



The effect of environmental,
physical, and nutritional factors on in
vitro fertilisation.

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ABSTRACT

Folliculogenesis, fertilisation and implantation of a human embryo requires unity of many different pathways. The literature review discusses folliculogenesis, implantation and the potential affect that endocrine disrupting agents (EDAs) and Vitamin D can have on infertility and Polycystic Ovary Syndrome (PCOS). Can physical and immune-modulating treatments such as Endometrial scratching (ES) and intralipid aid in the treatment of recurrent implantation failure (RIF)?

EDAs were detected in the PCOS and controls, only the polyfluoroalkyl-agent (PFAA) perfluorooctane sulfonate (PFOS) had a significantly higher concentration in the PCOS group, (4.11 ± 1.62 ng/ml vs. 3.11 ± 1.05 ng/ml, $p=0.03$). Whole group analysis showed PFAAs demonstrated significant positive correlations with testosterone. PFAAs, Polychlorinated Biphenyls and Dichlorodiphenyldichloroethylene (p,p-DDE) demonstrated significant positive correlations with cleavage rates ($p=0.01$ to 0.04), thus these chemicals may disrupt cell division in early embryo development. There was no correlation between EDAs and pregnancy in either group.

A pilot study was designed to see what effects Vitamin D levels had on IVF outcomes in PCOS and control subjects. A linear trend was observed between Vitamin D levels and fertilisation rates in the PCOS group suggesting a possible relationship between Vitamin D and oocyte maturation in this distinct population of women.

An observational study was designed to assess the effect of ES on women undergoing IVF. No increase in clinical pregnancy rates ($p=0.54$) was demonstrated in women with RIF, however clinical pregnancy rates were significantly reduced ($p=0.04$) in women undergoing their second cycle of IVF. These findings suggest that this treatment is not effective in the treatment of RIF.

The effect of intralipid to aid implantation in women with RIF has been postulated but not confirmed. A pilot study was designed to observe the effect of intralipid on NK-cell populations of women with RIF and controls undergoing IVF. The findings demonstrated no effect of intralipid on NK-cell populations in women with RIF.

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LIST OF PRESENTATIONS**Posters:**

Are polyfluoroalkyl chemicals associated with raised levels of serum testosterone in women with and without Polycystic Ovary Syndrome, undergoing controlled ovarian hyperstimulation IVF cycle. ESHRE, Helsinki, Finland, 3-6th July 2016.

Is a mock embryo transfer as effective as a formal endometrial scratch in an Unselected Population? Fertility, ICC Birmingham, 7th -9th January 2015

A Mock-Embryo Transfer is as effective as an endometrial scratching to aid implantation. ESHRE 31st Annual Meeting. Lisbon. 14th – 17th June 2015

Oral Presentations:

Endocrine Disrupting agents are present in the serum of women with and without polycystic ovary syndrome undergoing IVF in the United Kingdom. Oral Presentation. RCOG World Congress, Birmingham 20-22nd June 2016.

Does an Endometrial scratch the menstrual cycle prior to commencing IVF offer any more benefit than a Mock-Embryo Transfer or doing nothing at all? Oral Presentation. Yorkshire and Humber Deanery Academic Presentation Day. 3rd June 2015.

The relationship between Vitamin D levels in PCOS and non-PCOS patients undergoing subfertility treatment. 4th Annual HYMS Postgraduate Research Conference. 22nd May 2015

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AUTHOR'S DECLARATION

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources. I confirm that any patient information obtained to produce this piece of work has been appropriately anonymised.

CHAPTER ONE: Literature Review

1.1 Folliculogenesis

Folliculogenesis is the process in which a recruited primordial follicle grows and develops into a specialised graafian follicle with the potential to release its egg into the oviduct at mid-cycle where it may be fertilised or die by atresia.

Folliculogenesis is regulated by the central nervous system at the level of the hypothalamic pituitary axis, and by the ovary. The hypothalamus secretes pulses of gonadotropin-releasing hormone (GnRH) into the anterior portal veins, which act on the gonadotrophs within the anterior pituitary to release follicle stimulating (FSH) and luteinising hormone (LH). These gonadotropins act on the ovary to control folliculogenesis.

The primordial follicles originate in the fetal ovaries during development at between 6 and 9 months of gestation (Ohno et al. 1962). At birth the ovary contains approximately 2 million primordial follicles, of which about one million will contain an immature oocyte (Ganong. 2005). All the woman's oocytes are present from birth. The majority will undergo atresia and at puberty only approximately 300,000 remain (Faddy et al. 1992).

1.1.1 Timing

The process of folliculogenesis is long taking approximately 175 days from the primordial phase to the pre-ovulatory follicle (Gougeon. 1986). Folliculogenesis can be broken down into two phases; the pre-antral and antral phases (figure 1.1). The pre-antral (primary and secondary follicles) phase is the recruitment and development of a primordial follicle into a tertiary follicle and this stage is gonadotropin independent. The antral phase allows a cohort of class 5 (early tertiary) follicles continue to grow under the influence of FSH and develop antral cavities containing follicular fluid. These follicles secrete oestrogen and

inhibin that have a negative feedback effect on FSH, and the growth of those follicles with the fewest FSH receptors will slow and will undergo atresia. The last remaining follicle become the dominant follicle and will continue to grow to become the preovulatory follicle of approximately 20mm diameter.

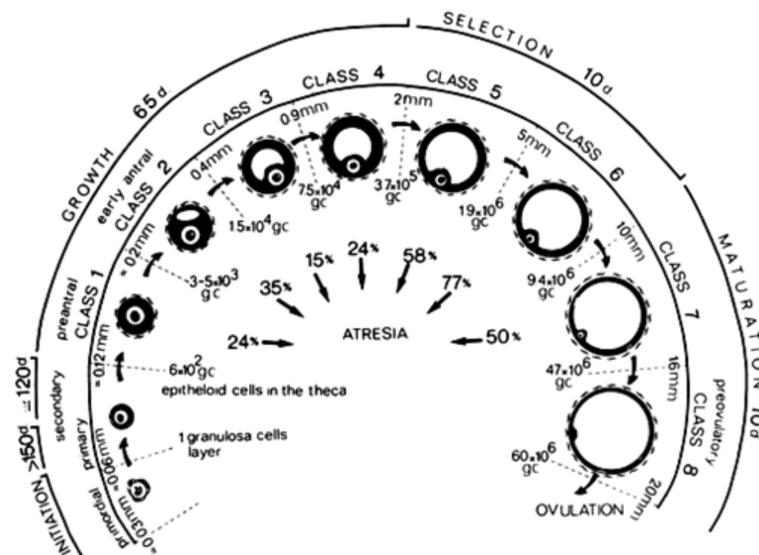


Figure 1.1 Chronology of human ovary folliculogenesis. Gougeon A. (1986).

1.1.2 The pre-antral follicle

During the pre-antral phase the flattened follicular cells of the primordial follicle differentiate into more cuboidal granulosa cells arranging themselves in a single layer surrounding the oocyte. These cuboidal granulosa cells begin to express FSH receptors. Granulosa-derived activin may play a role in the expression of FSH receptors via autocrine and paracrine mechanisms (Nakamura et al. 1993), (Figure 1.2), however they are gonadotrophin independent until the antral phase.

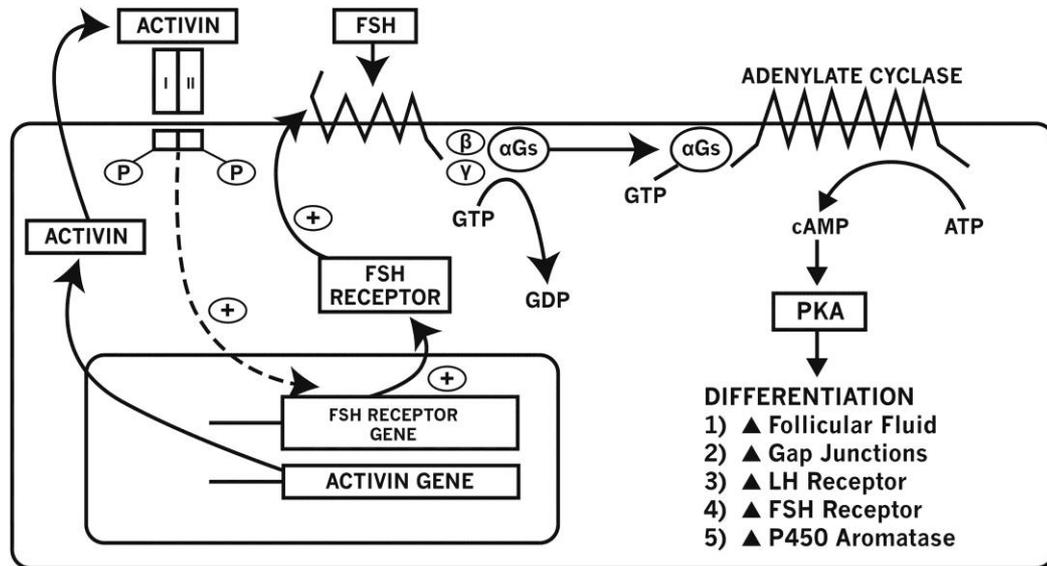


Figure 1.2 Proposed mechanisms for the autocrine control of FSH receptor expression in granulosa cells of preantral follicles, adapted from Erikson. (1997)

1.1.3 The secondary Follicle

The secondary follicle forms when further layers of granulosa cells surround the oocyte. Growth Differentiation Factor-9 (GDF-9) is vital for folliculogenesis following the primary stage and is present in the oocytes of recruited follicles (McGrath et al. 1995). Evidence for the involvement of GDF-9 is derived from the observation that follicular growth stops at the primary stage in GDF-9 deficient rats (Dong et al. 1996). Thecal cells begin to form from the differentiating basal lamina of the follicle and small blood vessels begin to form between the theca externa and theca interna. These are critical as they expose the follicle to the endocrine pathway. At this point the oocyte completes its growth (120um in diameter in the human) and is fully developed. The structure of the mature follicle is complete and the granulosa and theca cells will continue to undergo mitosis in response to the increase in the size and volume of the antral cavity, growing to a maximum diameter of 2cm or more (Mc Natty 1981).

1.1.4 The Antral and dominant Follicle

The antral phase is characterised by the formation of the antrum, a cavity filled with follicular fluid. LH induces androgen production by the thecal cells, mainly androstenedione. This LH-stimulated thecal cell androstenedione production is enhanced by cell, derived paracrine factors, including inhibins, Insulin-like Growth Factor 1 (IGF-1), and IGF-2, that stimulate thecal cell production.

All the granulosa cells within the follicle express FSH receptors, which allow a healthy follicle to continue to grow and differentiate to the pre-ovulatory stage. Follicles are most responsive to FSH when they reach a diameter of 2-5mm. When they reach a diameter of 6-8mm, the granulosa cells start to express aromatase, allowing androgens derived from the theca to undergo aromatization to form oestrogen. It is thought that the follicle with the highest number of FSH receptors will become the dominant follicle. As FSH levels rise, the antral follicles release oestrogen and inhibin that have a negative effect on FSH. Thus those follicles with fewer FSH-receptors stop growing and undergo atresia. During the midpoint of the menstrual cycle the dominant follicle reaches its maximal size. At this point the granulosa cells only have FSH receptors. The development of the LH receptors allows the granulosa cells of the dominant follicle to respond to LH stimulation during the pre-ovulatory surge of LH. LH enables the development of the site of follicle rupture and LH stimulates the meiotic maturation of the ovum (Erickson. 1997).

Following ovulation the follicle collapses and become the corpus luteum. Lutenisation is the process within the luteal cells of graafian follicles. The luteal cells become hypertrophied with vascularization and lipid accumulation and the follicles then become corpora lutea under the regulation of LH. These lutein cells produce large amounts of oestrogen and progesterone. If, however, fertilisation does not occur, the corpus luteum breaks down, a process referred to as "luteolysis."

This is an overview of the process of folliculogenesis in women with a regular menstrual cycle as demonstrated by figure 1.3. However 70-80% of women with Polycystic ovary syndrome (PCOS) are affected by oligomenorrhoea/anovulation (Broekmans et al. 2006). This anovulation is a result of disturbances in the process of folliculogenesis due to an imbalance of androgens, and insulin resistance. This leads to an endocrinological condition that culminates in anovulation, resulting in difficulties attempting to achieve a pregnancy. At this point it is important to discuss PCOS, then review the roles of androgens and insulin in both normal and disturbed folliculogenesis in the polycystic ovary.

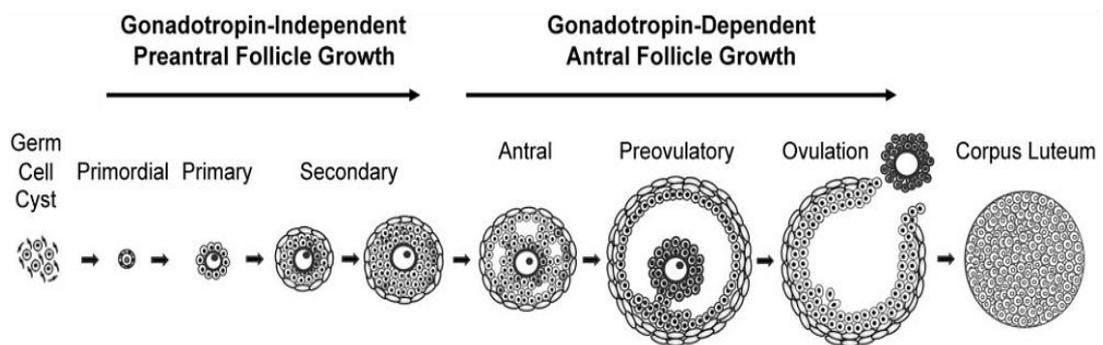


Figure 1.3. Schematic of folliculogenesis

1.2 Polycystic Ovary Syndrome

Polycystic ovary syndrome is one of the most common endocrine disorders affecting women. It is estimated that between 6-7% of the female population suffer from PCOS, which is one of the main causes of female infertility (Azziz et al. 2004). NICE (2013) state that one of the main causes of infertility that affects approximately 25% of couples is ovulatory disorders of which the majority suffer from PCOS (NICE, CG 156. 2013).

The most common symptoms are anovulation, excess androgenic hormones, and insulin resistance. Anovulation results in irregular menstruation, amenorrhoea, and ovulation-related infertility. Excessive biologically active androgens can result in acne and hirsutism. Insulin resistance is associated with

obesity, Type 2 diabetes, and high cholesterol levels (Franks 1995). Patients are diagnosed using the revised 2003 criteria from the Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group (ESHRE/ASRM 2004) which indicate PCOS to be present if any 2 out of the following 3 criteria are met:

1. oligo and/or anovulation
2. Clinical and/or biochemical signs of androgenism
3. Polycystic ovaries (either 12 or more peripheral follicles or increased ovarian volume (greater than 10 cm³))

Diagnosis of PCOS can only be made when other aetiologies have been excluded, such as thyroid dysfunction, congenital adrenal hyperplasia, hyperprolactinaemia, androgen-secreting tumours and Cushing syndrome.

The Rotterdam definition is broad, consequently including a large cohort of women without androgen excess. Critics say that findings obtained from the study of women with androgen excess cannot necessarily be extrapolated to women without androgen excess (Hart 2004). Barth et al. (2007), and Azziz. (2006a), feel the Rotterdam criteria are not sufficiently robust and are defined too vaguely for clinical and research practice. Moreover, the Rotterdam criteria allow diagnosis of PCOS to be made from two out of the three features stated above, creating the possibility of several phenotypes.

1.2.1 Phenotypes of PCOS

As a result, the prevalence of PCOS has been estimated as 20% (Wild et al. 2010) compared with the 6-7% as stated by Azziz et al. (2004). This has led to the proposal to use 4 PCOS phenotypes:

1. Classical:- oligomenorrhea, hyperandrogenism, and polycystic ovaries
2. Oligomenorrhea and hyperandrogenism but normal ovaries

3. Hyperandrogenism and polycystic ovaries without menstrual irregularities
4. Oligomenorrhea and polycystic ovaries, but no clinical or biochemical hyperandrogenism.

Women with the classical phenotype of oligomenorrhea, hyperandrogenism and polycystic ovaries have higher BMI, central obesity, more severe insulin resistance and a higher prevalence for dyslipidaemia compared to the other subgroups (Jovanovic et al. 2010; Wlitgen et al. 2010). An expert committee of the Androgen Excess and PCOS society (AEPCOS) have suggested that only the first three phenotypes should be included, with the exclusion of the fourth phenotype from the PCOS spectrum (Azziz et al. 2006b).

Biochemical abnormalities of PCOS include raised LH, raised testosterone, low SHBG, and a raised free androgen index. (Franks 1989). The raised LH levels, in combination with insulin, acting on the polycystic ovary result in an increased ovarian production of androgens. The raised insulin levels also increase adrenal production of androgens and reduce hepatic production of sex hormone binding globulin (SHBG), leading to a raised free androgen level. Insulin resistance is a common finding among women with normal weight, as well as in overweight women (Nafiye et al. 2010).

Adipose tissue possesses aromatase, an enzyme that converts androstenedione to estrone, and testosterone to estradiol. The excess of adipose tissue in obese women creates the paradox of having both excess androgens, which are responsible for hirsutism and virilisation, and oestrogens, which inhibit FSH via negative feedback. Fassnacht et al. (2003) demonstrated that women with PCOS had raised peripheral 5 α reductase activity, thus highlighting peripheral androgen activity again amplified by obesity.

At this point, the literature has demonstrated the imbalance of androgens in PCOS women and the aggravating effect of Insulin resistance. However the other key finding in PCOS women is the polycystic ovary. These are ovarian follicles that are in stasis at the early antral phase as a result of the imbalance of normal ovarian function. The PCOS ovary contains 2-3 fold the number of small 2-5mm follicles compared to the normal ovary (Hughesdon. 1982). This has been confirmed when PCOS ovaries have been biopsied to show higher densities of pre-antral and primary follicles compared to controls (Weber et al. 2003). Thus it is evident that the process of folliculogenesis in the PCOS ovary results in an exaggerated early follicular growth resulting in a large pool of preantral follicles and at this point there is a disruption in the process of selecting/recruiting a dominant follicle, resulting in the chronic anovulation that affects the majority of women with PCOS.

1.2.2 PCOS and In Vitro Fertilisation

There is a large volume of literature regarding PCOS and exogenous gonadotrophins within the In vitro Fertilisation (IVF) setting. However at this point it is essential to mention that a common feature of these women undergoing an IVF cycle is that they commonly produce large numbers of oocytes that are of poor quality (Sahu et al. 2008, Weghofer et al. 2007). PCOS is also a well-established risk factor for the development of OHSS (Humaidan et al. 2010). PCOS women exhibit an exaggerated response to exogenous gonadotrophins, resulting in the large number of oocytes retrieved unfortunately poor fertilisation rates are encountered due to oocyte immaturity as a result of stimulating large numbers of pre-antral follicles. PCOS women tend to show lower cleavage rates resulting in decreased implantation and pregnancy rates. So to what extent are androgen and insulin responsible for the disordered folliculogenesis in the PCOS ovary? I will give a brief overview of each, as the full subject matter is too great to include in this literature review. From this I will consider the effect that chronic exposure to persistent organic

pollutants or endocrine disruptors and Vitamin D concentrations could have on folliculogenesis and developmental competence in these women.

1.3 Androgens in Folliculogenesis

1.3.1 Sources of Androgens

The main sources of circulating androgens in women are the adrenal glands and the ovaries (Arlt 2006). Dehydroepiandrostone (DHEA) is produced by the zona reticularis of the adrenal glands and acts as a precursor of other androgens in the ovary (Labrie 2010). Androgens are also available from the conversion of precursors within other tissues such as fat, muscle and brain. The ovary normally synthesises three C19 androgens; androstenedione, testosterone and DHEA. The main ovarian androgens produced are testosterone and androstenedione produced in the thecal cells, both of which can be aromatised to oestrogens.

1.3.2 Biosynthesis of Androgens

All the androgens are synthesised from cholesterol, by a common pathway in which progestogens are precursors to androgens, which then act as precursors for the oestrogens (Figure 1.4). These lipid soluble hormones diffuse through cell membranes binding to the androgen and oestrogen receptors.

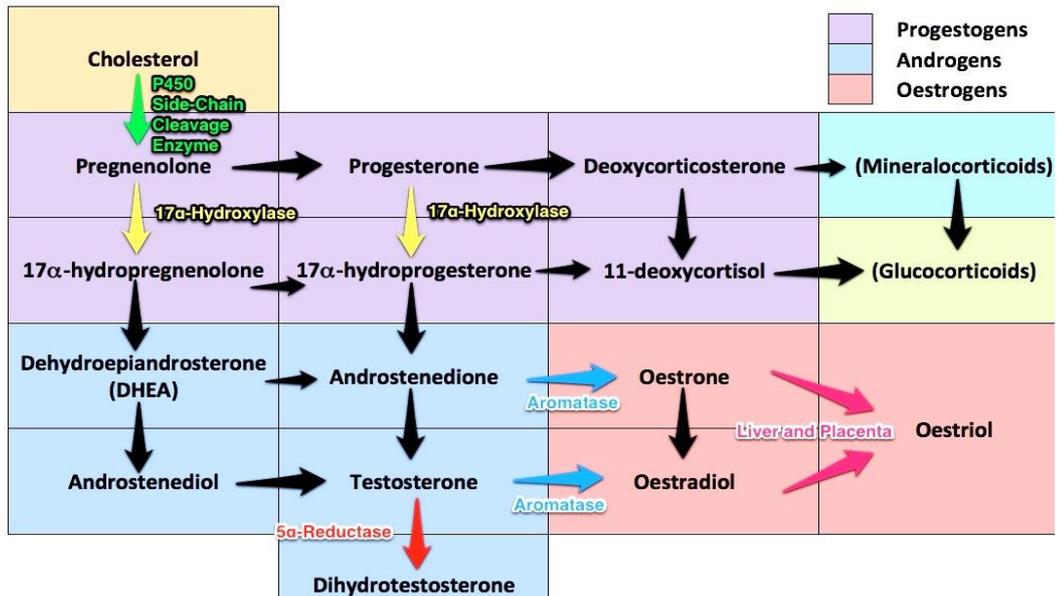


Figure 1.4: Schematic of Steroidogenesis

Androstenedione is synthesized in both the adrenal gland and ovary. ACTH governs adrenal androstenedione, whereas production of gonadal androstenedione is under control by gonadotropins and depends on both the time of day and the day in the menstrual cycle. There is a diurnal rhythm in androstenedione secretion. In the morning, the adrenal will produce approximately 80% of the androstenedione production, with a reduction in the evening (Lachelin et al. 1979). Early in the follicular phase of the menstrual cycle, the adrenals produce more androstenedione than the two ovaries, but as the follicle develops it secretes increasing amounts of androstenedione. This is reflected in an increase in the plasma concentrations usually observed in the mid-cycle and maintained in the luteal phase (Baird 1976).

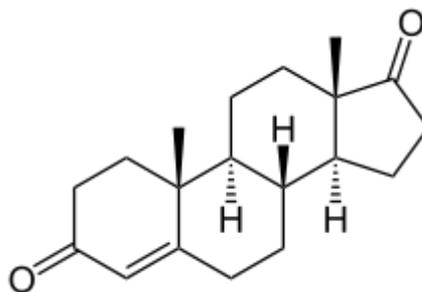


Figure 1.5 Chemical structure; Androstenedione

Testosterone is derived from cholesterol. It is synthesized in women by the theca cells of the ovaries, as well as by the zona reticularis of the adrenal cortex and even in the skin. Kirschner et al. (1976) demonstrated that 25% of testosterone is produced by the ovary, 25% by the adrenal gland and 50% from the peripheral metabolism of pre-hormones, mainly androstenedione from the liver, fat and skin.

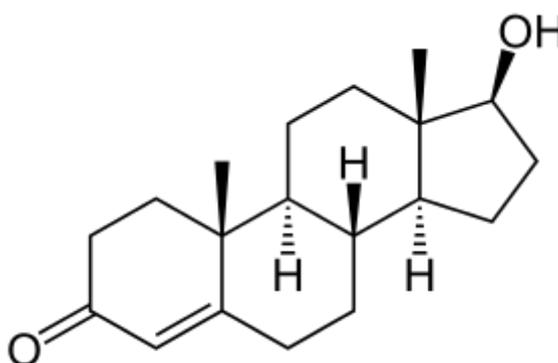


Figure 1.6 Chemical structure; Testosterone

DHEA is an important endogenous steroid hormone and the most abundant circulating steroid hormone in humans (Ganong 2005). It is produced in the adrenal glands, the gonads, and the brain where it functions predominantly as a metabolic intermediate in the biosynthesis of the androgen and oestrogen sex steroids (Mo et al. 2006). However, DHEA also has a variety of potential biological effects, binding to an array of nuclear and cell surface receptors (Webb et al. 2006). Although it predominantly functions as an endogenous precursor to more potent androgens such as testosterone and dihydrotestosterone (DHT), DHEA has been found to possess some degree of androgenic activity in its own right, acting as a low affinity ($K_i = 1 \mu\text{M}$), weak partial agonist of the androgen receptor (Chen et al. 2005; Gao et al. 2005).

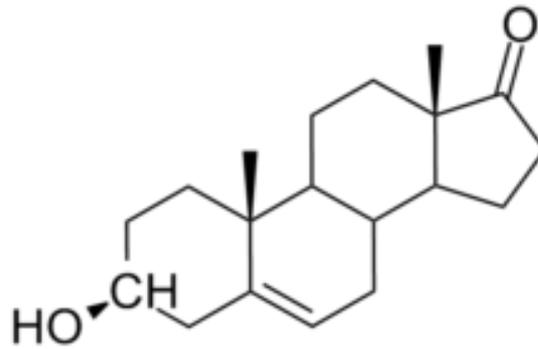


Figure 1.7 Chemical structure; DHEA

Oestrogens are produced from androgens, specifically testosterone and androstenedione, by the enzyme aromatase. The three major naturally occurring oestrogens in women are estrone (E1), oestradiol (E2), and estriol (E3). Oestradiol is the predominant oestrogen during the reproductive years, both in terms of absolute serum levels as well as in terms of oestrogenic activity. During menopause, estrone is the predominant circulating oestrogen and during pregnancy estriol is the predominant circulating oestrogen in terms of serum levels. Though estriol is the most plentiful of the three oestrogens it is also the weakest, whereas oestradiol is the strongest with a potency of approximately 80 times that of estriol (Tulchinsky et al. 1972).

