were collected from 48 women with

recurrent miscarriage and also eight healthy controls by biopsy curette. The main outcome measure was the production of IF-Ɣ, IL-10, transforming growth factor beta (TGF-β), IL-17, IL-6 and IL-8 by endometrial stromal cells when in the presence and in the absence of 1,25(OH)2 vitamin D3. Interestingly, this study found that the cytokine profile of endometrial cells of women with recurrent miscarriage had a tendency towards a T-helper 2 phenotype after treatment with 1,25(OH)2 vitamin D3. The authors suggest that treatment with vitamin D could make the endometrial cavity more amenable to embryo implantation in women with a history of unexplained recurrent miscarriage. The study concluded that more clinical studies should be done to investigate the beneficial effects of 1,25(OH)2 vitamin D3 on women with recurrent miscarriage.

Tavakoli's research group went on to investigate the expression of vitamin D receptors and 1 – a – hydroxylase and 25 – hydroxylase in the endometrium of women who had a history of recurrent miscarriage when compared to endometrium of healthy controls[144]. The expression of vitamin D receptors and enzymes required for vitamin D metabolism was found to be comparable in both groups. This study only investigated eight patients with recurrent miscarriage and eight healthy controls and therefore strong inferences could not be made. In addition, there were also no differences between the serum vitamin D levels between the group of participants with recurrent miscarriage and the group of health fertile controls. This would explain the comparable findings within the endometrium.

Murine experiments performed by Halhali et al., suggested a clear role of vitamin D in implantation[145]. The authors injected 1,25(OH)2 vitamin D3 into the horns of mice uteri and

found greater decidualisation in the horns that had received the injections compared to the controls.

In 2004, Bubanovic postulated that the immunomodulatory effects of vitamin D were vital in the prevention of recurrent miscarriage[115]. In a hypothesis article, the effect of vitamin D in establishing a T-helper 2 phenotype response, with a resultant increase in T-helper 2 cytokine production of IL-3, IL-4 and IL-10 is described. The increase of these cytokines is postulated to lead to a rise in the natural killer suppressor cells which reduce the activity of natural killer cells that cause an abortogenic immunological cascade when miscarriage occurs. The effect of vitamin D on the suppression of T-helper 1 cells subset and the subsequent decrease in IL-2 and IF-Ɣ with resultant reduction in natural killer activation of macrophages at the feto-maternal interface is also described. The author concludes that the use of vitamin D should be seen as a novel treatment of recurrent miscarriage.

Other author groups have reinforced the theory of cytokine augmentation by vitamin D. Evans et al., found that both 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 have a profound effect on cytokine production in human decidual cells[49,50]. When decidual natural killer cells were treated with bioactive 1,25-dihydroxyvitamin D3 or precursor 25-hydroxyvitamin D3 there was a reduction in cellular production of granulocyte-macrophage CSF2, TNF-α as wells as IL-6. The authors suggest that this allows for regulation of the acquired and innate immune responses at the fetal-maternal interface. Similar to the findings of Tavakoli et al., several enzymes of vitamin D metabolism (including 1-alpha-hydroxylase) were found within the maternal decidua of first trimester pregnancies.

Furthermore, the synthesis of 1,25-dihydroxyvitamin D3 was significantly higher in first trimester decidual cells when compared with third trimester decidual cells, suggesting an important role in early pregnancy and implantation.

Extravillous trophoblastic cells are equally as affected. Chan et al., and Canovas et al.,[146,147] demonstrated this in 2013. Isolated extravillous trophoblast cells from first trimester human placentae were treated with increasing doses of 1,25-dihydroxyvitamin D3. The degree of invasion was quantified and this study demonstrated that higher levels of 1,25-dihydroxyvitamin D3 (at a concentration of 0.1 nanomole to 1 nanomole) led to a two-fold increase in invasion into maternal endometrial tissues. This provided circumstantial evidence that 1,25-dihydroxyvitamin D3 promotes extravillous trophoblast invasion and that malplacentation may occur when serum 1,25-dihydroxyvitamin D3 levels are low. Although this study suggested that this finding was relevant for the pathogenesis of pre-eclampsia in pregnancy, abnormal placentation caused by low vitamin D levels could also affect the continuation of pregnancy in the first trimester in women with recurrent miscarriage

Discussion

This systematic review identified 19 articles that investigated the association between vitamin D and recurrent miscarriage. There were eight clinical studies and 11 basic science

articles. A clear biological plausibility has been investigated by several author groups but there are only a small number of clinical studies that have been published to confirm whether an association exists. According to the literature searches conducted in this review, this is the first systematic review investigating the association between vitamin D and recurrent miscarriage.

The present systematic review was strengthened by several factors. An extensive search strategy was used and a large number of citations were identified decreasing the possibility that relevant articles were missed and not included. No language restrictions were applied and the research databases were searched from date of inception. This means that our search is comprehensive.

However, only low level inferences can be made from the findings of this systematic review. The number of studies that were included in our results was low therefore firm conclusions cannot be drawn. In particular, there is a clear lack of clinical studies with only one small randomized controlled trial investigating the effectiveness of vitamin D treatment in women with a history of recurrent miscarriage[130]. This trial only recruited 40 women and the research group did not measure the participants' serum vitamin D and therefore it fails to answer the research question. Aside from this small trial there were only several observational studies that aimed to identify whether vitamin D deficiency was more common in women who had been diagnosed with recurrent miscarriage[131–134]. The findings from these studies were mixed, with some observational data suggesting a link between vitamin D deficiency and miscarriage and other studies finding no such association.

Additionally, the case control study conducted by Kashani et al., found that vitamin D levels were higher in women with recurrent miscarriage when compared to controls[138]. Therefore strong clinical conclusions cannot be made.

The biological plausibility for an association between vitamin D and recurrent miscarriage has been investigated more extensively with a larger number of basic science publications. Basic science findings have centred on the immunomodulatory effects of vitamin D[115,139–142] . In particular, authors have investigated the effects on cytokine production that vitamin D has when it is used to treat endometrial cells. The studies that were identified by this review have reported similar findings, confirming that there is a reduction in the production of pro-inflammatory cytokines (IL-1B, IL-6, IL-8, IL-10, IL-17, IF-Ɣ, TGF-β and TNF-α). These studies showed that higher vitamin D levels produce an immunological reaction within the endometrium more akin to a T-helper 2 phenotype. Additionally, vitamin D appears to steer the immune response away from a T-helper 1 phenotype due to modification of natural killer cell activity. The importance of natural killer cell activity in recurrent miscarriage has been further investigated previously[148].

In addition to the important immunomodulatory actions, the role of vitamin D in the implantation and maintenance of normal pregnancy has been identified by the presence of enzymes that are key in vitamin D metabolism within endometrial and decidual cells. Several authors have demonstrated that the presence of these enzymes is greatest in the first trimester[146,147]. Trophoblastic invasion has also been found to be greatly affected by the dose of vitamin D that trophoblastic cells lines are treated with[146].

The basic science studies identified in this review have suggested that there is a clear biological relationship between vitamin D and the processes of implantation, decidualisation, trophoblastic invasion and placentation. This association has been investigated more extensively in other conditions where these processes are defective (e.g. pre-eclampsia, intra-uterine growth restriction, IVF treatment) by the conduct of robust clinical research. As unexplained recurrent miscarriage also stems from these processes being faulty it would seem prudent to explore the association further by performing well designed clinical research. A large prospective cohort study investigating the prevalence of vitamin D deficiency in recurrent miscarriage populations and the outcomes of pregnancy would be an ideal starting point. If an association is found then a trial of vitamin D deficiency treatment would be the next logical step. For patients who have recurrent miscarriage, where there is currently no treatment which has been found to be effective, all possible research avenues must be explored. Vitamin D deficiency treatment is one of these avenues. Its treatment is simple, safe (with minimal side effects) and also cheap. Along with the biological plausibility found in laboratory research, the importance of vitamin D repletion in recurrent miscarriage patients is a research priority.

CHAPTER 5: THE USE OF ENDOMETRIAL FLUID FOR PREDICTING IN VITRO FERTILISATION TREATMENT SUCCESS – A SYSTEMATIC NARRATIVE REVIEW

Introduction

In some couples suffering with infertility the only viable treatment option in achieving pregnancy is ART. In the UK, 52,288 women had a total of 67,708 cycles of IVF or ICSI in 2014[31]. The number of IVF and ICSI cycles undertaken in the UK is increasing due to the rise in the prevalence of infertility. Despite advances in the technology used for IVF treatment the success rates for all cycles (regardless of the age of the woman undergoing treatment) remains approximately 35%[31]. This causes great emotional upset to many couples.

Failure of an IVF treatment cycle can be caused by a number of factors. It may be related to oocyte and embryo quality or implantation. Oocyte and embryo quality has been well researched with clear and ample evidence to guide selection of the best embryo with the highest potential to cause pregnancy[149]. However, investigation of the process of implantation has not been as fruitful and understanding of the processes required allowing for successful embryo implantation lags behind that of embryo biology. This is due to the delicate and intricate nature of the process of implantation and the limitations with studying it in vivo. Identifying the optimum environment for implantation by improving our understanding of the implantation process has the potential to increase the success rates from IVF treatment[150].

Implantation is dependent on three delicate processes. The first is apposition, when the blastocyst initially unites with the endometrium[6]. This is an unstable union formed by molecular bonding. The second is adhesion when firmer bonds are made that represent a stronger association between the blastocyst and the endometrium. Characteristically there

is increase in the vascularity at the basement membrane of the endometrium at the site of adhesion. Lastly, penetration occurs, whereby trophoblastic invasion reaches the maternal uterine vasculature[151].

Endometrium that is receptive to pregnancy implantation occurs physiologically, occurring during the mid-luteal phase for a short time window in every menstrual cycle. This short period of receptivity is known as the 'window of implantation' and occurs approximately six days after ovulation and lasts for four days[152,153]. This is when the endometrium is most receptive to implantation of a blastocyst. Outside of this time window the endometrium is not receptive and pregnancy is unlikely.

Similar to physiological menstrual cycles, in IVF treatment the uterine endometrium is dynamic. The endometrium responds to fluctuating concentrations of oestrogen and progesterone and will therefore change its receptivity to embryo implantation throughout the IVF treatment cycle. It is already understood that correct dating of the endometrium is key to successful implantation[152]. However, the only widely used method of gauging the receptivity of the endometrium is by measuring its thickness and examining its morphological appearances with ultrasonography[154,155]. Unfortunately, this does not provide accurate prognostic information[156,157]. A test that could allow the identification of markers of implantation could in some way complement the currently used methods or replace them entirely.

One method of characterising the potential of endometrial embryo implantation is to sample the endometrial fluid. This is the small volume of fluid that can be collected from the endometrial cavity[158]. Animal studies, investigating endometrial fluid, have identified a number of different markers that seem to signify that the endometrium has entered the window of implantation. This includes growth factors, cytokines, chemokines and lipids. In particular, epithelial growth factor, IL-6 and IL-11, leukaemia inhibitory factor (LIF), and prostaglandins have been studied as they show differential concentrations during the time of implantation when compared with other stages of the menstrual cycle[159,160].

From a reproductive medicine and IVF perspective, this data provides scope for further research. It has been found to be safe to collect the endometrial fluid close to the time of insertion of the embryo into the endometrial cavity (at embryo transfer)[161,162]. Moreover, sampling of endometrial fluid at this time could provide a snap shot of the uterine milieu and, importantly, allow clinicians to identify whether the endometrium is receptive. Sampling of endometrial fluid would not require endometrial biopsy, which has been found to have a deleterious effect on the endometrium and the process of implantation if performed at the time of embryo transfer[163].

The emergence of the "omics" disciplines has allowed for high throughput, real time and large scale identification of multiple prognostic markers[164]. There is no longer the need to search for one biomarker at a time. This could accelerate the potential discovery of a putative panel of markers predictive of successful embryo implantation, if the endometrial fluid is used as the examined bio-fluid.

Research studies have attempted to identify potential markers that are predictive of successful implantation and those that predict implantation failure in IVF treatment. Some research has aimed at identifying genes and epi-genetic markers that signify that the endometrium is conducive to embryo implantation (genomics)[165], whilst others have focused on the presence of particular peptides and proteins within the endometrial fluid (proteomics)[166]. The immunological constituents of the endometrial fluid as well as the microbiological nature of the endometrial fluid have also been studied[167].

The purpose of this systematic narrative review is to identify all published studies that have investigated the use of endometrial fluid in prediction of IVF success.

Methods

Literature Search

The aim of the literature search was to identify all studies investigating the use of endometrial fluid as a prognostic tool in IVF treatment. In particular, we wished to seek all studies that used endometrial fluid, sampled at the time of embryo transfer, to identify biomarkers predictive of IVF treatment success or failure.

The electronic databases EMBASE, MEDLINE and CINAHL (from inception to December 2015) were used to perform the literature search (see appendix 6) . The following keywords and MESH headings were used in the search strategy: 'endometrial fluid', 'endometrial

receptivity' and 'IVF treatment'. No language restrictions were applied in any of the searches or study selection.

Study Selection

Inclusion criteria were set prior to undertaking the literature search. Study selection was performed by two independent reviewers. The titles and abstracts of the electronic database search results were examined by both reviewers. The decision to include or exclude the study was made independently according to the pre-set inclusion criteria. In an effort to be as inclusive as possible, any disagreements regarding inclusion or exclusion between the two reviewers resulted in the inclusion of the study. The full manuscripts of the included titles and abstracts were retrieved for further examination. If there were duplicate studies, the most recent and complete versions were chosen. The full manuscripts were scrutinised further by both independent reviewers. Further exclusions were made if the full manuscripts did not fulfill the pre-determined inclusion criteria.

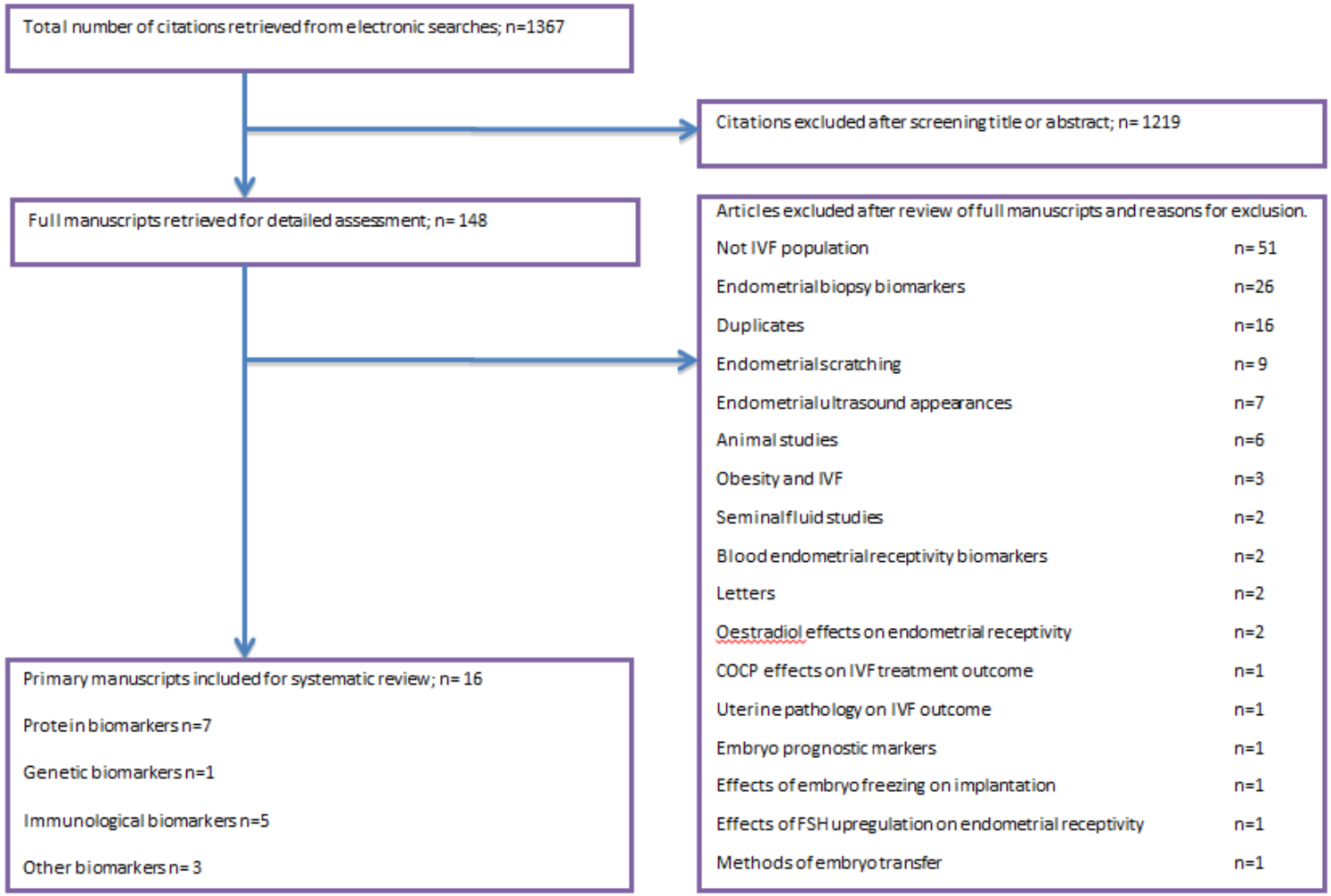
Studies that did not explicitly report results of the use of endometrial fluid as a prognostic tool were excluded. Additionally animal studies and manuscripts reporting on the non-IVF population were also excluded. Lastly, studies that did not report results from endometrial fluid sampled at the time of embryo transfer were excluded.

Results

Figure 14 shows the PRISMA flow diagram[52,53] of the review process. The search of electronic databases produced 1367 citations. One thousand two hundred and nineteen of

these citations were excluded after examination of the titles and abstracts as they did not meet the inclusion criteria. The full manuscripts for the remaining 148 citations were then

Figure 15. Study selection for review on the use of endometrial fluid for predicting in vitro fertilisation treatment success



retrieved. A further 133 citations were excluded after reading the full manuscripts. Fifty-one citations did not study the IVF population, 26 citations were excluded as they studied endometrial tissue biopsies, 16 citations were excluded as they were duplicate publications, nine citations investigated the effect of endometrial scratching on IVF treatment outcome, seven citations investigated the effect of endometrial ultrasound appearance on IVF treatment outcome, six studies investigated endometrial receptivity in animal models, three citations summarised the effects of obesity on IVF treatment outcome, two studies investigated seminal fluid, two studies investigated serum endometrial receptivity markers, two were letters, two studied the effects of serum oestradiol on endometrial receptivity, one study explored the effects of the combined oral contraceptive pill on the endometrium, one study investigated the effects of uterine pathology on IVF treatment outcome, one study investigated embryo prognostic markers, one citation explored the effect of embryo freezing on implantation, one citation studied the effect of FSH up-regulation on endometrial receptivity, and one study investigated the methods of embryo transfer. Therefore the total number of studies that fulfilled our inclusion criteria was 16.

The study characteristics of the 16 studies included in this review are presented in table 6. The 16 studies can be divided into several groups; those investigating protein biomarkers (seven studies), those investigating genetic biomarkers (one study), those investigating immunological biomarkers (five studies), and a miscellaneous group (lipid and microbiological biomarkers) (3 studies).

Table 6: Study characteristics of included studies. Endometrial fluid and IVF treatment							
Author (year)	**Study design**	**Study population**	**Method of endometrial fluid aspiration**	**Biomarker explored**	**Method of measuring biomarker**	**Results**	**Summary**
Bentin-Ley et al., (2011)	Prospective cohort	21 fertile women and 75 infertile women Denmark	Endometrial flushing 1 day and 7 days after ovulation in the natural cycle prior to start of IVF treatment cycle	Glycodelin PROTEIN	Glycodelin assay	Glycodelin concentrations were higher in infertile women with tubal causes compared to fertile women.	Receiver operator curve analysis showed that glycodelin concentrations were of no predictive value for IVF outcome.
Bhattacharya et al., (2012)	Prospective cohort	156 infertile women India	Endometrial fluid aspiration 7 days after ovulation in the natural cycle prior to start of IVF treatment cycle	Integrin beta 3 HOXA 10 gene expression PROTEIN and GENETIC	Intergin beta 3 assay PCR for HOXA 10 gene	Integrin beta 3 expression was higher in women with unexplained infertility who conceived in their IVF treatment cycle. HOXA 10 gene expression was higher in women of lower age groups.	Integrin beta 3 (an adhesion molecule) aids with embryo implantation. HOXA 10 gene expression is vital in pinopod formation increasing embryo receptivity of the endometrium. Poor expression of integrin beta 3 and HOXA 10 gene correlates with poor endometrial receptivity.
Damario et al., (2001)	Prospective cohort	101 infertile women USA	Endometrial fluid aspiration in a mock IVF treatment cycle prior to planned IVF treatment cycle	αvβ3 integrin and glycodelin. PROTEIN	Immunochemical staining of endometrial fluid for αvβ3 integrin and glycodelin	αvβ3 integrin and glycodelin staining intensity strongly correlate with endometrial dating and each other.	The putative window of implantation shows an increase expression of αvβ3 integrin and glycodelin
Das et al., (2007)	Prospective cohort	63 women with unexplained infertility India	Gentle suction of endometrial fluid on the day of HCG trigger during the index IVF	64-0-kDa human uterine fluid protein PROTEIN	Gel electrophoresis	64-0-kDa human uterine fluid protein correlated with endometrial thickness and IVF treatment pregnancy outcomes.	64-0-kDa human uterine fluid protein plays in important role in endometrial receptivity to support pregnancy.

			treatment cycle				
Halperin et al., (1995)	Prospective cohort	109 infertile women Israel	Endometrial fluid aspiration on the day of embryo transfer	Human decidua-associated protein 200 PROTEIN	ELISA	Participants were divided in to four groups dependent on the concentration of human decidua-associated protein 200. The group with the highest concentration of human decidua-associated protein 200 had the highest implantation and clinical pregnancy rates.	Human decidua-associated protein 200 may be involved with the implantation process and could be used as an endometrial marker of receptivity.
Heng et al., (2015)	Prospective cohort	N not provided Australia	Endometrial washings taken in women undergoing IVF treatment. Time of fluid collection not described	Proprotein covertase 5/6A PROTEIN	ELISA	Monoclonal antibodies developed to assay proprotein covertase 5/6A which was found to be higher in receptive endometrial fluid.	Proprotein covertase 5/6A ELISA can be an important tool in the development of non-invasive strategies to detect endometrial receptivity.
Ledee-Bataille et al., (2002)	Prospective cohort	33 infertile women France	Endometrial flushing at day 26 of the natural cycle prior to start of IVF treatment cycle	Leukaemia inhibitory factor (LIF) PROTEIN	ELISA	LIF was not detectable in women that became pregnant and detectable in women that did not become pregnant.	Low concentrations of LIF are predictive of embryo implantation and its use should be considered to predict IVF treatment outcome.
Parks et al., (2013)	Prospective cohort	24 infertile women undergoing frozen embryo transfer USA	Endometrial fluid aspiration immediately before transfer of embryo	29 cytokines and 229 micro RNAs GENETIC	Cytokine multiplex with tandem mass spectrometry panel and microRNA array	6 cytokines were found to correlate to pregnancy outcome from frozen embryo transfer treatment. These included IL-6, VEGF and IL-8 which are known to be involved in embryo-endometrial communication. MicroRNAs miR-891-a, miR-522 and miR-198 were absent from the endometrial fluid of women who did not become pregnant after frozen embryo transfer.	Abnormal microRNA expression may characterise implantation failure. Fine tuning may help increase the chances of treatment success.