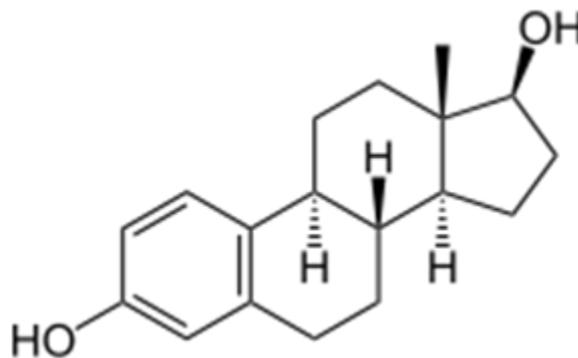


Figure 1.8 Chemical structure; Oestradiol

1.3.3 Androgens in the circulation

Only very small amounts of androgens are secreted in their active form. The majority of these hydrophobic hormones are carried in the circulation in an inactive state bound to albumin or SHBG. Albumin is the most abundant blood plasma protein and is produced in the liver. Albumin is important in regulating blood volume by maintaining the oncotic pressure, but can also carry molecules of low water solubility, including lipid soluble hormones, bile salts and free fatty acids. SHBG is produced in the liver and as stated earlier can be affected by androgens, obesity, and hyperinsulinaemia. Thus, changes in the levels of SHBG will affect the level of free androgen in the circulation. As discussed this can be seen in the case of where the hyperinsulinaemia of PCOS leads to the reduction in SHBG and thus an increase in the circulating levels of active testosterone, leading to hyperandrogenism and hirsutism in some cases. (Michelmore et al. 1999).

1.3.4 Androgens and normal follicle development

As discussed the primordial follicle is the smallest follicle and consists of a meiotically arrested oocyte surrounded by a single layer of squamous granulosa cells. The shape of the granulosa cells in a selected follicle change from squamous to cuboidal. The phosphoinositide 3-kinase (P13K) intracellular signaling pathway, is central in this process of primordial follicle selection (Reddy et al. 2008) and a basal degree of intra-oocyte P13K activation is required for survival throughout the dormancy of these primordial follicles (Reddy et al. 2010). At the same time P13K signalling is inhibited by several molecules such as, FOXO3, which ensures sustained dormancy of the primordial follicle pool (John et al. 2008).

In animal studies, testosterone induces by greater than 2 fold, the ratio of primary to primordial follicles via rapid stimulation of the intra-oocyte P13K/FOXO3 pathway in mouse primordial follicles (Yang et al. 2010). In rhesus

monkeys given supra-physiological doses of testosterone, pre-antral, and small antral follicles, are increased by 2.5 - 4.5 fold (Vendola et al. 1998). In the Rhesus monkey, the same group later reported that androgens are likely to act on folliculogenesis by increasing the number of FSH receptors expressed by the granulosa cells (Weil et al. 1999). This mechanism remains unclear since the androgen receptor is not detected or could be below the limit of detection in this follicle class (Weil et al. 1998). This may possibly be due to an indirect effect via increased IGF-1 and IGF-1 receptor gene expression shown in androgen treated monkeys (Vendola et al. 1999a,b).

Once the follicle is recruited, the primary follicle grows into the secondary follicle with 2 layers of granulosa cells. In bovine models testosterone stimulates the transition of primary to secondary follicle (Yang and Fortune 2006). In the rhesus monkey, administering large amounts of testosterone up-regulates the testosterone receptor in the granulosa cells (Weil et al. 1998).

As the antral follicle grows, the granulosa cells express FSH and LH receptors and become responsive to gonadotropins. At this point the theca cells differentiate and acquire steroidogenic function, allowing the follicle to interact with the endocrine signalling pathway. The thecal cell function is initially under neuro/paracrine control via GDF9 which is essential for inducing cytochrome P450c17 expression and androgen synthesis in the pre-antral follicles (Orisaka et al. 2009), while suppressing aromatase activity allowing an androgen rich environment. In the antral follicles granulosa cells produce activin that suppresses androgen synthesis and this is opposed by inhibin (Knight et al. 2012). Insulin in synergy with LH induces P450c17 and stimulates thecal androgen production (Franks et al. 1999).

Estrogen has a biphasic effect on LH/FSH during folliculogenesis. Lower levels of estrogen during the early follicular phase inhibits the gonadotrophins but

elevated levels of estrogen in the late follicular phase result in increased sensitivity of LH/FSH to GnRH. As more estrogen is secreted the theca cells produce more LH receptors producing more androgen, this androgen converts to estrogen, this spike in LH or the LH surge as a result of this positive feedback loop results in ovulation.

1.3.5 Androgens and Folliculogenesis in the PCOS Ovary

The androgen stimulates the growth of large numbers of small follicles in the early stages of follicle growth. Studies have demonstrated that the hyperandrogenaemia of PCOS reflects an abnormality in the theca cell function of the PCOS ovary (Gilling-Smith et al. 1994). There is an intrinsic defect in the enzymes within the theca cells that act on steroidogenesis resulting in an increased activity and exaggerated androgen production, such as CYP11A1, that converts cholesterol to pregnenolone, and CYP17 α 1 that catalyses the 17 α -hydroxylation of pregnenolone and progesterone (Nelsen et al. 1999).

This effect is amplified by the increased LH levels within the theca cells resulting in increased androgen concentrations that may interfere with follicular development by suppressing FSH. This affects the granulosa cell function resulting in premature granulosa cell luteinisation and follicular arrest in the small antral follicles. Consequently no dominant follicle develops, resulting in anovulation.

As a result of the excessive growth of small FSH independent follicles the excess androgens may interfere with the next step of folliculogenesis. During the FSH dependent stage, this enables the follicles to grow and mature usually between the 2-5mm stage. The larger follicles acquire aromatase activity, and as discussed earlier, oestradiol is produced, FSH levels decline in the late follicular phase and the most advanced follicle ovulates. However PCOS women have a lower FSH concentration that results in slow growth and a large pool of antral follicles (2-8mm), i.e., these follicles have undergone premature arrest and fail

to mature (Franks et al. 2008). Greisen et al. (2001) demonstrated that the androgen excess of PCOS inhibited aromatase activity contributing to the later stages of folliculogenesis and the inability to select a dominant follicle. Franks et al. (2008) also suggest that reduction in AMH expression in the small pre-antral follicles could contribute towards the disordered folliculogenesis. This is demonstrated by the high levels of AMH in PCOS women as it is the small antral follicles that are the greatest source of AMH hence the polycystic appearance of the ovary with high circulating levels of AMH.

Thus the hyperandrogenaemia of PCOS promotes early growth of the primordial pool resulting in an excess of small 2-5 mm follicles, while the excess of LH within the theca cells further promotes androgen excess and affects the FSH within the granulosa cells preventing the selection of a dominant follicle from this pool of immature follicles and resulting in anovulation.

1.3.6 Insulin and normal Folliculogenesis

Insulin is a peptide hormone produced by the beta cells in the pancreas, which primarily regulates the metabolism of carbohydrates and fats. It promotes the absorption of glucose from the blood to be stored as glycogen mainly in liver and muscle. Insulin receptors with protein kinase-A activity, have been identified on thecal cells and it has been demonstrated that insulin can stimulate the production of androstenedione. Insulin can produce androstenedione independently by this mechanism but can also enhance LH-driven production of androgen. (Barbieri et al. 1988; Nestler et al. 1998). It also induces the activity of cytochrome P450c17 α , a bi-functional enzyme that has 17 α -hydroxylase and 17,20-lyase activities, both of which are key enzymes in the synthesis of ovarian androgens. In the thecal cells, the P450c17 α converts progesterone to 17 α -hydroxprogesterone through the 17 α -hydroxylase, which then converts 17 α -hydroxprogesterone to androstenedione through its 17,20-lyase activity. Androstenedione is then converted to testosterone by 17 β -

reductase (Nestler et al. 1996). Cytochrome P450c17 α is necessary to cleave cholesterol to initiate the primary step of steroidogenesis (Figure1.4).

In vitro experiments have shown that together, LH and insulin stimulate androgen production in the thecal cells in a synergistic manner. Further work has identified interactions of LH and insulin on the thecal-cell expression of genes for intracellular sterol substrate delivery (steroidogenic acute regulatory protein, StAR) and subsequent utilisation in steroidogenesis (17- α hydroxylase/C17-20 lyase (CYP17), enabling potent signalling interfaces between these distinct hormonal agonists and thecal-cell steroidogenesis (Zhang et al. 2000). Testosterone and oestradiol circulate in the blood, bound mostly to the glycoprotein SHBG and to a lesser extent, serum albumin. SHBG is produced in the liver and is released into the blood. Approximately 1-2% of testosterone and oestradiol is unbound, and therefore biologically active, able to enter a cell and activate its receptor. SHBG inhibits the function of these hormones. Thus, the level of SHBG influences the bioavailability of sex hormones. Raised levels of insulin inhibit the production of SHBG by the liver resulting in elevated levels of free androgens (Nestler et al. 1990). Studies have shown that the hyperinsulaemia of PCOS may be pathogenic in hyperandrogenism by increasing androgen production in the ovary and reducing the levels of SHBG (Nahum et al. 1995; Nestler et al. 1991). In PCOS women treated by diazoxide (Nestler et al. 1990) or metformin (Velazquez et al.1994) and diet (Kiddy et al. 1989) have shown a reduction in free-testosterone and increase in SHBG levels.

1.3.7 Insulin and Folliculogenesis in the PCOS ovary

In PCOS, insulin promotes the secretion of androgens by the theca and stroma of the ovary. Thus insulin appears to trigger this dysfunction of steroidogenesis within the theca cells (Barbieri et al. 1984). Insulin works in synergy with LH by enhancing the effects of LH on the theca cells thereby increasing androgen production. A Cochrane review demonstrates that in women with PCOS weight

loss and lifestyle modification improves insulin sensitivity and androgens with metformin being less effective (Tang et al. 2012). In vivo studies have also demonstrated that insulin stimulates the enzyme P450c17 within the ovary via the phosphoinositide 3-kinase (P13K) pathway, which is activated in PCOS (Munir et al. 2004).

1.3.8 Insulin resistance

Insulin resistance is when the body produces insulin but cannot use this insulin effectively. This occurs when circulating insulin fails to or deficiently binds to insulin receptors, resulting in a failure to reduce glucose levels for that concentration of insulin (Ciaraldi 2000). It can be measured directly using the euglycaemic clamp which is the gold standard but invasive and labour intensive or the homeostatic model assessment which uses fasting plasma glucose and insulin and using a mathematical model to derive a level of insulin sensitivity. Insulin resistance can affect up to 40% of women with PCOS. The insulin resistance in PCOS results in the pancreas continuing to produce more insulin as a result of impaired insulin-mediated glucose transport (Dunaif et al. 1992). Insulin requires large transport mechanisms to carry glucose into the muscle and fat. Glucose transporter 4 (GLUT 4) mediates glucose transport stimulated by insulin. However in insulin resistance this transporter mechanism is defective resulting in reduction of glucose transport and insulin resistance as demonstrated by GLUT 4 knockout mice models (Zisman et al. 2000).

Diamanti-Kandarakis and Papavasiliou (2006), demonstrated insulin-signalling defects in PCOS women highlighted by impaired insulin, stimulated tyrosine phosphorylation of insulin receptor substrate 1 in adipose tissue, contributing to insulin resistance. The hyperinsulaemia results in hyperandrogenaemia due to an excess of ovarian androgen production, due to suppression of the production of SHBG by the liver resulting in elevated levels of free androgens resulting in menstrual disturbances and anovulation.

It is known that obese women with PCOS are at greater risk of developing Type II diabetes, metabolic syndrome and cardiovascular disease (Fauser et al. 2012). Thus, insulin resistance and hyperandrogenaemia are features of anovulatory infertility in the PCOS population and are key factors in the abnormal folliculogenesis in this population (Franks et al. 2008). However it is important to highlight that dietary modification and exercise improve the physical, metabolic and hormonal manifestations of PCOS (Moran et al. 2003).

1.3.9 AMH and the PCOS Ovary

Anti-Müllerian hormone (AMH), or Müllerian inhibiting substance is a member of the transforming growth factor- β (TGF β) super-family, and is a cause of follicle arrest. It is unclear, when, during folliculogenesis, AMH expression begins, with studies on primordial follicles producing equivocal results (Stubbs et al. 2005). However, Fleming et al. (2006) demonstrated a positive correlation with AMH levels and follicle size, with the highest expression of AMH found in preantral and small antral follicles. Pellatt et al. (2007) demonstrated that AMH production by the granulosa cells and follicular fluid was at its highest in small antral follicles and in low or undetectable concentrations in follicles ≥ 10 mm.

AMH has been heralded as a marker of ovarian reserve in humans (Van Roojj et al. 2002). Piltonen et al. (2005) demonstrated that young women of less than 25 years had AMH levels greater than women aged 35 years and older. These women were followed longitudinally for a period of between 1 and 7 years and a decline in serum AMH levels was noted, with levels becoming undetectable when menopause was reached (Piltonen et al. 2005).

As stated, AMH is present at highest level in smaller follicles and as follicles grow its level declines, an important step for the selection of a dominant follicles and ovulation. Thus AMH has an inhibitory role in the ovary, reducing both primordial follicle initiation and follicle sensitivity to FSH by inhibition of

aromatase. This is known from animal studies in which AMH knock-out mice demonstrate an increased pool of growing follicles resulting in an early depletion of the primordial pool and cessation of ovulation (Durlinger et al. 1999).

In women with PCOS, AMH is 2-3 fold higher in serum than in women with normal ovaries (Fallat et al. 1997). This was initially thought to be due to the increase in the number of small antral follicles. Pellatt et al. (2007) demonstrated that in women with anovulatory PCOS, AMH production was on average 75 times higher per granulosa cell than in granulosa cells from normal ovaries. Piouka et al. (2009) demonstrated that women with ovulatory PCOS who were equally hyperandrogenic had lower levels of serum AMH compared to anovulatory women with PCOS.

What effects do hyperandrogenaemia and hyperinsulinaemia have on AMH in the PCOS ovary? Women with hyperandrogenaemia and PCOS had higher concentrations of AMH compared to normogenic PCOS women (Eldar- Geva et al. 2005) and Gilling-Smith et al. (1994) showed that androgen production per theca cell was equally increased in anovulatory and ovulatory PCOS. Since women with anovulatory PCOS have greater follicle numbers, this may demonstrate why anovulatory PCOS women have significantly higher AMH production even compared to woman with ovulatory PCOS, whom have significantly higher levels, than the normal population.

Furthermore hyperinsulinaemia affects anovulatory more than ovulatory women (Conway & Jacobs 1993), and as discussed earlier, insulin enhances gonadotrophin-stimulated steroid production in the granulosa and theca cells. Thus, the resultant increase in androgen levels secondary to insulin resistance could raise AMH concentrations secondary to the effect of hyperinsulinaemia on androgen levels. This again shows that AMH, like insulin and androgens, has

affects on the normal growth and selection of follicles, but very high levels in PCOS can inhibit the normal process of folliculogenesis.

As discussed despite PCOS women having excess numbers of follicles, the ability to select a dominant follicle is impaired, commonly known as follicular arrest, which explains the anovulation of PCOS. In ovulating women FSH allows a cohort of follicles to grow. A leading follicle will proliferate developing both LH and FSH receptors. However in PCOS the women lack the inter-cycle FSH rise hence the exaggerated response in an IVF cycle. It has been well demonstrated that PCOS women have significantly higher levels of AMH compared to normals (Cook et al., 2002). Laven et al. (2004) demonstrated that the increase in AMH was related to the excess of 2-5mm follicle number. Thus it has been speculated that the excess AHM and lack of FSH induced aromatiase activity may result in the follicular arrest in women with PCOS.

To conclude, PCOS women comprise a population of women with disordered folliculogenesis as a result of excess insulin and androgen despite the process of steroidogenesis being unaffected (Rice et al. 2005). There are possible genetic abnormalities in women with PCOS, with altered gene expression affecting the pathways controlling steroidogenesis, and the action of insulin and gonadotrophins resulting in disordered folliculogenesis (Prapas et al. 2009). However could environmental factors be pathogenic in the process(es) leading to PCOS? Persistent organic pollutants are widespread present in the environment and can potentially disrupt the endocrine pathways in the body, such that long-term exposure in the follicular environment could be a factor in the development of PCOS.

1.4 Endocrine Disruptors

1.4.1 Definition of an Endocrine disruptor

Endocrine disrupting agents (EDAs) remain a controversial environmental issue. As a result this has made their potential effects a difficult concept to define.

There are numerous definitions of an endocrine disruptor, proposed by large Western Environmental Agencies such as the World Health Organisation (WHO). Such definitions have to consider the global problems which may be induced by EDAs, which will differ in geographical regions depending on the extent to which they use specific chemicals. For example, Dichlorodiphenyltrichloroethane (DDT) was banned in the USA and many westernised countries in the 1970s and early 1980s, but is still widely used in some developing countries such as India.

The United States Environmental Protection Agency defines an EDA as “an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes” (Kavlock et al. 1996).

The European Union definition states that “an EDA is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function. A potential EDA is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism” (Commission E. 1996).

The WHO definition states “an EDA is an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub) populations” (Damstra et al. 2002).

All these definitions include the same features i.e., that an external substance affects a healthy person by disrupting their hormonal balance but can also have far reaching effects by affecting their future offspring. In 2009 the Endocrine society, the world's largest organisation devoted to Endocrinology published a scientific statement on EDAs, defining them as "exogenous agents that interfere with the synthesis, secretion, transport, metabolism, binding action, or elimination of natural-borne hormones that are present in the blood and are responsible for homeostasis, reproduction and developmental process" (Diamanti-Kandarakis et al. 2009). This definition highlights how EDAs can affect the mechanisms of hormones of an individual but also their reproductive potential and the development of offspring. However, all these various definitions show how difficult it is to categorise such a large group of chemicals both synthetic and natural into a broad, over riding definition.

1.4.2 What are Endocrine Disruptors?

In 1962, Rachel Carson, an American biologist, wrote a highly significant book about the widespread use of DDT within America and catalogued its effects on the bird population. However, she questioned what affect the large quantities of pesticides had on the environment without a sufficient understanding of how they might affect both the wildlife population and the human population (Carson 1962).

With a series of studies highlighting the reduction in both male and female fertility over the last 50 to 60 years (Carlsen et al. 1992) it is unlikely that effects are genetic in origin (Marques-Pinto & Carvalho 2013). The 2007 Summit on Environmental Challenges to Reproductive Health and Fertility stated "Approximately 87,000 chemical substances are registered for commercial use in the United States, with ubiquitous human exposures to environmental contaminants in air, water, food, and consumer products. Exposures during critical windows of susceptibility may result in adverse effects with lifelong and

even intergenerational health impacts”(Woodruff et al. 2008). These adverse effects could result in defects in the development of the reproductive tract, hormonal imbalances that could result in reduced fertility and, fecundity (Woodruff et al. 2008).

Endocrine disruptors encompass an enormous array of compounds. They include: pesticides such as DDT and its metabolite Dichlorodiphenyldichloroethylene (p,p'-DDE); industrial lubricants and coolants such as polychlorinated biphenyls (PCBs); compounds used in the plastics industry eg, Bisphenol A (BPA); those used in household goods eg, Phthalates; fire retardant chemicals such as polybrominated diphenyl ethers (PBDEs); drugs (NSAIDS and ethinyl oestradiol, diethylstilbestrol); heavy metals (mercury, zinc, cadmium) and naturally occurring phytoestrogens (genistein).

This wide array of chemicals highlights the potential for exposure on a daily basis; from drinking from plastic bottles and sitting on furniture, to eating certain foods such as tofu that contain phytoestrogens. One study on indoor house dust in 16 homes demonstrated that the 22 most common congeners for PBDE were found at relatively high concentrations (Stapleton et al. 2005). Common organochloride and organobromide endocrine disrupting chemicals are highlighted in Table 1.1

Table 1.1 Common Organochlorine and organobromine endocrine disrupting compounds.

Endocrine Disruptor	Suffix	Usage
Organochlorine		
Pentaclorobenzene	PeCB	Fungicide
Hexachlorobenzene	HCB	Fungicide
γ - hexachlorocyclohexane,	Lindane (γ -HCH)	Pesticide
α - hexachlorocyclohexane	β -HCH	Pesticide
β -hexachlorocyclohexane	α -HCH	Pesticide
Trans(γ) Hexachlorocyclopentadiene	trans (γ) Chlordane	Pesticide
Cis(α) Hexachlorocyclopentadiene	Cis (α) Chlordane	Pesticide
Dichlorodipenyldichloroethylene	op-DDE	Pesticide
Dichlorodipenyldichloroethylene	pp-DDE	Pesticide
Dichlorodipenyldichloroethane	op-DDD	Pesticide
Dichlorodipenyltrichloroethane	pp-DDT	Pesticide
hexachlorocyclopentadiene	Mirex	Pesticide
Polychlorinated Biphenyl-28	PCB-28	Coolants and electrical apparatus, cooling fluid in machines
Polychlorinated Biphenyl-52	PCB-52	
Polychlorinated Biphenyl-101	PCB-101	
Polychlorinated Biphenyl-118	PCB-118	
Polychlorinated Biphenyl-153	PCB-153	
Polychlorinated Biphenyl-138	PCB-138	
Polychlorinated Biphenyl-180	PCB-180	
Organobromine Compounds		
Polybrominated diphenyl ether-28	BDE28	Flame retardant, plastics, furnishing electronic equipment
Polybrominated diphenyl ether-47	BDE47	
Polybrominated diphenyl ether-100	BDE100	
Polybrominated diphenyl ether-99	BDE99	
Polybrominated diphenyl ether-154	BDE154	
Polybrominated diphenyl ether-153	BDE153	
Polybrominated diphenyl ether-183	BDE183	
Hexabromocyclododecane- α	α -HBCDD	Flame retardant, Polystyrene, insulation, foam padding for seats

1.4.3 Routes of Exposure

Human exposure to these toxins can be through food and water chains, air, and absorption through the skin. Food consumption whether fresh or not is the mayor source of EDA exposure. EDA exposure from food from supermarkets has shown fish and other products to be contaminated with PBDE (Schechter et al. 2004).

Plastic packaging and the linings of tin cans are important sources of EDAs as they can leach out and contaminate the food within (Wagner & Oehlmann 2009; Gonzalez-Castro et al. 2011). This may be as a result of repeated exposure of these chemicals to heat, light and the acidic/alkaline nature of the food resulting in their breakdown and migration into the consumer's food and drink (Mercea 2009).

There is also chronic exposure to EDAs through the air and skin. It was often thought that food was the predominant source of exposure to BPA since it is rapidly cleared from the body. Thus, Stahlhut et al. (2009) investigated the effect of urinary BPA concentration and fasting time, expecting BPA levels to decrease with time. However results showed that this was not the case, suggesting substantial non-food exposure and accumulation of BPA in the body tissues. This chronic exposure is also highlighted by the fact that the banned pesticide DDT is still present in serum samples, due to the lipophilic nature of the molecule (Younglai et al. 2002).

1.4.4 Fecundity rates

Fecundity is the potential for reproduction of an organism or population as measured by the number of gametes (eggs), seed set. Fecundity is similar to fertility, which is the natural capability to produce offspring, however fecundity is under both genetic and environmental control, and is the major measure of fitness to reproduce. This is measured by the time to pregnancy (TTP), which is

the time between discontinuing contraception and clinical diagnosis of a pregnancy (Weinberg and Gladen. 1986).

Studies have suggested a link between reduced rates of female fecundity with endocrine disrupting chemicals as measured by a longer TTP (Axmon et al. 2006; Harley et al. 2010). The main chemicals studied included p,p'-DDE a metabolite of DDT, dioxins, PBDEs and PCBs. The majority of these studies were performed in populations where seafood was a major source of the diet as fish is a major source of EDA chemicals. The most recent and largest study, known as the LIFE study, examined the relationship between persistent organic pollutants (POPs) and TTP, and they showed there was a reduced fecundability in women of between 18-21% for PCBs and perfluorooctane sulfanimde and in men a 17-29% reduced fecundability for p,p'DDE and PCB. However despite these associations, the concentrations of the POPs were below the limit of detection, and to present there is no evidence supporting laboratory reference limits of POPs and fecundability.

1.4.5 Dichlorodiphenyltrichloroethane (DDT)

DDT is a colourless tasteless organochloride whose insecticidal action was discovered by a chemist, Paul Hermann Müller in 1939. In World War II it was used to control malaria. However, widespread agricultural use accelerated resistance among insect populations, in many cases reversing early successes against malaria-carrying mosquitos. In 2001 the Stockholm Convention on Persistent Organic Pollutants sought to ban 12 chemicals that persist in the environment, bio-accumulate in the food chain and pose health risks to humans. One hundred countries signed this and by 2008 160 countries had signed this agreement to restrict or eliminate the use of these chemical substances. Despite this DDT continues to be used in certain countries for the control of malaria (Van den Berg 2009). Following the Stockholm convention, studies have shown that the dietary intake of DDT and its metabolites have

reduced significantly (Agency for Toxic Substances and Disease Registry [ATSDR] 2002) and serum concentrations have dropped (Centers for Disease control and Prevention [CDC] 2003).

Commercial DDT is a mixture of several closely-related compounds. The two mayor components are p,p' DDT and o,p' isomer. DDT and its metabolite Dichlorodiphenyldichloroethylene (DDE) form as a result of dechlorination (Gold and Brunk 1982). These are also the major metabolites and breakdown products in the environment.

i. Endocrine disrupting effects of DDT

DDT has a strong bonding capability with the organic chemicals in soil and is virtually water insoluble. Wet life such as fish digest or absorb the DDT due to the lipophilic nature leaving little in the water. On land, food provides the major source. The highly lipophilic and low water solubility DDT readily binds to, and is stored in, fat with resultant bioaccumulation along the food chain (Kelly et al. 2004). In humans DDT has been demonstrated to have a long half-life of approximately 4 years, with DDE having a half-life that is probably even longer (Longnecker 2005).

Animal studies have supported the evidence that DDT is an endocrine disruptor. Studies of alligators born in contaminated lakes have shown altered steroid hormone profiles (Guillette et al. 1995). Further work by this group found alligators with multi-oocyte ovaries with polynuclear oocytes (Milnes and Guillette 2008). Studies in the granulosa cells of pigs have shown that DDE affects progesterone synthesis and high levels block progesterone production (Crellin et al. 1999; Crellin et al. 2001).

In humans, the DDT metabolite DDE acts as an androgen antagonist, but not as an oestrogen agonist. p,p'-DDT, has little or no androgenic or estrogenic activity

(Cohn et al. 2007). The o,p'-DDT has weak estrogenic activity. Cross sectional studies suggest that p,p'DDE serum levels in pregnant women were associated with delayed TTP (Axmon et al. 2006; Law et al. 2005). However a Study in a Mexican women did not find any correlation between p,p'DDE and p,p'DDT (Harley et al. 2008).

ii. DDT and the effects in IVF

The effects of DDT/DDE on the outcome of IVF are conflicting. Several studies have quantified the levels of DDT/DDE in serum and follicular fluid samples of patients undergoing IVF. Younglai et al. (2002) stated that 50% of women attending for fertility treatment had been exposed to environmental chemicals sufficient enough to produce detectable concentrations in their blood and follicular fluid, the most frequently being detected being p,p-DDE.

It has been demonstrated that the concentrations of p,p'-DDT and its metabolite p,p'-DDE were 2 to 3 times higher in the serum as opposed to the follicular fluid, despite considerable variability between subjects (Meeker et al. 2009). This was probably due to the higher lipid content in serum (0.6%) as opposed to follicular fluid (0.03%). This study was performed over two time periods and led to two interesting findings: a greater degree of reliability in the serum organochlorine concentrations and a decline in the serum concentrations possibly due to falls in environmental levels.