Boomsma et al., (2009)	Prospective cohort	210 infertile women Netherlands	Endometrial fluid aspiration immediately before transfer of embryo	IL-1β, IL-5, IL-6, IL-10, IL-12, IL-15, IL-17, IL-18, tumour necrosis factor-α, interferon-ϒ, macrophage migration inhibitory factor, eotaxin, interferon-ϒ protein-10, monocyte chemotactic protein-1, Dickkopf homolog-1, heparin-binding epidermal growth factor and vascular endothelial growth factor IMMUNOLOGICAL	Multiplex immunoassay	All biomarkers apart from interferon ϒ listed could be detected from endometrial fluid aspiration.	Cytokine profiling could be a useful non-disruptive and safe tool to analyse the uterine environment prior to embryo transfer.
Boomsma et al., (2009a)	Prospective cohort	210 infertile women Netherlands	Endometrial fluid aspiration immediately before transfer of embryo	IL-1β, IL-5, IL-6, IL-10, IL-12, IL-15, IL-17, IL-18, tumour necrosis factor-α, interferon-ϒ, macrophage migration inhibitory factor, eotaxin, interferon-ϒ protein-10, monocyte chemotactic protein-1, Dickkopf homolog-1, heparin-binding epidermal growth factor and vascular endothelial growth factor IMMUNOLOGICAL	Multiplex immunoassay	Monocyte chemotactic protein-1 and interferon-ϒ protein-10 were associated with failed embryo implantation after IVF treatment. IL-1β and tumour necrosis factor-α were associated with clinical pregnancy and were as predictive for pregnancy as embryo quality.	The identified cytokines may offer a profile that is can predict IVF treatment success.
Boomsma et al., (210)	Prospective cohort	198 infertile women Netherlands	Endometrial fluid aspiration immediately before transfer of embryo	Bacterial vaginosis IL-1β, IL-5, IL-6, IL-10, IL-12, IL-15, IL-17, IL-18, tumour necrosis factor-α, interferon-ϒ, macrophage migration inhibitory factor, eotaxin, interferon-ϒ protein-10, monocyte chemotactic protein-1, Dickkopf homolog-1, heparin-binding epidermal growth factor and vascular endothelial growth factor IMMUNOLOGICAL	Gram staining for bacterial vaginosis and multiplex immunoassay	Bacterial vaginosis was associated with IL-1β. No significant differences found in the ratios of pro and anti-inflammatory cytokine profiles.	Bacterial vaginosis is not associated with a pro-inflammatory cytokine profile in endometrial fluid. It is therefore unlikely to have an effect on endometrial receptivity.

Flyckt et al., (2012)	Prospective cohort	35 infertile women USA	Endometrial fluid aspiration immediately before transfer of embryo	IL-1a, IL-6, IL-8, IL-10, IL-1β , MCP1, GMCSF, TNF-α and VEGF IMMUNOLOGICAL	Multiplex immunoassay	High endometrial fluid concentrations of all the listed cytokines were found. MCP1 had an inverse correlation with clinical pregnancy	Endometrial fluid aspiration at the time of embryo transfer is a safe and effective method of exploring endometrial receptivity.
Ledee-Bataille et al., (2004)	Prospective cohort	133 infertile women France	Uterine flushing at the time of oocyte retrieval	IL-18 IMMUNOLOGICAL	ELISA	Pregnancy rate in the IL-18 positive group was 15%. Pregnancy rate in the IL-18 negative group was 37.9%. In patients who had a top quality embryo transferred, the pregnancy rate in the IL-18 positive group was 20% and the pregnancy rate in the IL-18 negative group was 51%.	IL-18 detectable in endometrial fluid is associated with poor fertility outcomes in women undergoing IVF treatment. IL-18 detection is a simple and non-invasive method to predict inadequate uterine receptivity, which is independent of embryo quality/
Moreno et al., (2015)	Prospective cohort	19 infertile women Spain	Endometrial fluid aspiration 24 hours prior to embryo transfer	Endometrial fluid microbiome MICROBIOLOGICAL	PCR	Analysis of the endometrial fluid microbiome established different profiles based on the bacteria found. These profiles were: 1. Normal (>90% Lactobacillus) 2. Pathological (>10% of pathogens) 3. Dysbiotic (>10% of bacteria changing the endometrial environment Patients with a pathological microbiome had the lowest embryo implantation rates. Reversion of pathological microbiome to normal resulted in better pregnancy rates	The data provides new information regarding the clinical relevance of the microbiological environment for embryo implantation. Treatment of a pathological microbiological environment may improve IVF treatment success rates.

Vilella et al., (2013)	Prospective cohort	173 women undergoing natural menstrual cycles, hormone replacement therapy, controlled ovarian stimulation and refractory endometrium induced by intrauterine device Spain	Endometrial fluid aspirated. IN women undergoing controlled ovarian stimulation, endometrial fluid was aspirated 24 hours prior to embryo transfer	PGE2 and PGF2α PROSTAGLANDINS	Liquid chromatography and tandem mass spectrometry	PGE2 and PGF2α concentrations were increased during the window of implantation in women with natural cycles and in women undergoing controlled ovarian stimulation.	PGE2 and PGF2α may be new non-invasive biomarkers of endometrial receptivity.
Braga et al., (2014)	Prospective cohort	22 infertile women undergoing hormone replacement therapy for frozen embryo transfers Brazil	Endometrial fluid collected at the time of embryo transfer	Lipid profiling LIPIDS	Tandem mass spectrometry	14 lipids were found to be of significant importance in women that achieved pregnancy from frozen embryo transfer. These were glycerolipids, glycerphospholipids and polyketides. Receiver operator curve analysis showed 98.7% area under the curve.	Mass spectrometry of endometrial fluid for lipid profiling is a useful method of investigating endometrial receptivity.

Endometrial fluid protein biomarkers to predict IVF treatment outcome

Proteomics is now considered to be an important method of biomarker research. This reflects the advances in mass spectrometry techniques, particularly the emergence of high-throughput technology. However, proteomic study is very complex, as protein synthesised by a particular tissue may not fully reflect the mRNA product from transcription. This is due to profound post translational and epigenetic changes that occur after transcription[168,169]. The endometrium is no exception. Nevertheless, proteomics has been used to search for biomarkers of receptive endometrium. Studies have investigated the proteome of endometrial tissue; however, the use of endometrial fluid uncomplicates analysis as the ever changing nature of cellular composition is not as much of an issue when examining the fluid when compared with tissue[170].

Initial research suggests that there is a protein profile in endometrial fluid that is characteristic of the window of implantation. Studies have been performed to understand the difference in endometrial fluid proteome of fertile women when compared to infertile women[167]. Additionally, the same types of experiments have been conducted to understand the difference in endometrial fluid proteome when comparing with endometriosis versus those that do not[171]. These studies found that there were clear differences in the expression of cytokines, growth factors, phosphatases and kinases[162,171,172]. Frustratingly, however, some of these proteins, as they are large molecules, are sometimes found fragmented despite careful endometrial fluid sampling[173]. In particular, this has been the case when trying to investigate the endometrial fluid for proteins that have been found to be highly

expressed in previous research, such as LIF and glycodelin[174]. Perhaps this is due to the fracturing of these proteins on endometrial fluid sampling.

In addition, another problem for proteomic study of the endometrial fluid is the abundance of plasma derived albumin and haptaglobins within the fluid. These large proteins must be removed so that the proteins derived from the endometrium can be better studied.

Our literature review identified seven studies that investigated protein biomarkers in endometrial fluid to provide prognostic information regarding IVF treatment outcome.

Bentin-Ley et al.,[175] performed a prospective cohort study in 21 fertile women and 75 infertile women to investigate the glycodelin expression in women undergoing IVF treatment. Endometrial fluid washings were sampled in the natural cycle immediately prior to commencement of IVF. The group found that the levels of glycodelin in endometrial fluid were higher amongst the infertile group who had tubal infertility. Additionally, their results found that women with unexplained infertility also had higher glycodelin levels when compared to the fertile controls. However, receiver operater curve analysis showed that endometrial fluid flushing glycodelin levels had no predictive value for IVF treatment outcome.

Bhattacharya et al.,[176] collected endometrial fluid samples from 156 women prior to IVF treatment. The aim of this study was to investigate the expression of integrin beta 3. This protein is a product of HOX10A gene, which must be highly expressed in order for embryo

implantation to take place. The group found that integrin beta 3 expression was similar through different age groups. Interestingly, integrin beta 3 expression was lower in women with unexplained infertility when compared to women with other causes of infertility. Additionally, the group observed that the integrin beta 3 expression was lower amongst women who did not succeed with their IVF treatment attempt. These findings did not reach statistical significance, however in endometrial fluid samples taken in the same cohort at different times of the menstrual cycle, integrin beta 3 expression was statistically significantly higher during the secretory phase of the menstrual cycle when compared to the proliferative phase ($p<0.001$).

Damario et al.,[177] controversially used mock IVF treatment cycles in 101 oocyte recipients to sample endometrial fluid. The investigated protein biomarkers were αvβ3 integrin (found to be decreased in women with endometriosis, hydrosalpinges and unexplained infertility) and glycodelin. Results showed that expression of αvβ3 integrin and glycodelin were strongly correlated to the age of the endometrium within the mock hormonal treatment. Also a strong inverse correlation was found between the expression of αvβ3 integrin and glycodelin.

Das et al.,[178] performed a prospective study of 81. Eighteen of these participants acted as parous controls, whilst 63 had unexplained infertility. The 63 women with unexplained infertility underwent no stimulation, clomiphene citrate ovulation induction or FSH injection ovulation stimulation. The primary outcome was the concentration of 64-0-kDa human uterine fluid protein, a protein that had previously been hypothesised by the same authors

as important in regulation of embryo implantation. The results showed that 64-0-kDa human uterine fluid protein correlated with endometrial thickness as well as pregnancy outcomes. The authors postulate whether 64-0-kDa human uterine fluid protein could play a role in endometrial receptivity.

Halperin et al.,[179] collected endometrial fluid samples from 109 women undergoing IVF treatment in a prospective cohort study. The endometrial fluid was sampled immediately prior to embryo transfer with a mock embryo transfer catheter used to check cervical patency. The aim was to examine the relationship between human decidua-associated protein 200, a glycoprotein thought to have function in embryo implantation, and affect the embryo implantation rate. The group's results indicated that human decidua-associated protein 200 had a significantly positive correlation to IVF implantation rate and clinical pregnancy rate. The 109 participants were divided in to four groups dependent on their human decidua-associated protein 200 endometrial fluid concentration. The group with the lowest human decidua-associated protein 200 concentration had an implantation rate of 2.3% and pregnancy rate of 5.6% when compared to the group with the highest concentration who had an implantation rate of 29% and pregnancy rate of 50% ($p=0.005$ and $p=0.003$ respectively).

Heng et al.,[180] reported their findings of an assay that is in development to detect proprotein covertase 5/6A. Previous research work had found that this enzyme is highly expressed in receptive endometrium and that the enzyme is also secreted into the endometrial fluid, with high concentrations suggestive of high endometrial receptivity. Monoclonal antibodies as

well as ELISA techniques have been used to develop this assay. Unfortunately, the group have not yet yielded any results. However, the objective for this research group is to develop a real time assay that can be used to provide prognostic implantation and endometrial receptivity information in women undergoing IVF treatment.

Lastly, Ledee-Bataille et al.,[181] studied the association between the concentration of LIF and embryo implantation in IVF treatment. Endometrial fluid was sampled in 33 women who were either undergoing IVF for premature ovarian failure or unexplained infertility. Previous research[182] had shown that LIF was absolutely necessary for murine implantation by monitoring the reproductive outcomes of LIF knockout mice. LIF protein is also only found in the luteal phase of the menstrual cycle[183]. The study by Ledee-Bataille et al., demonstrated contrasting findings, where median LIF concentrations were lower in the women that became pregnant when compared to those that did not become pregnant from the index IVF treatment (0pg/ml versus 203pg/ml, p=0.0013). The group recognised the dramatically different results and concluded that the contrasting results could be due to the measurement of the incorrect LIF transcript (as there are three different LIF proteins; LIF-D, LIF-M and LIF-T). Importantly, Ledee-Bataille et al., were able to reproduce consistent concentrations of LIF in subsequent cycles, suggesting that their method of endometrial fluid sampling provided consistent results. The group summarised their findings by stating that endometrial fluid could detect the unreceptive uterus before IVF treatment, to allow for normalisation of the environment before women embarked on treatment. However, a better comprehension of protein profiles was required before this approach could be used in clinical care.

Endometrial fluid genetic biomarkers to predict IVF treatment outcome

Developments in genetic technology have allowed the advancement of genomic association studies to identify single nucleotide polymorphisms associated with disease. These whole genome association studies have now become the most commonly used methodology to search for genetic loci to identify association between gene and disease state[164].

Genome wide studies on the endometrium and endometrial fluid have been more limited. Previous genomic studies have been performed to identify genetic associations in endometriosis[184,185] and endometrial cancer[186]. Results have been inconsistent due to heterogeneity in populations, techniques as well as small sample sizes. Large sample sizes are required to confirm that risk loci are associated with endometriosis and endometrial cancer so that they can then be further tested in different ethnic populations[187].

The clinical potential from genomic research carried out in the fields of endometriosis and endometrial cancer is substantial; providing risk potential data as well as targets for future disease therapy. Similarly, identifying single nucleotide polymorphisms that are associated with embryo implantation failure would also hold great potential. Many studies have been published to investigate the genomic expression and epigenetic micro RNA expression within receptive and non-receptive endometrium. Of special note, Diaz-Gimeno et al., have suggested that a gene array tool could be used to inform IVF specialists when to carry out embryo transfer, personalising transfer to the individual patient[188–191]. This gene array tool, called the endometrial receptivity array, is based on endometrial expression of 238 selected

genes that can be used to date the endometrium, ensuring that embryo transfer occurs at the optimal time after adequate endometrial priming. Initial studies have shown great promise with high sensitivity and specificity but it still requires further clinical validation[189]. In particular, the practical and clinical value of the test remains a hurdle to its use as it requires endometrial biopsy before the IVF cycle begins.

In this review, only one study was identified that investigated genetic biomarkers in endometrial fluid to provide prognostic information regarding IVF treatment outcome. Parks et al.,[192] conducted a study of 30 patients with recurrent implantation failure who were due to undergo frozen embryo transfer. Recurrent implantation failure is diagnosed when a patient has undergone two or more embryo transfers where the embryo has been graded as top quality. The aim of Parks et al., study was to investigate whether the microRNA present in endometrial fluid was associated with implantation. All of the participants underwent pituitary down regulation followed by oestrogen and progesterone replacement therapy. Uterine secretions were then collected by gentle aspiration 24 hours before FET or at the time of FET. Blinded uterine fluid analysis was performed using human microRNA array and tandem liquid chromatography mass spectrometry (LC-MS/MS). The study group found that 29 cytokines were associated with positive implantation with a p-value <0.05. Interestingly Park et al., found three microRNAs (miR-891am miR-522 and miR-198) were not present in any uterine fluid aspirated from those patients that did not have successful implantation. The group concluded that aberrant microRNA expression within uterine secretions may be the cause of implantation failure. Furthermore, predicting the maternal molecular microenvironment before transfer of embryo could mean that subtle

changes to the preparation of recurrent implantation failure patients could improve embryo implantation outcomes.

Endometrial fluid immunological biomarkers to predict IVF treatment outcome

A large proportion of the research performed to investigate the value of endometrial fluid in predicting IVF treatment outcome explored immunological biomarkers. These include cytokines, interleukins and chemokines. The interest in immunological mediators stems from the theory that receptive endometrium is immune-modulated with a resultant anti-inflammatory environment. This would allow for a 'guest embryo' to invade into the 'host' endometrium[193]. However, this theory of immunomodulation for successful implantation is probably too simplistic as it has also been found that some pro-inflammatory cytokines are essential for successful implantation[194]. These findings have led numerous research groups to search for a favourable immunological profile predictive of successful implantation in women undergoing IVF treatment.

Our literature review identified five studies that investigated immunological biomarkers in endometrial fluid to provide prognostic information regarding IVF treatment outcome.

Boomsma et al.,[195] aspirated the endometrial fluid of 210 women undergoing IVF treatment. The fluid was aspirated just before embryo transfer and then underwent multiplex immunoassay. The immune mediators assayed were IL-1β, IL-5, IL-6, IL-10, IL-12, IL-15, IL-17, IL-18, TNF-α, IF-ϒ, macrophage migration inhibitory factor, eotaxin, IF-ϒ protein-10,

monocyte chemotactic protein-1, Dickkopf homolog-1, heparin-binding epidermal growth factor and vascular endothelial growth factor. As a proof-of-principle study, the results showed that sufficient endometrial fluid was sampled in all but one participant and that all of the immune mediators were detectable with the multiplex assay technique apart from IF-ϒ. Importantly, Boomsma et al., were able to confirm that gentle aspiration of endometrial fluid just before embryo transfer is a safe technique as pregnancy rates from the 210 participants was comparable to age matched controls. The same research group went on to identify a cytokine profile which was predictive of pregnancy in IVF treatment[162]. Using multivariate regression analysis significant associations for failed embryo implantation were found for monocyte chemotactic protein-1 and IF-ϒ protein-10. Positive associations were found for clinical pregnancy and IL-1β and TNF-α. This predictive value of IL-1β and TNF-α for clinical pregnancy was strong, being equivalent to embryo quality. The group concluded that endometrial secretion cytokine profiling offers a new, safe approach to study embryo implantation and that it predicts pregnancy in women undergoing IVF treatment.

In a separate study population Boomsma et al.,[196] investigated whether bacterial vaginosis was associated with a pro-inflammatory cytokine profile in 198 women undergoing IVF treatment. Bacterial vaginosis has been postulated to reduce the chance of embryo implantation and increase the risk of miscarriage[197]. In this study, endometrial fluid was aspirated before embryo transfer and the same multiplex assay was performed. Seventeen women were diagnosed as carrying bacterial vaginosis, their endometrial secretions had a significant negative association for IL-1β. No significant differences were found in any other

pro or anti-inflammatory cytokine and therefore a cytokine profile for bacterial vaginosis was not found.

In a prospective cohort study, Flyckt et al.,[198] collected endometrial fluid from 35 women undergoing IVF treatment immediately before embryo transfer. Multiplex assay was performed to identify nine cytokines and growth factors from the endometrial fluid. The endometrial fluid of women achieving pregnancy was then compared to that of women that had failed treatment. Similar to the findings by Boomsma et al.,[162] monocyte chemotactic protein-1 was found to be strongly associated with failed IVF treatment. This finding reached statistical significance (p=0.045).

Ledee-Bataille et al., sampled endometrial fluid at the time of oocyte retrieval in 133 patients undergoing IVF treatment[199]. The group aimed to investigate whether the detection of IL-18 by ELISA in uterine luminal secretions could predict embryo implantation failure. Endometrial fluid was sampled by flushing the uterine cavity. The participants were divided into two groups dependent on whether IL-18 was detected in their endometrial fluid (the IL-18 positive group and the IL-18 negative group). The IL-18 positive group was found to have a clinical pregnancy rate of 15% whilst the IL-18 negative group had a clinical pregnancy rate of 37.9% (p=0.02). Ledee-Bataille et al., also performed sub-group analysis of 65 women who had a top quality embryo transferred, this showed that the difference in clinical pregnancy rates was even more pronounced (20% in the IL-18 positive group and 51% in the IL-18 negative group).

Endometrial fluid: Other biomarkers used to predict IVF treatment outcome

Microbiological endometrial receptivity biomarkers

In addition to the bacterial vaginosis and endometrial fluid study performed by Boomsma et al.,[196]. Other research has investigated the merit and usefulness of microbiological markers in predicting IVF treatment outcome. Moreno et al.,[200] studied the microbiome of endometrial fluid in 19 women undergoing IVF treatment. They sought to understand the functional relevance of different bacterial species in the human endometrium during the window of implantation. The group found that the microbiome within the endometrial fluid could be classified into normal (where lactobacillus constituted 90% of the microbiome), pathological (where the microbiome was comprised of pathogens such as Gardnerella) or dysbiotic (where the microbiome was made up of bacteria that subtly changed the endometrial environment). When Moreno et al., related this classification to IVF treatment outcomes, the group found that implantation rates were lowest in the pathological class. Additionally the miscarriage rate was also higher in the pathological class. Treatment of bacteria in the pathological class normalised the microbiome and subsequent cycles had a higher success rate. Given the low sample size, the authors concluded that further work needed to be explored, however, they recognised the new insights and hope that improvements can be made to implantation in women in the future.