A small Canadian study investigating POPs in women undergoing IVF in three different clinics within Canada found regional differences in the levels of DDE, but there were no effects on either the rate of fertilisation or cleavage (Jarrell et al. 1993). A large case controlled study of 617 women undergoing IVF measured the effects of DDT and its metabolites on implantation and pregnancy rates (Al-Salah et al. 2009). The levels of DDE were 1.646ug/L, and 0.407ug/L in serum and follicular fluid respectively, and the authors found no association between

p,p-DDE levels and implantation rates or pregnancy outcomes. Although the levels of p,p-DDE in this study were comparable to the studies above, p,p-DDE was detected in 77.7% of the subjects which was thought to be a cause for concern.

Mahalingaiah et al. (2012) studied 720 women undergoing IVF and the effect of DDE and DDT. Both DDE and DDT were detectable in all the women with median levels of 1.04ng/g and 1.12ng/g respectively. This was lower than the general population of many African and Asian countries where DDT is still in use. The authors again demonstrated no significant association between DDT/DDE levels and IVF outcomes.

These studies have shown that DDT/DDE is still detectable in the environment despite being banned for several decades. However the levels appear to vary depending on region and diet and only one study by Law et al. (2005) found a weak correlation between DDT/DDE exposure and time to pregnancy. More recent studies have shown that following the banning of these substances levels are dropping.

1.4.6 Brominated fire retardents (BFRs)

BFRs are used in industrial and consumer products and have resulted in a reduction in the incidence of fires by inhibiting the ignition of a flame. There are two groups of fire retardants, polychlorinated biphenyls (PCBs') and polybrominated diphenyl ethers (BDEs).

PCBs were widely used in electrical apparatus. However, due to PCBs' environmental toxicity and classification as a persistent organic pollutant, PCB production was banned by the United States Congress in 1979 and by the Stockholm Convention on Persistent Organic Pollutants in 2001. BDEs are chemically similar to the long banned PCBs and have been marketed as one of three compounds known as pentabrominated BDE, octabrominated BDE and

decabrominated BDE. Decabrominated BDE is the most widely used BDE in the world and is still manufactured in the USA and Europe. However pentabrominated BDE and octabrominated BDE have been banned in the European Union and the USA (Costa et al. 2007). In the manufacturing process PBDEs are added to the polymer product, as opposed to chemically bonded and as a result can leach from the product into the environment (Costa et al. 2008).

i. Endocrine disrupting Effects of Brominated fire retardants

Like PCBs and other persistent organochloride pollutants, BDEs are highly lipophilic compounds and are detected in soil, fish, and terrestrial animals. They bioaccumulate; a process which amplifies in each higher species resulting in significant levels being detected in humans (Hale et al. 2003; Law et al. 2006). The main sources of exposure are through the diet mainly from meat and dairy products high in fat, but also from fish and from house dust (Frederiksen et al. 2009).

A North American study highlighted the almost universal exposure of the US population to BDEs with 97% of people tested having detectable levels in their blood (Sjodin et al. 2008). The levels were almost 20 times the concentrations of PBDEs in their serum and fat compared with the European population 200ng/g vs 5ng/g lipid lipid (Lorber 2008). This study also found the greatest exposure to PBDE is through household dust, accounting for 82% of overall estimated intake. However while levels of PCBs have been decreasing in human serum, the levels of BDEs are increasing, especially the congeners, BDE-47, BDE-99, BDE-100 and BDE-153 which are the ingredients of penta-BDE (Sjodin et al. 2008; Horton et al. 2013). However as Horton et al. (2013) noted, despite these levels being lower than other US studies possibly due to legislation, they were almost 8 times higher than European levels (Foster et al. 2011).

One study has investigated a potential association between PBDE and fecundability in women by using Time to Pregnancy {TTP} (Harley et al. 2010). Again the authors found the most common congeners to be BDE-47, BDE-99, BDE-100 and BDE-153 in >97% of the women tested, and there was a significant decrease in fecundity in these women. The authors accept this is the first study to investigate fecundity in this context and only tested the female partner. Larger studies would be required to resolve this issue, however exposure to BDEs will continue to rise as a result the large amounts of household products that contain these chemicals.

ii. Animal studies on the Endocrine disrupting effects of Brominated fire retardants

The most common congeners of BDEs present in animals and humans are 2,2',4',-tetra-BDE (BDE-47), 2,2',4,4',5-penta-BDE (BDE-99) and 2,2',4,4',6-penta-BDE(BDE-100) (Sjodin et al. 2003). The major commercial BDE used in research is DE-71, which constitutes predominantly tetra-BDE-47 and penta-BDE-99 and 100. DE-71 has marked effects on the thyroid gland and the reproductive axis acting as agonists and antagonists to oestrogen, progesterone and androgen receptors (Mc Cormack et al. 1981; Lilienthal et al. 2006; Stoker et al. 2004; Stoker et al. 2005 and Darnerud 2008). DR-71 also disrupts the development of the reproductive system and folliculogenesis. It is important to remember that the thyroid is important for ovulation, fertilisation and maintaining pregnancy (Chevrier et al. 2010; Wang et al. 2008).

Bovine and porcine studies investigating BDE congeners BDE-77 and BDE-153 demonstrated reduced progesterone secretion in the follicular cells. However the mechanism remains to be determined. The other common congeners BDE-47 and BDE-100 are inhibitors of dihydrotestosterone-induced transcription activation of the human AR in vivo. Such an effect is not induced by BDE-99, which, by contrast, displays competitive binding to AR (Stoker et al. 2005).

Bovine studies on DE-47 (BDE-99, BDE-100 and BDE-47) demonstrated an elevated progesterone/testosterone ratio, but a reduced testosterone/17 β -oestradiol ratio in the ovarian follicle, suggesting premature luteinisation of the antral follicles.

Lilienthal et al. (2006) investigated the effects of low and high exposure to the common congener BDE-99, on rats' sexual development. It was found that puberty in the offspring was delayed or accelerated depending on the dose of BDE-99, but that the number of primordial follicles was reduced on a low dose (1mg/kg body weight), whereas the number of secondary follicles was reduced in a high dose group (10mg/mg body weight). This study also highlighted a significant reduction in oestrogen and testosterone levels in both the female and male offspring respectively, though the mechanism was not fully understood. These studies highlight the finding that BDE mixtures influence follicular homeostasis either by affecting local mechanisms in recruiting the primordial follicles or the growth of the developing follicles by interfering with androgen production. The anti-androgenic effect could result in excessive progesterone synthesis resulting in disruption to ovarian function, as androgens are critical in the production of oestrogen and in the suppression of progesterone secretion in the follicles.

iii. Human studies on the endocrine disrupting effects of Brominated fire retardants

Little work is available on BDEs and exposure in a human population. It is well known that BDEs are present in human milk (Law et al. 2006) although there has been a slight reduction since penta-BDE was banned (Sjodin et al. 2003). A Danish study in boys noted an association between BDE levels in breast milk and congenital cryptorchidism and serum LH levels with congeners BDE-47 and BDE-100. (Main et al. 2007). Deca-BDE, still commercially available, can be converted through exposure to sunlight into lower brominated BDEs, which are

more readily absorbed by the gut and stored within adipose tissue, where they bio-accumulate due their long half-lives. Whether this affects their endocrine disrupting effects is unknown (Watanabe and Tatsukama 1987).

iv. Brominated fire retardants and IVF

Studies undertaken in the IVF setting have demonstrated PBDE congeners in follicular fluid and serum (Petro et al. 2012; Meeker et al. 2011, Meeker et al. 2009; De Felip et al. 2004; Younglia et al. 2002; Johnson et al. 2012). A US study found that BDE-47, BDE-99, BDE-100, BDE-153 and BDE-154 were present at highest concentrations in both follicular fluid and serum (Johnson et al. 2012). The main congeners BDE-47, BDE-99, BDE-100 and BDE-153 found in these women are representative of other studies performed in the US (Sjodin et al. 2008). 39% of women, who had BDE-153 detectable in their follicular fluid, had elevated odds of failed implantation compared to those without detectable levels of BDE-153 (Johnson et al. 2012). However, the authors stated that, the concentrations of BDEs in serum may be a better marker of infertility, as there are problems in detecting BDEs in follicular fluid, and the great variability in ratios of BDEs in follicular fluid and serum between subjects could be due either to the exposure level or susceptibility between subjects. It was concluded that “serum measurements of PCBs were reliable measures of exposure to the oocyte” (Johnson et al. 2012).

Another recent study found BDE-153 to be the BDE congener in highest concentration in both follicular fluid and serum (Pedro et al. 2012). A strong association between a higher concentration of BDE-153 and reduced fertilisation rate was reported ($p < 0.00001$) and a reduction in the developmental capability of the oocyte to develop into a high-quality embryos ($p < 0.05$). This remained the case even when the results were adjusted for independent variables such as age, BMI, IVF/ICSI and male infertility. These results corroborate with a large prospective study of 765 women undergoing

IVF, which found the highest concentrations of the congener BDE-153 were associated with implantation failure (Meeker et al. 2011). Both Meeker et al. (2011) and Johnson et al. (2012) reported comparable levels of BDE-153 within follicular fluid, and the levels of contamination were two or more times lower than studies performed a decade earlier (De Felip et al. 2004; Younglia et al. 2002). It is clear that within the ovarian follicle there is possible disruption of this very fragile environment by these persistent polychlorinated pollutants.

1.4.7 Phthalates

Phthalates are used in a vast array of household, medical and industrial products. These include children's toys, detergents, paints, coatings for pharmaceutical tablets, cosmetics and food packaging, but their main use is as a plasticizer for the manufacture of polyvinyl chloride (PVC) (Heudorf et al. 2007). Phthalates are diesters of phthalic acid, a derivative from the oxidation and hydrolysis of naphthalene. Naphthalene is a polycyclic aromatic hydrocarbon derived from coal tar and is produced during the refining of petroleum.

Phthalates are not covalently bonded to the plastic products and can therefore leach out into the environment. This process can happen over time as the product ages, following ultraviolet light exposure and use. As a result, humans are exposed on a daily basis to this ever-present environmental contaminant (Thomas and Thomas. 1984). The most common phthalate within the environment is Di-(2-ethylhexyl) phthalate (DEHP) (ATDSR 2002). It has been proposed that the maximum daily exposure to DEHP is approximately 2mg/day, but occupational exposure may lead to, much higher levels (ATSDR 1993).

i. Endocrine disrupting effects of phthalates

Like the other POPs discussed, phthalates are water insoluble, but do not bioaccumulate within fat due to their short life, however their widespread availability causes toxic effects (Koch and Calafat 2009). The main route of

exposure in humans is ingestion (Fromme et al. 2007); they enter the food chain through the production and packaging of food (Xu-Liang 2010). Once ingested the phthalate diesters are hydrolysed in the gut to monoesters, which are further metabolised and excreted in the urine (Williams and Blanchfield 1975; Frederiksen et al. 2007). The most common monoester is monoethylhexyl phthalate (MEHP) the active metabolite of DEHP (Heudorf et al. 2007). In humans “phthalates are eliminated mostly within hours with excretion complete by a day or two: half-lives in the body are in hours” (Koch and Calafat 2009).

ii. Animal studies on the endocrine disrupting effects of phthalates

Animal studies have shown that phthalates act on the reproductive system in different ways. In vivo studies in rats demonstrated that DEHP significantly decreased oestradiol and aromatase mRNA (Xu et al. 2010). This suppression of ovarian production of oestradiol could result in an insufficient level to trigger the ovulatory surge of LH. In vitro studies have shown that DEHP and its metabolite MEHP again reduce oestradiol production and inhibit antral follicle growth (Gupta et al. 2010). Within the ovary the pre-ovulatory follicle is the site for oestradiol production and it has been suggested that the granulosa cells within the follicle are the targets of DEHP and MEHP (Lovekamp-Swan and Davis 2003). The enzyme aromatase within the granulosa cell is required for the production of oestradiol and MEHP can alter the availability of this enzyme in a dose-dependent manner (Lovekamp and Davis 2001).

iii. Human studies on the endocrine disrupting effects of phthalates

There have been few epidemiological and human studies on the effects of phthalates on human reproduction. Exposing human granulosa cells to MEHP suppressed oestradiol production and decreased aromatase mRNA activity (Reinsberg et al. 2009). There is an association between urinary biomarkers for phthalates and precocious puberty in girls and an association with higher levels

of phthalates and early thelarche and secondary breast development (Colon et al. 2000). A Chinese study showed that those girls with higher serum levels of DEHP had significantly larger ovaries and uteri compared to controls (Durmaz et al. 2010). However, a North American study found no difference between urinary biomarkers of phthalates between girls with precocious puberty and controls (Lomenick et al. 2010). These results could be confounded by ethnicity, age and locations of studies.

iv. Phthalates and IVF

Studies within the female population of reproductive age have shown that concentrations of phthalate metabolites are most frequently detected in urine (Hines et al. 2009; Hogberg et al. 2008) and were only found in <10% of saliva and breast milk samples (Hines et al. 2009). In a small study of 5 patients undergoing IVF, follicular fluid was collected at the time of ovum retrieval to discover if there was any evidence of accumulation of phthalates or their metabolites as a result of the brief exposure to medical plastics (Krotz et al. 2012). The phthalate levels were less than 15ng/mL which is below the level to disrupt the reproductive axis, but similar to serum levels published in previous studies (Hogberg et al. 2008). This suggests, that phthalates are present but not concentrated in follicular fluid possibly due to the short half-life of these compounds.

1.4.8 Bisphenol A

Bisphenol A (BPA) is a chemical primarily used in the production of polycarbonate plastic sheets and epoxy resins used in many consumer products, such as baby bottles and water bottles, sports equipment, CDs, and DVDs (Biles et al. 1999). Epoxy resins containing BPA are used to line water pipes, and act as coatings on the inside of many food and beverage cans (Bae et al. 2002).

The main source of BPA is through dietary intake. Despite being an inert plastic when exposed to heat, light and acidic/alkaline environments such as in food packaging, BPA can leach out and contaminate the food (Geens et al. 2012) The estimated dietary intake of BPA from polycarbonate plastic and epoxy resin food contact applications, based on the results of multiple migration studies with consistent results, is less than 0.000118 mg/kg body weight/day. The potential human exposure to BPA is more than 400 times lower than the maximum acceptable or "reference" dose for BPA of 0.05 mg/kg body weight/day established by the U.S. Environmental Protection Agency (Diamanti-Kandarakis. 2009).

An independent analysis by the European Commission's Scientific Committee on Food (ESCF), using a similar methodology, has confirmed the safety of polycarbonate plastic and epoxy resin food contact applications. The ESCF estimated total dietary intake of BPA from all food contact sources to be in the range of 0.00048 to 0.0016 mg/kg body weight/day, which is below the Tolerable Daily Intake set by the SCF of 0.01 mg/kg body weight/day.

The use of polycarbonate plastic and epoxy resins for food contact applications has been, and continues to be, recognized as safe by the U.S. Food and Drug Administration, the European Commission's Scientific Committee on Food, the United Kingdom Food Standards Agency (FSA), and other regulatory authorities worldwide. Recently, both the FDA and the European Food Safety Authority issued statements stating that they could not identify new evidence, which would lead them to revise their opinion that the presently known levels of exposure to BPA are safe.

i. Endocrine disrupting effects of Bisphenol A

BPA is an estrogenic EDA that binds to both the α - and β -oestrogen receptors (Hiroi et al. 1999) and has a molecular structure similar to diethylstilbestrol

(Dodds and Lawson 1936). Studies using both nuclear oestrogen receptor binding assays and transcriptional activation assays have demonstrated that BPA has a 10,000 fold lower affinity for the α - and β -oestrogen receptors than 17- β oestradiol (Zoeller et al. 2012). However, BPA can have an additive effect within the body, resulting in significant effects even at low doses (Rajapakse et al. 2002).

ii. Animal studies on the endocrine disrupting effects of Bisphenol A

Animal studies have demonstrated that exposure to BPA can reduce fertility by several mechanisms. Including oestrogen suppression, chromosomal abnormalities and modified oocyte maturation. In vivo studies in mice exposed to BPA have demonstrated a significant dose related increase in oocyte aneuploidy (Hunt et al. 2003). Further in vitro studies on mice exposed to BPA again reported further chromosomal abnormalities including meiotic spindle formation and dysfunction of the centromere (Lenie et al. 2008; Machtinger et al. 2011). Machtinger et al. (2013) repeated the animal studies with human oocytes collected from IVF patients and again observed a negative effect on spindle formation and chromosome function.

Studies on rat and porcine theca and granulosa cells exposed to BPA demonstrated dose dependent alterations in the levels of the sex steroids, with increased testosterone production in the theca cells and decreased progesterone and 17- β oestradiol production by the granulosa cells (McCormack et al. 1981; Zhou et al. 2008; Mlynarcikova et al. 2005). A more recent study in mice exposed to BPA indicated that high doses of BPA inhibited growth of antral follicles and reduced production of progesterone and 17- β oestradiol (Peretz et al. 2011).

iii. Human studies on the endocrine disrupting effects of Bisphenol A

BPA has been found in various human fluids including serum, follicular fluid and amniotic fluid (Ikezuki 2002). Several studies have shown increased levels of BPA in infertile women undergoing IVF. An Italian study demonstrated that infertile women had higher levels of BPA compared to healthy controls but also significantly higher expression of several nuclear receptors that regulate endocrine pathways (Caserta et al. 2013), including both the α - and β -oestrogen receptors and androgen receptors.

iv. Bisphenol A and IVF

In women undergoing IVF BPA is associated with a reduced 17- β oestradiol response during IVF (Ehrlich et al. 2012; Bloom et al. 2011). This would agree with the growing body of literature reporting reduced granulosa cell oestrogen synthesis response to BPA exposure. This reduction is secondary to inhibition of granulosa cell aromatase activity stimulated by FSH, which is necessary for the theca cell conversion of androgens to oestrogens. Bloom et al. (2011) did not find any effect of BPA concentrations and ovarian reserve parameters i.e. AFC, basal FSH and number of oocytes retrieved. However an earlier study did report an average 12% decrease (95%CI = -23%, -45) in the number of oocytes retrieved per log unit increase in urine total BPA (Mok-Lin et al. 2010).

v. Relationship between Bisphenol A and PCOS

Research by Fernandez et al. (2009) found that neonatal exposure to BPA in female rats had serious effects on the organisation of hypothalamic-pituitary axis. A further study by this group exposed neonatal rats to BPA at 500ug/ul (a dose higher than the lowest observed adverse effect), 50ug/ul or 5ug/ul (both lower than the lowest observed adverse effect level) from day 1 to 10 in the postnatal period (Fernandez et al. 2010). Exposure to the 500 and 50 ug/ul/day caused accelerated GnRH pulse frequency and a reduction in the inter-pulse intervals. There were elevated levels of testosterone production and multiple

ovarian cysts. They hypothesised that these findings could be linked to the development of a PCOS like syndrome both at low and high exposure to BPA. A recent study also found high doses of BPA led to multiple ovarian cyst production (Newbold et al. 2009).

A further study demonstrated a link between BPA and insulin resistance, mediated via the classical α - and β -oestrogen receptor pathway, occurring at both low and high doses of BPA. Chronic exposure resulted in hyperinsulinemia and altered glucose tolerance tests, revealing a possible association between environmental oestrogens and insulin resistance. (Alonso-Magdalena et al. 2006) However further work needs to be done to investigate whether BPA exposure in animal models causes a PCOS like condition or PCOS itself in order to make a relevant comparison in humans.

A few human studies have examined the effect of EDAs in PCOS. For example, a cross-sectional study compared BPA levels in PCOS patients and controls (Kandaraki et al. 2011). In this study the PCOS patients were divided into lean (BMI <25), overweight (BMI \geq 25) and obese. BPA levels were significantly higher in the PCOS group vs the controls (1.05 ± 0.56 vs 0.72 ± 0.37 ng/ml) though the study did not state whether such levels could cause a risk to human health. Multiple regression analysis for BPA showed a significant correlation with PCOS with a significant association with testosterone, androstenedione, and insulin resistance (Kandaraki et al. 2011). An earlier study found similar results when comparing BPA levels with the incidence of PCOS. Serum BPA levels were again significantly higher in both lean and obese women with PCOS when compared with lean and obese controls (Takeuchi et al. 2004). There was a significant positive correlation between testosterone and androstenedione.

These data could be a result of excess androgens affecting the clearance of BPA. It is known that patients with PCOS have reduced levels of SHBG possibility due

to obesity resulting in peripheral insulin resistance and hyperinsulaemia. The resultant hyperinsulaemia affects the hepatic production of SHBG, resulting in higher free testosterone levels. Certain environmental chemicals such as Bisphenol A also have the ability to bind to SHBG and thus to displace endogenous sex hormones.

SHBG is a glycoprotein that binds to sex hormones, specifically 17β -oestradiol and testosterone. Testosterone and 17β -oestradiol circulate in the bloodstream, bound mostly to SHBG and to a lesser extent serum albumin. Only a very small fraction of about 1-2% is unbound, or "free," and thus biologically active and able to enter a cell and activate its receptor. SHBG inhibits the function of these hormones. Thus, bioavailability of sex hormones is influenced by the level of SHBG. The relative binding affinity of various sex steroids for SHBG is dihydrotestosterone (DHT) > testosterone > androstenediol > oestradiol > estrone (Somboonporn and Davis 2004).

These studies demonstrate that although these environmental chemicals bind to SHBG and have a competitively binding activity for both testosterone and 17β -oestradiol, they have a very low binding affinity compared to the endogenous hormones, but are potent SHBG ligands (Dechaud et al. 1999). These endocrine disruptors could thus disrupt the androgen-oestrogen balance of the body resulting in PCOS like conditions. This could be relevant as many endocrine disrupting chemicals are lipid soluble and individuals could be exposed to them constantly from childbirth or before, especially as a result of accumulation in the food chain. The higher level of BPA in the PCOS population may arise as a consequence of BPA binding to the SHBG resulting in higher testosterone levels and a decreased clearance of BPA leading to higher concentration.

1.5 Vitamin D

Vitamin D is a fat-soluble steroid hormone responsible for calcium and phosphorous balance in the body. Its main target tissues are bone, the intestine and kidney. There are two forms of Vitamin D, ergocalciferol (D2) and cholecalciferol (D3). The body derives Vitamin D from two sources, exposure to ultraviolet light and diet. The precursor for Vitamin D is an intermediary in the cholesterol pathway called 7-dehydrocholesterol that is present in the skin (Holick 2007).

1.5.1 Synthesis of Vitamin D

D3 is produced from 7-dehydrocholesterol within the epidermis of the skin on exposure to ultraviolet light that accounts for the main source of Vitamin D approximately 80-90% (Holick 2006). D2 is produced in plants from ergosterol and supplies between 10-20% of Vitamin D via the diet (Bouillon et al. 1998).

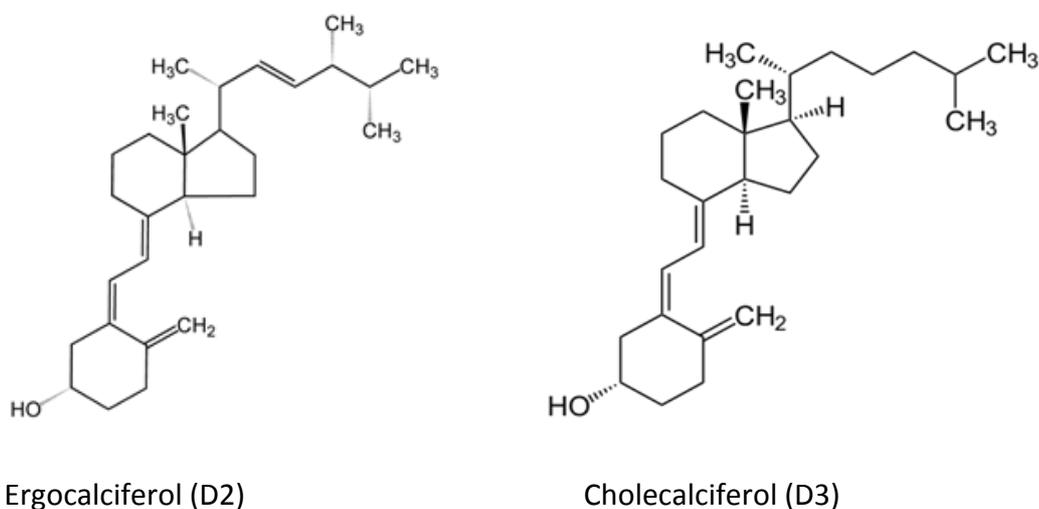


Figure 1.9 Chemical structure of Ergocalciferol and Cholecalciferol.

D2 and D3 the inactive forms of vitamin D are converted to 25-hydroxy-vitamin D (25(OH)D) by the enzyme 25-hydroxylase within the liver. The second step in the activation process occurs in the distal tubules of the kidney where the 25(OH)D is converted into the active form 1,25-dihydroxy-vitamin D

[1,25(OH)₂D₃] otherwise known as calcitriol, by the enzyme 1 α -hydroxylase (Figure 1.9). The enzyme 1 α -hydroxylase is also found in other tissues allowing for the local conversion of 25(OH)D to the active 1,25(OH)₂D₃ (Holick. 2007). The biological effects of Vitamin D occur through the vitamin D receptor (VDR), a member of the nuclear receptor family of transcription factors (Makishima et al. 2002). Once Vitamin D binds to the VDR this then heterodimerizes with the retinoid X receptor, which then binds to the vitamin D response element located in the promoter regions of the target genes (Jones et al. 1998).

The main sites for the VDR is within the Intestinal epithelial cells and osteoblasts where it mediates the actions of 1,25(OH)₂D₃ to promote intestinal calcium and phosphate uptake and promote musculoskeletal health (Hausler et al., 2008). VDR has been found in other tissues including the skin, cells within the immune system, colon, pancreas, endothelial and smooth muscle cells of the vasculature and may be implicated in immunosuppression, antimicrobial defence, and cardiovascular benefits by aiding oxidative stress reduction. VDR is expressed in the ovaries (Agic et al. 2007) and the endometrium, with one group demonstrating the endometrium capable of synthesising the active form of Vitamin D and that 1 α -hydroxylase was expressed within the endometrium (Vigano et al. 2006).

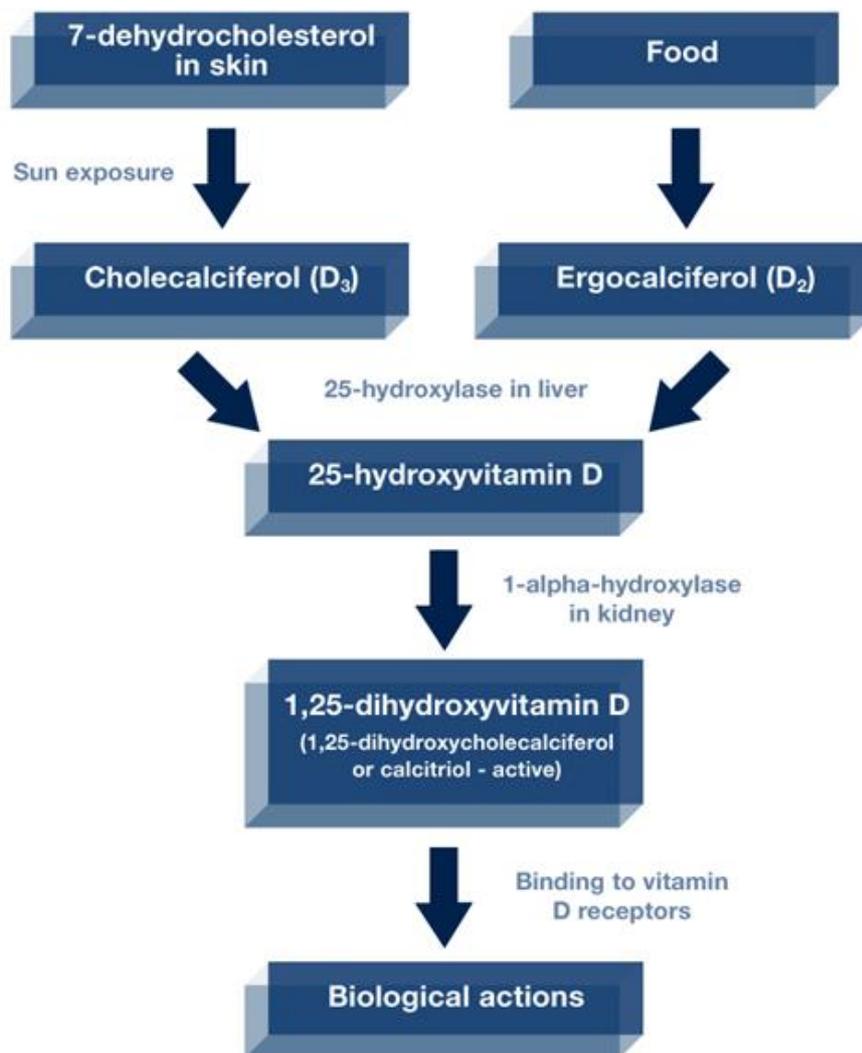


Figure 1.10 Steps of the activation process of Vitamin D

1.5.2 Vitamin D deficiency

Vitamin D deficiency is a very common problem. Vitamin D levels within the body are defined using serum levels of 25(OH)D; which has a longer circulatory half-life than the active metabolite 1, 25(OH)₂D₃ (Holick 2007). The Endocrine Society defines Vitamin D deficiency as ≤ 20 ng/ml, Vitamin D insufficiency as 20-30 ng/ml and Vitamin D replete as ≥ 30 ng/ml (Holick et al. 2011). To convert ng/ml into nmol/L multiply by 2.496. Thus deficiency refers to levels less than 50 nmol/L, insufficiency refers to levels between 50 – 75 nmol/L. and sufficiency refers to levels above 75 nmol/L.

Causes of deficiency include lack of exposure to ultraviolet radiation to the skin (Chen et al., 2007), increased skin pigmentation (Clemens et al. 1982), application of sun creams which can reduce the production of Vitamin D by the skin (Matsuoka et al. 1987), latitude (the further from the sun, the less cholecalciferol is formed) (Holick. 2003) and medical conditions such as malabsorption disorders including Crohn's, liver and renal disease. Obesity can result in Vitamin D deficiency, as described by Wortsman et al. (2000) who concluded "Obesity-associated vitamin D insufficiency is likely due to the decreased bioavailability of vitamin D₃ from cutaneous and dietary sources because of its deposition in body fat compartments." Thus clearly living in a Northern latitude, with long winters with more frequent use of sun cream in the summer months combined with increasing obesity rates make Vitamin D deficiency endemic in the UK population. Studies suggest that between 30-50% of both the adult and adolescent population are at risk of vitamin D deficiency (Tangpricha et al. 2002, Gorgon et al. 2004).

1.5.3 Animal studies of Vitamin D deficiency and infertility

Over 70 % of vitamin D deficient mice have reduced fertility and 30% reduced litter sizes (Halloran and DeLuca. 1980). Animal studies have demonstrated that calcium participates in the activation and maturation of oocytes suggesting a role in folliculogenesis. However, it has been postulated that the restoration of fertility in these Vitamin D deficient animals is as a result of calcium supplementation (Johnson and DeLuca. 2001). The effect of the Vitamin D deficiency results in disrupted calcium regulation within the reproductive tract and thus diminished fertility. Thus fertility is restored not by normalising the hypocalcaemia in vitamin deficient mice, but requires Vitamin D (Kwieceński et al. 1989).

Studies in which VDR and 1 α -hydroxylase was deleted resulted in infertility with decreased oestrogen and progesterone, and elevated LH and FSH (Panda et al.

2001; Sun et al. 2010). VDR knockout female mice showed impaired folliculogenesis, with no follicles developing past the secondary stage, and decreased aromatase expression thus confirming the importance of Vitamin D in folliculogenesis (Kinuta et al. 2000; Yoshizawa et al. 1997). The 1α -hydroxylase mice also demonstrated impaired follicular development and uterine hypoplasia (Sun et al. 2010). The VDR and 1α -hydroxylase knockout mice conceived infrequently, having smaller litters of offspring. However fertility was partially corrected by calcium supplementation in the VDR knockout mice although litters remained small with underweight offspring (Sun et al. 2010). In the 1α -hydroxylase deficient mice fertility was corrected when feed a diet high in calcium. These results would suggest that the infertility demonstrated in these animals is not a direct action of Vitamin D deficiency but possibly mediated by calcium.

1.5.4 Vitamin D and Polycystic Ovary Syndrome PCOS

Between 67-85% of PCOS patients have low serum levels of 25(OH)D (Thomson et al. 2012). Observational studies have suggested that Vitamin D may affect the metabolic features of PCOS namely insulin resistance and FAI (Ngo et al. 2011; Wehr et al. 2009). These studies, despite being inconsistent, suggest there is an inverse relationship between Vitamin D, insulin resistance and FAI, however what affect Vitamin D has on infertility in PCOS women is unclear.

1.5.5 The effect of Vitamin D on AMH

The granulosa cells of the follicle express AMH. AMH is involved in the recruitment of follicles from the primordial pool, and limiting the formation of these primary follicles by inhibiting excessive follicular recruitment by FSH (Dewailly et al. 2014). Research has shown that environmental factors including vitamin D can alter its expression (Merhi et al. 2014). Wojtusik and Johnson (2011) tested the effect of $1,25(\text{OH})_2\text{D}_3$ regulated AMH mRNA in the granulosa

cells of chickens and found enhancement of granulosa cell proliferation of 3-5mm and 6-8mm follicles incubated in $1,25(\text{OH})_2\text{D}_3$.

AMH has its effect through the AMH receptor (AMHR-II). Thus, AMH is unregulated at the time of primordial follicle development but down regulated at the time of transition from primordial to primary follicle through expression of the AMHR-II. There is evidence to show that $1,25(\text{OH})_2\text{D}_3$ could have an inhibitory effect on AMHR-II expression, thus inhibiting the inhibitory effect of AMH on the granulosa cells. This would thus allow follicles to mature and ovulate. A study on human granulosa cells attained from patients undergoing IVF, were cultured with or without Vitamin D. An inverse relationship between follicular fluid $25(\text{OH})\text{D}$ and AMHR-II gene expression was found (Merhi et al. 2014). Those patients who were deficient in $25(\text{OH})\text{D}$ ($<30\text{ng/ml}$), had a twofold increase in AMHR-II expression compared with those replete in $25(\text{OH})\text{D}$ ($>30\text{ng/ml}$). Those granulosa cells cultured with $25(\text{OH})\text{D}$ had a statistically 32% decrease in AMHR-II mRNA levels (Merhi et al. 2014).

Advanced glycation end-products (AGEs), which are pro-inflammatory molecules, are involved in the pathogenesis of PCOS and accumulate in the theca and granulosa cells of the ovary (Merhi et al. 2013). A study of PCOS and control vitamin D deficient women was performed to see what effect Vitamin D supplement for 8 weeks would have on the soluble receptor for AGEs (sRAGE) and AMH (Irani et al. 2014). sRAGE is a soluble receptor that binds to AGEs and prevents their harmful effect on the follicle (Merhi et al. 2013). Vitamin D supplementation increased serum sRAGE ($P = .03$) and decreased serum AMH levels ($P < .001$) in the PCOS patients but not the controls. The increase in serum sRAGE positively correlated with the increase in serum $25(\text{OH})\text{D}$ after supplementation in the PCOS group ($r = 0.6$, $P = .01$).

AMH has a repressive effect inhibiting the loss of the oocyte pool by inhibiting the recruitment of primordial follicles and slowing down growth that is followed by follicular atresia and death. In PCOS patients the high AMH reflects stasis in the oocyte pool culminated in high numbers of primary and pre-antral follicles and classic polycystic ovarian morphology (Mason 2001). Optimal levels of vitamin D may have the ability to counteract the repressive effects of the AMH on the granulosa cells thus allowing the multiple follicles in the PCOS ovaries to mature. AMH, like Vitamin D undergoes seasonal changes. Studies have shown that seasonal variation in AMH is correlated with the seasonal changes to circulating Vitamin D. Supplementing patients with Vitamin D can stop the seasonal changes in both the Vitamin D and AMH (Dennis et al. 2012).

1.5.6 Vitamin D, insulin resistance (IR) and PCOS

Vitamin D may be associated with IR in PCOS patients. Many studies associate IR with obesity in PCOS however many lean women with PCOS have IR. Studies have reported lower Vitamin D levels in obese PCOS women compared to normal weight controls (Hahn et al. 2006). A review which performed a univariate regression analysis revealed that an increase in serum vitamin D levels was significantly associated with a reduction in HOMA-IR in PCOS and controls, however after multivariate regression analysis with Vitamin D and BMI as independent variables serum Vitamin D was no longer an independent predictor of IR in PCOS, but remained significant for the control patients (Kruel-Poel et al. 2013).

Besides obesity how else can Vitamin D deficiency be associated with IR. Vitamin D regulates intra- and extra-cellular calcium that is essential for the insulin mediated processes in insulin sensitive tissue such as skeletal muscle (Pittas et al. 2007). Low circulating Vitamin D may induce an inflammatory response resulting in insulin resistance (Shoelson et al. 2007).

1.5.7 The Effect of Vitamin D on Hyperandrogenism in PCOS

There has been conflicting evidence regarding the effects of Vitamin D and hyperandrogenism in PCOS. Studies have demonstrated an association between 25(OH)D with FAI and SHBG but not with total testosterone, androstenedione, dehydroepiandrosterone sulphate (DHEAS) and oestradiol. (Hahn et al. 2006; Wehr et al. 2009). However Hahn et al. (2006) made no adjustments for BMI. Yildizhan et al. (2009) reported a correlation between 25(OH)D levels with testosterone and DHEAS.

1.5.8 Vitamin D supplementation and its effect on PCOS

Vitamin D and/or calcium supplementation can help normalise menstruation (Thys-Jacobs et al. 1999; Rashidi et al. 2009) improve insulin resistance and reduce androgens levels associated with PCOS (Selimoglu et al. 2010; Kotsa et al. 2009).

A recent study demonstrated that Vitamin D supplementation in PCOS patients resulted in a significant drop ($P=0.003$) in AMH levels but had no effect in non-PCOS patients. This again indicates that in PCOS Vitamin D has a role in the metabolic disturbances in PCOS. This study also highlighted that, as the BMI increases, the effect of supplementation is smaller requiring higher doses of supplemented Vitamin D, probably due to its deposition in body fat (Irani et al. 2014).

From this survey of the literature it is clear that the majority of PCOS patients are Vitamin D deficient. There are small pilot studies stating this may contribute to the metabolic consequences of PCOS. However there is no clear evidence from properly powered randomised controlled trials to confirm these answers. The supplementation studies again show a positive effect of optimising Vitamin D concentrations in these patients, but these are small, uncontrolled observational studies with no clear link into how and why this happens.

However it is clear that if these patients are optimised prior to conceiving either naturally or through assisted technology i.e. weight loss or normalisation of Vitamin D concentrations, these may have a positive outcome by lowering AMH eliminating insulin resistance resulting in better follicular function and/or restoration of ovulation.

1.5.9 Vitamin D and IVF outcome

A few observational studies have been published seeking to correlate 25(OH)D levels in follicular fluid with IVF outcomes. One such study found 25(OH)D levels within serum and follicular fluid to be highly correlated ($r=0.94$) and that those patients with higher serum and follicular fluid levels of 25(OH)D had significantly higher clinical pregnancy rates ($p=0.04$). Multivariable logistic regression analysis demonstrated 25(OH)D as an independent variable for IVF success; each 1ng/ml increase in follicular fluid 25(OH)D increased the likelihood for achieving a clinical pregnancy by 6%. (Ozkan et al. 2010). A further study demonstrated higher clinical pregnancy rates in Caucasian patients undergoing IVF with normal serum 25(OH)D levels, but an inverse relationship in Asian patients who had higher pregnancy rates with lower 25(OH)D levels, demonstrating a possible genetic influence (Rudick et al. 2012).

The same authors performed a further study looking at the relationship between recipient 25(OH)D levels and pregnancy rates in donor-recipient IVF cycles. They demonstrated that those patients who had normal levels of 25(OH)D had higher rates of clinical pregnancies compared with 25(OH)D deficient patients [78% vs 38%] (Rudick et al. 2014). Thus, in donor cycles it was the recipient's 25(OH)D concentration that was important in having a localised effect on the endometrium. However this study did not investigate the donors' vitamin D levels which could affect embryo quality and the study included several ethnic populations which added further confounding factors. It is known that VDR is present in the glandular cells of the endometrium and a randomised

double blinded, placebo controlled study of PCOS patients receiving Vitamin D supplementation during their infertility treatment reported significantly thicker endometrium ($p=0.003$) versus placebo, however there was no significant difference in pregnancy outcomes (Asadi et al. 2014).

Other studies have found no correlation between serum and follicular fluid levels of 25(OH)D and IVF outcomes (Anifandis et al. 2010; Aleyasin et al. 2011; Firouzabadi et al. 2014). One study reported that elevated levels of Vitamin D ($>30\text{mg/ml}$) resulted in significantly poorer embryo quality, and a higher level of follicular fluid 25(OH)D resulted in a poorer IVF outcome (Anifandis et al. 2010). These studies highlight that although Vitamin D may have a role in IVF outcomes, the literature is at present conflicting. As previously stated, there are numerous confounding factors within these studies ranging from patient ethnicity, to sperm, egg and embryo quality.

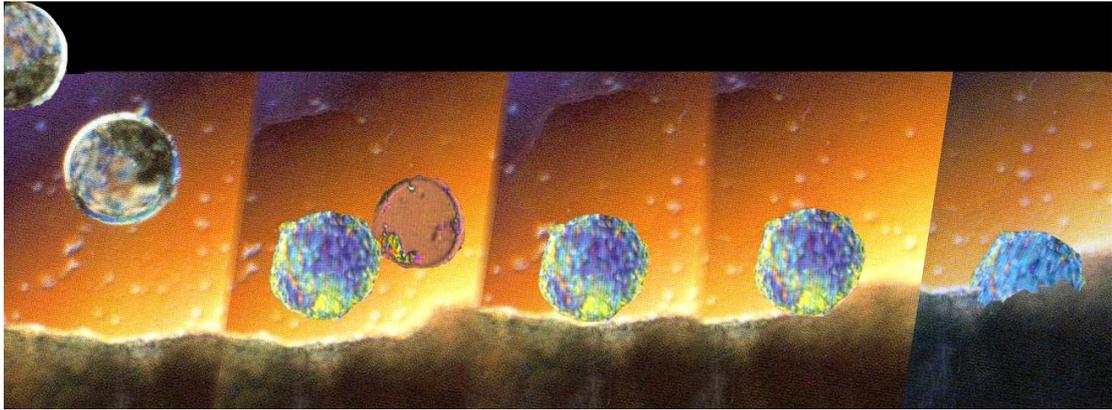
A recent large cohort study compared serum vitamin D levels and pregnancy rates in women undergoing fresh IVF cycles (Polyzos et al. 2014). They found that clinical pregnancy rates were significantly lower in women who were vitamin D deficient ($p=0.05$ and $p<0.001$). Interestingly logistic regression analysis demonstrated that Vitamin D was an independent variable in clinical pregnancy. Polyzos et al. (2014), found no differences in the embryological data between the vitamin D deficient and those with Vitamin D levels $\geq 20\text{mg/ml}$. This study has been the only one to assess pregnancy rates using single embryo transfer at blastocyst stage and, Vitamin D was noted to be independently associated with pregnancy rates, $p=0.02$ (Polyzos et al. 2014). However, Franasiak et al. (2015) retrospectively reviewed 527 IVF cycles and found Vitamin D was unrelated to pregnancy outcome. The results of these two recent studies again contradict each other as to whether or not Vitamin D may play a role in reproduction and that it may be an independent variable.

There is obviously clinical benefit of optimising women's Vitamin D levels prior to commencing a cycle of IVF. Within the clinical setting, the effect of Vitamin D on the process of folliculogenesis is unclear. Could there be any association with what effect Vitamin D has on the process of folliculogenesis in both normal women and those with PCOS and could this be observed in women undergoing a fresh IVF cycle?

1.6 Implantation

Implantation is the process by which the embryo attaches and migrates into the endometrium (Figure 1.10). It remains the main rate-limiting step in an IVF cycle with between 25-40% of embryos successfully implanting (de los Santos et al. 2003; Coughlan et al. 2014; Ferra et al. 2002). The most recent data on outcomes, according to ESHRE, gives an implantation rate of 32% for fresh embryo transfers resulting in a clinical pregnancy (Ferraretti et al. 2013). It is thought that this is due to a combination of factors such as poor uterine receptivity, embryo developmental potential and interaction of the embryo and endometrium.

This process of implantation whereby a viable embryo reaches a receptive endometrium can only occur over a short period of time called the Window of Implantation (WOI); this is usually during the mid-secretory phase (days 20-24) of a regular 28 day menstrual cycle. Thus for implantation to be successful a receptive endometrium is required, as well as an embryo that has the ability to attach and form a sound interaction between the two. Uterine receptivity is responsible for two thirds of implantation failures and the embryo for the other third (Simon et al. 1998; Ledee-Bataille et al. 2002)



Orientation

Hatching

Apposition

Adhesion

Invasion

Figure 1.11 Stages of Implantation

The stages to implantation include;

i. Orientation and Hatching

Before apposition, when the blastocyst is in close contact with the epithelial layer of the endometrium, but has yet to adhere, the inner cell mass of the embryo is oriented towards the endometrial epithelial lining and the zona pellucida is shed in a process called hatching. The failure of the embryo to release from the zona pellucida has been identified as a possible cause of implantation failure in IVF, especially in older women and those with repeated unsuccessful (2 or more) cycles of IVF (Seif et al. 2006).

Assisted hatching is an artificial technique in which the embryologist creates a small hole in the zona pellucida, on the fourth day of embryo development when the embryos contain an average of six to eight cells to aid in the implantation process. Seif et al. (2006) performed a meta-analysis on studies of assisted hatching where the zona pellucida is artificially disrupted, suggest that while pregnancy rates were significantly improved, there was no effect on live birth rates.

ii. Apposition

This is the point where the embryo and receptive endometrium first interact. This is established by L-selectin, which mediates the interaction between the uterus and trophoblast cells necessary in establishing pregnancy (Genbacev et al. 2003). Heparin-binding EGF (HBEGF) is another protein that mediates the process of apposition (Lim 2009). It is highly expressed at the WOI in both the mouse and human endometrium and is localised on the luminal epithelium (Yoo et al. 1997). HBEGF is also associated with zona-hatching (Das et al. 1994)

iii. Adhesion

Adhesion is when the embryo attaches itself to the endometrial basal lamina and stromal extracellular matrix. At this point the embryo is adherant to the endometrium and cannot be dislodged. Adhesion molecules include troponin, cadherins and integrins, are expressed by the endometrium and embryo (Singh et al. 2011). Osteopontin (OPN), a ligand that these molecules bind to, expression is increased at the time of WOI and plays an important role in establishing the embryo-endometrium interaction (Johnson et al. 2003). Research has demonstrated that OPN increases the adhesiveness of the blastocyst by binding to its surface receptors (Chaen et al. 2012).

iv. Invasion

The embryo penetrates through the epithelial layer. Thin folds of trophoblastic cells intrude between the endometrial epithelial cells. At the tips, integrins anchor the trophoblast to the basement membrane. This binding triggers the secretion of proteases, which digest the basement membrane. uNK (uterine Natural Killer) cells release factors that trigger the endometrial stroma to produce cytokines/chemokines such as IL-15, IL-8 that induce trophoblast migration (Germeyer et al. 2009). Some trophoblastic cells fuse to form syncytia, which proliferate and invade the endometrial extracellular matrix. The trophoblastic cells, now called cytotrophoblastic cells, migrate between the

syncytia followed by the fetal stoma, leading to the formation of the placental villi.

1.6.1 The Endometrium

The endometrium is the mucosal lining of the uterine cavity and is composed of 2 layers simple columnar epithelium, overlying the myometrium (figure 1.12).

1) Stratum functionalis (functional layer): This undergoes cyclical changes in response to progesterone and oestrogen and is shed during menstruation. The cells bordering the lumen are epithelial in type.

2) Stratum basalis (basal layer): This layer remains attached to the myometrium and forms the base for the new functional layer after menstruation.

It receives its blood supply from the uterine arteries that form the arcuate arteries in the myometrium and the radial branches in the endometrium (Figure 1.12). These divide further in the endometrium to form the straight arteries in the basal layer and the spiral arteries in the functional layer.

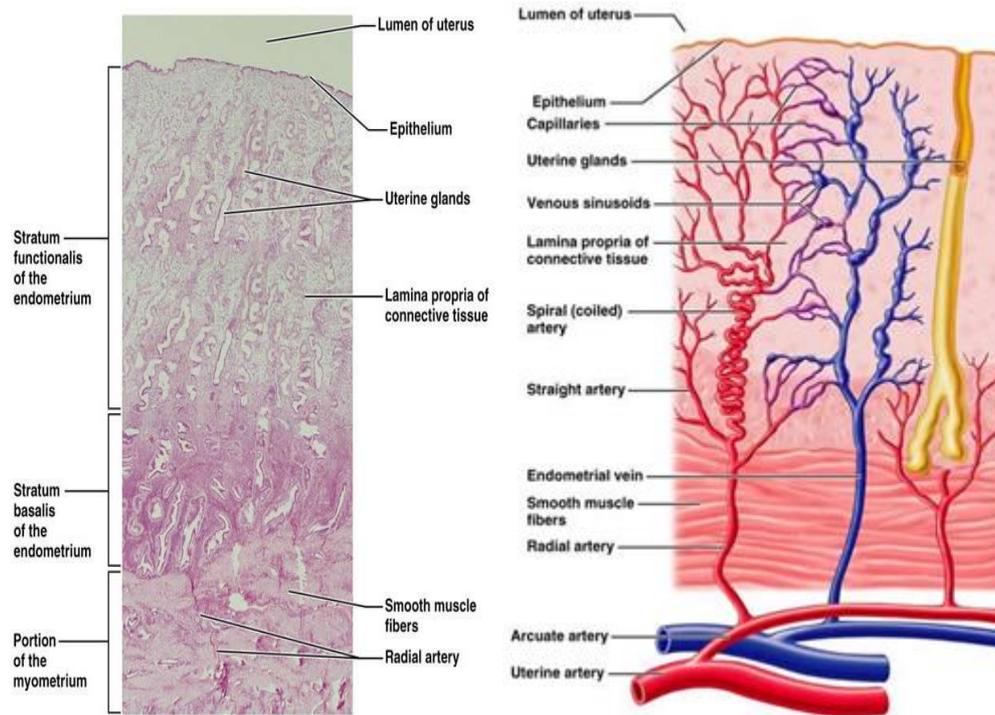


Figure 1.12 Histological slide demonstrating the functional and basal layer of the endometrium and blood supply of the endometrium.

The functional layer undergoes 4 phases during each menstrual cycle excluding menstruation, which usually spans the first 5 days of a menstrual cycle. These include resurfacing, proliferation, secretory and ischaemic phases. During resurfacing (days 5-6) the remnants of the glands in the basal zone of the mucosa proliferate and migrate to cover the raw surface of the endometrium.

During the proliferative/follicular phase (days 7-14) the endometrium thickens in response to rising oestrogen levels (Figure 1.13). The mucosal glands within the basal layer become longer and thicker and become coiled. The stroma between these glands increases by proliferation of connective tissue cells. There is evidence to suggest that implantation will not occur if endometrial thickness is less than 6mm (Abdalla et al. 1994).

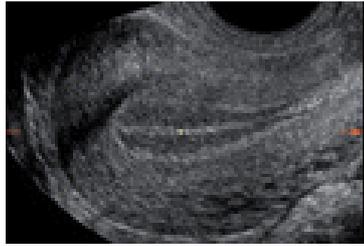


Figure 1.13 Trilaminar appearance of late proliferative endometrium

Following ovulation the corpus luteum is formed and the endometrium enters the secretory/luteal phase (days 15-27) induced by progesterone. The glands stop growing and distend and secrete abundant amounts of glycogen and glycoproteins and by the mid-luteal phase stromal oedema develops together with proliferation of the spiral arterioles.

It is known that the endometrium is composed of differing cell types, including luminal and glandular, epithelial cells, stroma, blood vessels and immunological cells. The original work by Noyes et al. (1975), through biopsies taken at different times in a menstrual cycle demonstrated that during the follicular phase of the menstrual cycle, endometrial architecture was characterised histologically by glandular cell mitosis and in the luteal phase by, stromal oedema and gland tortuosity and secretion. Ovarian steroids provide overriding endocrinological control throughout the menstrual cycle, while cytokines and growth factors work at the cellular level as paracrine and autocrine mediators of steroid action. Highly coordinated events, involving receptors, cell adhesion molecules within these epithelial cells and stroma aid uterine receptivity and allow the process of implantation.

1.6.2 Endometrial Receptivity to allow Implantation

i. Structural

Pinopods: These are small protrusions that are on the apical surface of the epithelium. They lie above the microvilli and project into the uterine lumen

(Martel et al. 1987). They are thought to play a role in the apposition and attachment of the embryo. Only several micrometres in size, they were detected by electron microscopy (Martel et al. 1987). Their expression is episodic and coincides with the window of receptivity (Nikas et al. 1995). There is evidence showing blastocyst attachment to the top surface of pinopods suggesting receptors required for blastocyst adherence are located in that region (Bentin-Ley et al. 1999).

Extracellular Matrix: This substance lies outside of the cells and is formed from secreted proteins and glycoproteins. Within this matrix is Mucin 1 (MUC1) a large glycoprotein that may act as an anti-adhesive molecule and prevent apposition. MUC1 interferes with both cell to cell and cell to matrix communication by a steric hindrance phenomenon (Wesseling et al. 1996). The apical surface of the majority of epithelial cells is covered with a glycocalyx composed of mucins to protect the cell surface from pathological agents (Strous and Dekker 1992). However within the endometrium the MUC1 sits above this glycocalyx, resulting in MUC1 being the first compound the embryo will encounter when attempting to attach to the endometrium. Animal studies suggest that MUC1 is downregulated prior to implantation (Surveyor et al. 1995) and that high levels of progesterone during the secretory phase may inhibit MUC1 allowing implantation (Surveyor et al. 1995).

ii. Biochemical mechanisms

a) Progesterone and Oestrogen

Oestradiol is secreted from the developing follicles. This initiates growth and cell division resulting in hypertrophy and hyperplasia of the endometrium during the proliferative phase. Following ovulation, progesterone modifies the endometrium into a secretory tissue to support early pregnancy.