Prostaglandin as a biomarker of endometrial receptivity

The effect of prostaglandin concentrations in endometrial fluid has also been studied. Aberrant prostaglandin synthesis has been associated with poor implantation in animal[201] as well as human models[202]. In the field of reproductive medicine, it has also been linked with recurrent implantation failure[203]. Vilella et al.,[204] obtained endometrial fluid from women who had natural menstrual cycles, women using hormone replacement therapy and women who were undergoing controlled ovarian stimulation in IVF treatment. Lastly, the group obtained endometrial fluid from women who had an intrauterine contraceptive device in situ, where the endometrium was least likely to be favourable for implantation. The group compared the prostaglandin E2 and prostaglandin F2α concentrations between these groups and found that prostaglandin E2 and F2α were significantly raised in the window of implantation in women having natural menstrual cycles and also women undergoing IVF where treatment was successful. They postulated that prostaglandin concentrations could be used as a non-invasive marker for endometrial receptivity.

Lipid endometrial receptivity biomarkers

Lastly, Braga et al.,[205] collected the endometrial fluid of 22 women undergoing hormone replacement therapy for FET. The endometrial fluid was collected at the time of embryo transfer and mass spectrometry was used to analyse the fluid. Participants were divided in to two groups; those that achieved pregnancy and those that did not. The lipid profiles of the pregnant group were compared to that of the failed treatment group. Fourteen lipids were identified as potential biomarkers as they were detected at higher concentrations in women who achieved pregnancy after FET. These lipids included glycerolipids, glycerphospholipids and polyketides. Receiver operator curve analysis demonstrated an

area under the curve of 98.7% suggesting that the lipid profile identified in women with a receptive endometrium was very predictive. Braga et al., concluded that the use of mass spectrometry on endometrial fluid for lipid profiling was a novel and non-invasive method of predicting endometrial receptivity but conceded that further work with a larger sample size was required to validate their findings.

Discussion

The use of ART is increasing along with the prevalence of couples affected by infertility. Embryo implantation and endometrial receptivity represents an area of research that is novel and exciting. It is also an area of research that needs further advancements to ensure that the chances of fertility treatment success are maximised for patients. Additionally, funding restrictions mean that optimising the chances of pregnancy is paramount to couples that may not be able to self-fund multiple attempts at IVF treatment.

The advantages of perfecting a non-invasive method to investigate the endometrial environment are clear. It offers the opportunity to provide clarity for patients' prognosis and more excitingly may offer a method to identify women that could benefit from treatment to increase the chances of successful treatment. Sampling endometrial fluid at or close to the time of embryo transfer would offer the closest opportunity both in time and space for this assessment to be performed. Furthermore, aspiration of endometrial fluid has been found to be safe and does not cause detrimental effects to the chances of successful treatment[161]. The use of endometrial fluid as a predictive tool has been investigated by

many study groups and will be further researched in years to come. This review has identified the publications that have used this method in the IVF population.

This review was strengthened by several factors. It employed a comprehensive literature search, using applicable literature databases. The review was conducted with inclusivity in mind, meaning that all relevant citations were identified. Specifically, no language or publication date limitations were applied to aid with the comprehensiveness of the literature search. The major weakness in this narrative review is the small number of publications that have been identified. As the publications have investigated different biomarkers, it has not been possible to perform any form of meta-analysis to strengthen findings. However, this was expected prior to beginning the literature search given the paucity of research that has been performed making the use of endometrial fluid as a prognostic tool for IVF treatment outcomes.

It is anticipated that as technological advances in systems biology 'omics' disciplines are made, biomarker research will grow and more publications will result. This same trend is likely to be evident with the use of endometrial fluid as the bio-fluid for endometrial receptivity biomarker discovery. The major strength of the 'omics' disciplines is in the high-speed throughput that is achievable. Further advances in technology may allow IVF physicians of the future to be able to sample fluid prior to embryo transfer, process it for a biomarker panel and delay or proceed with embryo transfer dependent on the result.

As implantation represents such a complex nature, it is unlikely that a single biomarker will be found that is strongly predictive however a panel may achieve high prediction. Selection of the panel in itself is a difficult process, this review has demonstrated several areas (proteins, genetics, immunological etc.) which have been investigated without offering a clue as to which area may be the most likely to reveal best to perform future research. However, glycodelin, adhesion molecules (integrin β3 and integrin αvβ3) have been investigated thoroughly and are providing exciting results. HOX10-A gene expression has also been investigated and has been shown to have an association with endometrial receptivity. Cytokine profiling of endometrial fluid also represents an exciting research area, where IL-8, IL-10, vascular endothelial growth factor and TNF-α have been shown to offer high predictive value. Unfortunately and disappointingly, none of these promising biomarkers have had any practical applications so far, mainly due to low sample sizes. Perhaps a combination of these biomarkers may offer the best prognostic panel available. However, this scatter gun approach to biomarker discovery may not be the best method to identify important biomarkers indicative of receptive endometrium. Perhaps a better approach would be to fully investigate healthy, fertile women first to identify and validate the best biomarkers that confirm receptivity. In that way, a targeted approach could then be adopted in the IVF population. Further validation of any proposed biomarker panel would also be required to test its predictive value in large sample sizes. This may require international collaborations of differing omics disciplines and research groups to establish a global dataset of biomarker discovery from endometrial fluid. Further refinement of endometrial fluid sampling is also required so that biomarker assay can be standardised to achieve precise and reliable

results[150]. Lastly, a quick turnaround from endometrial fluid sampling to result would be ideal to allow for maximal impact in clinical use.

Performing this narrative review did not reveal any publications investigating the metabolomics profile of endometrial fluid. Metabolomics is the 'omics' discipline which best represents the phenotype of any bio-fluid. This would be an exciting area that has not yet been researched and potentially could unlock the black box that is embryo implantation.

CHAPTER 6: ENDOMETRIAL FLUID METABOLOMIC PROFILING IN WOMEN UNDERGOING IN VITRO FERTILISATION TREATMENT – A PROSPECTIVE COHORT STUDY

Introduction

Infertility affects 10-15% of couples and is a disorder with emotional, psychological and occasionally physical consequences. The prevalence of infertility is growing. In 2011, 61726 IVF or ICSI cycles were performed on 48147 women in the UK. The latest figures show that 24.5% of these cycles led to live births in 2011[31].

Through technological advances, success rates have improved since the first successful IVF cycle was performed in 1978. However, there is still great room for improvement both in treatment outcomes and in our understanding of fertilisation and implantation of the embryo. In IVF or ICSI treatment the goal is to produce a high quality embryo with artificial fertilisation. Following this, embryo transfer is performed with the aim of causing embryo implantation in the uterine cavity, which achieves clinical pregnancy. Embryo implantation is a complex process, and abnormal implantation leads to a high frequency of pre-clinical pregnancy loss (where trophoblastic invasion never occurs)[4]. It is embryo implantation, which remains the rate-limiting step in improving IVF treatment success. This has led to much research aimed at investigating the peri-implantation environment within the uterus, and the factors that determine receptivity to implantation and the initial maternal-embryo interface, as this is still not well understood.

In a physiological menstrual cycle, the human endometrium is receptive during the mid-luteal phase. During this implantation window the endometrium is primarily determined by the sex steroids oestrogen and progesterone[201], which affects the concentration and expression of gene transcription factors, growth factors and cytokines. The exact nature of

this receptive endometrial environment in physiological cycles has not been fully investigated. However, this knowledge could prove vital in mimicking the environment prior to embryo transfer for women undergoing IVF treatment. Therefore, research groups have studied a range of different factors including; endometrial genetic expression[164,206,207], uterine immunology[195,196,204,208,209], the importance of the endometrial cavity microbiological environment[196] and endometrial protein expression[188,210,211]. The findings from these studies were discussed in the previous thesis chapters but broadly they all have the aim of identifying and hopefully replicating the optimum environment for embryo implantation.

The emergence of the "omics" disciplines has allowed the study of the maternal-embryo interface in much greater detail. Omic strategies are designed to investigate complex biological interaction as a whole instead of targeting specific compounds[212]. Over the past fifteen years, genomics (the study of the whole genetic compartment) has led to the development of other omics disciplines; proteomics (proteins), lipidomics (lipids) and metabolomics (metabolites).

Metabolomics provides a snapshot of the interaction between genotype and the environment in a biofluid. It is therefore considered to be the closest representation of tissue phentotype[213] and has been used in IVF research in recent years, particularly in fertilisation. Studies have been performed to use metabolomic techniques to compare the profile of follicular fluid aspirated during oocyte retrieval. The metabolome of the follicular fluid is assessed to investigate whether certain metabolites could provide an indication of embryo quality[214,215]. In addition, other research groups have sought to identify the ideal

metabolome of spent embryo culture media in order to identify differences in molecular secretions between embryos that implant and those that do not[216–218].

Both of these areas of research (follicular fluid and spent embryo culture media metabolomics) aim to help embryologists' select the embryo, which has the greatest pregnancy and implantation potential. Metabolomics has not yet been applied to the study of the interface between embryo and endometrium. Ideally, endometrial secretions collected at the time of embryo transfer in IVF treatment could undergo metabolomics analysis. This would provide a true representation of the micro-environment within the uterus at the time of embryo implantation.

Researchers have shown that the sampling of endometrial fluid immediately before embryo transfer is safe. Van der Gaast et al.,[161] published a cohort study of 66 patients in 2003 where trans-cervical endometrial fluid aspiration was performed prior to embryo transfer. Importantly, the study found that when compared to matched controls, the implantation rate in the study group was not reduced. Following this study Boomsma et al.,[162] confirmed the safety of this technique by conducting a similar cohort study of 210 women undergoing IVF treatment. Once more, endometrial fluid was aspirated trans-cervically immediately before embryo transfer. When treatment outcomes were compared to matched controls, no reduction in treatment success was observed in the study group. Endometrial fluid aspirated by Boomsma et al., was investigated for a pre-selected panel of immunological biomarkers. The research group was able to find significant associations between monocyte chemotactic protein-1 and IL-10 cytokine levels and implantation and between IL-1β and

TNF-α and clinical pregnancy. However, this research group only examined 17 soluble regulators of implantation and the metabolome was not investigated.

The research studies described above have prompted others to consider that endometrial fluid aspiration before embryo transfer offers a novel tool for assessing endometrial receptivity during treatment cycles without affecting implantation rates[166]. This current proof of principle cohort study was conducted with the aim of identifying whether endometrial fluid sampling at the time of embryo transfer for the purposes of metabolomics sampling is possible. Furthermore, the discovery of a putative metabolic biomarker panel, which predicts successful IVF treatment was a secondary aim. Lastly, this study aimed to improve the knowledge and understanding of how the endometrial fluid milieu affects implantation, and how this could lead to novel diagnostics, therapies to achieve better treatment outcomes.

Methods

Ethical approval was obtained from the Centre of Human Reproductive Sciences (ChRS) department, a HFEA Research Centre (#0209) and approved tissue bank (NRES REC 13/EM/0272) at the Birmingham Women's Fertility Centre (see appendix 7). Participants consisted of patients undergoing IVF or ICSI treatment between May and September 2015. Only participants with normal ovarian reserve were considered for inclusion into the study as these were perceived to be the women most likely to complete treatment with an embryo transfer and the least likely to have their IVF treatment cancelled due to under or over response to recombinant follicular stimulating hormone injections.

Patients identified to have a uterine cavity abnormality from ultrasonography of the pelvis or hysteroscopy in their initial infertility investigations were excluded. Potential participants were approached after their nurse led injection tutorial appointment. A brief counseling consultation was undertaken with each participant regarding the nature of the study. A participant information sheet was provided for the participant to take home (see appendix 8). At their next appointment (the first follicle tracking ultrasound appointment) each participant was re-approached and, if willing, provided two-stage informed consent to participate in the study (see appendix 9).

Between May and September 2015, 20 participants were recruited into the study. All participants were attending for their first or second IVF or ICSI treatment cycle.

IVF and ICSI Treatments

All participants undergoing fresh cycle IVF or ICSI underwent pituitary suppression using either a short GnRH antagonist (Cetrotide, Merck Serono, France) down regulation protocol or a long GnRH agonist (Buserelin, Sanofi-Aventis, France) protocol injected subcutaneously. Complete pituitary suppression was confirmed with ultra-sonogropahic evidence of little or no follicular activity and an endometrial thickness of less than 5mm. Following down regulation, participants underwent ovarian stimulation to encourage growth of multiple ovarian follicles with daily subcutaneous injections of purified urinary gonadotrophin (Follicular stimulating hormone-luteinising hormone: Menopur, Ferring, France) at a dose of 150-450 international units per day. Starting doses depended on the patient's age. The starting doses were 150iU of menopur if women were less than 35 years, 225iU is women

were 36-38 years, 300iU if women were 39-42 years and 300 to 450iU in the woman was over the age of 42. Additionally, if baseline FSH was greater than 10iU/ml in women less than 31 years, the start dose of menopur would be increased to 225iU. In women with a baseline FSH greater than 10iU/ml over 31 years, the start dose of menopur would be increased to 300iU. In women with anovulatory cycles with polycystic ovarian syndrome, an individualized stimulation protocol was planned. Previous poor responders would also have a higher starting menopur dose.

According to the ovarian response (monitored by transvaginal ultrasonography) the dose of follicle stimulating hormone was altered to optimise ovarian stimulation. When three follicles or more had reached a diameter of at least 17mm, 5000 IU of Human Chorionic Gonadotrophin (Ovitrelle, Merck Serono, France) was used as a trigger for ovulation and final maturation of the oocytes. Thirty-six hours after trigger injection, trans-vaginal oocyte retrieval was performed under ultrasound guidance by needle aspiration to obtain oocytes for fertilisation and embryo culture. After oocyte retrieval, all participants received micronized progesterone (Cyclogest, Actavis, UK) (400mg daily, per vagina) for endometrial preparation. Grading of embryos was performed to select the best quality embryo for uterine transfer. Fresh embryo transfer was undertaken in line with the Birmingham Women's Fertility Centre unit embryo transfer policy. Endometrial fluid sampling was performed immediately before embryo transfer.

Endometrial fluid collection

All participants had endometrial fluid sampled immediately before embryo transfer. With the patient in the lithotomy position, a Cusco speculum was inserted to visualize the cervix. The cervix was cleaned and an empty embryo transfer catheter (Rocket Embryon, Rocket Medical, UK) was inserted into the cervical canal of each participant under ultrasound guidance. The outer catheter occupied the cervical canal to a length of 4cm, whilst the inner catheter was inserted a further 2cm to a total length of 6cm. This meant that the inner catheter was inside the endometrial cavity swathed in endometrial secretions. No suction was applied to the embryo transfer catheter. The inner catheter was then withdrawn to lie inside of the outer catheter tubing to protect it from cervical mucus and the whole embryo transfer catheter was then removed from the uterus and cervix. The inner catheter was then placed in a sterile 15ml centrifuge tube (Falcon, Corning, USA) ready for sample processing. The participant then proceeded with their embryo transfer with an embryo transfer catheter loaded with the embryo, under ultrasound guidance.

Figure 16. Positioning of embryo transfer catheter to obtain endometrial fluid

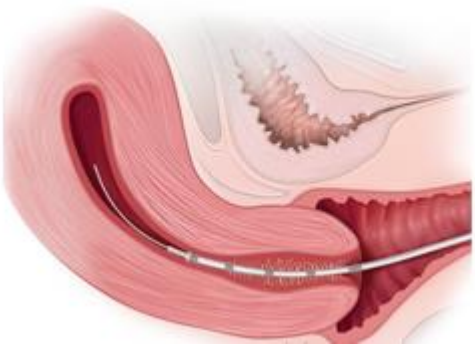


Figure 17. The Rocket Embryon embryo transfer catheter

Processing of endometrial fluid sample

With the inner catheter of the embryo transfer catheter in the 15ml centrifuge tube, 2ml of 80:20 methanol: water mix was injected into the centrifuge tube to render the endometrial fluid sample cell free. A 2ml syringe was then used to flush and aspirate the catheter a total of ten times to ensure that all of the endometrial fluid was left in the solution. The embryo transfer inner catheter was then discarded. The endometrial fluid sample was then vortexed within the 15ml centrifuge tube for a total of two minutes. The sample was then centrifuged at 100G for 30 minutes. After centrifuge the sample was poured into a 2ml micro-centrifuge tube (Eppendorf, Sigma-Aldrich, USA), labeled with a study identification number and stored at -20°C. Three control samples were processed with unused embryo catheters implementing the technique outlined above, these unused embryo catheters had not been in contact with any endometrial fluid and were processed directly from their packaging. The three unused embryo transfer catheter samples were used for quality control.

Metabolic profiling

Metabolic profiling of all samples was performed by applying Direct Infusion Mass Spectrometry (DIMS) analysis followed by statistical data analysis to define metabolic changes associated with (1) IVF outcome and (2) age (below and above 35 years of age).

Direct Infusion Mass Spectrometry (DIMS) analysis.

DIMS analysis was undertaken by coupling a Triversa NanoMate ion source (Advion Biosciences, Ithica) to an LTQ-Orbitrap Elite (Thermo Scientific Ltd, UK). Prior to analysis, samples were dried under a nitrogen gas stream, reconstituted in 70µL of 80/20 methanol: water (v/v) with 20mM ammonium acetate, vortex mixed for 15 seconds and centrifuged at 13,500G for 15 minutes at 4 °C. Samples were then loaded onto a 384 well plate (Thermo Scientific Ltd, UK) for analysis in positive ion mode. NanoMate conditions consisted of a nitrogen backing pressure of 0.3psi, with a voltage of +1.7kV applied to the nanoESI chip. The total sample volume aspirated from each well was 6µL with total time infused through the nanoESI chip of 3.2 minutes. The capillary inlet for the mass spectrometer was set at 200°C. MS data was collected in two scan windows (50 – 450 Da and 250 – 1000 Da) in positive ion mode. Mass resolution was set at 240,000 (FWHM defined at m/z 400) with a scan speed of 0.4 seconds and an AGC setting of $5x10^5$.

Data pre-processing.

DIMS data was exported from Thermo Xcalibur software and processed using an in-house developed R script (Thomas Lawson) to construct a data matrix, which allowed further data analysis. The data matrix was composed of all detected mass-to-charge (m/z) ratios as rows and each sample as a column. For a specific m/z, which was detected in a specific sample a response (proportional to the metabolite concentration) was reported.

Univariate and multivariate analysis.

Processed data was analyzed in 'R' applying the unsupervised multivariate principal components analysis (PCA), supervised multivariate Partial Least Squares-Discriminant Analysis (PLS-DA) and univariate non-parametric Mann Whitney U test. A critical p-value of <0.05 was applied with no correction for false discovery rate applied. The fold change (median peak area before treatment/median peak area after treatment) was calculated. Metabolites were manually annotated by calculation of all molecular formula matching to the accurately measured m/z value within a +/-5ppm mass error and subsequent matching of molecular formula to metabolites present in the Human Metabolome Database[219].

Participant data collection

The primary outcome was clinical pregnancy rate. This was defined as the appearance of fetal heart activity at pelvic ultrasonography seven weeks after embryo transfer was performed. Additionally, participant baseline characteristics (age, ethnicity, body mass index, smoking status), fertility history (cause and duration of infertility), IVF or ICSI treatment cycle variables (baseline early follicular phase follicular stimulating hormone, number of previous IVF treatments and outcomes, parity, index treatment [IVF or ICSI], treatment protocol, follicular stimulating hormone starting dose, total number of follicular stimulating hormone ampoules used, endometrial thickness at last follicle tracking ultrasound, number of oocytes retrieved, fertilisation rates, day of embryo transfer, and embryo quality) and biochemical pregnancy rate (positive pregnancy test two weeks after embryo transfer) were collected from patient hospital records seven weeks after each participant's embryo transfer.

Results

Endometrial fluid samples were collected on all 20 recruited participants. As planned, the mock embryo transfer catheters were inserted into the endometrial cavity and then processed as described above. Table 7 shows the demographic data of the cohort. The pregnant and non-pregnant groups were similar in age, BMI, ethnicity and smoking status. No discomfort above what was experienced during the real embryo transfer was reported by any of the participants.

All 20 participants had fertility treatment data collected, as there were no abandoned IVF or ICSI treatment cycles. The flow of participants is shown in figure 15. Six women (30%) achieved a clinical pregnancy. Additionally, one participant achieved a biochemical pregnancy, where a urinary pregnancy test performed two weeks after embryo transfer was positive, however a clinical pregnancy was not achieved. We performed a univariate analysis comparing the IVF cycle characteristics between the pregnant and non-pregnant group (table 8). This showed that the IVF cycle characteristics were comparable between the two groups. The group of women that achieved clinical pregnancy and the group that did not were similar in duration of infertility, type of infertility (primary or secondary), cause of infertility, base line follicular stimulating hormone, treatment type (IVF or ICSI), starting dose of follicular stimulating hormone, number of follicular stimulating hormone ampoules administered, endometrial thickness at last monitoring ultrasound scan, the proportion that received a day five embryo transfer, the proportion of women having single or double embryo transfer, and the proportion of women having a top grade quality embryo transferred.