Oestrogen receptors (ER α and ER β), progesterone receptors (P-A and P-B), and androgen receptors are all expressed in the human endometrium. ER α play the major roles in endometrial development. This is demonstrated in the mouse model where those mice deficient in ER α had hypoplastic uteri that could not support implantation whereas implantation was normal in ER β mice (Cha et al. 2012). The mediator leukaemia inhibitory factor (LIF) is the predominant mediator for oestrogen. Stewart et al. (1992) demonstrated in the mouse model that LIF is indispensable for implantation and some human trials have demonstrated low levels of LIF is associated with unexplained fertility (Wu et al. 2013). Oestrogen up-regulates both ER α and the progesterone receptors within the epithelial and stromal cells in the proliferative phase, but these are downregulated by progesterone in the secretory phase (Lessey et al. 1988).

Progesterone is the dominant hormone in the secretory phase, secreted by the corpus luteum and stimulates the differentiation of the endometrium following oestrogen priming (Gnainsky et al. 2014). Progesterone is critical in the converting a non-receptive endometrium to its receptive state. Progesterone has 2 receptors (P-A and P-B). It is P-A that is necessary for fertility, while P-B promotes proliferation and differentiation of the mammary gland (Graham et al. 2002). Immunophilin (FKBP52) is a main regulator of progesterone function and rodent studies have demonstrated FKBP52 knock-out mice have implantation failure due to impaired endometrial responsiveness (Tranguch et al. 2005).

While the endocrine pathway represents the main route for oestrogen and progesterone actions on the endometrium, there is evidence that progesterone can act via a paracrine pathway acting through the endometrial stroma (Lessey 2003) as demonstrated from Figure 1.14.

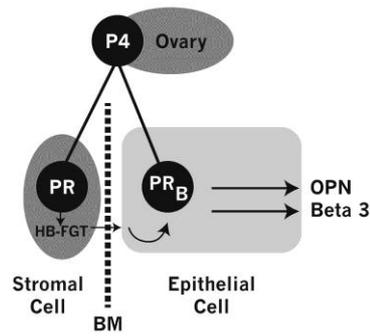


Fig 1.14 Progesterone has a direct influence on endometrial epithelium via the progesterone receptor (PR-B) and an indirect influence via the endometrial stromal cells adapted from Lessey. (2003)

PR-B, acting via the endometrial epithelium, allows the production of osteopontin, a glycoprotein that has binding sites on the maternal and embryonic epithelium (trophoblast). It is secreted by the glandular epithelium during the mid-secretory phase and is thought to act as a bridging ligand between the surface receptors on the endometrium and the embryo thus aiding apposition (Figure 1.15). A paracrine effect via the stromal cells produces heparin-binding epidermal growth factor (HBEGF) that stimulates the $\beta 3$ integrin subunit production needed for apposition.

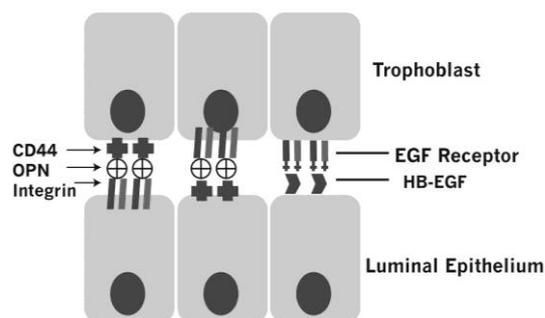


Figure 1.15 Schematic of Apposition. OPN; Osteopontin, HBEGF; heparin-binding epidermal growth factor

b) Integrins

Integrins are transmembrane receptors that are the bridges for cell-cell and cell-extracellular matrix interactions. They are members of the cell adhesion molecule (CAM) family that also includes selectins, cadherins and immunoglobulins. Integrins have two different chains, the α (alpha) and β (beta) subunits. In mammals, there are eighteen α and eight β subunits that, when paired, form 24 distinct obligate heterodimers. Many integrins are expressed within the endometrium throughout the menstrual cycle (Lessey et al. 1994). Three integrins ($\alpha 1\beta 1$, $\alpha 4\beta 1$ and $\alpha v\beta 3$) are co-expressed during days 20-24 of the menstrual cycle i.e. during the window of implantation (Lessey et al. 2000). However, the luminal epithelium only expresses $\alpha v\beta 3$ at the apical pole, suggesting a role in attachment (Lessey et al., 1996). The loss mid-luteal expression of $\alpha v\beta 3$ has also been associated with unexplained infertility (Lessey et al. 1995)

c) Cytokines

Cytokines are a broad group of small proteins (~5–20 kDa) that are important in cell signalling. Cytokines include chemokines, interferons, interleukins, lymphokines, and tumour necrosis factor. They are released by cells and affect the behaviour of other cells. Two cytokines of interest in relation to implantation are leukemia-inhibitory factor (LIF) and Interleukin-1 (IL-1).

LIF peak expression is during the time of implantation in animal studies (Bhatt et al. 1991). Fertile women demonstrate a 2.2 fold increase in LIF secretion between the proliferative and secretory phase (Hambartsoumain 1998). While in those women with unexplained fertility, LIF secretion increases only weakly. IL-15 is expressed in both the epithelial and stroma cells of the epithelium (Kitya et al. 2000). Studies have shown that the expression of IL-15 is different in patients with failed implantation after IVF/ICSI compared with fertile controls (Leddee et al. 2011; Mariee et al. 2012). Both studies demonstrated a

correlation with local uNK recruitment and stromal IL-15 suggesting a role in the control of uNK-cell function within the endometrium (Leddee et al. 2011; Mariee et al. 2012).

d) Prostaglandins

Prostaglandins are secreted by decidual cells and are necessary for necessary for endometrial receptivity and successful implantation (Song et al. 2002). The Cyclooxygenases-2 (COX-2) is responsible for prostaglandin synthesis under the control of progesterone (Kotani et al. 2005). These and other enzymes required for prostaglandin synthesis such as the secretory phospholipase A2 group: IIA, V, and IB (sPLA2-IIA, sPLA2-V, sPLA2-IB), glypican-1, PG-E synthase, PG-E receptors, and lysophosphatidic acid receptor 3 (LPA3) have been measured in RIF women with 85% having very low levels of COX-2 and over expression of sPLA2-IIA in response (Achache et al. 2010). Thus the COX pathway is crucial for successful implantation (Figure 1.16).

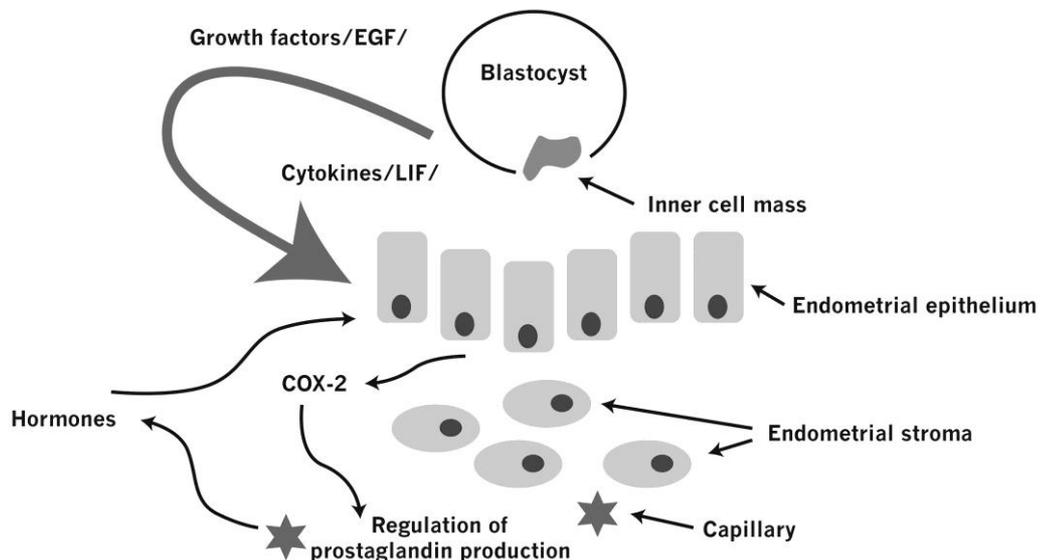


Figure 1.16 Schematic of prostaglandins and implantation adapted from Timeva et al. (2014)

1.6.3 Natural Killer cells and Implantation

The immune system is in part to play for recurrent implantation failure. Natural Killer (NK) cells have been implicated in women not only with recurrent implantation failure, but also with recurrent miscarriage (three or more consecutive miscarriages). Although the possible relationship between NK cells and reproductive failure is controversial as it has been used to guide immunological treatments (Rai et al. 2005) despite no clear consensus on what is an abnormal concentration of NK cells.

NK cells are a type of granular lymphocyte that are derived from haematopoietic progenitor cells in the bone marrow and differentiate and mature in the bone marrow, spleen, tonsils and lymph nodes that belong to the innate immune system i.e. providing immediate defence against infection. They comprise approximately 15% of the lymphocytic population and in humans they usually express the surface markers CD16 (FcγRIII) and CD56 where CD3 are absent (Robertson and Ritz. 1990). NK cells provide rapid responses against viral-infected cells and respond to tumour formation. Immune cells detect the major histocompatibility complex (MHC) presented on infected cell surfaces, triggering cytokine release, resulting in lysis or apoptosis. However NK cells have the ability to recognize stressed cells in the absence of antibodies and MHC, allowing for a much faster immune reaction. NK cells elicit their cytolytic effects through specific cells surface markers that are deficient in class I molecules of the MHC. This is essential when pathogenic cells have no MHC 1 and thus would not be detected by other immune cells, such as T lymphocyte cells.

i. Activation of NK cells

NK cells exhibit their cytotoxicity in several ways inducing cell lysis or apoptosis:-

1. Small granules in the NK cell cytoplasm contain proteins such as perforin and proteases known as granzymes. Upon release in close proximity to a cell targeted for killing, perforin forms pores in the cell membrane of the target cell, creating an aqueous channel through which the granzymes and associated molecules can enter, inducing either apoptosis or osmotic cell lysis (Figure 1.18). Apoptosis and cell lysis are different functions. Lysing a virus-infected cell could potentially release the virions inside the cell, while apoptosis leads to destruction of the virus inside.

2. Cytokines are stress molecules released by cells upon viral infection; they serve to signal to the NK cell the presence of viral pathogens in the affected area. Cytokines involved in NK activation include IL-10, IL-12, IL-15, IL-18, interferon-gamma (IFN- γ) and TNF- α (Figure 1.17). NK cells are activated in response to interferons or macrophage-derived cytokines.

3. Antibody-dependent cellular cytotoxicity. Infected cells are marked with antibodies for detection. These antibody-antigen complexes are recognised by the CD16 (Fc γ RIII) expressed on the NK cells causing activation and the release of cytotoxic granules and lysis/apoptosis.

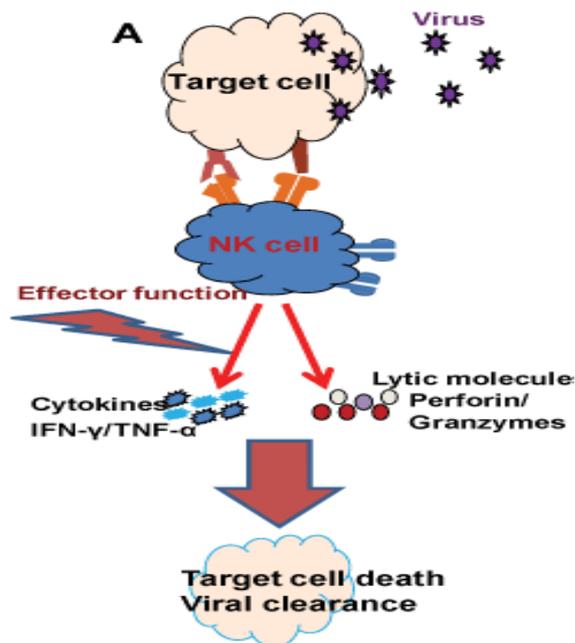


Figure 1.17 Schematic demonstrating NK-cell killing

ii. Subtypes of NK-cells

As stated the NK cells represent the dominant lymphocyte population of the innate immune system. Peripheral blood NK cells constitute 4-10% of the total lymphocyte population. There are 2 distinct subtypes of NK cells characterised by the expression of CD56 on the cell surface.

1. CD56dim account for ~90% of the peripheral blood NK cells and are potent cytotoxic cells (Kwak-Kim and Gilman-Sashes 2008). They express high levels of perforin and granzymes that induce lysis or apoptosis of the target cell via FasL-Fas interaction (Cooper et al. 2001). CD56dim express CD16 (Fc γ RIII) involved in antibody-dependent cell mediated cytotoxicity (Cooper et al. 2001).

2. CD56bright CD16 (CD16dim/-ve) account for ~10% of the peripheral blood NK cells. These NK cells are the primary source of NK cell derived cytokines and thought to be an important inflammatory or regulatory subset

(Kwak-Kim and Gilman-Sachs 2008). CD56^{bright} produce more IL-10 than CD56^{dim}. CD56^{bright} and CD16^{dim} make up between 70%-90% of the total lymphocytes within the endometrium. This demonstrated that the role of the NK cell within the endometrium is mainly inflammatory and regulatory. These uterine Natural Killer (uNK) cells are thought to be less cytotoxic and responsible for trophoblast invasion (Lash et al. 2006).

Thus the majority of uNK cells are CD56⁺/CD16⁻ whereas the majority of peripheral NK cells are CD56⁺/CD16⁺. Both these subgroups of NK cells differ phenotypically and functionally. The uNK cells have little cytotoxic activity; and have an immunoregulatory role in the process of implantation. They are recruited to the implantation site during decidualisation inducing vascular growth and spiral artery remodelling within the decidua (Bulmer and Lash 2005). They maintain immune tolerance and suppress inflammation and allogenic T helper 17 (TH17) by secretion of IFN- γ . However the peripheral NK cells are highly cytotoxic with established antiviral and neoplastic functions and it has been proposed that women with a history of reproductive failures such as implantation failure and recurrent miscarriage have elevated numbers of peripheral NK cells compared to normal fertile women.

iii. NK cytotoxicity and infertility

Within the IVF/subfertility spectrum NK cells have been used to guide differing types of immunotherapy (Rai et al. 2005) for those women with recurrent implantation failure, despite these treatments themselves being controversial. What level of peripheral NK cells within the blood constitutes a cytotoxic response in women with recurrent implantation failure?

Beer et al. (1996) was the first group to demonstrate an elevated percentage of peripheral NK-cells in women with recurrent implantation failure. They regarded a level of peripheral NK-cells 12% to be a cut off mark for raised and

normal NK cell levels. Thus those women with NK-cell levels <12% were much more likely to take a pregnancy to term compared to those with a level >12%.

Another group defined the cut off for abnormal NK-cell activity as 10% (Roussev et al. 2007). This group used this figure to define what effects various immunological treatments had on NK cell activity using this arbitrary marker (Roussev et al. 2007). However, King et al. (2010) identified a higher percentage of NK-cells (>18%) for women with recurrent implantation failure. This demonstrates there is no standardised range in what a normal/abnormal NK-cell count, which is concerning when it is being used as a basis to commence immunosuppressive treatment. Within each study there were different inclusion/exclusion criteria for study and control populations. This incorporated with the analysis of the NK cells, routinely via flow cytometry however some studies perform immunohistochemistry and manually count NK-cells. With flow cytometry counts can vary depending on how the lymphocyte gates are set on the flow cytometer resulting in further confounding factors (Rai et al. 2005). Other variables such as stress and exercise can change peripheral NK-cell expression, which has not been taken into account when defining what an abnormal NK-cell percentage is (Timmons and Cieslak 2008).

iv. Research to Present

Numerous studies have investigated the effects of NK cell counts on women with RIF. However two recent large meta-analysis (Seshadri and Sunkara 2014; Tang et al. 2011) have reviewed the literature in this divisive topic. These studies both agreed that using measurements of peripheral NK-cells did not predict events such as implantation failure and miscarriage. Tang et al. (2011) discussed limiting factors in the studies, which included problems such as underpowered studies and the heterogeneity between the studies. Seshadri and Sunkara. (2013) performed subgroup analysis on these studies to tackle this

issue and again found no difference in the percentage of NK cells between the infertile subjects and infertile controls.

Seshadri and Sunkara. (2013) concluded that, the immune system is a constant and ever changing environment and that one variable such as the NK-cell does not reflect the overall changes to the immune system in response to a pregnancy. It is also important to state that IVF is a very emotionally and physiologically stressful time for women and thus this may further elevate the NK-cell level leading to further stress if they are informed of high NK-cell levels that may affect their chances of a successful IVF cycle. This study would agree with Tang et al. (2013) by making a direct comparison with the Cochrane review on immunotherapy for recurrent miscarriage stating, “a specific assay to diagnose immune-mediated early pregnancy loss and a reliable method to determine which women might benefit from manipulation of the maternal immune system are urgently needed” (Porter et al. 2006). Thus a large multicentre RCT is required with a standardised protocol to measure peripheral NK-cells. But prior to this a greater understanding of this specific lymphocyte is required on the physiology of implantation/implantation failure and/or recurrent miscarriages, are they one and the same or different processes.

v. NK cells and Immunotherapy Intervention

Immunotherapy has been offered to women with high levels of peripheral NK-cells and those where no clear diagnosis has been identified. These immunological therapies such as intralipid (Roussev et al. 2007; Roussev et al. 2008), intravenous immunoglobulins (IVIg), (Roussev et al. 2007; Moraru et al. 2012) and prednisolone, (Quenby et al. 2005) have used peripheral NK-cells to demonstrate treatment effect and improve clinical outcomes. A recent meta-analysis on treatments to improve reproduction in women with raised peripheral NK-cell levels (Polanski et al. 2014). However the studies were of such poor quality that only 3 from 215 articles were of quality to include in the

meta-analysis. The conclusion was the use of adjuvant treatments in women found to have elevated peripheral NK-cells undergoing IVF, could not be supported. (Polanski et al. 2014). The RCOG state there is little evidence supporting any test or immunomodulatory treatment in couples with reproductive failure (RCOG, Scientific Impact Paper No.5).

1.6.4 The Embryo

There are a number of factors to consider regarding the embryo in an assisted reproductive cycle. These include maturity of embryos, the number retrieved, their genetics and when it is best to replace them, i.e., at the cleavage stage or the blastocyst stage. It has been suggested that those women who respond poorly to exogenous gonadotropins in an IVF cycle produce fewer numbers of oocytes and that they are of poorer quality (Ferraretti et al. 2011). At the other end of the spectrum, women who have an excessive response to gonadotrophins can produce high numbers of immature oocytes (Ferraretti et al. 2011). Age is another important determining factor with declining oocyte numbers and quality a major reason for poorer IVF outcomes with advancing age. The national average live birth for 40-42 yrs being 13.8% and 43-44 yrs being 4.8% compared to 33.7% for under 35 yrs (HFEA 2016).

Smoking has been associated with many health problems but there are many conflicting studies as to what affect it may have in women of reproductive age. Smoking has been associated with reduction in ovarian reserve (Zenzes. 2000) and contamination of follicular fluid with cotinine a metabolite of nicotine (Rosevear et al., 1992). However with so many confounding factors the precise effects of smoking on fertility is difficult to directly measure. Wright et al. (2006) retrospectively analysed the IVF cycles of 389 smokers and found no significant effect on oocytes collected, embryo quality and pregnancy outcome.

There are high levels of genetic abnormalities in human embryos conceived naturally or via assisted conception. In couples with recurrent miscarriage, chromosomal abnormalities of the embryo account for 30–57% of further miscarriages (Carp et al. 2001). The risk of miscarriage resulting from chromosomal abnormalities of the embryo increases with advancing maternal age (Ogasawara et al. 2000). Mosaicism (aneuploidy and euploid cells in the embryo) are reported to be present in as many as 70% of cleavage stage embryos and 90% of blastocyst-stage IVF embryos derived although the clinical relevance remains unclear and would depend on the chromosome involved (Taylor et al. 2014).

Over the last decade there have been improvements in extended culture to allow embryos to be grown to day 5, i.e., the blastocyst stage (Gardner et al. 1998) which is the stage at which early embryos would normally be present in the uterus (Nyboe et al. 2004). The advantages of blastocysts are therefore a better correlation between morphology and euploidy status and better synchronization with an endometrium already affected by an IVF stimulated cycle (Papanikolaou et al. 2008). However there is evidence to suggest that those embryos cultured in different media demonstrate different kinetics of cleavage, compaction, and hatching. There was greater implantation rate for those embryos only cultured to day 3 compared to day 5 indicating that prolonged culture could alter the phenotype of the embryo (Van Langendonck et al. 2001).

1.6.5 Grading an Embryo

The cleavage stage embryos (Figure 1.18) and blastocysts (Figure 1.19) are graded to allow the selection of the most viable embryo that may proceed to implantation (Cutting et al. 2008). However, like all grading techniques, there is inter-observer variability. The number in the grading classification explains the degree of expansion of the blastocyst cavity and its progress of hatching out of

the zona pellucida on a scale from 1-6; as the embryo expands the degree of expansion increases. The first letter is grading on a scale from A to E (A as the highest) to determine the quality of the inner cell mass, which potentially becomes the cells which form the body of the embryo after implantation. The second letter is also on a grading scale from A to C (A is the highest) and this grade determines the quality of the trophectoderm, which are the cells to give rise to the placenta and extraembryonic tissues after implantation (Cutting et al. 2008).

Cutting et al. (2008) stated that, using the grading scheme, any embryos with a grading of 3AA or above are suitable for single embryo transfer whatever the women's age with a high implantation rate even in older women. (Cutting et al. 2008). However the IVF unit does offer 2 embryo transfer to those women with poor quality embryos or who were 37 years or older.

Blastomere number	
Blastomere size	4 = regular, even division
	3 = <20% difference
	2 = 20-50% difference
	1 = >50% difference
Fragmentation	4 = <10% frags by volume
	3 = 10-20%
	2 = 20-50%
	1 = >50%

Figure 1.18 Cleavage stage embryo grading system (Cutting et al., 2008)

Expansion Status	1= Early blastocyst; blastocyst less than half the volume of the embryo, little or no expansion in overall size, zona pellucida (ZP) still thick
	2 = Blastocyst; blastocoel more than half the volume of the embryo, some expansion in overall size, ZP beginning to thin.
	3 = Full blastocyst; blastocoel completely fills the embryo
	4 = expanded blastocyst: blastocoel volume now larger than that of the early embryo. ZP very thin.
	5 = Hatching blastocyst; trophoectoderm has started to herniate through the ZP
	6 – Hatched blastocyst, the blastocyst has evacuated the ZP
ICM Grading	A = ICM prominent, easily discernible and consisting of many cells, cells compacted tightly and adhered together.
	B = Cells less compacted so larger in size, cells loosely adhered together; some individual cells may be visible.
	C = Very few cells visible, either compacted or loose, may be difficult to completely distinguish from trophoectoderm
	D = Cells of ICM appear degenerate or necrotic
	E = No ICM cells discernible in any focal plane
Trophoectoderm	A = Many small identical cells forming a completely continuous layer
	B= Fewer larger cells, may not form a completely continuous layer
	C = Sparse cells, may be very large, very flat or appear degenerate

Figure 1.19 Blastocyst grading system. (Cutting et al., 2008)

A Cochrane meta-analysis reported there was no difference in live birth or pregnancy outcomes between Days 2–3 and 5–6 transfers of embryos (Blake et al. 2007). The most recent systematic review and meta-analysis by Papanikolaou et al. (2008) of over 1600 patients (including those in Blake et al.

2005) demonstrated the live birth rate after fresh IVF is significantly higher after blastocyst-stage embryo transfer as compared to cleavage-stage embryo transfer when equal number of embryos are transferred in the two groups compared. However the cancellation rate per patient (OR: 2.21, 95% CI: 1.47–3.32; P = 0.0001) was significantly higher in patients with a blastocyst-stage embryo transfer. The cryopreservation rate was significantly higher in the cleavage-stage group (OR: 0.28, 95% CI: 0.14–0.55; P < 0.0002), highlighting the technical difficulties in the cryopreservation/thawing process of blastocysts.

Recent advances in technology to monitor embryo development include timelapse imaging. This technology takes regular images of the embryo, allowing embryologists to inspect the developing embryos without removing them from the incubator thus exposing them to environmental changes and aiding in the selection of top quality embryos. However the consensus of opinion is that RCTs need to be performed regarding the efficacy of timelapse technology and its ability to improve implantation and live birth rates. (Bolton et al. 2015).

1.7 Recurrent Implantation Failure

Recurrent implantation failure (RIF) has no formal definition. Do we define it as the number of cycles undertaken and/or how many embryos replaced in total, or per cycle? The two main problems arise from (i) the many variables within each IVF cycle (ii) different IVF clinics using different protocols for the use of exogenous gonadotropins. This has led to a number of definitions of RIF. The term RIF has been used since the 1980s. Early definitions defined RIF as more than 12 embryos being transferred in multiple procedures without achieving a pregnancy (Coulam et al. 1995a). More recent publications have been unable to reach agreement on the number of unsuccessful cycles or numbers of embryos transferred (Rinehart 2007; Simon and Laufer 2012). Rinehart. (2007) defined

RIF as the transfer of 8 or more, 8-cell stage embryos or 5 or more 5 blastocysts. They do not mention the cycle numbers in this definition.

With best practice today advising only a single embryo transfer to reduce the multiple pregnancy rates the definitions above are unfeasible. ESHRE defined RIF as a failure to achieve pregnancy after ≥ 3 unsuccessful transfers of high quality embryos or a total of ≥ 10 embryos in multiple transfers (Thornhill et al., 2005). With the move to limit the numbers of embryos transferred in a single cycle, the definition of RIF became the failure to achieve a pregnancy after 3 embryo transfers with good quality embryos (Margalioth et al. 2006). Cutting et al. (2008) define a good quality embryo, as an embryo having the correct number of cells corresponding to the day of its development and day 5 embryos (blastocysts) are graded according to expansion and the quality of the inner cell mass and trophectoderm.

Tan surveyed the 79 (at the time) HFEA licenced IVF centres in the UK regarding how they defined RIF. They had an 82% response rate, with the most common definition being three unsuccessful cycles ranging from 2 – 6 cycles, of which 19% included frozen cycles (Tan et al. 2005). This illustrates the considerable variation between clinics. However Tan recommended not including frozen ET (FET) in this definition as at the time the success rates were inferior to fresh embryo transfers. Since then, vitrification has been developed. This faster freezing method decreases the chances of embryos developing ice crystals, which has greatly increased post thaw survival rates compared to traditional slow freezing at both the cleavage and blastocyst stage (Loutradi et al. 2008). AbdelHafez et al. (2010) demonstrated clinical pregnancy rates were $>50\%$ compared to traditional slow freezing.

Despite this there continues to be papers published regarding this very topic with no agreement on the best definition (Laufer and Simon. 2012; Penzias

2012). A recent review by Coughlan et al. (2014) defined RIF “as the failure to achieve a clinical pregnancy after transfer of at least 4 good-quality embryos in a minimum of three fresh or frozen cycles in a woman under the age of 40 years.” This definition takes into account both single embryo transfer and frozen cycles as evidence has shown implantation rates to be equivocal in frozen-thawed embryos compared to fresh (Shapiro et al. 2011). However, as all previous studies have discussed, there needs to be an internationally agreed definition on RIF such as that for PCOS (Rotterdam ESHRE/ASRM – Sponsored PCOS Consensus Workshop Group, 2004).

With recent definitions incorporating the transfer of good quality embryos, so does this make RIF an endometrial problem? However we are aware of other factors that could lead to RIF.

Anatomical factors can affect implantation such as uterine abnormalities, polyps, endometriosis, fibroids (Sanders. 2006), and hydrosalpinges (Strandell. 2000).

Endocrine disorders such as, hypothyroidism, diabetes (Unane et al. 2011).

Immunological factors such as NK cells (Rai et al. 2005).

Male factor issues such as DNA fragmentation, however this would result in poor embryo quality (Avendano et al. 2010).

Regarding the endometrium is this a problem with the physiology of the developing endometrium or a communication issue between the endometrium and embryo.

1.8 Treatments to aid the receptivity of the Endometrium

There are several treatments potentially available to aid the receptivity of the endometrium and possibly dampen any immunological response to aid implantation. These include endometrial scratching, intralipid and intravenous Immunoglobulin (IVIG).

1.8.1 Endometrial Scratch

In considering this question, it is important to consider the following issues:

The physiology of the endometrium

The methods available

The evidence for efficacy and safety

1.8.2 The Inflammation Theory

It is thought that endometrial scratching causes inflammation within the endometrium thus aiding implantation. During the window of implantation there are changes within the cells of the endometrium including the transformation of the stromal cells into decidual cells (Dunn et al. 2003). There is an influx of blood vessels and up-regulation of various cytokines, growth factors and prostaglandins under the regulation of oestrogen and progesterone all precisely timed to aid implantation (Gnainsky et al. 2014). It is thought that mechanical trauma to the endometrium initiates an inflammatory response that increases the receptivity of the endometrium. Loeb (1907) scratched the inner lining to the uterus of guinea pigs during the progestational phase of the estrous cycle and discovered this provoked a rapid growth of decidual cells.

Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of leukocytes, the up-regulation of cytokines and chemokines, and recruitment of immune cells from the blood into the injured tissue (Feghali et al. 1997). At the time of implantation, the levels of these cells increase (Kelly et al. 2001). As discussed earlier, specific cytokines (IL-6, IL-8, TNF α) are released at the time of adhesion and invasion with their expression unregulated under the control of oestrogen and both by the endometrial cells and by NK-cells from the immune system (Hirata et al. 2008).

Gnainsky et al. (2010) performed a day 21-endometrial scratching on two groups of patients during their menstrual cycle with the experimental group having a scratch performed during the proliferative phase of the same cycle. They found elevated levels of pro-inflammatory cytokines, TNF α , growth regulated oncogene- α and macrophage inflammatory protein 1B (MIP-1B) in a day 21 biopsy of the experimental group and a positive correlation between the levels of these cells and pregnancy outcome. Previous studies had shown that TNF α is expressed at the time of implantation, however this study Gnainsky et al. (2010) hypothesized that TNF α increased the expression of MIP-1B, suggesting that inflammation had a role in the development of a receptive endometrium. The same group also found an increase expression of OPN that helps the embryo adhere to the endometrium.

An Endometrial scratch is a mechanical method proposed to aid implantation in women with recurrent implantation failure. The two ways in which an endometrial scratch can be performed, are either under direct vision with a hysteroscope or by using an endometrial biopsy Pipelle. However the Cochrane review on endometrial scratch prior to commencing IVF lists endometrial curettage or biopsy not hysteroscopy (Nastri et al. 2012).

1.8.3 Endometrial Scratch Procedures

i. Hysteroscopic Endometrial Biopsy

The hysteroscope is the gold standard method for visualising the uterine cavity (Pundir and El-Toukhy 2010)

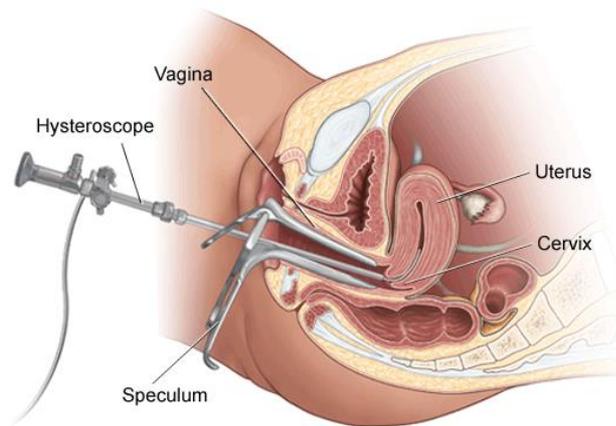


Figure 1.20 Sagittal Image of hysteroscopy. Krames Staywell. 780 Township Road, Yardley, PA 19067,267-685-2500

However there is great variation as to how the endometrial scratch procedure is carried out. Some studies have used claw forceps within a fine flexible hysteroscope causing only a single site-specific injury site (Huang et al. 2011). Others used 5.5mm rigid scopes (Makrakis et al. 2009) or 3-5mm rigid scopes (Rama Raju et al. 2006) and created a furrow with the hysteroscopes. The question is what benefit is there in performing this invasive procedure in the cycle prior to commencing IVF in patients commencing their first cycle of IVF or should it be reserved for patients with recurrent unsuccessful implantation.

ii. Hysteroscopy Prior to the 1st IVF Cycle

Some studies have examined the effect of hysteroscopy prior to commencing a first cycle of IVF (Doldi et al. 2005; Kilic et al. 2013; Yu et al. (2012). Unfortunately these studies demonstrated considerable variation in methodology regarding types of hysteroscope used and what happened during the procedure. Two studies performed endometrial biopsies at the time of hysteroscopy (El-Nashar and Nasr 2011; Doldi et al. 2005), both of which demonstrated significantly higher clinical pregnancy rates in the hysteroscopy group compared to the controls. This added a further confounding variable; that

the endometrial biopsy itself may be the reason for the increased pregnancy rates as opposed to the hysteroscopy. El-Nashar and Nasr. (2011), performed operative hysteroscopy in those patients diagnosed with an intrauterine pathology at the time of their hysteroscopy and endometrial biopsy. With several variables it is difficult to conclude whether the hysteroscopy, the endometrial biopsy or the operative part of the hysteroscopy procedure that resulted in these increased clinical pregnancy rates.

Yu et al. (2012) reported that those patients who had a diagnostic hysteroscopy only to view the endometrial cavity had no significant difference in clinical pregnancy rates compared to the controls. However those patients that underwent an operative procedure at the time of hysteroscopy had significantly higher live birth rates compared to the non-operative hysteroscopy group (51.2% vs. 33.6%, $P=0.02$). This again demonstrates that the treatment of any uterine pathology or performing the endometrial biopsy at the time of hysteroscopy is the determining factor for the increase in pregnancy and birth rates. However this was a small non-randomised single centre study, which concluded that routine hysteroscopy itself had no effect on outcomes demonstrating it is not the procedure itself but the operative procedure on pathology that has the effect on pregnancy outcomes.

iii. Hysteroscopy with Recurrent Implantation Failure

Some small randomised control trials showed benefit of the hysteroscope if it was performed the cycle preceding their IVF cycle in women with repeated cycle failure of IVF (Demirel and Gurgan. 2004; Rama Raju et al. 2006; Makrakis et al. 2009). The results of these small studies contradict each other, with some showing benefit of hysteroscopy (Rama Raju et al. 2006; Makrakis et al. 2009), and others showing no benefit (Almog et al. 2010). A small, randomised control trial showed statistical significance towards a benefit but the Confidence Interval was close to unity (Demirel and Gurgan 2004). The study with site-

specific injury to the endometrium during an on-going IVF cycle had a 100% pregnancy rate vs. 46% in the control group but this study only had 6 patients in the treatment arm (Haung et al. 2011).

These studies had wide variation in the age of patients recruited; from 26-30 years in one study to 42 years in another. Different media were used to fill the uterine cavity. Glycine in some and normal saline in others. The studies also replaced more than 2 embryos. Interestingly large numbers of patients in these studies had uterine pathology that was treated at the time of hysteroscopy raising the question that treatment of the pathology resulted in the increased pregnancy rates. For example, it should be noted that, hysteroscopy was developed to visualise the uterine cavity and treat uterine pathologies not to cause scratching despite furrows occasionally being created when the scope was not kept within the centre of the cervical canal or if the uterus was acutely anteflexed or retroverted.

However the pre-IVF hysteroscope debate was laid to rest at the ESHRE conference in 2015 which reported the large multicentre randomised TROPHY trial which included over 700 women <38 years, with between 2-4 failed IVF cycles. Exclusion criteria included BMI >35, uterine fibroids and untreated hydrosalpinx. There was no difference in live birth rates between the two groups, 31% in the hysteroscopy group and 29% in the control group (El-Toukhy et al. ESHRE 2014).

iv. Endometrial Scratch by Endometrial Biopsy Pipelle

An endometrial scratch performed by an endometrial biopsy pipelle is a relatively easy procedure, which may be performed in the outpatient setting. However, it can be uncomfortable to the patient as recorded on a visual analog scale (Nastri et al. 2013). The procedure requires the patient to be in the lithotomy position and the cervix is visualised by using a Cusco speculum. The

pipelle is inserted through the cervix into the uterine cavity to the level of the uterine fundus. The inner sheath of the pipelle is withdrawn to create negative pressure and the pipelle is moved up and down the endometrial cavity several times and rotated gently at the same time (Figure 1.22). This movement causes trauma to the endometrium and causes the endometrial “scratch”. When the pipelle is removed the negative pressure causes the tissue that has been scratched, to be sucked into the body of the pipelle. Patients are advised they may have some spotting after the procedure. On occasions; a cervical dilatation may be required to dilate the cervical canal to allow the pipelle to pass into the uterine cavity.

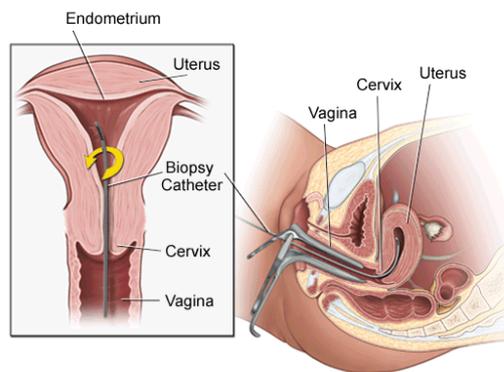


Figure 1.21 Sagittal Image showing Endometrial Biopsy with Pipelle Catheter.
Krames Staywell. 780 Township Road, Yardley, PA 19067, 267-685-2500

The first study investigating the effect of endometrial scratching on pregnancy outcome in an IVF population was performed by Barash et al. (2003). This prospective study randomly selected 45 good responders from a cohort of 134 patients. A good responder was defined as “patients that responded positively to hormonal stimulation producing fertilisable oocytes” (Barash et al. 2003). The patients in this group had their endometrial scratching performed on 4 occasions (days 8, 12, 21 and 26) of the menstrual cycle prior to their IVF cycle. Significant differences in both clinical pregnancy rates (66.7% vs. 30.3%, $p=0.00009$) and live birth rates (48.9% vs. 22.5%, $p=0.16$) were claimed for the scratching group. However was this was due to the number of times the

procedure was performed, the transfer of 3 or more embryos per cycle or the fact that the majority of these patients had only one previous IVF cycle and therefore could not be defined as having recurrent implantation failure.

A small RCT by Narvekar et al. (2009) randomised 100 women with at least one failed cycle of IVF who again responded well to hormonal stimulation. This study however only performed the scratching 2 times (once in the follicular phase and once in the luteal phase) in the menstrual cycle before commencing the IVF cycle. Again this small study demonstrated a significantly higher clinical pregnancy rate of (32.7% vs. 13.7%) in the scratching group, but large numbers of embryos were transferred (>3).

A better quality RCT by Karimzadeh et al. (2009) comprised 115 patients diagnosed with recurrent implantation failure defined as per Tan (2005) definition (i.e., having 2-6 unsuccessful cycles of IVF-ET and the transfer of at least 10 high grade embryos without the achievement of a clinical pregnancy). In this study the scratching was only performed in the luteal phase in the menstrual cycle before commencing their IVF. The clinical pregnancy rate was significantly higher in the scratching group (27.2% vs. 8.9%, $p=0.02$). However 9 patients were lost to follow-up, there were 9 cancelled cycles due to poor response to the exogenous gonadotrophins and 13 patients were excluded due to poor embryo quality, thus these results were provided by 93 patients.

In all the studies described above, the endometrial scratch was performed in the cycle prior to commencing IVF with one RCT performing the procedure on the day of oocyte retrieval (Karimzade et al. 2010). The groups had similar demographics and were all on their first cycle of IVF. The clinical pregnancy rate was significantly lower in the endometrial biopsy group ($p<0.05$). Karimzade et al. (2010) concluded that this procedure had a negative effect on implantation, possibly by affecting the environment within the uterus.

Two studies have found no effect on clinical pregnancy rates with endometrial Pipelle scratching. The first was a small, randomised placebo trial of 48 patients with recurrent implantation failure (Baum et al. 2012). The average number of failed cycles in both the treatment and control group was 8.5. 18 patients underwent on endometrial pipelle and 18 controls had a cervical pipelle on the cycle preceding their IVF. They found the clinical pregnancy rate was significantly lower in the treatment group (0% vs. 31.25%, $p < 0.05$). A larger retrospective study looked into the effects of endometrial scratching on ovum recipients obtaining donor eggs from young women aged 23-30 with proven fertility (Dain et al. 2014). They found no differences in clinical pregnancy rates in the 122 women who underwent the endometrial scratch compared to the 615 controls. These women were significantly older than the controls, and received significantly more embryos in each cycle (2.9 ± 0.7 vs. 2.5 ± 0.6 , $p < 0.0001$).

Systematic reviews and meta-analysis regarding endometrial scratching using endometrial biopsy catheters have been published indicating a benefit of endometrial scratching in the cycle prior to commencing a fresh IVF cycle (El-Toukhy et al. 2012; Nastri et al. 2012) One of these meta-analysis contained 4 randomised control trials (Nastri et al. 2012) and the other only 2, both of which were included in the other meta-analysis, and 6 non-randomized or unpublished studies (El-Toukhy et al. 2012). However there were severe methodological differences in the studies selected; differing numbers of embryos replaced and the inclusion of patients who did not have recurrent implantation failure. Simon and Bellver (2014) reviewed these papers and noted that the RCTs selected for these systematic reviews were published in journals with no or a very low impact factor, and that the inclusion criteria were insufficiently robust (Simon and Bellver 2014).

A recent RCT reported the effect of endometrial scratching using a Pipelle biopsy catheter on an unselected group of 300 patients undergoing IVF (Yeung et al. 2014). There were no differences in characteristics between the two groups and no difference in pregnancy rates between the two groups (26.7% scratching group vs 32% in the control group $P=0.375$). However 69.7% of the cohorts were undergoing their first cycle of IVF, which could be seen as a limiting factor since the procedure is claimed to benefit those with recurrent implantation failure. Subgroups analyses on patients in their first IVF cycle showed that the endometrial scratch had no significant difference on ongoing pregnancy rates, and in those patients with recurrent cycles the ongoing pregnancy rate was significantly lower in the “scratching” group. However the numbers of patients with repeated cycle’s 1 or more was only 91 of which 46 received an endometrial scratch. So despite being a high quality study that is adequately powered, it does not answer the question regarding endometrial scratching and recurrent implantation failure, except for showing that this procedure is not needed for all patients. There remains a need for a large randomised controlled multicentre study to address the question whether this procedure is of any benefit and if so, how many times should it be performed and at what stage in the menstrual cycle.

1.8.4 Immunological therapy and Recurrent Implantation Failure

It is thought that immunotherapy could help women with recurrent implantation failure whether they have elevated levels of NK-cells based on invalidated techniques, or where no clear cause has been identified. The mainstays of immunotherapies are intravenous Immunoglobulin (IVIG) and intralipid using NK-cells as a measure of treatment efficacy.

1.8.5 intravenous Immunoglobulin (IVIG)

IVIG is made of immunoglobulin fractions extracted from the plasma of blood donors and it has been suggested that it can modify NK-cells, autoantibodies,

complement, cytokines and regulatory T lymphocytes (Schwab and Nimmerjahn 2013). It has been used off licence to treat women with recurrent implantation failure in an attempt to modify the immune response.

Early meta-analysis concluded that IVIG does not offer any more benefit over placebo in women with RIF (Hutton et al. 2007; Ata et al. 2011). The most recent meta-analysis by Polanski et al. (2014) reviewed 403 citations but only included 3 studies into the analysis due to the large heterogeneity between studies. They again concluded with such poor studies they could not advocate the use of IVIG in women with elevated absolute numbers or activity of NK-cells undergoing ART (Polanski et al. 2014). Christiansen et al. (2015) performed a powered double-blind placebo controlled RCT on 82 patients with at least 4 miscarriages. There was no difference in live birth rates between the IVIG and placebo group 23/42 (54.8%) vs, 20/40 (50%).

1.8.6 Intralipid

Early work by Loo et al (1982); who analysed the effects of intravenous Intralipid on cellular toxicity on herpes simplex virus. They demonstrated that the high circulating levels of intralipid may “interfere with antiviral immunity in humans” however this may “predispose hosts who are already compromised to severe viral infections.”

Soon after intralipid was introduced into clinical practice in the intensive care environment, concerns were raised regarding its immune-compromising effects and the increased risk of infections (Utermohlen and Tucker 1986; Wan et al. 1988). Today, there is consistent evidence from human and animal in vitro and ex vivo studies that demonstrate soy bean oil (intralipid) inhibits lymphocyte proliferation (Soyland et al. 1993; Calder et al. 1994), decreases natural and lymphokine activated killer cell activity (Monson et al. 1988; Yaqoob et al. 1994), decreases chemotaxis and random migration of granulocytes (Fischer et

al. 1980; Cury-Boaventura et al. 2006) and negatively affects the reticulo-endothelial system (Katz et al. 1991).

Battistella et al. (1991) randomised 60 trauma patients with a severity score of 27 and an Acute Physiology and Chronic Health Evaluation (APACHE II) score of 23, to 10 days of postoperative parental nutrition with or without intralipid. Patients who received the lipid emulsion had a longer hospital stay (39 vs 27 days), a longer stay in intensive care (29 vs 18 days) and more days of mechanical ventilation (27 vs 15). The intralipid group also had a significantly reduced natural and lymphokine activated killer cell activity and a higher number of infections (72 vs 39).

At present within the Intensive care setting, intralipid may form part of parenteral nutrition however as these soybean-oil lipid emulsions have been associated with increased rates of infection, the soybean-oil has been substituted with other lipid emulsions that have reduce infection rates and oxidative stress (Calder et al., 2010). Intralipid is still used to treat local anaesthetic toxicity by removing the lipophilic anaesthetic from the affected tissues reversing the cardiac and neurological effects of the local-anaesthetic toxicity (Mazoit er al., 2009). There are no randomised trials in humans only case reports (Foxall et al., 2007), however both the American and British Anaesthetic Societies have guidelines using intralipid to treat suspected local anesthetic toxicity

These results demonstrate that in severely ill patients within the intensive care setting intralipid has the ability to reduce natural killer cells at the cost of making the patients more prone to infection, not forgetting these patients are already overcome with massive sepsis. However in the IVF setting our patients are otherwise well and the aim is to dampen down the immune response

enough to allow a pregnancy to implant without risking the patients natural immunity thus making them more vulnerable to infections.

Matsubayashi et al. (2001) examined the NK cell number of 94 infertile women who were unable to conceive despite treatment. The NK cell activity of the infertile women was $40.2\% \pm 14.7$, which was significantly higher than the control group ($31.5\% \pm 11.9$, $P < 0.0001$). This was not associated with either age or duration of infertility.

Roussev et al. (2007) reported that intralipid along with intravenous immunoglobulin (IVIg) and soluble human leukocyte antigen-G suppressed NK cell activity. They decided to base their study on earlier work that women with reproductive failure have significantly elevated numbers of peripheral (CD56+/CD16+) NK cells compared to the fertile population (Coulam et al. 1995b; Kwak et al. 1995). This large study analysed blood samples from 275 women who had suffered recurrent implantation failure with IVF and those with recurrent pregnancy loss, of which 126 had abnormal NK cell killing (>10% killing). They based normal NK cell killing activity as ($\leq 10\%$ killing) and abnormal NK cell killing as ($\geq 10\%$ killing) using the equation below.

$$\% \text{ Changes in NK cytolysis} = \frac{(\text{Natural NK lysis} - \text{NK cytolysis after IVIg/Intralipid/sHLA-G}) \times 100}{\text{Natural NK cytolysis}}$$

The patient's blood samples were mixed with intralipid, IVIG and soluble human leukocyte antigen-G. They found that in the groups the IVIG, intralipid and soluble human leukocyte antigen-G all had equal suppressing effect on the NK cells. However the authors were unable to answer the question as to how the treatments had suppressed the NK cells and more importantly could it be used in a clinical environment to treat those women with RIF associated with elevated NK cells?

Further work by this group investigated the duration and level of intralipids suppressive effect on NK cell function (Roussev et al. 2008). This small study of fifty patients with RIF had abnormal levels of NK cells. All subjects received an intralipid infusion and blood samples were taken one week later. They based normal NK cell killing activity as $\leq 10\%$ killing using 55 control subjects. They divided the subjects into two groups, those with abnormal NK cells above 15% killing and those with $<15\%$ killing. 89.3% of those with NK cell $<15\%$ killing had normalized their NK cell to below the normal NK cell activity of $<10\%$ following one infusion of intralipid. Overall 78% showed NK cell suppression to within the normal range the first week after infusion. 22% showed suppression but this was still above the baseline 10%. Following a second infusion 2 weeks later 10 of these 11 subjects had normal levels of NK cell activity the following week and after a third infusion all had normal levels of NK cell activity. The duration of suppression lasted between 6-9 weeks for 47 subjects, 5 weeks for 2 subjects and only 4 weeks in 1. Do these results demonstrate that intralipid could create an artificial window of immune suppression to allow an embryo to implantation, develop and maintain a pregnancy at this critical phase? The only way to answer this would be to perform large-scale prospective RCT trials.

The most recent work on the clinical effects of intralipid was a small non-randomised trial that demonstrated a 50% pregnancy rate and a 46% clinical pregnancy rate achieved in patients with RIF who had an elevated TH1 cytokine response (Ndukwe et al. 2011). They infused intralipid once between days 4 and 9 of stimulation and the patients had a further infusion within seven days of a positive pregnancy test. In all 50 patients the TH1:TH2 activity ratio was decreased following the intralipid treatment. This demonstrates that there is a reduction in the TH1:TH2 ratio. But is this as a result of the intralipid, the IVF process or a natural occurrence that was discovered by chance that requires more detailed work before offering intralipid treatment to all patients with RIF?

Shreeve and Sadek. (2012) have reviewed all the relevant data on intralipid and concluded that all findings to date should be treated with caution. They conclude “no newly proposed immunological therapy for pregnancy loss should be taken seriously until it has been proven in a well designed RCT, in accordance with CONSORT guidelines.”

1.9 Thesis Summary and Objectives

The literature review has demonstrated that the process of folliculogenesis is a very complex process, commencing during fetal development. It is under the influence of numerous endocrine, paracrine and autocrine pathways leading to the release of a dominant follicle after a long journey of growth and development lasting almost a year. If conceptus happens this oocyte may fertilise becoming an embryo that will require a healthy endometrium to implant and develop otherwise it will demise and the whole process will reset during menstruation and continue.

Unfortunately, infertility affects 1 in 7 heterosexual couples (NICE, CG 156. 2013). The exact mechanism for almost 25% of these couples is unknown. Other causes for infertility such as tubal pathology and male factor infertility we have been able to successfully treat using surgery and assisted reproductive techniques such as IVF and ICSI. Other pathologies such as PCOS we have demonstrated a greater understanding through in vivo and in vitro research both in animal and human models the role of androgens and insulin in folliculogenesis. Research has demonstrated there are several phenotypical subgroups with those with severe symptoms to others whose physiology lie just outside what is deemed normal. Recent changes to the diagnostic criteria of PCOS, has increased the numbers of women diagnosed with the condition. Studies of the polycystic ovary and its exaggerated response to exogenous gonadotrophins has increased our knowledge in treating these women safely whilst undergoing a controlled IVF cycle with exogenous gonadotropins to

reduce the incidence of Ovarian Hyper-stimulation Syndrome (OHSS). Thus the polycystic ovary appears to be a very sensitive organ, to androgen, insulin and gonadotropins. Anovulation in many women, responds appropriately to weight loss enabling many overweight women to ovulate spontaneously by affecting androgen and insulin status, and in the world of assisted conception the PCOS ovary is an incredibly sensitive organ that displays an overwhelming response to exogenous gonadotrophins.

In the 21 century we are exposed to numerous chemicals every second of every day. This can be in our homes, cars, work, and in the food that we consume. As discussed in the literature review folliculogenesis is a very fragile process under strict endocrine, paracrine and autocrine control. It has been demonstrated that in PCOS the altered androgen status ultimately leads to insulin resistance and in many an anovulatory status. We hypothesize that women are exposed to endocrine disrupting chemicals persistent in the environment, and are present in the serum of women in the United Kingdom. These chemicals may affect the outcomes of a controlled ovarian hyperstimulation cycle and they may be an underlying factor for the altered androgen status in PCOS.

Vitamin D has been postulated to have many influential effects within the body not just within the musculoskeletal system. The majority of women with PCOS are overweight or obese which can affect absorption and influence of Vitamin D in the body. This cumulated with people avoiding direct sunlight, increased sunscreen application; obese people tend to cover in the sun and living in the northern hemisphere result in lower vitamin D levels. We hypothesize that Vitamin D may have an influence in the outcomes of IVF cycles in women undergoing IVF with and without PCOS.

Within IVF despite achieving good theoretical and practical experience in achieving fertilization the implantation rate remains low. It is thought that the

most important factor affected implantation is the endometrium. Unfortunately for many women going through the process of assisted conception there can be an ongoing problem with implantation leading to the phrase, recurrent implantation failure. The process of the stages of the developing endometrium is too vast for this thesis, but instead we are looking into treatments that may help the developing embryo to implant in women with recurrent cycles of IVF where implantation has failed to take place. An endometrial scratch has been demonstrated to help implantation in women with RIF by causing trauma to the endometrium allowing the developing embryo to implant. The mechanism on why this procedure may aid implantation is not fully understood but possibly by the release of cytokines and growth factors creating a more favourable environment for implantation. In the Hull IVF unit we offer a Mock-ET that may cause some trauma to the endometrium prior to commencing an IVF cycle, but this is not performed in second and subsequent cycles. We hypothesise that the Endometrial Scratch performed the cycle prior to commencing an IVF/ICSI may offer more benefit than a Mock-ET in a first cycle of IVF/ICSI or doing nothing at all in second and subsequent IVF/ICSI cycles.