Table 7: Patient demographic data – Endometrial fluid metabolomics profiling in women undergoing IVF treatment

Parameter	Clinical pregnancy outcome		p-value
	Pregnant (n=6)	**Non-pregnant (n=14)**	
Age-years(SD)	32.8 (3.13)	34.0 (4.35)	0.562
BMI	24.3 (4.50)	24.0 (3.76)	0.866
Ethnicity			
White(%)	6/6 (100.0)	3/14 (21.4)	0.219
South Asian (%)	0/6 (0)	11/14 (78.6)	
Smoking			
Non-smokers (%)	3/3 (50.0)	14/14 (100.0)	0.061
Smokers(%)	3/3 (50.0)	0/14 (0)	

Figure 18: Participant flow chart - Endometrial fluid metabolomics profiling in women undergoing IVF

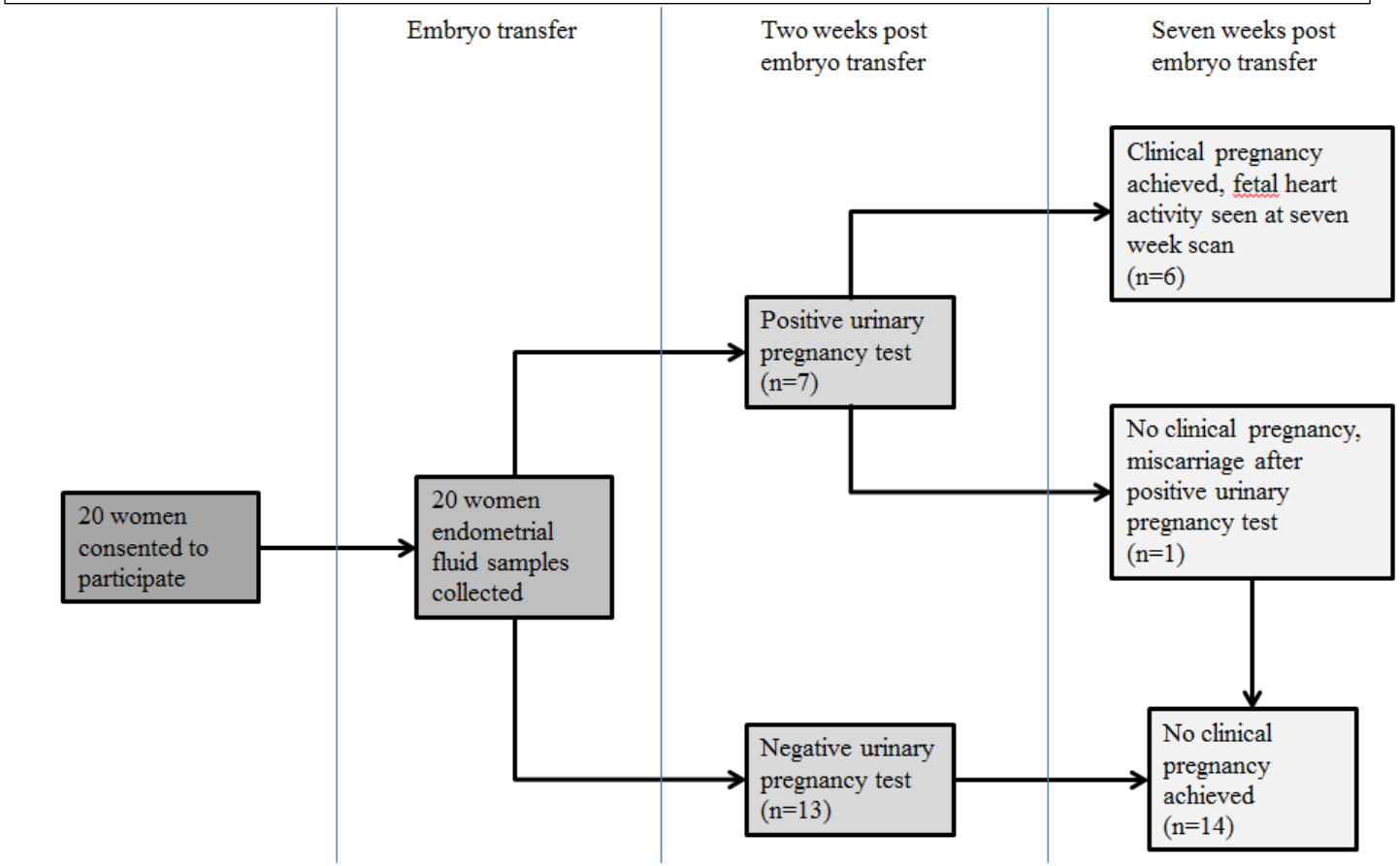


Table 8. IVF cycle variables of pregnant and non-pregnant women			
	Clinical pregnancy outcome		
IVF cycle parameter	**Pregnant (n=6)**	**Non-pregnant (n=14)**	**p-value**
Duration of infertility in months - Median (IQR)	48 (36-72)	42 (30-72)	0.688
Type of infertility			
Primary (%)	6/6 (100)	13/14 (92.9)	0.752
Secondary (%)	0/6 (0)	1/14 (7.1)	
Cause of infertility			
Unexplained (%)	1/6 (16.7)	6/14 (42.9)	0.420
An ovulatory (%)	1/6 (16.7)	1/14 (7.14)	
Tubal disease (%)	2/6 (33.3)	2/14 (14.3)	
Diminished ovarian reserve (%)	1/6 (16.7)	1/14 (7.14)	
Male factor (%)	1/6 (16.7)	4/14 (28.6)	
FSH (iU/ml) – mean (SD)	7.60 (2.05)	7.73 (3.78)	0.939
Treatment type			
IVF (%)	4/6 (66.7)	8/14 (57.1)	0.690
ICSI (%)	2/6 (33.3)	6/14 (42.9)	
Starting FSH dose			
150 or less (%)	2/6 (33.3)	6/14 (42.9)	

225-300	2/6 (33.3)	3/14 (21.4)	0.941
300 or more	2/6 (33.3)	5/14 (35.7)	
Number of FSH ampoules-mean (SD)	48.2 (20.3)	50.9 (17.1)	0.757
Endometrial thickness-mean mm (SD)	10.5 (1.54)	11.0 (2.34)	0.656
Num. of oocytes retrieved- mean (SD)	11.8 (4.96)	10.1 (5.18)	
Embryo transferred on day 5			
No (%)	1/6 (16.7)	7/14 (50)	
Yes (%)	5/6 (83.3)	7/14 (50)	0.163
Mean number of embryos transferred	1.67	1.43	0.435
Num. of embryo transferred			
Single embryo (%)	2/6 (33.3)	8/14 (57.1)	
Double embryo (%)	4/6 (66.7)	6/14 (42.9)	0.329
Top grade embryo transfer			
No (%)	4/6 (66.7)	8/14 (57.1)	
Yes (%)	2/6 (33.3)	6/14 (42.9)	0.690

Endometrial fluid metabolomics analysis

Sufficient bio-fluid was collected for metabolomic analysis in all 20 endometrial fluid samples collected from the participants. The three control samples taken using unused embryo transfer catheters enabled baseline metabolomics profiling to be calibrated for metabolomics analysis of the study samples.

Metabolomic analysis was performed in the study samples to identify statistically significant fold changes in metabolic concentrations of all metabolites found in the endometrial fluid samples. Fold changes were detected by dividing the 20 participants by age (those above the age of 35 years and those below 35 years) and also by clinical pregnancy outcome. This was conducted to ensure adequate blinding during sample metabolic analysis.

Endometrial fluid analysis by age

A total of 29 different metabolites were identified to have significant fold changes when metabolomics analysis was performed according to age group (Table 9). The most significant fold change was a metabolite with the molecular formula C26H35NO7. This produced a fold change of 35.97 ($p<0.005$). Unfortunately, this metabolite is yet to be identified. A further 24 unidentified metabolites were also found to have significant` fold changes when participants were stratified according to their age. A significant fold change of 0.420 ($p=0.03$) is found in metabolite molecular formula C16H22O4, which represent 3-carboxy-alpha-chromanol, 4-prenylphlorisovalerophenone or alpha-cyano-4-hydroxycinnamic acid. Similarly, a fold change of 4.09 ($p=0.04$) was found in metabolite C22H26O8 representing

syringaresinol (a lignan), euparotin acetate or liatrin (a lactone). Triethanolamine ($C_6H_{15}NO_3$), an organic amine, was found to have a fold change of 0.54 ($p=0.05$). Lastly, didesmethyl tocotrienol ($C_{25}H_{36}O_2$), which is an antioxidant, was found to have a fold change of 3.44 ($p=0.05$).

Endometrial fluid analysis by IVF treatment outcome

When the participants were stratified according to IVF treatment outcome, more metabolites were identifiable when compared with analysis by age (table 10). A total of 33 metabolites were found to have a significant fold change. Of these 26 were identifiable. The metabolite with the most statistically significant fold change of 1.71 ($p=0.001$) is currently unidentified. Six further unidentified metabolites also showed significant fold change differences between participants who achieved clinical pregnancy versus those that did not. Identified metabolites with significant fold changes between the two reproductive outcome groups included those with the following molecular formulae; $C_8H_8O_2$, $C_{11}H_{14}O_3$, $C_5H_8O_2$, $C_{13}H_{22}O$, $C_{10}H_{16}O_2$, $C_{13}H_{22}O$, $C_{10}H_{18}O_3$, $C_8H_6O_2$, $C_5H_{12}O_4$, $C_{12}H_{22}O_2$, $C_4H_{10}O_3$, $C_{12}H_{22}O_3$, $C_8H_{14}O$, $C_4H_{10}O_3$, $C_{12}H_{24}O_3$, $C_{16}H_{26}O$, $C_7H_{14}O_5$, $C_{13}H_{22}O$, $C_7H_6O_2$, $C_{10}H_{16}O_2$, $C_{17}H_{26}O_2$, $C_{16}H_{24}O_2$, $C_{10}H_{14}O$, $C_5H_8O_3$, C_3H_4O and $C_7H_{12}O$. The metabolites that these molecular formulae represent can be grouped into aromatic compounds (such as Toluate, Phenylacetic acid, hydroxyacetophenone and 3,4-Dihydroxystyrene), short chain organic acids (angelic acid, tiglic acid and seneciocic acid), carbohydrates (D-apiitol), fatty alcohols (hexadecatrienal) and fatty acids (hydroxyl-dodecenoic acid and oxo-dodecenoic acid).

Table 9: Metabolite fold changes stratified by age (>35 years and <35 years)– Endometrial fluid metabolomics profiling in women undergoing IVF treatment

Metabolite ID	Fold change	p-value	Ion type	Molecular formula	Metabolite(s)
474.2481	35.966	0.0048481	[M+H]+	C26H35NO7	currently unidentified
342.29225	2.41	0.014544			currently unidentified
450.3783	0.30557	0.014544			currently unidentified
450.8801	2.9829	0.014544			currently unidentified
476.3345	2.9635	0.014544			currently unidentified
486.9088	3.2925	0.014544			currently unidentified
522.93755	3.5893	0.014544			currently unidentified
558.4645	3.824	0.014544			currently unidentified
283.11735	3.9615	0.0202			currently unidentified
83.0489	0.44901	0.027311			currently unidentified
301.1407	0.42008	0.027311	[M+Na]+	C16H22O4	3'-carboxy-alpha-chromanol; 4-prenylphlorisovalerophenone; Alpha-CEHC
427.1338	0.60273	0.027311			currently unidentified
427.1426	0.61657	0.027311			currently unidentified
149.0231	0.79709	0.036522			currently unidentified
277.1407	2.784	0.036522			currently unidentified
332.3308	4.5399	0.036522			currently unidentified
419.1696	4.0941	0.036522	[M+H]+	C22H26O8	(+)-Syringaresinol; Euparotin acetate; Liatrin
440.3058	3.903	0.036522			currently unidentified
96.9215	0.50877	0.047673			currently unidentified

98.9186	0.5108	0.047673			currently unidentified
104.9926	0.14444	0.047673			currently unidentified
150.1123	0.54394	0.047673	[M+H]+	C6H15NO3	Triethanolamine
238.8803	0.4788	0.047673			currently unidentified
285.1822	0.64852	0.047673			currently unidentified
387.3082	4.6537	0.047673			currently unidentified
391.26025	3.4399	0.047673	[M+Na]+	C25H36O2	didesmethyl tocotrienol
441.1515	2.4852	0.047673			currently unidentified
492.9597	0.64128	0.047673			currently unidentified
530.981	2.9712	0.047673			currently unidentified

Table 10: Metabolite fold changes stratified by clinical pregnancy outcome – Endometrial fluid metabolomics profiling in women undergoing IVF treatment						
Metabolite ID	**Fold change**	**p-value**	**Ion type**	**Molecular formula**	**Metabolite(s)**	**Type of metabolite**
109.1009	1.7175	0.0019392	UNIDENTIFIED	UNIDENTIFIED	unidentified	Other
137.0595	1.3712	0.010343	[M+H]+	C8H8O2	Hydroxyacetophenone 3,4-Dihydroxystyrene 3-methoxybenzaldehyde 3-Methylsalicylaldehyde 3-Vinylcatechol 4-Hydroxyphenylacetaldehyde Benzylformate methylbenzoate o-Toluate p-Anisaldehyde Phenylacetic acid Toluate	Aromatic compounds
195.1014	1.4288	0.010343	[M+H]+	C11H14O3	unidentified	Other
101.0594	1.6464	0.010343	[M+H]+	C5H8O2	2-Ethylacrylic acid 3-methyl-4-cis-hydroxy-2-butenal 3-Methylbutyrolactone 5-Valerolactone Allyl acetic acid Angelic acid beta-ethyl acrylic acid beta-penteic acid Isopropenylacetic acid Methyl methacrylate Pentane-2,4-dione Senecioic acid Tiglic acid	Short chain organic acids
212.20075	1.7683	0.010343	[M+NH4]+	C13H22O	unidentified	Other

169.1222	1.5264	0.014544	[M+H]+	C10H16O2	(+)-exo-5-Hydroxycamphor (+)-Iridodial (1S,4R)-1-Hydroxy-2-oxolimonene 6-Isopropenyl-3-methyl-2-oxo-oxepanone Isopropenyl-7-methyl-2-oxo-oxepanone (Z)-3,5-Hexadienyl butyrate 1,2-Campholide decadienoic acid 8-Epiiridodial Aleprestic acid Ascaridole Chrysanthemic acid cis, cis-stillingic acid cis-2-Methyl-5-isopropylhexa-2,5-dienoic acid Diosphenol Geranic acid hydroxy-beta-cyclocitral Iridodial Iridomyrmecin Lilac aldehyde nerolate Tiglyl tiglate trans, cis-stillingic acid trans-2-Methyl-5-isopropylhexa-2,5-dienoic acid	Mixed classes
217.0057	1.6955	0.014544	UNIDENTIFIED	UNIDENTIFIED	unidentified	Other
195.1742	1.8669	0.014544	[M+H]+	C13H22O	unidentified	Other
187.1327	1.8956	0.014544	[M+H]+	C10H18O3	(2s,4s)-Alpha-Campholinic Acid 6-Hydroxy-3-isopropenyl-heptanoate 6-Hydroxy-5-isopropenyl-2-methylhexanoate 1,3S-dihydroxy-8E-decen-5-one hydroxy-decenoic acid Oxodecanoic acid	Short/medium chain organic acids

135.0439	1.9834	0.014544	[M+H]+	C8H6O2	Octene-2,4-diynoic acid phenylglyoxal	Mixed classes
159.0626	2.0631	0.014544	[M+Na]+	C5H12O4	D-Apiitol	Carbohydrate
199.1691	1.4307	0.0202	[M+H]+	C12H22O2	Menthyl acetate dodecenoic acid Citronellyl acetate Linderic acid methyl-undecenoic acid	Mixed classes
107.07	1.4477	0.0202	[M+H]+	C4H10O3	Diethylene glycol	Other
215.164	1.4707	0.0202	[M+H]+	C12H22O3	hydroxy-dodecenoic acid oxo-dodecanoic acid	Fatty acids
127.1115	1.8203	0.0202	[M+H]+	C8H14O	octenal Sulcatone	Mixed classes
124.0966	1.3475	0.027311	[M+NH4]+	C4H10O3	Diethylene glycol	Other
217.1796	1.3569	0.027311	[M+H]+	C12H24O3	hydroxy-dodecanoic acid	Fatty acid
235.20545	1.6544	0.027311	[M+H]+	C16H26O	hexadecatrienal	Fatty alcohol
201.0732	1.9934	0.027311	[M+Na]+	C7H14O5	Methyl Fucose beta-D-Digitalopyranose D-Thevetose	Carbohydrate
217.1561	2.1261	0.027311	[M+Na]+	C13H22O	unidentified	Other
238.9821	0.41094	0.036522	UNIDENTIFIED	UNIDENTIFIED	unidentified	Other
321.1491	0.41168	0.036522	UNIDENTIFIED	UNIDENTIFIED	unidentified	Other
123.0438	1.3623	0.036522	[M+H]+	C7H6O2	Hydroxybenzaldehyde Benzoate Salicylaldehyde Tropolone	Aromatic compounds

186.1487	1.4917	0.036522	[M+NH4]+	C10H16O2	(+)-exo-5-Hydroxycamphor (+)-Iridodial (1S,4R)-1-Hydroxy-2-oxolimonene 6-Isopropenyl-3-methyl-2-oxo-oxepanone 4-Isopropenyl-7-methyl-2-oxo-oxepanone (Z)-3,5-Hexadienyl butyrate 1,2-Campholide decadienoic acid 6-Oxocineole 8-Epiiridodial Aleprestic acid Ascaridole Chrysanthemic acid cis, cis-stillingic acid cis-2-Methyl-5-isopropylhexa-2,5-dienoic acid Diosphenol Geranic acid hydroxy-beta-cyclocitral Iridodial Iridomyrmecin Lilac aldehyde nerolate Tiglyl tiglate trans, cis-stillingic acid trans-2-Methyl-5-isopropylhexa-2,5-dienoic acid	Mixed classes
173.1171	1.6182	0.036522	UNIDENTIFIED	UNIDENTIFIED	unidentified	Other
263.2003	1.3133	0.047673	[M+H]+	C17H26O2	heptadecadiynoic acid	Fatty acids
249.1847	1.3196	0.047673	[M+H]+	C16H24O2	hexadecatetraenoic acid 4-[3]-ladderane-butanoic acid	Fatty acids

151.1116	1.3957	0.047673	[M+H]+	C10H14O	(-)-Isopiperitenone (+)-menthofuran (+)-Sabinone 2,4,7-decatrienal 2-sec-Butylphenol 2-tert-Butylphenol 3-tert-Butylphenol 4-Isopropyl-3-methylphenol 4-n-Butylphenol 4-sec-Butylphenol 4-tert-Butylphenol adamantanone Carvacrol Chrysanthenone Isopiperitenone Menthofuran Myrtenal p-Cumic alcohol Perillyl aldehyde Pinocarvone Piperitenone safranal Thymol Umbellulone Verbenone	Mixed classes
227.164	1.4805	0.047673	UNIDENTIFIED	UNIDENTIFIED	unidentified	Other
139.0364	1.6766	0.047673	[M+Na]+	C5H8O3	2-Methylacetoacetic acid Oxopentanoic acid 3-Methyl-2-oxobutanoic acid Levulinic acid Methylacetoacetic acid Tetrahydrofuran-2-Carboxylic Acid	Short chain organic acids

74.0597	1.7283	0.047673	[M+NH4]+	C3H4O	2-Propyn-1-ol Acrolein	Mixed classes
113.0958	1.9514	0.047673	[M+H]+	C7H12O	2-Cyclopropylmethylenepropanal heptenal	Mixed classes
225.102	3.7061	0.047673	UNIDENTIFIED	UNIDENTIFIED	unidentified	Other

Discussion

This small cohort study shows that the metabolic profile of endometrial fluid collected at the time of embryo transfer is different in women who achieve pregnancy compared to women who do not achieve pregnancy from IVF treatment.

Review of the published literature shows that this is the first study of its type. No other study has ever been performed to use metabolomics testing in the endometrial fluid of women undergoing IVF treatment. Other studies have used metabolomics in IVF patients in the past, however metabolomics profiling was conducted on spent embryo culture fluid or follicular fluid collected at the time of oocyte retrieval[220–222].

As this is a small cohort study of 20 women, firm conclusions cannot be drawn from the results. However, this study demonstrates that it is possible to sample endometrial fluid at the time of embryo transfer from women undergoing IVF using a mock embryo transfer catheter inserted into the uterine cavity immediately prior to embryo transfer. Importantly, the technique used to sample endometrial fluid appears to be safe, with the 20 women studied achieving a clinical pregnancy rate (30%) that is representative of the overall IVF and ICSI treatment success rates at the Birmingham Women's Fertility Centre. This suggests that the endometrial fluid sampling used in this study does not adversely affect the reproductive treatment outcomes of women undergoing the sampling. The safety exhibited in this cohort study supports what has been demonstrated by other author groups who have also sampled endometrial fluid at the time of embryo transfer[161,195,196]. Most excitingly, the study shows

that the small volume of fluid, in the order of microliters, collected by passing a mock embryo transfer catheter trans-cervically into the uterine cavity is suitable for metabolomics analysis, with all recruited participants' endometrial fluid samples yielding data. All of the above suggests that the technique used for collection of endometrial fluid could represent a clinically useful method of studying the endometrial factor in embryo implantation.

The data yielded from this study demonstrates significant differences in the fold changes of metabolites in the endometrial fluid from women achieving pregnancy versus the endometrial fluid in the women that did not get pregnant. This is despite the demographic data and the IVF treatment cycle variables of the pregnant and non-pregnant groups being comparable. Of particular note, statistically significant differences in fatty acid and carbohydrate concentrations were found in the two groups. One potential explanation for this may be the difference in energy substrate required for the metabolism of an implanting or non-implanting embryo. It has been well described that an implanting embryo must be metabolically quiet. Implantation is most likely to be achieved, if the metabolic processes within the embryo are occurring at a lower rate compared to embryos with higher metabolic rates[223–225]. Given that the endometrium plays a key role in implantation, the differences in metabolic substrate seen in the endometrial fluid could represent the contrast between an endometrial environment that is amenable to embryo implantation and one that is not.