Finally research has shown that a subtype of white blood cell called the natural Killer (NK-cell) may interfere with the process of implantation as they are cytotoxic to the embryo, and a supplement of 20% Intralipid may aid in reducing the concentration of these cells in the blood. However there are few studies within a patient population to verify this hypothesis. The fourth and final hypothesis is that intravenous Intralipid will reduce the concentration of Natural Killer cells in the blood of women with recurrent failed cycles of IVF.

The objectives of this thesis were

1. To conduct a prospective study investigating whether Endocrine Disrupting chemicals were detectable in the serum of a population of women with and without PCOS residing, and do these chemicals have

any effects on pregnancy rates and aspects of a controlled ovarian hyperstimulation IVF cycle. (Chapter 2)

2. To conduct a pilot study to see what effect Vitamin D levels have on controlled ovarian hyperstimulation during an IVF cycle in women with and without PCOS.

(Chapter 3)

3. To conduct an observational study to see the effect of endometrial scratch on In vitro Fertilisation in an unselected group of women.

(Chapter 4)

4. To conduct a pilot study to see what effect intravenous Intralipid had on Natural Killer cells in women with recurrent failed cycles of IVF and compare this against a cohort of women undergoing their first cycle of IVF.

(Chapter 5)

CHAPTER TWO:

Are endocrine disrupting chemicals detectable in the serum of sub-fertile women with and without Polycystic Ovary Syndrome, are the levels of these chemicals associated with pregnancy rates and aspects of a controlled ovarian hyperstimulation IVF cycle.

2.1 Abstract

Background: Endocrine Disrupting Agents (EDAs) are ubiquitous chemical substances that are used in agriculture as pesticides, and in the production of household goods, and have the ability to interfere with the natural hormone balance within a healthy person. These chemicals can agonise or antagonise the effects of endogenous hormones, having the potential to interfere with the natural endocrine pathways such as the reproductive axis. Polycystic ovary syndrome (PCOS) is a common endocrine condition resulting in, hyperinsulinaemia, hyperandrogenaemia and subfertility. This study was conducted to see whether there was any association between EDAs and this group of women.

Method: Serum samples were collected from 59 (29 PCOS and 30 controls) women undergoing IVF/ICSI. The samples were analysed using gas chromatography combined with mass spectrometry to measure the concentration of polychlorinated biphenyls (PCBs), organochlorine pesticides, polybrominated diphenyl ethers and polyfluoroalkyl agents (PFAAs). Statistical analysis was performed to assess for potential associations with the levels of serum contamination of these EDAs, pregnancy rates and various characteristics of an IVF cycle between the PCOS and control groups.

Results: The polyfluoroalkyl congeners PFOS, PFOA, PFHxS and PFNA, the PCBs 118, 138, 153, 180, were detected in all participants. The levels of EDAs in the

serum were comparable in each group with only PFOS having a significantly higher concentration in the PCOS group, (4.11 ± 1.62 ng/ml vs. 3.11 ± 1.05 ng/ml, $p=0.03$). The PFAAs had significant positive correlations with testosterone in the overall patient population ($p=0.001$) and in both the control ($p=0.02$) and PCOS ($p=0.03$) subgroups. The PFAAs, PCBs and p,p-DDE demonstrated significant positive correlations with cleavage rates ($p=0.04$, 0.01 , and 0.04 respectively). There was no correlation between the levels of EDAs and pregnancy in either the PCOS and control groups.

Conclusion: This study demonstrates that EDAs are detectable within subfertile women and that PFOS is significantly higher in PCOS women. There is evidence that these chemicals may disrupt not only endocrinological pathways but also affect the cleavage stage in early embryo development.

2.2 Introduction

Endocrine Disrupting Agents (EDAs) are external substances (man made or naturally occurring) that have the potential to interfere with the natural hormone balance within a healthy person. The chemicals can antagonise or agonize the effects of endogenous hormones and interfere with the natural endocrine pathways resulting in adverse effects in that individual whether homeostatic, reproductive or developmental. (Kavlock et al., 1996, Diamanti-Kandarakis et al., 2009; Younglai et al., 2005).

Exposure to these substances is through environmental contaminants in the air, water, food, consumer products and, in some cases, occupational exposure. Food is a major source of exposure to some of these compounds, whether from packaging or from the consumption of contaminated meat (Schechter et al., 2004). Research has shown that many of these compounds are highly lipophilic and the levels bio-accumulate in higher species. (Kelly et al. 2004; Frederiksen et al. 2009).

Research into wildlife exposed to these endocrine-disrupting chemicals has demonstrated changes in both male and female fertility including gonadal development, maturation of germ lines, fertilisation and pregnancy. Blood samples from these studies have shown altered steroid hormone profiles supporting this. (Guillette et al 1995; Milnes and Guillette 2010).

Within the human population there have been links to the effects of endocrine disruptors on both male and female infertility as well as time to pregnancy, however no strong relationship has been made (Axmon et al., 2005; Fei et al., 2009; Harley et al., 2010). In the UK today, 1 in 7 heterosexual couples is affected by infertility, of which 25% fall under the umbrella of unexplained infertility i.e., having no identified male or female cause following all investigations (NICE Clinical Guidance 156). The only treatment at this point is

through the means of IVF, with the most recent data on outcomes giving an implantation rate of 32% for fresh embryo transfers resulting in a clinical pregnancy (Ferraretti et al., 2013).

NICE (CG156) estimated 25% of patients suffer from anovulatory infertility. PCOS (WHO Group 2 anovulatory women) would account for 80% of women presenting with anovulation. It is one of the most common endocrine disorders affecting women (Azziz et al., 2004). The most common symptoms are anovulation and excess androgens resulting in ovulation-related infertility. These patients have large markers of ovarian reserve as demonstrated by both their AMH and high antral follicles count as a result the hyper-androgenic environment.

Studies have shown a possible link with PCOS and endocrine disrupting chemicals demonstrating correlations with androgens and insulin resistance (Kandaraki et al., 2011). Endocrine disruptors could thus disrupt the androgen-oestrogen balance of the body resulting in PCOS like conditions. This could be relevant as many endocrine disrupting chemicals are lipid soluble and individuals could be exposed to them constantly. This could result in germ cell lines being affected prior to fertilisation through to exposure in the perinatal period and from childbirth.

2.2.1 Serum and Follicular fluid to assess endocrine disruptor concentrations

Studies have been able to detect endocrine disruptors in both serum and follicular fluid samples. It has been demonstrated that serum levels are best at detecting overall exposure of that individual to the particular contaminant. However, Johnson et al. (2012) hypothesized that the concentrations of endocrine disruptors in follicular fluid might be a more relevant dose metric for examining associations with infertility. However, detection rates for the examined chemicals in follicular fluid were lower than in serum, and significant

variability in ratios between follicular fluid and serum between subjects was observed. They concluded that serum measurements of polychlorinated biphenyls (PCBs) within their study context were reliable measures of exposure to the oocyte (Johnson et al., 2012). We applied this reasoning to the full suite of compounds examined in the current study.

The objective of this pilot study was to assess if there is evidence of endocrine disrupting chemicals in the blood of sub-fertile women with and without PCOS undergoing a short antagonist IVF protocol. Secondary objective was to determine if there was any relationship between EDAs and whole group clinical parameters.

2.3 Materials and Methods

The NRES Committee Yorkshire & The Humber – Humber Bridge Ethics Committee in November 2013, approved the study. Women whom had been diagnosed with subfertility and requiring IVF or ICSI-IVF at the Hull IVF Unit from 2014 were invited to participate in the study, before commencing on their IVF or ICSI-IVF cycle.

A patient information sheet was created for this study and was passed by the NRES Committee Yorkshire & The Humber – Humber Bridge Ethics Committee. A member of the research team was present at the monthly group session held at the East Riding Medical Education Centre within Hull Royal Infirmary to give a short presentation regarding the study and ask questions. A patient information sheet was given to all patients after the group session. The patients could then contact the unit if they were willing to take part in the study. We attained most of our patient's through the group sessions and thus the majority of our patients were undergoing their first cycle of IVF.

We planned to collect 60 subjects for our study. Power and sample size for pilot studies has been reviewed by Birkett and Day. (1994). They concluded that a minimum of 20 degrees-of-freedom was required to estimate effect size and variability. Hence, we intend to recruit 30 patients allowing for drop-outs and covariate adjustment.

Twenty-nine patients with PCOS were recruited. PCOS was diagnosed using the revised 2003 criteria from the Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group (ESHRE/ASRM 2004), indicating PCOS to be present if any 2 out of 3 criteria are met:

1. oligo and/or anovulation
2. clinical and/or biochemical signs of androgenism
3. polycystic ovaries (either 12 or more peripheral follicles or increased ovarian volume (greater than 10 cm³))

Diagnosis of PCOS can only be made when other aetiologies have been excluded (thyroid dysfunction, congenital adrenal hyperplasia, hyperprolactinaemia, androgen-secreting tumours and Cushing syndrome). The other 30 patients were women undergoing IVF without PCOS.

2.3.1 Sample collection

Women who expressed an interest to take part in the study were asked to attend the IVF unit at the time of their review appointment. This appointment occurred in the luteal phase of their menstrual cycle the cycle prior to commencing their IVF. At this appointment the patients completed their Human Fertilisation and Embryology Authority (HFEA) consent forms, underwent their Mock embryo transfer and were instructed on injection technique.

The patients were invited to come fasted from midnight and to attend their appointment 30 minutes early so informed written consent could be taken and

fasting blood samples could be collected without affecting their review appointment. The bloods were put on ice and transferred to the Michael White Diabetes Centre, Anlaby Road, Hull approximately 5 minutes' walk from the IVF unit. At this point the bloods were centrifuged at 3500g for 15 minutes and 5°C placed into aliquots and frozen at -80°C.

The assays were processed at the biochemistry laboratory at Hull Royal Infirmary, Anlaby Road, Hull.

FSH:- Architect analyser (Abbott laboratories, Maidenhead, UK)

Total Testosterone:- (Abbott laboratories, Maidenhead, UK)

SHBG:- immunometric assay with fluorescence detection on a DPC Immulite 200 analyser (Euro/DPC, Llanberis UK)

Insulin:- competitive chemiluminescent immunoassay performed on a DPC Immulite 200 analyser (Euro/DPC, Llanberis UK)

Plasma Glucose:- Synchron LX20 analyser (Beckman-Coulter)

FAI:- this was calculated by dividing the Total cholesterol by SHBG, and then multiplying by a 100.

Endocrine Disrupting Chemicals, 40-50ml of serum was collected and stored at -80°C.

2.3.2 The IVF Cycle

All patients underwent a standard IVF antagonist protocol. The patients commenced their rFSH stimulation on day 2 of their menstrual cycle using either Merional (Pharmasure) or Gonal-F (Merck Serono) for women with PCOS. A GnRH antagonist (Cetrotide: Merck Serono) was used to prevent a premature LH surge.

The patients underwent ultrasound scans from day 7 to observe the ovarian response to stimulation and were repeated every 48 hours. The scans were used to measure the diameters of the follicles thus observing response and follicle numbers. The hCG trigger Buserelin (Sanofi-Aventis, Frankfurt, Germany) or Pregnyl (Merck Sharp and Dohme) was administered when the leading follicle was $\geq 18\text{mm}$.

Oocyte retrieval was performed 36 hours later. This was carried out under sedation. The follicles were aspirated under ultrasound guidance by the consultants, nurse consultant or Clinical Research Fellow. Luteal support was provided with micronised progesterone (Uterogestan; Besins Iscovesco Laboratories, Paris, France) at a dose of 600mg each night commencing on the day of oocyte retrieval. The embryologists at this point according to whether the patient was undergoing IVF or ICSI-IVF inseminated the oocytes directly or the oocytes were stripped and had sperm injected 4 hours after retrieval. Embryo transfers were performed on day 3 or ideally at day 5 (blastocyst) to give the best chance for implantation.

Transcervical embryo transfer was performed in the lithotomy position according to the plan documented at the mock embryo transfer. The embryo transfer catheters used in this period were Rocket Soft 18cm (Rocketmedical PLC, Washington, United Kingdom) and the Sydney IVF Embryo Transfer Set (Cook Medical, Limerick, Ireland). The embryos were suspended in 20-25uL of culture medium (Quinns Advantage. Blast, SAGE, USA).

A pregnancy test was performed 14 days following oocyte retrieval. If this was positive the patient would continue the progesterone (Uterogestan; Besins Iscovesco Laboratories, Paris, France) for luteal support. They would attend the IVF unit for a trans-vaginal Ultrasound scan to assess, for an intrauterine

pregnancy, viability, and number of fetuses. Clinical pregnancy was defined as a fetal heartbeat on a trans-vaginal ultrasound between cycle days 42 and 49.

2.3.3 Analysis of endocrine disrupting chemicals in serum

All blood samples were analysed for 14 polyfluoroalkyl congeners, 7 Polychlorinated Biphenyl (PCB) congeners, 7 Polybrominated diphenyl ether (BDE) congeners, hexabromocyclododexanes (α -HBCDD, β -HBCDD, γ -HBCDD), and the pesticides perclorobenene (PeCB), hexachlorobenzene (HCB), hexachlorocyclohexanes (γ , α , and β -HCH), chlordanes (trans (γ) chlordane, Cis (α) Chlordane), dichlorodiphenyltrichloroethane (p,p-DDT) and its metabolites (op-DDE, pp-DDE, op-DDD), and Mirex. Analyses were conducted at the National Research Centre for Environmental Toxicology (ENTOX) at the University of Queensland in Brisbane, Queensland, Australia.

Liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)

This technique was used to measure the detectable levels of endocrine disruptors in the serum. Established analytical methods are available for many of the endocrine disrupting chemicals. Extraction of these chemicals from liquid samples such as water is based on liquid-liquid extraction followed by gas chromatography or high performance liquid chromatography (Ling et al., 2007). Most developed countries such the US have established regulatory authorities such as the United States Environmental Protection Agency and Agency for Toxic Substances and Disease Registry for testing pesticides, metals, industrial chemicals and PCBs in food and environmental matrices. As a result of differing organisations analyzing these chemicals using various analytical methods, suitable instrumental techniques or standard methods of analysis are lacking. As a result if different techniques are used to assess the presence of these compounds within human tissue samples differing limits of detection are published throughout the world. We are the first group to study the UK serum

levels of these endocrine disrupting chemicals in an infertile population and are aware of the limitations when discussing values.

i) Analysis of Polyfluoroalkyl Substances in Serum

Reagents and sample preparation Ammonium acetate (>97%) was purchased from Chem-Supply (Gillman, SA, Australia), and methanol (99.8%, LiChrosolv®) and acetonitrile (99.9%, LiChrosolv®) from Merck (Darmstadt, Germany). All water used was laboratory produced ultra-pure water. A native standard mix containing native PFAAs (Table 2.1), and a mass labeled internal standard mix (13C4-PFBA, 13C2-PFHxA, 13C4-PFOA, 13C5-PFNA, 13C2-PFDA, 13C2-PFUnDA, 13C2-PFDoDA, 18O2-PFHxS, and 13C4-PFOS) was from Wellington Laboratories (Guelph, Ontario, Canada).

Table 2.1 Native PFAA and their acronyms.

Native PFAA	Acronym
perfluorobutanoic acid	PFBA
perfluoropentanoic acid	PFPeA
perfluorohexanoic acid	PFHxA
perfluoroheptanoic acid	PFHpA
perfluorooctanoic acid	PFOA
perfluorononanoic acid	PFNA
perfluorodecanoic acid	PFDA
perfluoroundecanoic acid	PFUnDA
perfluorododecanoic acid	PFDoDA
perfluorobutanesulfonate	PFBuS
perfluorohexane sulfonate	PFHxS
perfluorooctane sulfonate	PFOS
Perfluorodecane sulfonate	PFDS

An aliquot of 200 μ l serum was transferred to a 2 ml eppendorf tube followed by addition of the internal standards. Acetonitrile was used to precipitate the proteins and ultrasonication and vortex mixing facilitated the extraction. After centrifugation, the supernatant was filtrated into a LC vial through a 2 μ m GHP membrane (Pall, East Hills, NY, USA) and reduced to 200 μ l using nitrogen, after which 300 μ l 5 mM ammonium acetate in water and the performance standards 13C8-PFOS and 13C8- PFOA were added (Wellington Laboratories).

HPLC-MS/MS Analysis

The analytical system consisted of a Nexera HPLC (Shimadzu Corp., Kyoto, Japan) coupled to API5500QTRAP mass spectrometer (AB/Sciex, Concord, Ontario, Canada) with electrospray ionization (ESI) interface operating in negative mode. Chromatographic separation of the analytes was achieved with a 4 micron 50 \times 2.0mm, 4 μ m C18 Gemini column (Phenomenex, Torrance, CA), maintained at 45 $^{\circ}$ C, with a flow rate of 0.3 mL/min and injection volume of 5 μ L. Mobile phases consisted of methanol:water (1:99, v/v) (A), and methanol:water (95:5, v/v) (B), with 5mM ammonium acetate in both phases. An isolator column (Phenomenex) was included inline directly after the mobile phase mixing chamber to delay the elution of solvent-derived background PFC contamination. Data acquisition and processing was carried out using Analyst[®] TF 1.6 and MultiQuant[™] software (AB Sciex). Quality assurance, the limit of reporting (LOR) was determined by quality of peak shape and using a minimum signal-to-noise ratio of 10:1 in matrix. Contamination was assessed by inclusion of procedural blanks (milli-Q water, n=3) extracted in parallel with unknown samples. Linearity was established from 0.05 to 25ng/mL serum using non-extracted aqueous standards, with R² values >0.99. Analytes were identified based on retention time (<2% RSD from standard), and confirmed by a second MRM transition where possible (confirmation transitions were not available for PFPeA, PFBS, PFDA, PFUnDA, PFDoDA, and PFBA). Quantification was performed using isotope dilution mass spectrometry. PFHxS, PFOS, PFHxA, PFOA, PFNA,

PFDA, PUnDA and PDoDA were quantified against their respective mass-labelled analogues; PFBuS was quantified against 18O2-PFHxS; PFPeA and PFHpA against 13C2- PFHxA; and PFDS against 13C4-PFOS. Method precision and accuracy were assessed using a certified reference material, SRM 1957 (National Institute of Standards and Technology, NIST).

ii) Analysis of Organochlorine and Organobromine Substances in Serum

The Polychlorinated Biphenyl (PCB) congeners, Polybrominated diphenyl ether (BDE) congeners, Hexabromocyclododexanes (α -HBCDD, β -HBCDD, γ -HBCDD), and the pesticides Perchlorobene (PeCB), Hexachlorobenzene (HCB), hexachlorocyclohexanes (γ , α , and β -HCH), chlordanes (trans (γ) Chlordane, Cis (α) Chlordane), Dichlorodiphenyltrichloroethane (p,p-DDT) and its metabolites (op-DDE, pp-DDE, op-DDD), and Mirex were investigated in this study.

Extraction

6 mL of serum was aliquoted into 50 mL Falcon tubes. For targeted analysis of PBDEs, organochlorine pesticides (OCPs), PCBs and HBCDDs, samples were spiked with 5 ng of each of $^{13}\text{C}_{12}$ -labelled BDEs -28, -47, -99, -100, -154, -153 and -183, $^{13}\text{C}_{12}$ -labelled α -, β - and γ -HBCDDs, $^{13}\text{C}_{12}$ -labelled PCBs -28, -52, -101, -118, -138, -153 and -180, and $^{13}\text{C}_6$ -labelled Hexachlorobenzene. For non-targeted analysis samples were also spiked with a suite of deuterated pharmaceuticals and personal care products containing 40ng of each of atenolol-d₇, atrazine-d₅, caffeine-d₃, carbamazepine-d₁₀, chlorpyrifos-d₁₀, diazinon-d₁₀, diclofenac-d₄ and metolachlor-d₆.

After vortexing the samples for 1 minute, 6 mL acetonitrile, 3 mL milliQ water, 5 g anhydrous MgSO_4 and 1 g NaCl were added along with a ceramic homogenizer. Samples were manually shaken for 1 minute, before being centrifuged at 4700 RPM for 8 minutes at 10 °C. The supernatant layer (approximately 6 mL acetonitrile) was collected and transferred to 12 mL glass

vials. An aliquot of 200 μL was collected and cleaned-up for non-targeted analysis. The remainder of the extract was cleaned-up for targeted analysis.

Non-target clean-up

For non-target analysis, 200 μL of the extract was collected and diluted with 400 μL of Acetonitrile (0.1% formic acid) to ensure destruction of all proteins. The solution was passed through a Captiva ND lipid filter cartridge (3 mL, Agilent). A 300 μL aliquot was then transferred to an autosampler vial and evaporated to near-dryness under a gentle stream of nitrogen and reconstituted in 100 μL of a 20% methanol in water solution.

Target clean-up

For targeted analysis, the remainder of the initial extract was evaporated under a gentle stream of nitrogen to near-dryness and reconstituted in 1-2 mL hexane. 1 mL >98% concentrated sulfuric acid was added and the sample was vortexed for 30 seconds. The aqueous and hexane layer were left to separate overnight at $<4\text{ }^{\circ}\text{C}$. The supernatant (hexane) layer was transferred directly onto a silica SPE cartridge (Supelco LC-Si, 3 mL/500 mg) preconditioned with 6 mL dichloromethane (DCM) and 6 mL hexane. Target compounds were eluted into a 15 mL glass collection tube using 6 mL hexane followed by 8 mL DCM. Samples were evaporated to near-dryness and reconstituted in 100 μL methanol containing 2.5 ng $^{13}\text{C}_{12}$ -PCB-141 and $^{13}\text{C}_{12}$ -TBBPA as a recovery standard.

Instrumental Analysis

HBCDDs (α -, β - and γ -) were measured in serum samples using an AB/Sciex API 5500Q mass spectrometer (AB/Sciex, Concord, Ontario, Canada) coupled to a Shimadzu Nexera HPLC system (Shimadzu Corp., Kyoto, Japan). The mass spectrometer (MS) was operated in multiple reaction monitoring (MRM) mode using negative electrospray ionisation (ESI). A volume of 5 μL was injected. Separation was achieved using a Kinetex XB C18, 50 x 2.0 mm 1.7 μm column

(Phenomenex, Torrance CA) using a mobile phase gradient of 85% methanol, ramping up to 100% methanol over 6 min and then holding for 4 min at a flow rate of 0.3 mL/min. Full MS parameters are detailed in the supporting information (Table 2.2).

Table 2.2 MRM values and LC-ESI-MS/MS parameters for analysis of HBCDD

Compound	Precursor (m/z)	Fragment (m/z)	Declustering Potential	Entrance Potential	Collision Energy	Cell Exit Potential
HBCDD	640.6	78.9	-75 V	-8 V	-70 eV	-10 V
	640.6	80.9				
¹³ C ₁₂ ⁻ HBCDD	652.6	78.9	-75 V	-8 V	-70 eV	-10 V
	652.6	80.9				
¹³ C ₁₂ ⁻ TBBPA	554.7	78.9	-110 V	-10 V	-115 eV	-10 V
	554.7	80.9				

BDEs, PCBs and OCPs were analysed by high resolution gas chromatography coupled with high resolution mass spectrometry (HRGC/HRMS). A Thermofisher TRACE 1300 gas chromatograph was coupled to a Thermofisher DFS mass spectrometer. The injector was operated in splitless mode with separation achieved on an Agilent DB-5ms column (30 m length x 0.25 mm in diameter x 0.25 µm film thickness). Experiments were conducted in MID mode at 10,000 resolution (10% valley definition). The inlet, transfer line and source were held at 250 °C, 280 °C and 280 °C respectively. The flow rate was maintained at 1.0 mL/min. Similar ramp rates were used for both the PBDE injection and for OCPs and PCBs. An initial temperature of 80 °C was held for two minutes before ramping to 180 °C at 20 °C/min and hold for 0.5 min. The temperature was then increased to 300 °C at 10 °C/min and held for 5 minutes.

For non-target analysis, chromatography of the polar analytes was achieved using a Shimadzu Nexera X2 UHPLC system equipped with a binary pump. A

reverse-phase XDB-C18 analytical column (4.6 mm × 50 mm and 1.8 μm particle size) (Agilent Technologies, Santa Clara, CA) was used for the negative mode and a XDB-C18 column (2.1 mm × 100 mm and 1.8 μm particle size) (Agilent Technologies, Santa Clara, CA) for the positive mode. Compounds were analysed in negative ionization mode with 1% methanol in MilliQ water as mobile phase A and 10% MilliQ water in methanol as mobile phase B, with 5 mM ammonium acetate in both phases. The initial gradient (10% B) was held for 0.20 min. A gradient ramp followed over 6.50 min to 100% B, which was held for 3 min, then returned to the initial composition in 0.10 min, followed by equilibration for 2.20 min. The injection volume was 10 μL. The column unit was held at 45 °C and the flow rate was 0.6 mL/min. For analysis in positive ionization mode mobile phases A and B were MilliQ water and methanol with 0.1% formic acid in both phases. A linear gradient ramped from 5% B to 100% B in 10 min, and then kept for 4.50 min at 100% B, then returned to the initial conditions in 0.10 min, followed by equilibration for 2 min. The flow rate was 0.4 mL/min, the column oven was held at 50°C. An injection volume of 5 μL was used.

The UHPLC was coupled to a hybrid quadrupole time-of-flight mass spectrometer, TripleTOF 5600 System (AB Sciex) with electrospray ionization (ESI) interface working in positive and negative ionization modes. The ion source parameters were optimized to the following final conditions: ion spray voltage floating (ISVF), -4500 V and 5500 V for negative and positive ionization, respectively; temperature (TEM), 600°C; curtain gas (CUR), 35 L/min and ion source gas (GS1 and GS2) at 70 psi. High purity nitrogen was used as the nebulizer gas, curtain gas and collision gas. The MS was operated in full-scan TOF-MS (m/z 100-950) and MS/MS mode (m/z 30-950) through Information Dependent Acquisition (IDA) in single run analysis. The parameters, declustering potential (DP) and collision energy (CE) were (-)80 V and (-)10 eV in the full-scan TOF-MS experiment.

The acquisition method developed via IDA simultaneously combined TOF-MS survey scan and dependent MS/MS scans (programmed with a maximum of 10 candidate ions to monitor per cycle). The criteria for the IDA experiment were: (i) an intensity threshold of 500 counts per second (cps) (units); (ii) a mass range of 30 to 950 Da and; (iii) a mass tolerance of 10 ppm. The CE was ramped over an interval by entering a CES value. Both the CE and CES were set at (-)35 and 15 eV, respectively (which means 35 ± 15 eV) to achieve optimal fragmentation of the precursor ion and subsequent sufficient structural information in MS/MS mode. Dynamic background subtraction (DBS) criteria by IDA was applied.

Mass calibration and resolution adjustments were performed automatically using a 10^{-5} mol/L solution of poly (propylene glycol) introduced via a syringe pump and connected to the interface. The instrument was calibrated in full-scan TOF-MS and MS/MS modes. The mass spectrometer was operated with a resolution power (RP) of approx. 30000 FWHM (full width at half maxima) at m/z 956. This QTOF uses an automated external calibration system for mass accuracy with an injector system (CDS, calibrant Delivery System Status) and an internal auto calibration by means of an interactive recalibration tool based on the common background ions found.