Previous studies that have reported the collection of endometrial fluid at the time of embryo transfer have analysed the fluid for immune biomarker assays[195,196]. Significant differences were seen in the mean concentration of individual immune mediators. IL-1β and TNF-α

were found to be predictive of pregnancy whereas a significant negative correlation was found between monocyte chemotactic protein-1 and embryo implantation[195]. Although these results are exciting, the search for an individual biomarker that is predictive of IVF treatment outcome is likely to represent an over-simplification of the endometrial fluid properties that would be conducive to embryo implantation. It is more likely that a systems biology approach to identify a panel or profile of biomarkers will eventually be clinically useful.

In the present study, significant differences in metabolite profile were also seen when the 20 participants were divided in to two groups according to their age. Importantly, it should be noted that the use of mass spectrometry in any analytical process is very sensitive and this is the likely reason that the significant fold changes in the metabolites were demonstrated when age was stratified. Very significant p-values (<0.005) must be used for data interpretation when using liquid chromatography and mass spectrometry as there is always an inevitably high false discovery rate[226]. However, the effect that age has on the fold changes of metabolites could be a significant finding in itself. In older women undergoing IVF treatment, one of the main focuses of clinical management is to reduce the effects of ageing ova on reproductive treatment outcomes. Older oocytes are more likely to hold chromosomal abnormalities leading to abnormal fertilisation and failed implantation[227]. This is why in older women, who have had previously unsuccessful attempts at IVF treatment, the option of acting as an ova recipient (using the oocytes from a younger egg donor) is offered. The significance in the differences in the endometrial fluid collected from older women compared to younger women undergoing IVF treatment should not just be

dismissed and attributed to the use of highly sensitive mass spectrometry. One may argue that the difference in endometrial fluid metabolome between the two age groups may in fact be a product of the effect of age on the endometrium itself.

Metabolomics is a relatively new format of systems biology. Great advances have been made in the systems biology field of genomics, whereas metabolomics is relatively more junior in its development as a field of science. Because metabolomics is still an omics discipline that is still developing, many of the metabolites that have significant fold change differences between the pregnant group and the non-pregnant group, seen in this cohort study, are still to be identified. In fact, the metabolite demonstrating the greatest fold change with the highest level of statistical significance between the pregnancy outcome groups remains unidentified. Furthermore, at certain selected mass and charges, there are multiple compounds that the metabolite could represent. Therefore, more work must be performed to improve the metabolome library before mechanistic research can be conducted to understand the biological mechanisms by which the change in metabolites affects reproductive treatment outcomes when endometrial fluid is studied in this context.

Future work in this field would involve the use of a much larger sample size. This would allow more confidence in the identification of potential metabolite profiles that are predictive of fertility treatment success. After this biomarker discovery phase, targeted metabolomics could then be conducted to verify that the putative metabolite profile was indeed predictive of IVF success or failure. Another potential research direction could be to study the metabolomics of endometrial fluid of fertile women who are ovulating

physiologically. This could allow the identification of a pattern of metabolites that appear during the physiological implantation window that may become the target to mimic in women undergoing IVF treatment.

In summary, although the results of this cohort study are unable to suggest a clinically useful metabolome of endometrial fluid predictive of IVF treatment outcome, promising results have been achieved. Further research in this area may unlock the exciting potential in the understanding of implantation. Additionally, if a predictive endometrial fluid metabolome can be identified, this could be a great aid to all infertile women and reproductive medicine physicians in the future, by assisting in the decision making required for successful embryo transfer.

CHAPTER 7: SALPINGOSTOMY IN THE TREATMENT OF HYDOSALPINX – A SYSTEMATIC REVIEW AND META-ANALYSIS

This work has been published in Human Reproduction[30] (see appendix 10)

Chu J, Harb HM, Gallos ID, Dhillon R, Al-Rshoud FM, Robinson L, Coomarasamy A.

Salpingostomy in the treatment of hydrosalpinx: a systematic review and meta-analysis.

Hum Reprod. 2015 Aug; 30(8):1882-95.

Introduction

Female infertility can be caused by a number of different pathologies. Fallopian tubal disease is the causative factor in female infertility in roughly one quarter of these patients[228]. The vast majority of infertility caused by tubal disease is the consequence of ascending infection from sexual transmitted infections (such as Chlamydia trachomatis and Neisseria gonorrhoeae). The ascending infection leads to infection and subsequent inflammation in the pelvis causing pelvic inflammatory disease (PID)[229]. Unfortunately, if PID is untreated, it may lead to chronic inflammation of the fallopian tubes. Distal tubal inflammation can cause obstruction to the distal tube, which then causes fluid to collect inside the tubal lumen. This is called hydrosalpinx and can be diagnosed by hysterosalpingogram, ultrasound or at laparoscopy.

Current research evidence suggests that hydrosalpinx should be treated before the use of IVF or ICSI treatment. Treatment involves sterilizing surgery, which can involve placing clips to the fallopian tubes (tubal occlusion) or removing the hydrosalpinges altogether (salpingectomy). If this surgical treatment is not undertaken, hydrosalpinx approximately halves the chance of successful IVF treatment and clinical pregnancy[230–242]. How hydrosalpinx causes this deleterious effect on IVF treatment outcome is not fully understood. However, it is thought that the hydrosalpingeal fluid exerts two effects on the embryo that is transferred into an endometrial cavity that is affected by this fluid. The first effect is that the hydrosalpingeal fluid is toxic to the embryo, reducing the chances of the embryo adhering to the endometrium to achieve implantation[231,243]. The second effect is

more mechanical, it is postulated that the fluid within the fallopian tubes can wash the embryo out of the endometrial cavity, thereby causing failed IVF treatment[232].

Prior to the widespread availability of IVF and ICSI treatment for tubal infertility, reproductive surgeons corrected tubal disease. Tubal surgery was a specialised domain of gynecological surgery with reconstruction and normalisation of tubal anatomy the goal. Tubal surgery was delicate and placed emphasis on minimizing trauma to fallopian tissue to preserve tubal function. Great importance was also placed on haemostasis to reduce the risk of adhesions post-surgery, which may in turn cause further tubal obstruction. Tubal surgeons performed peri-tubal adhesiolysis to free fallopian tubes of scarring, fimbriostomy to correct mild distal fallopian tube obstruction and salpingostomy in more severe forms of hydrosalpinx[233,244–246].

As the use of IVF and ICSI has increased and its availability has widened, tubal surgery such as those listed above have become less common. In more severe tubal disease, which has caused tubal infertility, the preference has shifted to performing sterilising surgery, such as salpingectomy and tubal occlusion, to ensure that hydrosalpingeal fluid is kept separate from the endometrial cavity where the embryo is transferred. This ensures that the fluid cannot have its embryotoxic and mechanical washout effects. Indeed, there is evidence that using these sterilising surgical techniques doubles the chances of IVF or ICSI success[28,232,247]. Consequently, it has now become common clinical practice in reproductive medicine to perform sterilising surgery prior to the initiation of IVF and ICSI treatment when hydrosalpinx has been identified.

The main disadvantage of sterilising surgery is that the patient with bilateral hydrosalpinges (where bilateral tubal disconnection or bilateral salpingectomy is performed) is then reliant on IVF or ICSI treatment for all future attempts at achieving pregnancy. There is no option to ever try for natural conception after sterilising surgery unless the couple self-funds a tubal re-anastamosis, which may not even be possible. As the funding restrictions in the UK mean that many couples are only eligible to one cycle of IVF or ICSI treatment, many women with hydrosalpinx may be left with very few therapeutic options to become pregnant if the IVF or ICSI cycle should fail. Even if the one funded IVF or ICSI treatment is successful, couples are required to fund further cycles of treatment if they wish to have more children.

An alternative management strategy, which conserves a patient's fallopian tubes by correcting tubal pathology, would be to perform salpingostomy followed by a trial of natural conception. If pregnancy is not achieved, women can then be offered IVF treatment with or without sterilising surgery.

The aim of this systematic review was to investigate the chances of natural pregnancy after salpingostomy is performed for hydrosalpinx. Furthermore, the aim was also to explore the chances of live birth, miscarriage and ectopic pregnancy after salpingostomy treatment of hydrosalpinx.

Methods

Literature Search

The population of interest consisted of women who underwent salpingostomy for hydrosalpinx. The primary outcome of interest was natural clinical pregnancy. Secondary outcome measures included live birth rates, ectopic pregnancy and miscarriage rates in women that had undergone salpingostomy for hydrosalpinx.

A literature search strategy was developed based on the following key words and/or MeSH terms: tubal surgery, salpingectomy, salpingostomy, in vitro fertilisation, intracytoplasmic sperm injection, assisted reproductive techniques, hydrosalpinx, fallopian tube disease and pregnancy. The search strategy was used on several electronic medical literature databases including: MEDLINE, EMBASE, Cochrane Central Register of Controlled Trials and CINAHL (from inception to March 2015). The reference lists of all primary research study articles and review articles were examined to ensure that all relevant articles that were not captured by the electronic medical literature database searches. No language restrictions were applied in any of the searches or study selection.

Study Selection

Criteria for inclusion in the study were established prior to the literature search. In particular, studies that reported the reproductive outcomes of women that had undergone salpingostomy for hydrosalpinx were included. Studies that did not explicitly report results from salpingostomy for hydrosalpinx were excluded. In particular, we excluded studies that

did not provide clarity in their description of the surgical treatment performed and those studies that did not report specifically on salpingostomy. Additionally, studies that did not report on the primary outcome of natural clinical pregnancy were also excluded.

Study selection was carried out by four independent reviewers. Firstly, the titles and abstracts of research articles identified by the electronic searches were examined. Each title and abstract were included or excluded independently according to the predefined inclusion and exclusion criteria; any disagreements regarding inclusion were resolved by a further reviewer. The full manuscripts of the titles and abstracts that were considered to be relevant for inclusion were obtained. In cases of duplicate publication, the most recent and complete versions were selected.

Data were extracted from full manuscripts by two independent reviewers.

Validity Assessment

Validity assessment was performed by two reviewers who completed the quality assessment independently. The Newcastle-Ottawa Quality Assessment Scales for Observational studies were used to ensure that each study was of good quality. The Newcastle-Ottawa Quality Assessment Scale for Observational studies quality checklist awards one star as maximum for all items except comparability where a maximum of two stars can be awarded. An arbitrary score was used based on the assumption of equal weight of all items included in the Newcastle-Ottawa Scale. This score was used to give a quantitative appraisal of overall

quality of the individual studies. The total score ranged from 0 to 9, with a score of either 0 or 1 for each item.

From each included study, relevant outcome data were extracted by two reviewers independently.

Statistical Analysis

The clinical pregnancy, ectopic pregnancy, live birth and miscarriage rates were extracted from each of the included studies. The log of the ratio and its corresponding standard error for each study was computed. Meta-analysis using inverse-variance weighting was performed to calculate the random-effects summary estimates. The square root of this number is the estimated SD of the underlying effects across studies. Because we had relative measures of effect, the confidence intervals were centered on the natural logarithm of the pooled estimate and the limits exponentiated to obtain an interval on the ratio scale. Forest plots were created for each outcome, showing individual study proportions with confidence intervals (CIs) and the overall DerSimmonian-Laird pooled estimate. Heterogeneity of the treatment effects was assessed graphically with forest plots and statistically analysed using the χ^2 test. Statistical analyses were performed using Stata 12.0 (StataCorp, College Station, TX).

Lastly, we performed a stratified analysis splitting studies from 1972 to 1999 and from 2000 to 2014 to explore the temporal effects on clinical pregnancy rates.

Results

The PRISMA flow diagram[52,53] of the review process is presented in figure 16. The search strategy yielded 14396 citations from the electronic databases. Of these, 14231 publications were excluded because it was clear from the title or abstract that they were not relevant to the search criteria outlined above. Therefore, full manuscripts of 165 articles were retrieved. One hundred and forty-three of these publications were excluded because 63 were review articles, opinion letters, case reports or questionnaires; two were duplicate articles; 17 articles did not specify the nature of the tubal disease and 46 performed tubal surgeries other than salpingostomy e.g. salpingectomy, essure or transvaginal drainage of hydrosalpinx; six reported outcomes that were not of interest e.g. ovarian response after tubal surgery or endometrial receptivity; two articles reported salpingostomy in the IVF population and seven articles reported data that could not be extracted. The remaining 22 articles were suitable for inclusion into this review[244,246,248–267]. All of these articles were observational in design.

Study characteristics

The study characteristics of the 22 articles are presented in table 11. The earliest date of publication was 1972. The most recent article was published in 2014. There was also variation in sample size; the smallest sample size was 10, the publication with the largest sample size presented results from 467 women.

Figure 19. Study selection for review on salpingostomy treatment for hydrosalpinx and natural conception

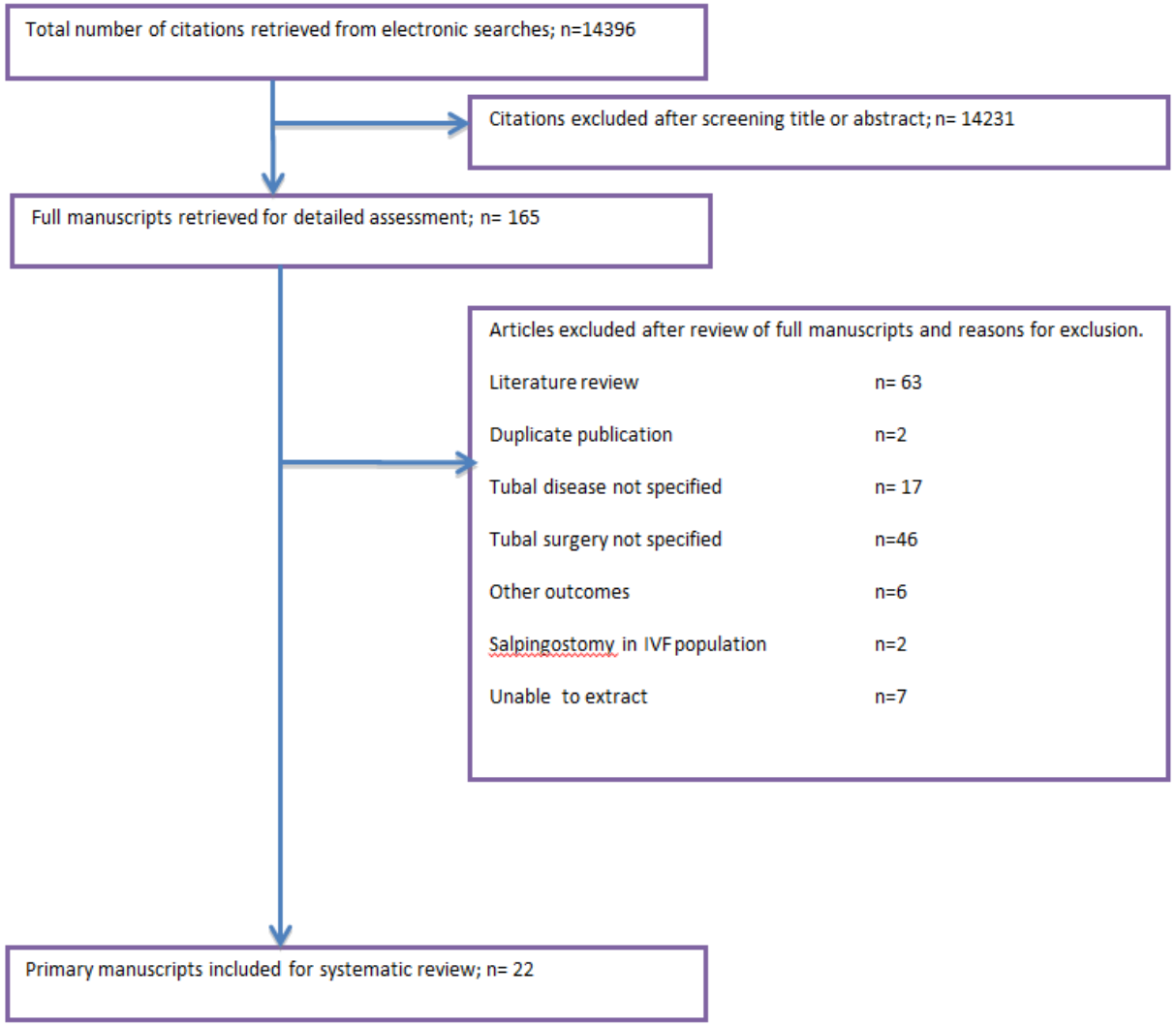


Table 11. Study characteristics of included studies for review on salpingostomy treatment for hydrosalpinx and natural conception

Author (year)	Study design	Study population	Age of study population	Method of diagnosis	Salpingostomy group	Unilateral or bilateral disease	Classification of hydrosalpinx	Surgical technique	Outcomes (pregnancy rate)	Duration of follow up	Cumulative pregnancy data	Mean time to conception
Audebert et al., (1980)	Retrospective Cohort	172 patients with hydrosalpinx 1976-1979 France	Range 20-40 years	laparoscopy	96 salpingostomy to ampulla, isthmic, type 1 and type 2.	Mixture	By stage of hydrosalpinx	Laparotomy and microsurgery	IUP 29/96 (30.2%)	24 months	1-3 month 1/96 (1.0%) 4-6 months 7/96 (7.3%) 7-9 months 14/96 (14.6%) 10-12 months 22/96 (22.9%) 12-23 months 27/96 (28.1%) At 24 months 29/96 (30.2%)	Not reported
Audebert et al., (2014)	Retrospective Cohort	434 patients with distal tubal occlusion 1988-2000 France	Range 21-42 years	HSG and laparoscopy	434 patients underwent everting neosalpingostomy	Mixture	Classified according to mucosal appearances and adhesions	Laparoscopy	IUP 125/434 (28.8%) LBR 106/434 (24.4%) EP 43/434 (9.9%)	60 months	At 10 months half of all IUP achieved At 21 months three quarters of all IUP achieved Specific numbers not reported	Not reported
Bayrak et al., (2006)	Retrospective Cohort	40 patients hydrosalpinges 1999-2002 USA	Mean age 35.5+/-5.5 years Range 21-42 years	HSG	40 patients underwent cuff salpingostomy Authors noted high age of study population.	Bilateral 30/40 (75.0%) Unilateral 10/40 (25.0%)	By size of the hydrosalpinx, presence of rugae and adhesions.	Laparoscopy 32/40 (80.0%) Laparotomy 8/40 (20.0%)	IUP 2/40 (5.0%) EP 1/40 (2.5%)	22 months	At 4 months IUP 1/40 (2.5%) At 7 months IUP 2/40 (5.0%) At 22 months IUP 2/40 (5.0%)	Not reported
Beyth et al., (1982)	Retrospective Cohort	31 patients with hydrosalpinx 1976-1979 Israel	Range 20-43years	HSG and laparoscopy	31patients underwent salpingostomy	Mixture	By size of hydrosalpinx	Laparotomy and microsurgery	IUP 5/31 (16.1%)	42 months	Not reported	Not reported

Boer-Meisel et al., (1986)	Retrospective Cohort	108 patients with hydrosalpinx tubal infertility 1974-1981 The Netherlands	Mean age 27.5 years Range 20-40 years	HSG and laparoscopy	108 patents underwent terminal salpingostomy	Mixture Bilateral 74/108 (68.5%) Unilateral 34/108 (31.5%)	Classified by size of hydrosalpinx, thickness of tubal wall and condition of endosalpinx	Laparotomy	IUP 50/108 (46.3%) term pregnancy 24/108 (22.2%) EP 19/108 (17.6%) miscarriage 7/108 (6.5%)	Unable to extract data	Not reported	Not reported (only median time of 10.5 cycles reported)
Bontis et al., (2000)	Retrospective cohort	258 patients with hydrosalpinx Greece	Not reported	Not reported	258 patients underwent surgery for salpingostomy	Not reported	Classified by degree of severity Stages I-IV	39/258 (15.1%) patients laparoscopy 219/258 (84.9%) patients laparotomy and microsurgery	IUP 44/258 (17.1%) EP 23/258 (8.9%)	36 months	At 36 months IUP 44/258 (17.1%)	Not reported
Channelles et al., (2011)	Retrospective Cohort to assess a management protocol	81 patients with hydrosalpinx managed with a tubal surgery protocol 2003-2007 France	Mean age 32.6 years +/- 4.7 years	HSG or USS and laparoscopy	10 patients underwent unilateral salpingostomy	Unilateral	Classified by mucosal and tubal stages I-IV	Not reported	IUP 3/10 (30%)	12 months	At 12 months IUP 3/10 (30%)	2 months
Chong et al., (1991)	Retrospective Cohort	34 patients with bilateral hydrosalpinx 1982-1988 USA	Mean age 30.2	HSG	19 patients underwent cuff technique salpingostomy 15 patients underwent Bruhat technique salpingostomy	Bilateral	Not reported	Laparotomy and microsurgery	IUP 9/34 (26.5%) EP 2/34 (5.9%)	Not reported	No reported	Not reported

Cohen et al., (1972)	Retrospective cohort	706 patients undergoing tubal surgery France	Not reported	Not reported	188 patients underwent unilateral salpingostomy: 70 terminal salpingostomy 68 medio-ampullar salpingostomy. 279 patients underwent bilateral salpingostomy: 104 terminal salpingostomy, 122 medio-ampullar salpingostomy.	Mixture Bilateral 188/467 (40.3%) Unilateral 279/467 (59.7%)	Not reported	Laparotomy	IUP 89/467 (19.1%) EP 46/467 (9.9%)	18 months	At 18 months IUP 89/467 (19.1%)	Not reported
Dubuisson et al., (1994)	Retrospective cohort	81 infertile women with hydrosalpinx 1986-1991 France	Mean age 30.1+/-4.7 years Range 20-39 years	HSG	81 women underwent unilateral or bilateral salpingostomy	Mixture Bilateral 39/81 (48.1%) Unilateral 42/81 (51.9%)	Classified by severity of disease. Stages I-IV	Laparoscopic salpingostomy	IUP 26/81 (32.1%) EP 4/81 (4.9%)	24 months	IUP cumulative At 12 months 26.4% At 18 months 28.7% At 24 months 29.8%	Not reported
Dubuisson et al., (1995)	Retrospective cohort	123 infertile women with hydrosalpinx 1986-1993	Mean age 28.5+/-4.9 years Range 19-39 years	HSG	123 underwent laparoscopic salpingostomy	Not reported	Classified by severity of disease. Stages I-IV	Laparoscopic salpingostomy	IUP 34/123 (30.4%) EP 9/123 (8%)	Over 24 months	IUP cumulative 23.5% at 12 months 26.0% at 15 months 27.7% at 18 months 28.6% at 24 months	Not reported

Jansen et al., (1980)	Retrospective cohort	107 patients with hydrosalpinx 1966-1975 Australia	All patients <40	Not reported	91 patients underwent bilateral salpingostomy 16 patients underwent unilateral salpingostomy	Mixture Bilateral 91/107 (85.0%) Unilateral 16/107 (15.0%)	Not reported	Laparotomy	IUP 24/107 (22.4%)	Unable to extract	Unable to extract	Unilateral salpingostomy 104 weeks Bilateral salpingostomy 61 weeks
Kosasa et al., (1988)	Retrospective cohort	93 patients with hydrosalpinx 1981-1986 Hawaii	Mean age 31 years Range 21-39 years	Laparoscopy	93 patients underwent microsurgical everting salpingostomy	Mixture Unilateral 27/93(29.0 %) Bilateral 66/93 (71.0%)	Not reported	Laparotomy and microsurgery	Term pregnancy 34/93 (36%) EP 13/93 (14%) Miscarriage 3/93 (3%)	Not reported	Not reported	Not reported
Mage et al., (1983)	Retrospective cohort	68 patients with hydrosalpinx 1977-1981 France	Range 20-38 years	HSG and laparoscopy	30 patients underwent salpingostomy by electrosurgery 38 patients underwent salpingostomy by CO_2 laser	Mixture	Not reported	Laparotomy and microsurgery	Term pregnancy 14/68 (20.6%) EP 6/68 (8.8%) Miscarriage 3/68 (4.4%)	Not reported	Not reported	Not reported
McComb et al., (2001)	Retrospective cohort	23 patients with unilateral hydrosalpinx and a patent contralateral fallopian tube 1988-1997 Canada	Mean age 31.9 years Range 25-39 years	Laparoscopy	23 Unilateral salpingostomy underwent laparoscopic salpingostomy 18salpingostomies sutured, 5 not sutured	Unilateral	Not reported	Laparoscopy	IUP rate 10/23 (43.5%) EP rate 1/23 (4%)	6 years	At 72 months 10/23 (43.5%)	13.4 months

Milingos et al., (2000)	Retrospective cohort	61 patients with hydrosalpinx 1990-1997 Greece	Mean age 31+/-3.9 years Range 23-38 years	HSG and laparoscopy	61 patients underwent laparoscopic bilateral salpingostomy	Bilateral	Classified using the AFS scoring system of distal tubal occlusion	laparoscopy	IUP rate 14/61 (23.0%) EP rate 2/61 (3.3%)	2 years	Unable to extract	Not reported
Singhal et al., (1991)	Retrospective cohort	97 patients with hydrosalpinx 1983 – 1989. U.K	Mean age 30.4 years Range 20-42 years	HSG and laparoscopy	97 patients underwent salpingostomy.	Mixture of unilateral and bilateral	Classified by size of hydrosalpinx	Laparotomy and microsurgery	LBR 28/97 (28.9%) IUP 33/97 (34.0%) EP 6/97 (6.2%) Miscarriage 5/97 (5.2%)	Mean duration of follow up 2.8 years (range 10 months to 6 years)	At 12 months 28% At 36 months 40%	Not reported
Smalldridge et al.,(1993)	Retrospective cohort	30 patients with hydrosalpinx 1986-1990 New Zealand	Not reported	HSG and laparoscopy	30 patients undergoing salpingostomy	Not reported	Not reported	Laparotomy and microsurgery	IUP 9/30 (30.0%) EP 3/30 (10.0%) Miscarriage 0/30 (0.0%)	9-36 months	Unable to extract	Not reported
Taylor et al., (2001)	Retrospective cohort	139 patients with hydrosalpinx 1984-1998 Canada	Mean age 30.9 +/-4.1 years Range 21.4 to 41.1 years	HSG and laparoscopy	139 patients underwent laparoscopic salpingostomy	Unilateral in 86/139 Bilateral in 53/139	AFS classification system of distal tubal occlusion	Laparoscopic salpingostomy	LBR 25/139 (18.0%) IUP 34/139 (24.5%) EP 23/139 (16.5%) Miscarriage 9/139 (6.5%)	36 months	Of the patients who conceived: - 55.2% within 12 months - 84.5% within 36 months	17.7 months (range 0.5-86.4 months)
Teoh et al., (1995)	Retrospective cohort	96 women with bilateral hydrosalpinges 1982-1991 Ireland	Not reported	laparoscopy	96 women underwent bilateral salpingostomy	Bilateral	Not reported	Laparotomy and microsurgery	LBR 19/96 (19.7%) EP 3/96 (3.1%) Miscarriage 1/96 (1.0%)	10 years	Unable to extract	Not reported

Tulandi et al., (1984)	Retrospective cohort	91 women with bilateral hydrosalpinx Canada	Range 20-37 years	HSG	23 women underwent salpingostomy with CO2 laser 22 women underwent salpingostomy with microdiathermy needle 46 women underwent salpingostomy by cold dissection	Bilateral	Classified by mucosal appearance, thickness of tubal wall and peritubal adhesions	Laparotomy and microsurgery	IUP 24/91 (26.4%) EP 6/91 (6.6%)	At 5 years IUP 24/91 (26.4%) EP 6/91 (6.6%)	At 5 years IUP 24/91 (26.4%) EP 6/91 (6.6%)	23.5+/-4.3 months (range 6-60 months)
Winston et al., (1991)	Retrospective cohort	388 patients with bilateral hydrosalpinx 1971-1988 U.K	Mean age 31.5 years Range 19 to 44years	HSG and laparoscopy	323 women underwent primary salpingostomy	Bilateral	Modified Boer-Meisel classification. Classified by thickness of tubal wall, appearances of endosalpinx mucosa, tubal adhesions and ovarian adhesions	Laparotomy and microsurgery	LBR 74/323 (22.9%) IUP 106/323 (32.8%) EP 32/323 (9.9%) Miscarriage 32/323 (9.9%)	Variable follow up duration. Longest 10 years. 12% of participants lost to follow up and reported as not pregnant	At 1 year IUP 55/323 (17.0%) At 4 years IUP 106/323 (32.8%) Cumulative rates displayed graphically	Not reported

Not all of the studies reported the ages of the women that had undergone salpingostomy. In total, 13 out of the 22 studies reported the mean age of their sample. Twelve of the studies that reported their sample's mean age reported results from a younger population with a mean age ranging from 27.5 to 32.6 years. The remaining study had a higher mean age of 35.5 years.

The methods used to diagnose hydrosalpinx also differed between the included studies. Five of the studies used hysterosalpingogram alone to diagnose hydrosalpinx, whereas four studies used laparoscopy to identify hydrosalpinx. The remaining 13 studies used a combination of hysterosalpingogram and laparoscopy to diagnose hydrosalpinx.

The surgical approach used to perform salpingostomy varied between the included studies. Thirteen of the 22 studies used an open laparotomy approach followed by microsurgery to perform salpingostomy. Six studies reported a minimal access laparoscopic approach to perform salpingostomy with the remaining three studies using a mixture of open and laparoscopic approaches.

Only 14 out of the 22 included studies made an attempt to classify the severity of tubal disease encountered during surgery. The remaining eight studies made no reference to the severity of tubal disease of the women that they performed salpingostomy on.

The majority of the studies (15 studies) reported the reproductive outcomes of women with a mixture of unilateral and bilateral disease. Two studies reported outcomes after salpingostomy was performed in women with unilateral hydrosalpinx and five studies reported outcomes on women with bilateral hydrosalpinx where bilateral salpingotsomy was performed.

The length of follow up varied widely in the included studies. Follow up was only 12 months in some studies with the longest follow up period being 71 months.

Included study assessment

The Newcastle-Ottawa Quality Assessment of the included studies is presented in table 12. All studies scored highly on the Newcastle-Ottawa Quality Assessment Scale achieving scores between six and eight.

Clinical pregnancy

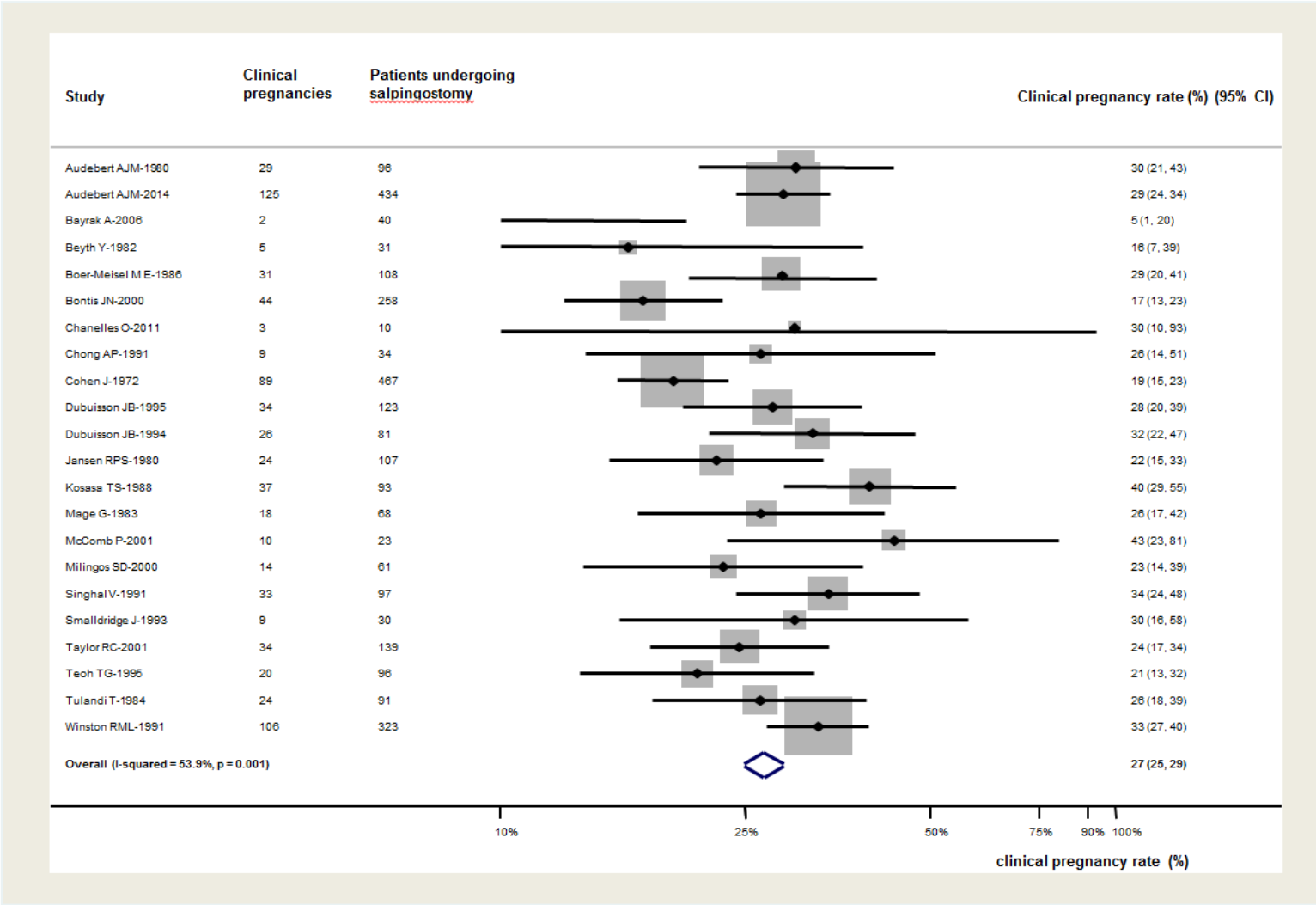
The primary outcome of this review was natural clinical pregnancy after salpingostomy. All 22 studies reported the clinical pregnancy rates achieved as an outcome (figure 17). When the clinical pregnancy rates of the 22 studies were pooled, the overall clinical pregnancy rate was 27% (95% CI 25 to 29%). There was a substantial level of heterogeneity in these studies indicated by an I^2 value of 53.9%, p=0.001.

Table 12. Appraisal of methodological quality (Newcastle-Ottawa Scale) of included studies. Salpingostomy for hydrosalpinx								
Study	**Case-cohort representative**	**Ascertainment of exposure**	**Outcome negative at start**	**Comparability by design or analysis**	**Outcome assessment**	**Duration of follow up**	**Adequacy of follow up**	**Score**
Audebert AJM et al., -1980	*	*	*	*	*	*	*	7
Audebert AJM et al., -2014	*	*	*	*	*	*	*	7
Bayrak A et al., -2006	*	*	*	*	*	*	*	7
Beyth Y et al., -1982	*	*	*	*	*	x	*	6
Boer-Meisel M et al.,-1986	*	*	*	*	*	x	*	6
Bontis JN et al., -2000	*	*	*	*	*	x	*	6
Chanelles O et al., -2011	*	*	*	x	*	*	*	6
Chong AP et al., -1991	*	*	*	*	*	x	*	6
Cohen J et al., -1972	*	*	*	x	*	*	*	6
Dubuisson JB et al., -1995	*	*	*	**	*	*	*	8
Dubuisson JB et al., -1994	*	*	*	**	*	*	*	8

Jansen RPS et al., -1980	*	*	*	x	*	*	*	6
Kosasa TS et al., -1988	*	*	*	*	*	x	*	6
Mage G et al., -1983	*	*	*	*	*	x	*	6
McComb P et al., -2001	*	*	*	*	*	*	*	7
Milingos SD et al., -2000	*	*	*	*	*	*	*	7
Singhal V et al., -1991	*	*	*	*	*	*	*	7
Smalldridge J et al., -1993	*	*	*	*	*	*	*	7
Taylor RC et al., -2001	*	*	*	*	*	*	*	7
Teoh TG et al., -1995	*	*	*	*	*	x	*	6
Tulandi T et al., -1984	*	*	*	*	*	*	*	7
Winston RML et al., -1991	*	*	*	*	*	*	*	7

*Indicates that a feature is present. X that a feature is absent. However for comparability by design or analysis this checklist awards the maximum of two stars (**), one (*) or none if the feature is completely absent (x).

Figure 20. Natural pregnancy rates after salpingostomy treatment for hydrosalpinx



Clinical pregnancy rates by publication date

The included studies were grouped according to their publication date. The first group was the articles published before 2000. The second group was the articles published after 2000. In the studies published before 2000, the pooled clinical pregnancy rate was 28% (95% CI 25 to 30%, figure 18). In the articles published after 2000, the pooled clinical pregnancy rate was 25% (95% CI 22 to 29%, figure 18).

Cumulative clinical pregnancy rates

Ten of the included studies reported cumulative pregnancy data. The cumulative natural clinical pregnancy rates over the follow up period are displayed graphically in figure 19. The cumulative clinical pregnancy rates at six months were provided by three studies. Pooling of this data showed a natural clinical pregnancy rate of 8.7% (95% CI 6.6-11.5%) at six months. The same three studies reported cumulative pregnancy data at nine months. The pooled natural cumulative pregnancy rate at nine months was 13.3% (95% CI 10.6-16.7%). Seven studies reported their natural clinical pregnancy rates at 12 months after salpingostomy. The pooled cumulative pregnancy was 20.0% (95% CI 17.5-22.8%) at 12 months. Four studies reported their clinical pregnancy rates 18 months after surgery. The pooled cumulative pregnancy rate was 21.2% (95% CI 18.6-24.1%) at 18 months. Lastly, five studies

Figure 21. Natural pregnancy rates after salpingostomy treatment for hydrosalpinx by date of publication

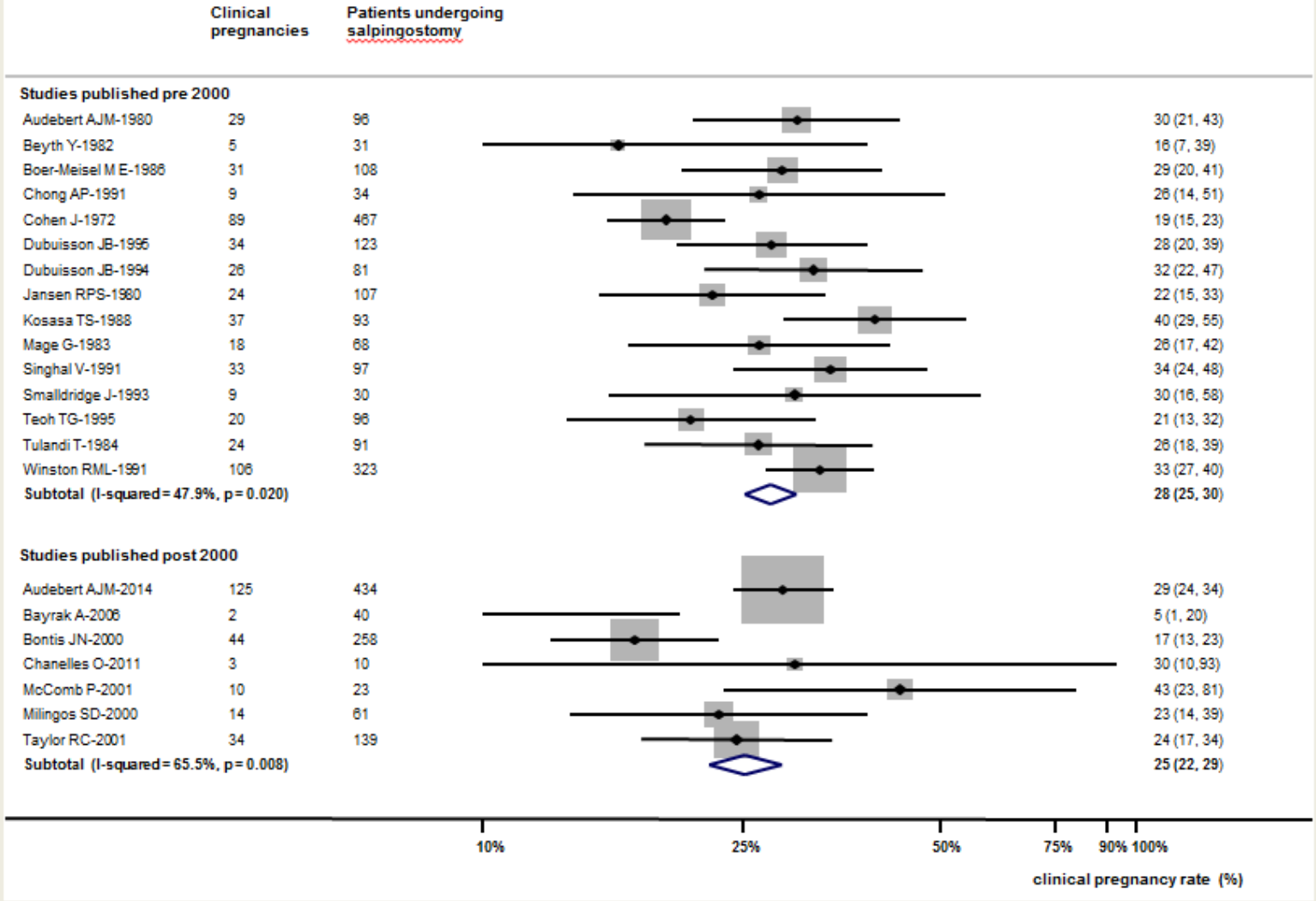
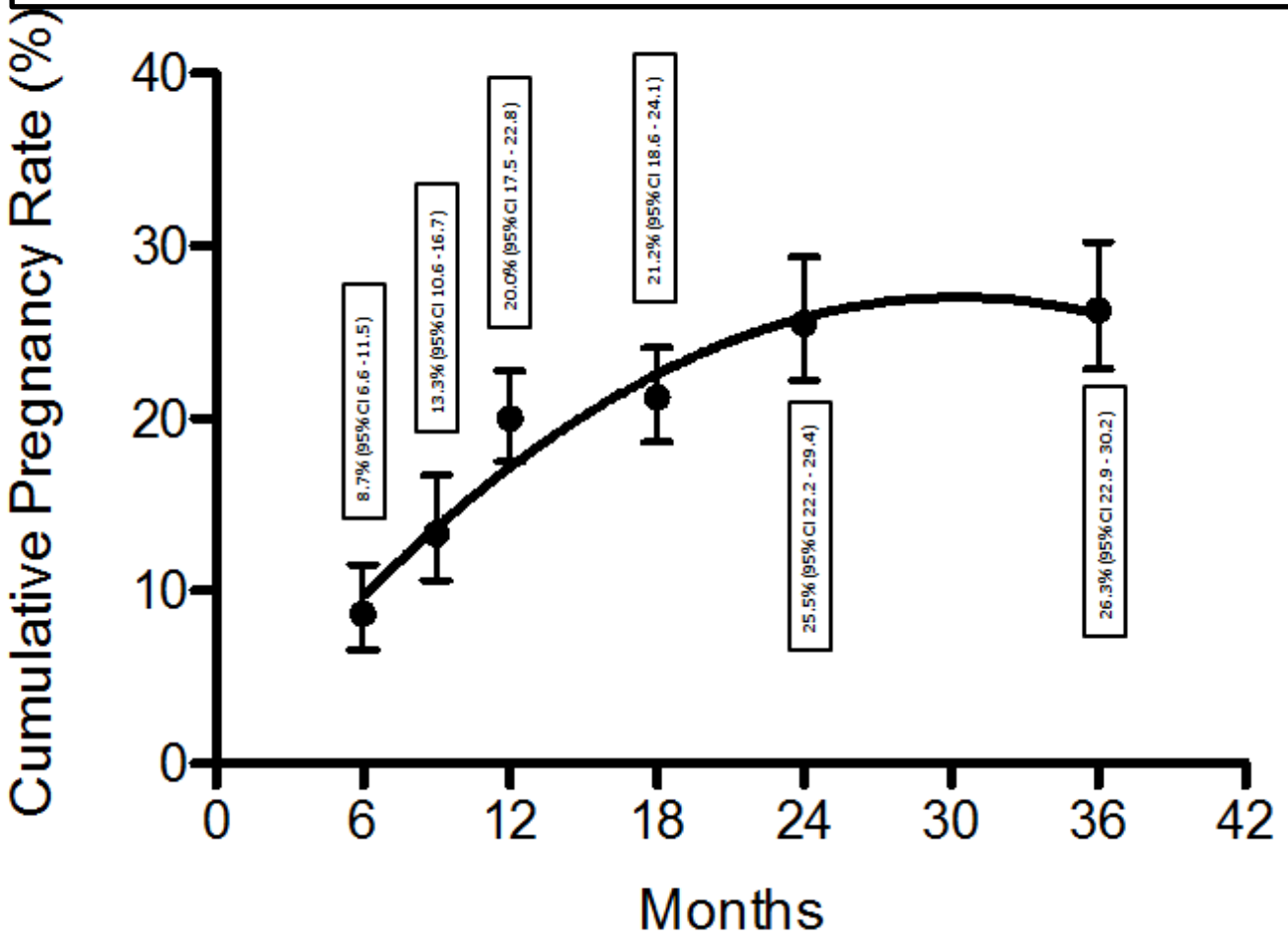


Figure 22. Cumulative natural pregnancy rates after salpingostomy treatment for hydrosalpinx



reported their results at 24 months. The pooled cumulative pregnancy rate was 25.5% (95% CI 22.2-29.4%) at 24 months.

Figure 19 demonstrates that there is a plateau in the cumulative natural pregnancy rate achievable by salpingostomy at 24 months.

Clinical pregnancy rates in women with bilateral hydrosalpinges

A post-hoc analysis was performed on the five studies that reported the natural clinical pregnancy outcome in women with bilateral hydrosalpinges that underwent salpingostomy. In this sub-population, one would expect that without any surgical treatment the natural clinical pregnancy rate would be very low or nil. The pooled clinical pregnancy rate from the five studies was 29.0% (95% CI 25 to 34%, figure 20). There was statistical heterogeneity between these five studies as indicated by an I^2 value of 17.8%, p=0.301 .

Live birth

Only ten out of the 22 included studies followed their patients up to live birth. This is displayed in figure 21. These 10 studies showed that a pooled live birth rate of 25% (95% CI 22 to 28%) can be achieved when salpingostomy is performed for hydrosalpinx. These ten studies showed a moderate level of heterogeneity indicated by an I^2 value of 28.8%, p=0.180.

Figure 23. Natural pregnancy rates after bilateral salpingostomy treatment for bilateral hydrosalpinges

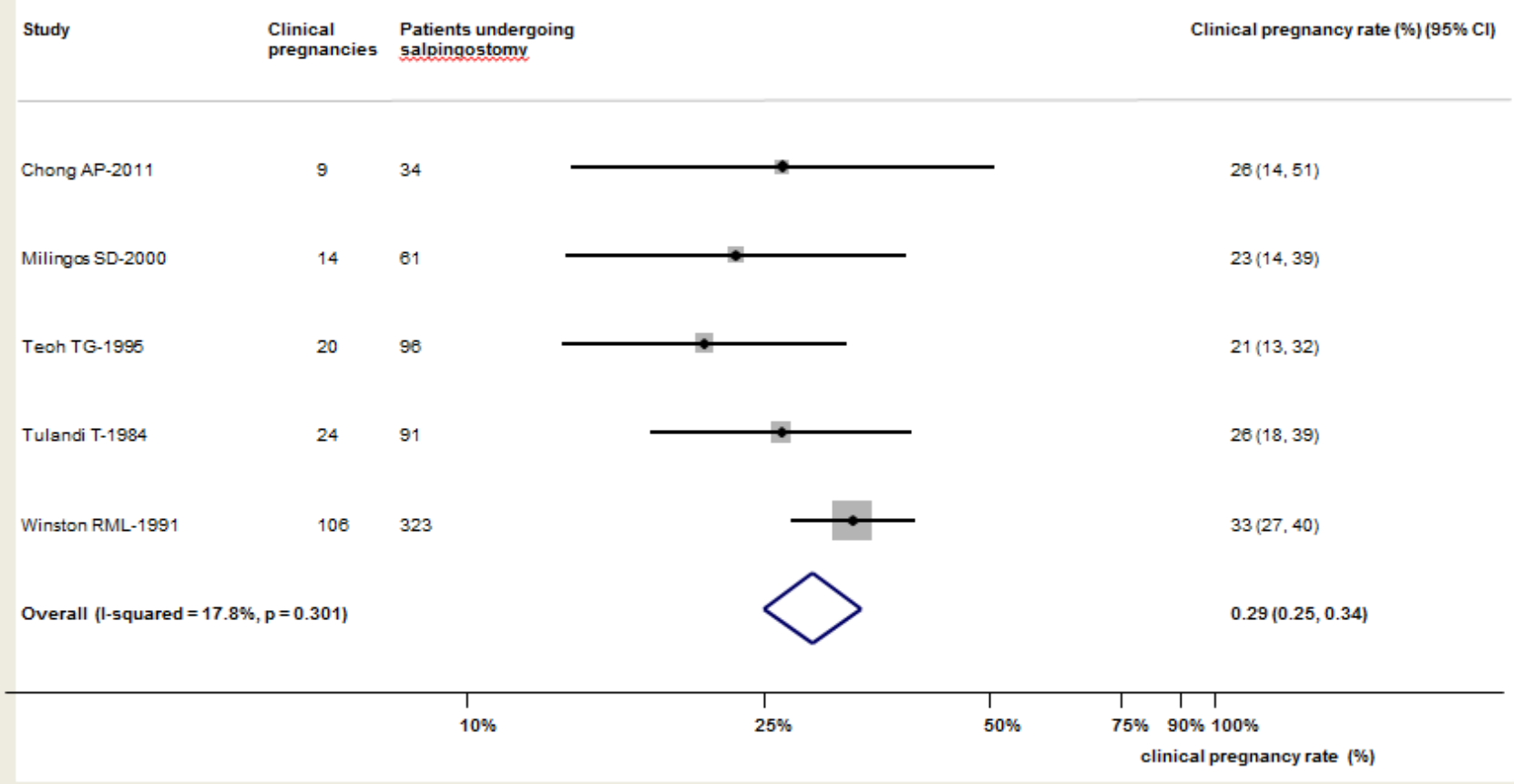
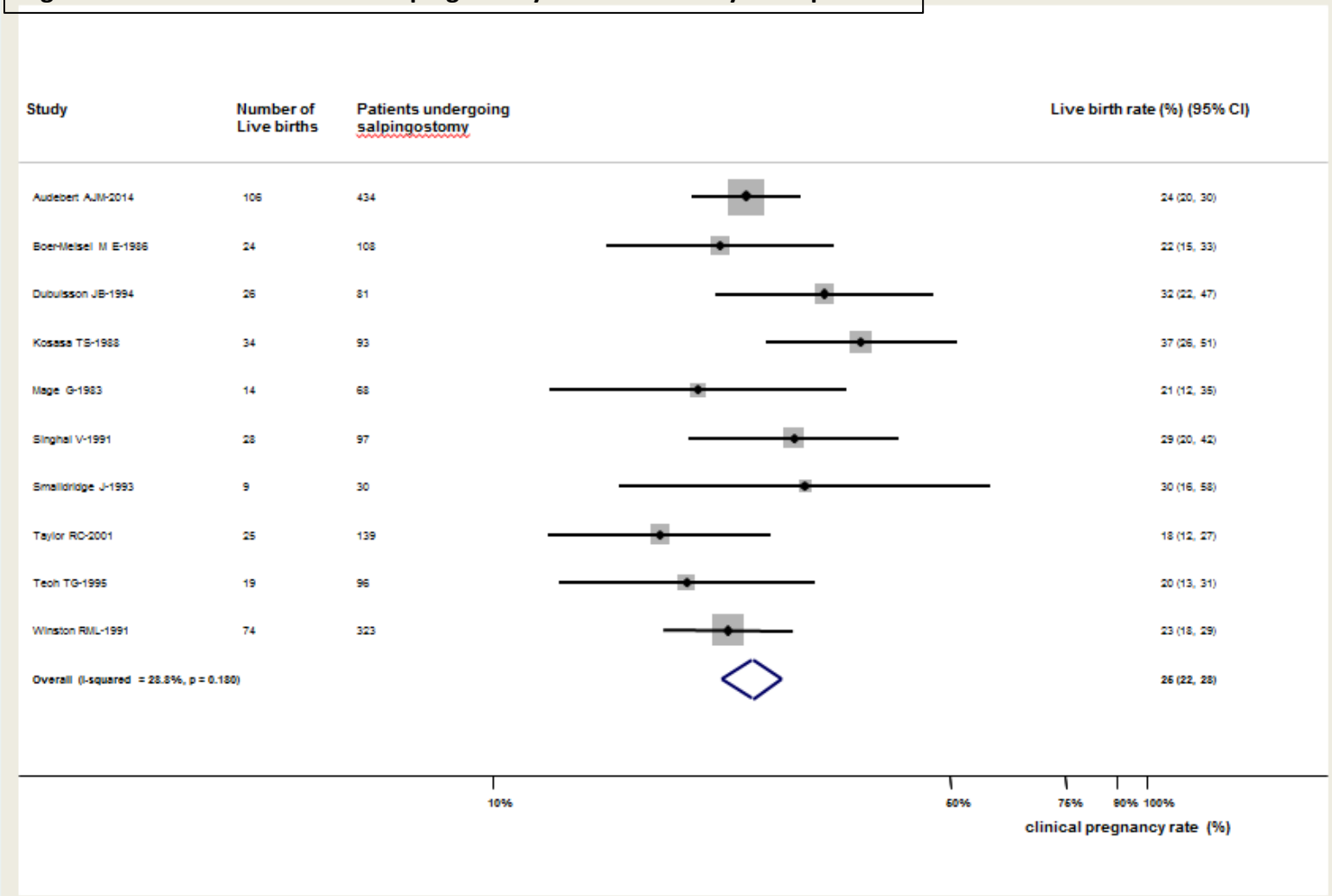


Figure 24. Live birth rates after salpingostomy treatment for hydrosalpinx



Ectopic Pregnancy

Nineteen of the included studies reported the ectopic pregnancy rates after salpingostomy is performed for hydrosalpinx (figure 22). Meta-analysis of the data from the 19 studies showed a pooled ectopic pregnancy rate of 10% (95% CI 9 to 11%). These studies showed a moderate level of heterogeneity indicated by an I^2 value of 41.8%, p=0.029.

Miscarriage

Seven of the included studies reported the outcome of miscarriage from their cohorts (figure 23). Pooling of the results from the seven studies showed a miscarriage rate of 7% (95% CI 6 to 9%). There was moderate heterogeneity between these studies with an I2 value of 42.1%, p=0.110.

Figure 25. Ectopic pregnancy rate after salpingostomy treatment for hydrosalpinx

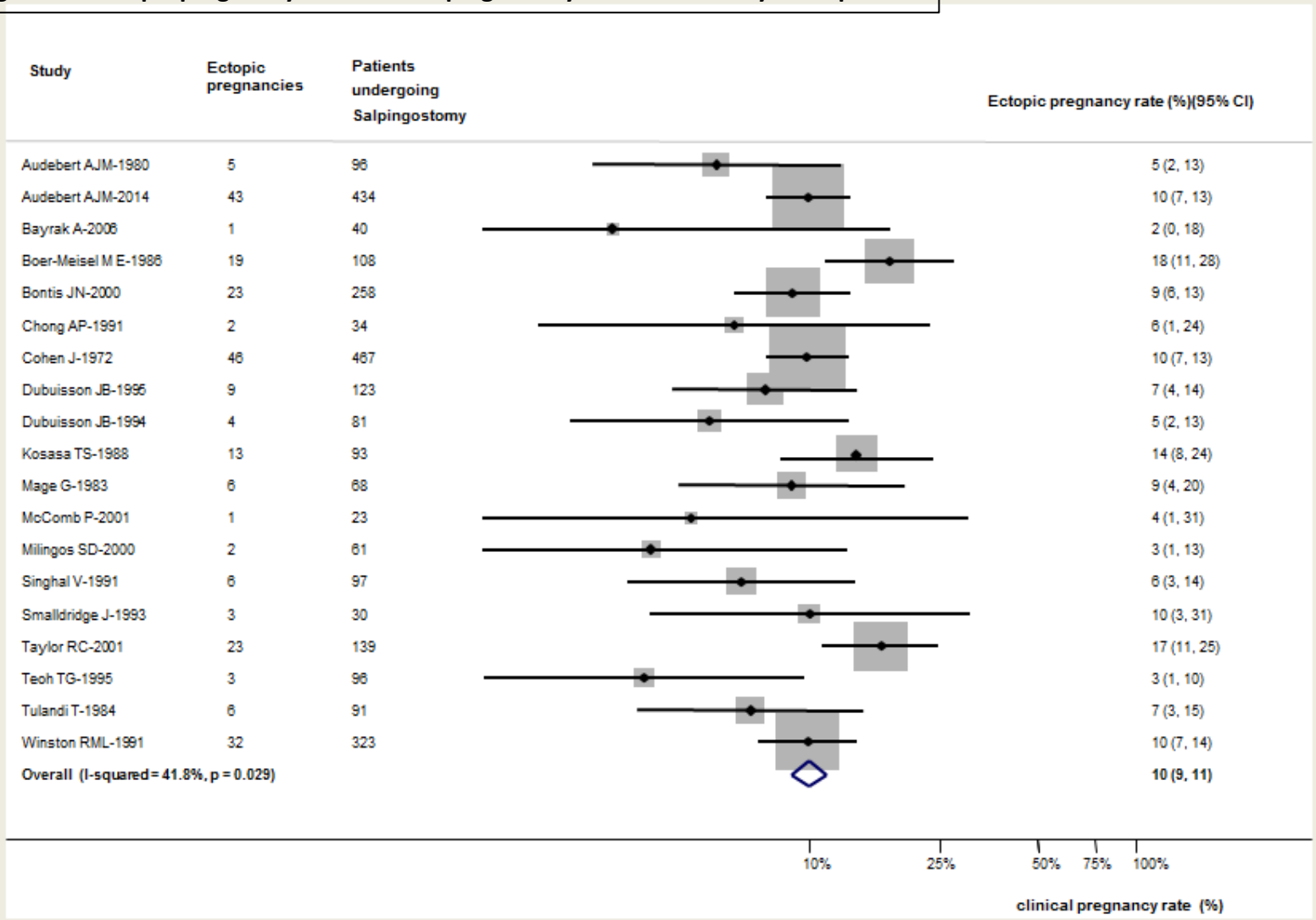
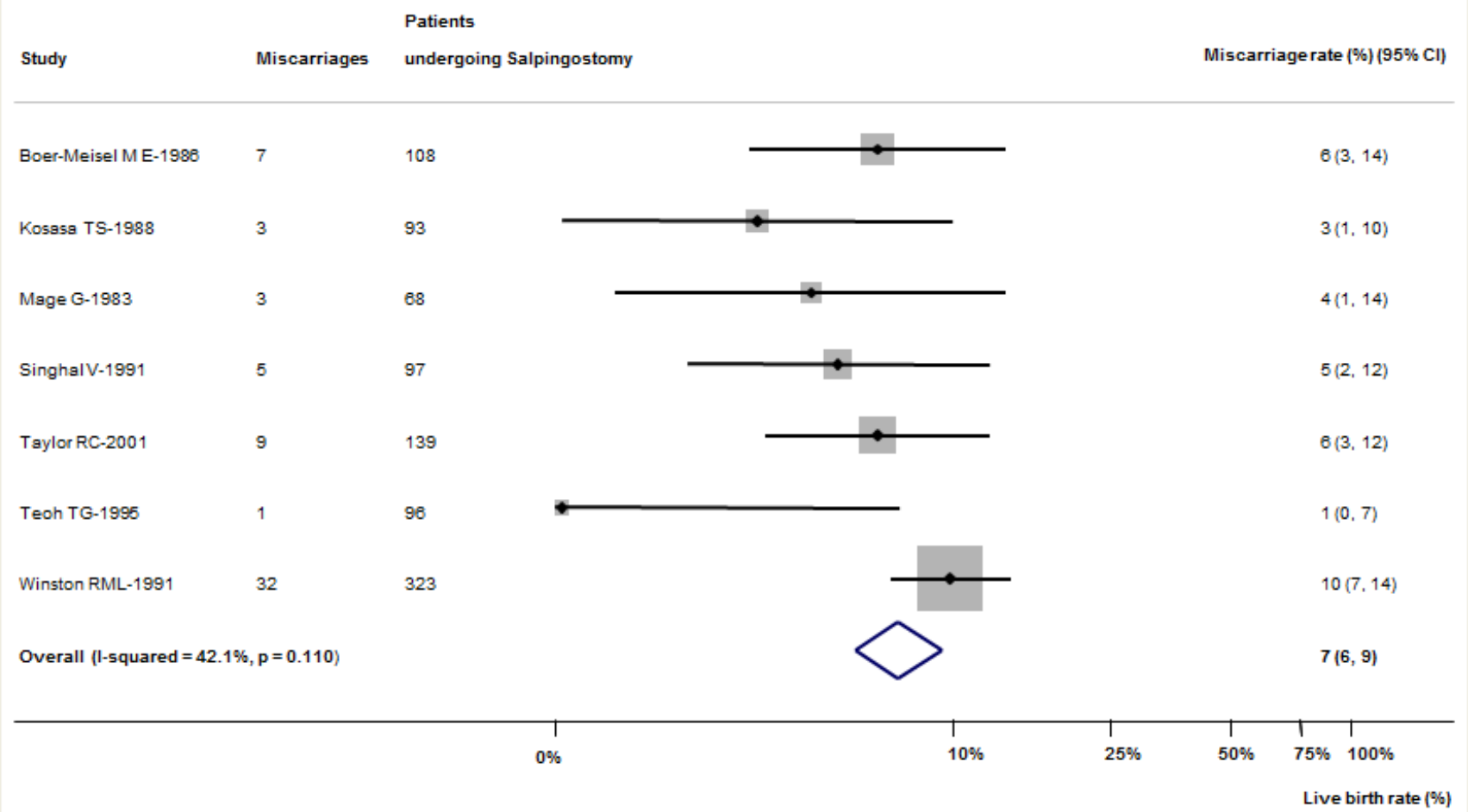


Figure 26. Miscarriage rates after salpingostomy treatment for hydrosalpinx



Discussion

This systematic review identified 22 studies reporting on the reproductive outcomes of women with hydrosalpinx undergoing salpingostomy. The data from these studies suggests that in these women, the chance of achieving a natural clinical pregnancy is 27%. Furthermore, treating hydrosalpinx with salpingostomy carries a 25% chance of live birth. However, salpingostomy is not without its risks, as there is a 10% risk of ectopic pregnancy if a pregnancy is achieved after surgery.

This systematic review has been strengthened by several factors. An extensive search strategy was employed. Independent reviewers were used to identify studies for inclusion, which validates our methods of data synthesis. No language restrictions were applied to our electronic data searches and the search strategy was wide, therefore there is a high level of confidence that all relevant articles were identified. A valid quality assessment was applied to the included studies in the form of the Newcastle-Ottawa Quality Assessment Scale. The included studies scored highly on this scale meaning a low risk of bias.

However, there are also limitations to this systematic review. There is a high degree of clinical heterogeneity of the included studies. In the surgical studies that are included, some clinical heterogeneity must be expected as surgical techniques develop and evolve with time. Furthermore, the methods of diagnosis of hydrosalpinges will have changed dramatically with the introduction of laparoscopic surgery and the widening availability of

ultrasound. The management and attitudes of tubal surgeons towards the treatment of hydrosalpinx will have also changed with time. However, the clinical heterogeneity between the studies in this review increases the generalisability and therefore may not weaken the results.

The variation in publication dates of the included studies is wide. The oldest study was published in 1972 and the most recent study that was included was published in 2014. The surgical techniques implemented to perform salpingostomy have changed greatly over the last four decades. There have been major advancements in technology and equipment that are available for tubal surgery[268]. In the studies that are included in this review that were published earlier, the surgery will have been performed through an open incision in the abdomen. Microsurgical techniques will have then been used to carry out the delicate tubal surgery. This is in contrast to the more recent studies that have been included, where the surgery has mainly been performed through a laparoscopic approach. With the advancements in surgical technology and equipment, one might have expected that the clinical pregnancy rates achieved to be higher in the more recent publications (those published after 2000). However, the data shown in this systematic review demonstrates the opposite, with higher pregnancy rates achieved in the older publications. One possible reason for this is in the difference of the patients that were chosen pre-2000 and post-2000. Before IVF and ICSI became widely available, tubal surgeons may have selected patients with milder tubal disease whom they would expect more favourable outcomes. Furthermore, before 2000 tubal surgery was more popular as a treatment strategy for infertile women with hydrosalpinx. This would mean that tubal surgeons had greater experience and greater

expertise and would therefore have achieved better success rates with improved reproductive outcomes. In theory, one would expect that laparoscopic salpingostomy would yield better clinical pregnancy rates when compared to laparotomy and microsurgical salpingotomy as there is less chance of adhesion formation from a minimal access approach. However, as laparoscopic surgery requires more time to gain higher levels of competence, it may be several decades before the change in surgical technique is reflected in the achieved clinical pregnancy rates.

Inevitably, there will have also been large differences in the expertise of the surgeons that reported their findings. Furthermore, the tubal surgeons also reported the used of differing surgical techniques. For example, some tubal surgeons preferred cold knife dissection, whereas other tubal surgeons preferred to use electrosurgical techniques to create the stoma in the fallopian tube to drain the hydrosalpingeal fluid. Moreover, some surgeons attempted to reduce the chances of the salpingostomy healing and closing by everting the edges of the stomas created and then suturing the everted edges back. In contrast, other surgeons did not possess the necessary skill to do this and would leave the stoma edges un-everted. These differing surgical techniques would also be expected to yield a range of natural clinical pregnancy rates.

The majority of the studies that were included reported the reproductive outcomes of women of similar ages. As mentioned above, twelve of the studies reported the clinical pregnancy rate of women where the mean age ranged from 27.5 to 32.6 years. The pooled clinical pregnancy rate of these 12 studies was 31%. One study, published by Bayrak et al.,[250]

had a higher mean age (35.5 years) and had the lowest clinical pregnancy rate (5%). Although this was a small study of only 40 women, the contrasting clinical pregnancy rates achieved in younger women compared to that achieved in the older women in the Bayrak et al., study may suggest that performing salpingostomy on hydrosalpinx and waiting for natural pregnancy may not be the be the best strategy in older women with hydrosalpinx.

In some of the studies identified by this review, women had unilateral hydrosalpinx and would have undergone unilateral salpingostomy. In other studies, the sample population was all women with bilateral hydrosalpinges. It would be expected that women with unilateral hydrosalpinx may have a more mild form of tubal disease. However, when analysed as a sub-population, the five studies that reported on pregnancy outcomes of women with bilateral hydrosalpinges still showed favourable outcome, with a pooled clinical pregnancy rate of 29% (n=605). Reassuringly, the I^2 value was low suggesting minimal statistical heterogeneity between these five studies.

There was variation in the methods with which tubal surgeons reported the severity of hydrosalpinx in their study samples. Indeed, some studies did not report on the severity of the tubal disease at all, whilst others classified the degree of severity encountered. This is important as this increases clinical heterogeneity within study and between studies further as better fertility outcomes would be expected in those with milder forms of hydrosalpinx.

The duration of follow up of women in the included studies varied widely. In studies where the follow up duration was short (just 12 months in some studies) there may have been

under-reporting of the natural clinical pregnancy rates, as women may have become pregnant after the follow up duration was complete.

The time interval between salpingostomy and natural pregnancy is an important clinical factor. This is certainly a key issue that must be discussed when counselling a woman who may be considering salpingostomy for hydrosalpinx. These women who are choosing to have tubal conserving surgery would want to know the duration of time that they should attempt natural conception before considering further treatment in the form of sterilising surgery and IVF or ICSI treatment. The cumulative natural clinical pregnancy data presented in this review suggests that there is a plateau at approximately 24 months. This is therefore the amount of time that a woman should be counselled to try conceiving naturally after salpingostomy is performed for hydrosalpinx.

In 2010, A Cochrane review published by Johnson et al.,[28] concluded that surgical sterilising treatment (in the form of salpingectomy or tubal occlusion) should be offered for all infertile women with hydrosalpinges prior to IVF treatment to improve the chance of successful treatment. Importantly, this review did not find any randomised trials that investigated the effectiveness of salpingostomy versus salpingectomy or tubal occlusion prior to IVF treatment. To date there have still been no studies to investigate this. Presumably, this is due to the fact that most IVF clinicians prefer sterilising surgery and IVF over tube conserving surgical strategies and would not wish to conduct this randomised trial. The Cochrane review recommended that further research is required to assess the value of tubal restorative surgery as an alternative (or as a preliminary treatment) to IVF. This systematic

review adds further weight to this recommendation from the Cochrane review, as our results demonstrate that reasonable clinical pregnancy rates can be achieved with salpingostomy in carefully selected patients.

Salpingostomy may be an important alternative option for treatment of hydrosalpinx in carefully selected populations. This is particularly the case in women who may wish to have the option to have salpingostomy to continue to attempt natural conception. If this does not result in natural pregnancy then more definitive sterilising surgery in the form of salpingectomy can be performed. This may be preferred by certain groups of women who may not wish to be reliant on ART with no further opportunities to try to conceive naturally. Another sub-group that may prefer tubal restorative surgery in the form of salpingostomy may be younger women who have more time to conceive. In older women or in women with more severe hydrosalpinges it may be more advisable to proceed directly to salpingectomy and IVF treatment as time and chances of natural conception are more limited.

In the reproductive medicine setting, there has been a general trend for the removal or disconnection of fallopian tubes in women who have hydrosalpinx since the increasing availability of IVF treatment[269]. It is likely that the ability of reproductive specialists to restore normal tubal anatomy has been reduced due to an over reliance on tubal removal or occlusion followed by IVF treatment. The reduced abilities for tubal restorative surgery may also be the result of de-skilling in the surgical expertise required for such surgery. Importantly, women with hydrosalpinx may not be given the full range of treatment choices

in the clinical setting and are then left with no other option than to pursue and fund IVF treatment. Women who opt to have tubal restorative surgery must be counselled fully regarding the risks involved with salpingostomy. There was a 10% risk of ectopic pregnancy in the included studies, and this must be conveyed to the women. A balanced consultation must be delivered with the potential success rates of IVF treatment, tubal conservative surgery, their risks and repercussions should be discussed.

The findings of this systematic review have re-introduced the possibility of considering tubal restorative surgery for a carefully selected group of women with hydrosalpinx. Further research is required to identify the women who would benefit from tube conserving surgery as opposed to the current management of tube removal. It may also be beneficial to investigate the best technique to perform salpingostomy in these selected patients.

CHAPTER 8: THESIS CONCLUSIONS

Thesis Conclusion

This thesis has sought to explore a number of strategies to improve the process of implantation in women attempting to conceive using ART or with natural conception. Four strategies have been investigated;

1. The importance of vitamin D in IVF treatment
2. The importance of vitamin D in women with recurrent miscarriage
3. The role of endometrial fluid as a prognostic tool for women undergoing IVF treatment and the potential use of metabolomic profiling in this context
4. The role of salpingostomy in the treatment of hydrosalpinx.

In this summary the findings from each of these domains is presented. The potential direction of future work in these research fields will also be discussed.

Vitamin D and IVF treatment

Interesting findings resulted from the vitamin D and IVF treatment systematic review and meta-analysis. The studies identified by the rigorous search strategy found conflicting evidence from differing research groups. It is also clear that this is a relatively immature but promising and exciting field of research with all of the studies being published after 2010 and seven of the 13 studies published since 2014 (see table 1). The search also did not identify any well-conducted trials investigating the potential merits of vitamin D deficiency treatment in IVF patients for inclusion into the review. However, when the data from each cohort study was meta-analysed, the overall risk ratios calculated show that vitamin D status in women undergoing IVF treatment is an important factor in the outcome of treatment (RR

0.79 (95% CI 0.66-0.93). There was clinical heterogeneity between the studies that were included into the meta-analysis. Specifically, the use of differing vitamin D status categorisation by different authors, however the comparison of IVF treatment outcome in women that are replete in vitamin D versus those that are non-replete is clear when the Endocrine Society reference ranges for vitamin D status are used[41].

The findings from the vitamin D and IVF treatment systematic review and meta-analysis were mirrored in the vitamin D and IVF treatment cohort study presented in chapter three. The cohort study was the first to be conducted in the UK and the sample of patients used is representative of the country as a whole. Vitamin D deficiency and insufficiency prevalence was found to be high (84.0% of all participants). This is an important finding in its own right. Vitamin D is now implicated in many obstetric complications such as pre-eclampsia, gestational diabetes and intra-uterine growth restriction[106,270,271] and the results of the cohort study suggest that a high proportion of women that are commencing IVF treatment are, in fact, deficient in vitamin D. Most importantly, the cohort study showed that vitamin D deficiency reduces the chances of obtaining a positive pregnancy test, achieving a clinical pregnancy as well as a live birth when compared to women with replete vitamin D. As the data generated is observational in nature, ascertainment of whether this association is evidence of a causal relationship between replete vitamin D status and fertility treatment success is a question that remains unanswered[272].

The findings of both the systematic review and cohort study have wider implications. The high prevalence of vitamin D deficiency in the infertile population suggests that there is a

high prevalence of vitamin D deficiency in the general population as a whole. Many studies have already demonstrated this[273,274]. Importantly, this is a cause for concern in women of reproductive age. Given the proposed mechanisms that have been postulated for the importance of vitamin D in implantation (immunomodulation of the endometrium[5]), vitamin D may be an important health issue for all women that are trying to conceive. Potentially, this could mean that vitamin D could be assessed in all pre-conceptual women, both attempting conception via natural methods and also with ART. Vitamin D treatment could then be implemented to aid implantation to optimize the efforts at natural conception. This could have the potential to reduce the need for fertility treatments before they are required. These conclusions may be far-fetched, however the prospect of a simple oral treatment, that is relatively safe and cost effective that could improve the chances of pregnancy would be a desirable therapy for clinicians and more importantly women that are desperate to start a family.

It is important to note that many pre-conceptual vitamin and mineral supplements contain vitamin D and that a significant proportion of women self-administer these preparations. However, the vitamin D in these preparations is at a supplementary dose and not at treatment doses[275]. It is well recognized that vitamin D deficiency treatment must be at therapeutic levels to achieve a replete vitamin D status and therefore its desired effect[41].

All of the above points suggest that a trial is required to test the effectiveness of vitamin D deficiency treatment in improving attempts at conception. The IVF population, specifically women planning IVF treatment offers an easily reachable population to investigate. Vitamin

D status could be screened once a patient is referred for IVF treatment. If required, vitamin D deficiency could then be treated. However, this simplistic view of conducting the necessary trial becomes more complex when trial design is considered. In order to fully investigate the effectiveness of vitamin D deficiency treatment on IVF treatment outcomes, a placebo controlled trial would be the ideal design[276]. This would require women to either receive vitamin D deficiency treatment or placebo. This poses an ethical dilemma as the health benefits of replete vitamin D status are not solely through its role in optimising embryo implantation. As mentioned above, there are numerous obstetric complications linked with vitamin D deficiency as well as other health benefits e.g. prevention and treatment of diabetes, asthma and cancer[277–280]. Therefore trial design for an individual placebo-controlled randomised study may be complicated. One potential method to circumvent this ethical dilemma is to perform a cluster trial. This would involve the use of several similar IVF centres, with half of centres offering screening and treatment of vitamin D deficiency whilst the other half of centres do not[281]. A stepped wedge design[74] could also be employed, where all centres begin with no screening and treatment of vitamin D deficiency and at pre-defined time intervals, IVF centres would be chosen randomly to start screening and treating their patients for vitamin D deficiency. These cluster trial methodologies would involve collection of centre specific data rather than individualized patients, which is a disadvantage as the effectiveness of vitamin D deficiency treatment may become diluted[74,281].

Whichever trial methodology is employed, a well-conducted trial for vitamin D is certainly required in women undergoing IVF treatment. Any means to improving fertility treatment

outcome should be fully explored not only to aid women in their wishes for pregnancy but due to the funding restrictions that apply in the UK. Many couples are not in the financial situation to have multiple attempts at IVF treatment if this is the therapy required. This leads to many couples having only one treatment cycle (the NHS funded cycle). Consequently, the optimisation of any correctable condition that improves the chance of successful fertility treatment is vital. If trial findings are positive, concluding that vitamin D deficiency treatment does lead to improved fertility treatment outcomes, the next logical step would be to perform a similar trial in women attempting natural conception. All of the above contributes to an exciting research field.

Vitamin D and recurrent miscarriage

The nature of the data generated from the vitamin D and IVF treatment systematic review and meta-analysis prompted the narrative review investigating the association between vitamin D and recurrent miscarriage. One would logically expect that if vitamin D bears an association with implantation that it may also bear an association with recurrent miscarriage. However, this association could not be confirmed. Using a robust search strategy, only a small number of research articles could be found linking vitamin D deficiency and recurrent miscarriage. The speculative association between vitamin D deficiency and recurrent miscarriage carries sound biological plausibility and is certainly an area of recurrent miscarriage research that will grow in the future.

In particular, the basic science studies identified by the systematic narrative review share a common theme. This focuses on the immunomodulatory role of vitamin D on the endometrium. Interestingly, specific pro-inflammatory cytokines have been found to be reduced when endometrium from women with recurrent miscarriage is treated with vitamin D producing a less hostile environment for the successful completion of the delicate implantation process[143]. A sound mechanism by which vitamin deficiency may contribute to unexplained recurrent miscarriage is therefore presented.

From a clinical research perspective, the association between vitamin D deficiency and recurrent miscarriage has yet to be proven or disproven. This is an area of clinical research that is very much in its infancy with little interest recently. All but one of the eight identified clinical research publications was observational in nature and the findings were conflicting. The remaining study was interventional and aimed to investigate whether vitamin D treatment would be of any benefit in women with recurrent miscarriage[130]. However, this trial was weak in its methodology. Firstly the sample was too small to answer the research question (40 women) and none of the women diagnosed with recurrent miscarriage had their vitamin D status checked prior to vitamin D treatment. Consequently, vitamin D treatment would not have been targeted on the deficient population.

Identification of whether an association between vitamin D and recurrent miscarriage exists remains an interesting research area. Much like in the IVF population, a safe and cost effective treatment that could reduce the risk of miscarriage in any further pregnancies represents an important avenue to explore. Although recurrent miscarriage affects a small

proportion of the general population (1%), the women affected have profound health impact. Recurrent miscarriage holds psychological and physical health implications and therefore, any treatment that carries sound biological plausibility should be fully investigated. The first step would be to conduct a robustly designed observational cohort study to identify the prevalence of vitamin D in the recurrent miscarriage population. The outcome of any achieved pregnancy after vitamin D assessment could then be tracked and data collected.

The use of endometrial fluid as a prognostic tool in women undergoing IVF treatment

The use of endometrial fluid as a prognostic tool is still at a very early research stage. The narrative review conducted demonstrates this, as only a small number of publications were identified. However, the research published thus far is very exciting, with several groups proposing several biomarkers that could be of clinical use. In particular, the genetic, protein and immunological factors that have been suggested have promising potential. Currently, there are still no endometrial fluid tests that show whether a woman will achieve pregnancy or not whilst undergoing IVF treatment. However, it would be anticipated that such a test could become available soon. The tool that is closest to becoming clinically useful is the Endometrial Receptivity Array that assesses the expression of 238 selected genes in the endometrium[190,191]. An endometrial biopsy is taken the month prior to the IVF treatment cycle. This aids clinicians predict when the endometrium is most receptive and offers data to assist with a personal embryo transfer that has the maximum potential for implantation. This test is currently being used in the private health sector at a substantial cost.

Additionally, the test may need to be repeated on multiple occasions to identify the most receptive day that embryo transfer should be performed.

Perhaps the most exciting part of this area of research is the potential to collect endometrial fluid at close proximity to implantation with respect to time and space. A number of studies identified in the narrative review collected fluid before IVF treatment commenced, whilst others collected endometrial fluid at the time of oocyte retrieval. One could argue that the use of endometrial fluid aspirated at the time of embryo transfer as a prognostic tool offers the greatest interest with regards to increasing the level of understanding towards embryo implantation. This is due to the fact that the fluid collected is closely representative of the uterine environment that the embryo will be transferred into and is therefore the closest to the implantation event itself. The identified literature seems to suggest that this is a safe technique that does not disrupt the mechanisms required for embryo implantation to occur and the research published by Boomsma et al., highlights this[162].

As technology advances, it may become possible to collect endometrial fluid just prior to embryo transfer so that a prognostic result can be provided to guide clinicians as to whether the embryo transfer should be performed or whether the embryos should be cryopreserved and transferred at a later date. The test and technology required would need to offer a quick turnaround to allow a decision to be made that would be clinically important and feasible to undertake.

The endometrial fluid metabolomics cohort study presented in chapter six has provided some novel and exciting results. The study demonstrated that the novel technique of sampling endometrial fluid without active aspiration is safe with an expected number of clinical pregnancies achieved by the 20 women recruited that underwent fluid sampling. Furthermore, the technique adopted allows for sufficient fluid to be sampled in order to conduct metabolomics profiling, which had not been performed before in the context of endometrial fluid sampled from women undergoing IVF treatment. As a proof-of-principle study this was the primary aim. However, the data generated suggested that several metabolites could be used to differentiate the endometrial fluid of women that achieved clinical pregnancy versus the endometrial fluid of women that did not. Unfortunately as this was a proof-of-principle study the sample size was not large enough for any firm conclusions to be found. But a larger sample size may well yield useful metabolites that indicate a receptive endometrium. The significance of these endometrial fluid metabolites must also be investigated and the mechanisms behind their importance understood.

The future direction for the use of endometrial fluid in IVF treatment is a hopeful one. There is a need for the identification of a clinically useful panel of biomarkers that are capable of differentiating endometrial fluid that is receptive to embryo implantation versus endometrial fluid that is non-receptive. In the first instance, it would seem logical to explore these biomarkers in the endometrial fluid in a large sample of fertile women during the receptive mid-menstrual cycle to identify those biomarkers that were consistently present in fertile and receptive endometrial fluid. Once the biomarkers are identified the panel could then be tested on women that were having IVF treatment. Metabolomic profiling would

seem an attractive avenue for further enquiry as a testing tool. The reason for this is that it is the omics discipline that is furthest downstream of the endometrial tissue. It would therefore offer the most representative test of the phenotype of endometrial fluid when compared with genomics or proteomics for example.

Salpingostomy in the treatment of hydrosalpinx

The clinical pregnancy rates achieved by the studies identified by the systematic review and meta-analysis performed in chapter seven are noteworthy. Before IVF treatment became commonplace and available widely it was the role of gynaecologists to perform tubal surgery with the aim of achieving the possibility of natural conception in women with tubal infertility. These surgical strategies have now been overtaken in popularity by IVF treatment. In women with hydrosalpinx, current practice involves sterilising surgery to prime the uterus and endometrium to optimise the chances of implantation. The aim of the sterilising surgery (whether this be tubal disconnection or salpingectomy) is to reduce the deleterious effect of the fluid within the hydrosalpinx causing toxicity to the endometrium or from washing out the embryo inserted into the uterine cavity. Although this does improve the chances of IVF treatment success[282] it renders the woman sterile and reliant on IVF treatment for any further attempts at pregnancy conception, which may be at significant financial cost to the infertile couple, as well as the significant emotional and physical distress caused by repeated attempts at ART.

The findings of the salpingostomy and hydrosalpinx systematic review demonstrate that the clinical pregnancy rates achieved by conservative tubal surgery are comparable to those achieved by IVF treatment today. This is an important finding as it brings to the fore the effectiveness of tubal surgery that may have been considered redundant by many working in the field of reproductive medicine. At least in a well-selected population, conservative tubal surgery to drain the fluid from hydrosalpinx should be offered so that future attempts at natural conception can be made. Critically, the review identified that there was a plateauing of clinical pregnancy rate 24 months post-surgery. This means that if women have not achieved natural pregnancy two years after salpingostomy, then sterilising surgery followed by IVF treatment would seem the most sensible treatment strategy.

More research is required to identify the exact sub-population where salpingostomy should be used. However, it would be expected that tubal conserving surgery for hydrosalpinx would be best offered to younger women who have more time and ovarian reserve compared to those that are older. Additionally, salpingostomy would be more suited to women with more mild forms of hydrosalpinx where the degree of tubal anatomical distortion is proportionately less than those with severe hydrosalpinx[282].

The review also demonstrated that the risk of ectopic pregnancy in women undergoing salpingostomy is relatively high, which is a potential downfall of tubal conserving surgery. However, it should be noted that IVF treatment is not in itself risk free, with the risks of ovarian hyper-stimulation and the surgical risks of oocyte retrieval being rare but present nonetheless. With appropriate counseling and careful management of patients'

expectations a fuller range of treatment options can be offered to women with hydrosalpinx with a balancing of risk of either strategy.

A surgical trial would be difficult to perform given the heterogeneity of surgical technique used to perform salpingostomy by different surgeons. However, a trial has been recommended by the Cochrane review published by Johnson et al., in 2010[28]. The Cochrane review calls for a trial to compare the cumulative pregnancy rates achieved by salpingostomy in the surgical treatment of hydrosalpinx versus the use of tubal occlusion or salpingectomy followed by IVF treatment. The findings presented in thesis chapter seven reinforce this need.

The promising clinical pregnancy rates achieved by salpingostomy in correcting distal fallopian tubal occlusion could also inspire interest in other forms of tubal restorative surgery. A similar review is currently underway investigating the effectiveness of tubal catheterisation for proximal tubal occlusion, which is mainly treated with IVF treatment currently.

As with many facets of clinical medicine, trends in therapy selection rise and fall with time. Treatments that had become unpopular (such as fallopian tube conserving surgery) may become popular once again. Importantly, this could be the case with tubal conserving surgery, allowing for more treatment options and information to be available to patients with tubal infertility.

Summary

As a subspecialty, reproductive medicine is a relatively young area of expertise. Its use of high technology treatments means that it also lies at the cutting edge of science and will be the subject of many advances in the future.

Over the past four decades, real cultural changes have occurred with regards to how women in the UK view fertility. Prior to the advent of IVF treatment it was not uncommon for women to be told that they would be unable to become pregnant and have children. With improvements in IVF care more women are now leaving child bearing until later on in their reproductive years. Unfortunately, this may be due, in some part, to false perceptions of the results that modern IVF treatment can achieve. There have been clear advancements in reproductive medicine; however, there remains a plateau in progress concerning the improvements in success rates of IVF treatment. Much of this is due to the imperfect knowledge that there is regarding embryo implantation.

Further research work is desperately required to advance embryo implantation knowledge. Additionally, the development of novel tools to help identify the correct environment for implantation will hopefully continue to improve pregnancy rates both in natural efforts at conception as well as in IVF treatment.

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APPENDICES