Post-acquisition data processing.

Post-acquisition data processing was carried out for the identification of target compounds and the structural characterization of non-target and unknown polar compounds. Data obtained with LC-ESI-QTOF/MS were processed with PeakView[®] software (AB Sciex), which incorporates tools to display, filter, and process data-dependent MSⁿ acquisition (IDA), along with XIC Manager and MS Library tools. XIC Manager and the MS library were used for rapid screening and identification of target compounds and for other compounds not included a priori in the analytical method (considered as non-target compounds in this study). The library of accurate mass information and MS/MS spectra from AB

Sciex was utilized, which includes approx. 3000 compounds (mainly pesticides and pharmaceuticals). In addition to this, a new library database containing both TOF MS and MS/MS spectra of target compounds selected in this study at CE (-)35±15 eV was made.

Confirmation of target analytes was based on the retention time, the accurate mass measurement of the (de)protonated molecule (mass error), the isotopic pattern and by automatic MS/MS library searching. For the positive identification of the target compounds with the automatic searching the following values were selected: retention time ± 0.5 min, isotope score > 60%, library score >70%, mass error <5 ppm and mass error score > 80%. The default threshold for the XIC (extracted ion chromatogram) was 1000 cps, S/N < 5 and peaks > 500 counts. For the identification of non-target analytes included in the commercial library the same criteria were used, except for the retention time. The confirmation of non-target analytes and structural characterization of unknown analytes (analytes not included in the MS/MS libraries) was performed using PeakView software in this study). The library of accurate mass information and MS/MS spectra from AB Sciex was utilized, which includes approx. 3000 compounds (mainly pesticides and pharmaceuticals).

Confirmation of target analytes was based on the retention time, the accurate mass measurement of the (de)protonated molecule (mass error), the isotopic pattern and by automatic MS/MS library searching. For the positive identification of the target compounds with the automatic searching the following values were selected: retention time ± 0.5 min, isotope score > 60%, library score >70%, mass error <5 ppm and mass error score > 80%. The default threshold for the XIC (extracted ion chromatogram) was 1000 cps, S/N < 5 and peaks > 500 counts. For the identification of non-target analytes included in the commercial library the same criteria were used, except for the retention time. The confirmation of non-target analytes and structural characterization of

unknown analytes (analytes not included in the MS/MS libraries) was performed using PeakView software and Formula Finder tool and based on the accurate mass measurement (mass error < 5 ppm), elemental composition assignment, isotopic pattern distribution, ring and double bonds factor and MS/MS spectrum interpretation. The MOL File in-silico fragmentation application, inside the PeakView software, was used for the structural elucidation of MS/MS fragments of unknown analytes. This data processing includes the matching of the experimental MS/MS spectrum with a list of theoretical fragment masses, which was performed by applying a mass tolerance threshold, lower than 20 ppm. Mass errors observed in MS/MS fragment ions are typically over 5 ppm, probably because of the highly selective operation mode leading to a greater reduction in the background signals which are used as internal auto-calibration.

The quantification of targeted compounds was performed using MultiQuant™ software (AB Sciex), allowing the extraction and processing of mass spectral data using the LC retention time and the molecular ions accurate mass.

2.3.4 Data analysis and Statistics

Statistical analysis was performed using SPSS (v22, Chicago, Illinois). Descriptive data is presented as mean \pm SD for continuous data and n (%) for categorical data. T-tests were used to compare means for normally distributed data and Mann Whiney tests for non-normally distributed data.

Because of the relative low number of samples in the study and the detection of outliers, the Wilcoxon matched-pairs signed-rank test was used to examine the difference in compound concentrations between the PCOS and control groups. Spearman's rank correlation was performed to assess between the detectable levels of the organochlorines, organobromines and polyfluoralkyl chemicals and androgen, insulin status and ovarian reserve (AMH and total antral follicle

count) of PCOS and control subjects and against the outcomes of the IVF cycle (oocyte number at ovum retrieval, fertilisation and cleavage rates and average embryo quality). A p-value of <0.05 was considered to indicate statistical significance.

Following the review of the literature the decision was taken to follow the analysis taken by Petro et al (2012). All those chemicals in which <50% of the results were above the limits of quantification (LOQ) were excluded from the statistical analysis. However in chemicals where >50% of the samples tested above the LOQ, those samples with levels below the LOQ were assigned a value of $f \times \text{LOQ}$ with f being the detection frequency or the ratio between the number of samples detected above the LOQ and the total number of samples analysed (Voorspoels et al. 2002).

Principal components analysis (PCA), a data reduction technique, was performed to reorganise the original variables into 5 new variables namely OCPs, PCBS, BDES, HBCDD and polyfluoroalkyl agents for the 59 subjects. Correlations were assessed between these variables and the outcome variables, ovarian responsiveness, embryological response, and endocrine profile.

2.4 Results

59 women were recruited into the study from patients attending the Hull IVF unit. There were 30 controls and 29 women with PCOS as diagnosed through the Rotterdam Consensus Criteria. The mean age and BMI of the controls and PCOS women were similar (32.6 ± 4.70 vs. 30.9 ± 4.80 , $p=0.14$ and 25.5 ± 3.60 vs. 26.0 ± 3.80 , $p=0.56$ respectively). Of the controls, the majority had a male factor infertility diagnosis ($n=15$, 50%), the remaining were unexplained ($n=6$ 20%), tubal ($n=5$, 16.66%), anovulatory ($n=2$, 6.66%) and endometriosis ($n=2$, 6.66%). 56% of the subjects underwent IVF and the other 34% underwent ICSI.

Table 2.3 Demographics for Control and PCOS patients

	Control			PCOS			p-value
	Mean	SD	n	Mean	SD	n	
Age	32.6	4.7	30	30.9	4.8	29	0.14
IVF cycle	1.2	0.6	30	1.2	0.7	29	1.00
Menarche	13.1	1.9	30	12.9	1.2	29	0.63
Years trying	3.8	1.7	30	3.4	1.6	29	0.36
BMI (kg/m ²)	25.5	3.6	30	26.0	3.8	29	0.56
AMH (pmol/l)	23.44	13.30	30	56.39	14.23	29	0.0001 ^{***}
Total Antral Follicle Count	17.2	6.8	30	38.4	17.8	29	0.0001 ^{***}
Endo at OR	10	2	30	11	2	28	0.42
Follicles Aspirated	11	5	30	16	5	28	0.002 ^{**}
Eggs Retrieved	8	5	30	11	5	28	0.04 [*]
No. Fertilised	5	3	28	8	4	28	0.004 ^{**}
Fertilisation rate	0.66	0.27	28	0.75	0.20	28	0.19
No. Cleaved	4.7	2.7	28	7.3	4.4	23	0.01 [*]
Cleavage rate	0.94	0.19	28	0.82	0.31	23	0.35
No abnormal fertilised	0.5	1.70	28	1.1	3.10	23	0.39
≥G 3 day 3	3.0	2.30	27	4.2	3.50	23	0.16
Prop top Quality embryo	0.38	0.29	27	0.36	0.22	23	0.79
Fasting Glucose (nmol/L)	4.81	0.35	29	4.49	0.82	29	0.06
Insulin Result (uIU/ml)	7.68	4.01	30	8.13	4.69	29	0.69
HOMA-IR	1.69	1.00	30	1.97	1.58	29	0.97
Testosterone (nmol/l)	0.79	0.36	30	1.12	0.52	29	0.005 ^{**}
SHBG (nmol/l)	114.23	80.86	30	60.45	51.47	29	0.004 ^{**}
FAI	1.35	0.57	30	4.21	2.91	29	0.0001 ^{***}
Positive pregnancy Test		11			10		1.00
Clinical Pregnancy		10			7		0.57

(*p<0.01, **p<0.001, ***p<0.0001)

Table 2.3 displays the mean demographic, IVF cycle, and biochemical results. There was significant difference in ovarian reserve parameters AMH and total antral follicle count between the control and PCOS groups, (AMH 23.44 ± 13.00 pmol/l vs. 56.39 ± 14.23 pmol/l, $p=0.0001$; and antral follicle count 17.2 ± 6.80 vs. 38.4 ± 17.8 , $p=0.0001$, respectively).

There was no significant difference in fasting insulin between the groups ($p=0.69$) and the mean HOMA-IR in the control and PCOS groups was similar (1.69 ± 1.00 vs. 1.97 ± 1.58 , $p=0.97$). However there was significant difference in androgen status between the control and PCOS groups (testosterone 0.79 ± 0.36 nmol/l vs. 1.12 ± 0.52 nmol/l, $p=0.005$ and FAI 1.35 ± 0.57 vs. 4.21 ± 2.91 , $p=0.0001$).

Regarding the IVF results, there was no difference in endometrial thickness between the control and PCOS groups at the time of ovum retrieval (10.00 ± 2.0 vs. 11 ± 2.0 , $p=0.42$ respectively, Table 2.4). As expected the PCOS group had a significantly greater numbers of follicles aspirated and eggs retrieved compared to the controls (16 ± 5.0 vs. 11.0 ± 5.0 , $p=0.002$ and 11.0 ± 5.0 vs. 8.0 ± 5.0 , $p=0.03$ respectively). Despite having a significantly higher number of ovum that fertilised in the PCOS group ($p=0.004$) there was no significant difference in fertilisation rates between the groups ($p=0.19$). There was no difference in the quality of the embryos at day 3 ($p=0.16$).

Two PCOS and one-control woman developed OHSS and their embryos were frozen. One PCOS subject was cancelled during stimulation due to having over 80 follicles and unresponsive to the stimulation. Four subjects failed to achieve fertilisation (3 PCOS, 1 control). These women were not excluded from the final analysis despite not continuing to proceed in their treatment to achieve an embryo transfer. There was an overall clinical pregnancy rate of 35.6%, of which 10 (33.3%) of the control and 7 (24.1%) of the PCOS subjects achieved a clinical

pregnancy. There was no significant difference ($p=0.84$) in clinical pregnancy rates between the groups.

2.4.1 Endocrine Disrupting Chemicals

The polyfluoroalkyl congeners PFOS, PFOA, PFHxS and PFNA, the PCBs 118, 138, 153, 180, HCB, p,p-DDE, BDE 47 were detected in nearly every serum sample. Additional chemicals that were detected in over 50% of the serum samples were PFDA, bHCH, BDE 100, BDE 99, BDE 153, α -HBCDD and p,p-DDT, and these were also included in the statistical analysis (Table 2.4). The remaining compounds were detected less frequently or not at all (Table 2.4).

Table 2.4 Detection frequencies (DF) and mean concentrations of EDAs within the PCOS and control subjects.

Analyte	L.O.D	PCOS		Control		p-value
		Detection n (%)	Mean \pm S.D.	Detection n (%)	Mean \pm S.D.	
PFOS	0.5 ng/ml	29 (100)	4.11 \pm 1.62	30 (100)	3.33 \pm 1.05	0.03*
PFOA	0.1 ng/ml	29 (100)	2.95 \pm 1.74	30 (100)	2.56 \pm 1.42	0.35
PFHxS	0.05 ng/ml	29 (100)	1.59 \pm 1.82	30 (100)	0.99 \pm 0.44	0.09
PFBA	0.5 ng/ml	0 (0)	0.00 \pm 0.00	0 (0)	0.00 \pm 0.00	NA
PFBeA	0.5 ng/ml	15 (50)	0.82 ± 0.39	13(43)	0.87 ± 0.34	0.72
PFHxA	0.5 ng/ml	0 (0)	0.00 \pm 0.00	0 (0)	0.00 \pm 0.00	NA

Table 2.4 Detection frequencies (DF) and mean concentrations of EDAs within the PCOS and control subjects.

PFDS	0.5 ng/ml	0 (0)	0.00 ± 0.00	0 (0)	0.00 ± 0.00	NA
PFHpA	0.1 ng/ml	9 (31)	0.24 ± 0.19	1 (3)		NA
PFNA	0.2 ng/ml	29 (100)	0.72 ± 0.41	30 (100)	0.56 ± 0.24	0.07
PFDA	0.2 ng/ml	(15) 51	0.35 ± 0.25	15 (50)	0.26 ± 0.13	0.21
PFDODA	0.5 ng/ml	0 (0)	0.00 ± 0.00	0 (0)	0.00 ± 0.00	NA
PeCB	1.5 ng/g	11 (38)	3.91 ± 7.92	12 (40)	3.04 ± 6.18	0.77
a-HCH	0.5 ng/ml	0 (0)	0.00 ± 0.00	0 (0)	0.00 ± 0.00	NA
HCB	1.6 ng/ml	26 (90)	9.83 ± 8.23	30 (100)	8.56 ± 4.86	0.48
b-HCH	0.5 ng/ml	18 (62)	3.77 ± 6.19	21 (70)	2.46 ± 1.84	0.36
Lindane	0.5 ng/g	4 (14)	0.20 ± 0.53	3 (10)	0.17 ± 0.36	0.94
Trans Chlordane	0.5 ng/g	7 (24)	0.27 ± 0.64	7 (23)	0.39 ± 0.86	0.77
Cis- Chlordane	0.25 ng/g	12 (41)	0.66 ± 0.88	16 (53)	0.85 ± 0.91	0.58
p'p-DDE	1.0 ng/g	29 (100)	48.89 ± 71.54	30 (100)	31.66 ± 45.90	0.27

Table 2.4 Detection frequencies (DF) and mean concentrations of EDAs within the PCOS and control subjects.

p'p-DDT	1.0 ng/g	22 (76)	4.20 ±	22 (73)	3.25 ±	0.29
			3.53		2.18	
Mirex	1.1 ng/g	9 (31)	1.02 ±	15 (50)	1.63 ±	0.40
			1.60		1.72	
PCB-28	1.0 ng/g	8 (28)	0.91 ±	7 (23)	0.60 ±	0.73
			2.06		1.19	
PCB-52	1.0 ng/g	4 (14)	0.92 ±	0 (0)	0.00 ±	NA
			4.31		0.00	
PCB-101	0.80 ng/g	9 (31)	0.81 ±	17 (57)	1.55 ±	0.20
			1.27		1.4	
PCB118	1.0 ng/g	29 (100)	5.76 ±	30 (100)	5.29 ±	0.38
			2.35		1.73	
PCB138	1.0 ng/g	29 (100)	11.51 ±	30 (100)	12.20 ±	0.64
			6.64		4.64	
PCB153	1.0 ng/g	29 (100)	14.71 ±	30 (100)	16.15 ±	0.51
			9.70		6.95	
PCB180	1.0 ng/g	29 (100)	12.32 ±	30 (100)	14.28 ±	0.24
			7.02		5.52	
BDE-28	0.1 ng/g	8 (28)	0.04 ±	11 (37)	0.06 ±	0.59
			0.06		0.09	
BDE 47	0.3 ng/g	23 (79)	0.47 ±	30 (100)	0.78 ±	0.11
			0.57		0.75*	
BDE 99	0.1 ng/g	18 (62)	0.17 ±	20 (67)	0.17 ±	1.0
			0.20		0.17	
BDE 100	0.1 ng/g	18 (62)	0.24 ±	23 (77)	0.24 ±	1.0
			0.42		0.27	

Table 2.4 Detection frequencies (DF) and mean concentrations of EDAs within the PCOS and control subjects.

BDE 153	0.3 ng/g	13 (45)	1.39 ±	22 (73)	1.32 ±	0.92
			2.68		1.24	
BDE 154	0.3 ng/g	0 (0)	0.00 ±	0 (0)	0.00 ±	NA
			0.00		0.00	
BDE-183	0.4 ng/g	0 (0)	0.00 ±	0 (0)	0.00 ±	NA
			0.00		0.00	
α	0.3 ng/g	19 (66)	2.12 ±	19 (63)	1.51 ±	0.40
HBCDD			2.58		1.77	
β	0.9 ng/g	0 (0)	0.00 ±	1 (3)	0.10 ±	NA
HBCDD			0.00		0.53	
α	0.3 ng/g	9 (31)	0.26 ±	11 (37)	0.50 ±	0.46
HBCDD			0.47		0.85	

* Indicates significant difference P <0.05.

PCOS vs. Control Women

No significant difference was demonstrated in the organochlorine compounds between the groups. The only compound that demonstrated any significant difference was PFOS having a significantly higher concentration in the PCOS group, (4.11 ± 1.62 ng/ml vs. 3.11 ± 1.05 ng/ml, $p=0.03$, Figure 2.1). This may be an incidental finding in view that multiple comparisons have been made resulting in a possible Type1 statistical error.

The levels of p,p-DDE were 31.66 ± 8.38 ng/g and 48.89 ± 13.29 ng/g, for the control group and PCOS group respectively but there was no significant difference $p=0.93$. These levels were considerable lower than Petro et al (2014) and Al-Salah et al (2009), the most recent publications on DDT levels in women undergoing IVF.

We were unable to establish any associations between these low levels of p,p-DDE and fertilisation and pregnancy rates after controlling for age and BMI. Our study agrees with the numerous studies that DDT exposure has no effect on fertilisation and adverse pregnancy outcomes. Jarrell et al.(1993) found that p,p-DDE had no effect on fertilisation rates and cleavage in a population in which the levels of p,p-DDE were 3 times higher than in the current study. Younglia et al (2003) again found no correlation with p,p-DDE levels and fertilisation and cleavage rates. A more recent study by and Law et al. (2005) and found weak associations between p,p-DDE and time to pregnancy.

Within this study significant positive correlations between p,p-DDE and cleavage rates ($r=0.28$, $p= 0.04$) and a negative correlation with abnormal fertilisation($r=-.411$, $p=0.003$) in the study population as a whole. This effect is not noted in the control group sub-analysis but remained significant for the PCOS group with a significant positive correlation for cleavage rates ($r.60$, $p=0.002$) and negative correlation for abnormal fertilization($r=-.62$, $p=0.002$).

HCB was detected in 95% of serum samples. The levels in control and PCOS groups were similar ($8.56 \pm 0.89\text{ng/g}$ vs. $9.99 \pm 1.49\text{ng/g}$, $p=0.59$). These levels were similar to Petro et al (2014), $p=0.17$ and significantly lower than Muhalingaiah et al (2011) $p<0.04$. Our study like Muhalingaiah et al (2011) found no significant association between HCH and pregnancy rates.

PCB-118, 138, 153 and 180 were detected in the serum samples of all 59 subjects. There was no significant difference between the control and PCOS groups in the level of contamination with the individual PCBs. PCB-138, PCB-153 and PCB-180 demonstrated a significant positive correlation, $p=0.01$ with cleavage rates and only PCB-153 having a significant negative correlation with abnormal fertilisation rates in the total group. However this relationship was only maintained for PCB-118 and PCB-180 in the PCOS group.

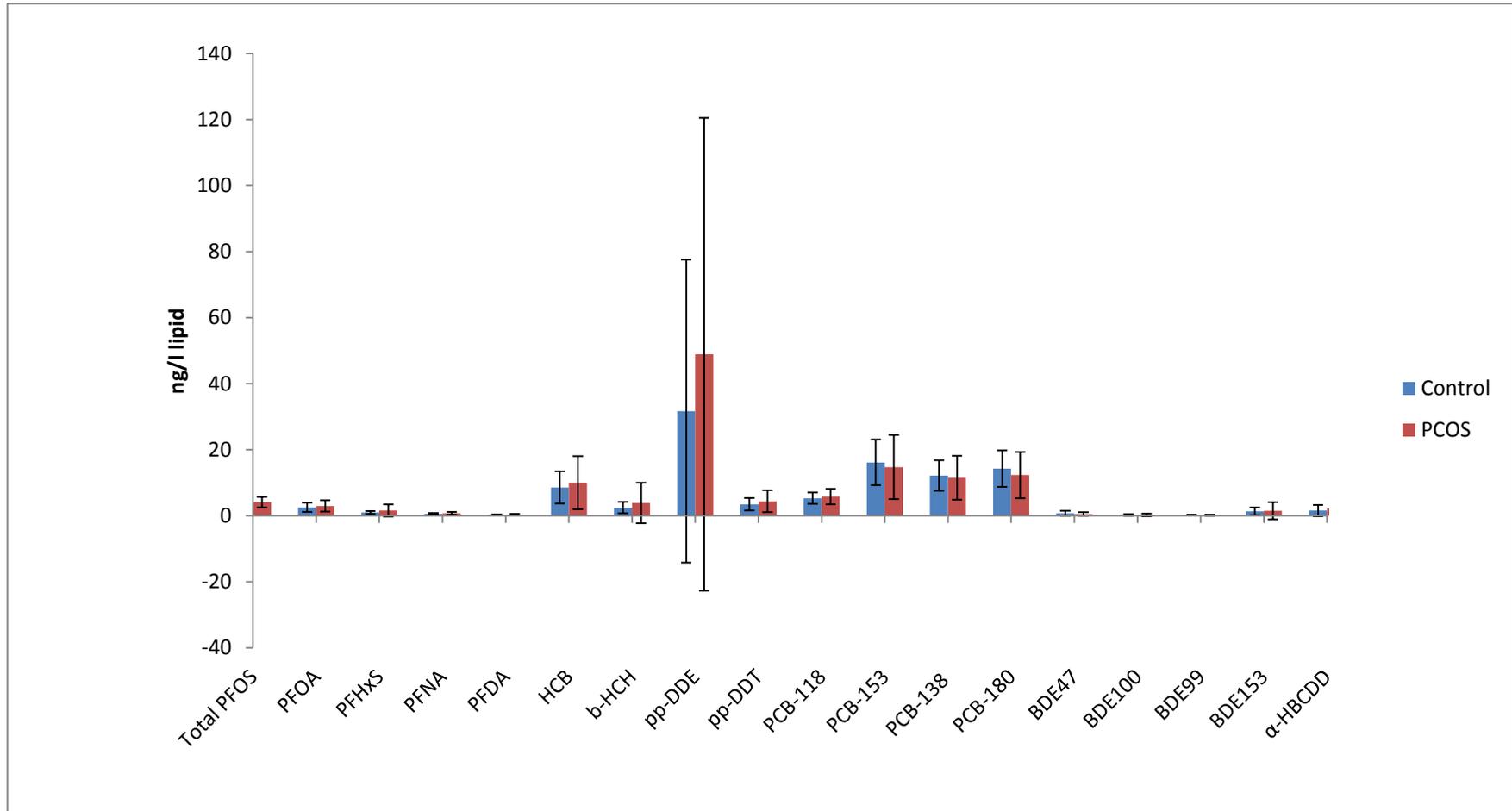


Figure 2.1 Mean levels of EDAs for both controls and PCOS groups. Error bars extend to +/- 1 SD.

2.4.2 Clinical Pregnancy Rate and EDAs

Mann Whitney U tests were performed to compare the mean of the concentrations of each compound between pregnancy outcome within each group (PCOS and controls). This demonstrated no association with the EDA compounds and pregnancy outcomes whether positive or negative (Table 2.5). It was decided not to perform regression analysis due to the small numbers of subjects.

Table 2.5 Demographics (Mean and SD) and Mann Whitney U-test for pregnancy rates for Control and PCOS patients.

	Clinical Pregnancy									
	No				Yes					
	Group				Group				Mann Whitney	
	Control		PCOS		Control		PCOS		U test (p-value)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Control	PCOS
Total PFOS	3.16	0.98	4.05	1.45	3.34	1.03	4.11	1.46	0.80	0.88
PFOA	2.44	1.59	2.85	1.56	2.64	1.29	2.56	1.63	0.56	0.59
PFHxS	0.94	0.44	1.27	0.74	1.06	0.47	1.39	0.97	0.59	0.98
PFNA	0.53	0.24	0.62	0.30	0.57	0.23	0.76	0.35	0.72	0.21
PFDA	0.26	0.16	0.26	0.14	0.25	0.08	0.43	0.27	0.87	0.56
HCB	9.27	4.91	13.03	9.22	6.39	4.47	8.29	5.14	0.57	0.17
b-HCH	2.81	1.39	3.63	4.24	1.50	2.05	5.35	9.65	0.72	1.0
pp-DDE	36.91	58.33	32.76	34.32	19.86	10.89	75.07	96.68	0.23	0.76
pp-DDT	3.70	1.72	3.39	1.77	2.63	2.03	6.58	4.98	0.38	0.16
PCB-118	5.26	1.63	6.05	2.95	4.80	1.58	5.94	1.59	0.59	0.55
PCB-153	16.89	6.03	16.29	11.44	12.60	6.49	16.42	7.37	0.10	0.55
PCB-138	12.63	3.96	13.14	8.10	10.11	4.85	11.78	3.96	0.21	0.88
PCB-180	14.93	4.47	13.21	7.44	11.16	5.12	14.50	7.13	0.57	0.55
BDE47	0.87	0.92	0.51	0.40	0.69	0.37	0.39	0.17	0.72	0.83
BDE100	0.30	0.30	0.30	0.45	0.21	0.20	0.20	0.35	0.41	0.56
BDE99	0.21	0.17	0.17	0.13	0.16	0.10	0.17	0.13	0.79	0.88
BDE153	1.33	0.96	1.56	2.88	1.30	1.65	2.11	3.04	0.44	0.81
α -HBCDD	1.74	1.86	1.95	1.77	1.56	1.57	3.08	4.09	1.0	1.0

2.4.3 IVF outcomes and EDAs

As the data was not normally distributed a Spearman's rank coefficient was used to assess potential associations between the EDAs and outcomes during an IVF cycle. This was performed on all the subjects together, and then divided into the PCOS and control groups. The IVF parameters considered were:

- 1) Ovarian responsiveness in terms of antral follicle count, follicles aspirated, and eggs retrieved
- 2) Embryological response in terms of number eggs fertilised, fertilisation rate, cleavage rate, number abnormality fertilised and top quality embryos.
- 3) Endocrine profile. HOMA, FAI,

As a group overall, only BDE-47 demonstrated a significant negative correlation with AFC ($R=-0.29$, $P=0.03$). Regarding embryological data, total PFOS, p,p-DDE, PCB-138, 153 and 180 demonstrated a significant positive correlation with the cleavage rate of embryos, but p-p,DDE and PCB 153 were negatively correlated with abnormal fertilisation rate. Regarding endocrinological data the PCB-138, 153, 180 and BDE-153 had a significant negative correlation with FAI. Interestingly it was only the PFAAs substances that had a significant positive correlation with testosterone at both the $p=0.05$ and 0.01 level and only the fire retardants demonstrating a significant positive correlation with SHBG.

In the control group there was no correlation demonstrated between the EDAs, ovarian responsiveness and embryological data in the IVF cycle. Regarding the endocrinological data there was a significant negative correlation between FAI and the majority of the EDAs (total PFOS, HCB, b-HCH, p,p-DDE, PCB-118, 138, 153, 180, and BDE 47). All of these EDAs except total PFOS, b-HCH, and p,p-DDE however had significant positive correlation with SHBG, a component of the FAI.

The PCOS group only b-HCH had any significant correlation with ovarian responsiveness in an IVF cycle demonstrating a statistically negative correlation

with antral follicle count and numbers of eggs retrieved, $r=-.37$ $p=0.04$ and $r=-.40$ $p=0.035$ respectively. On the embryological front only α -HCBHH had a significantly negative correlation with numbers of embryos fertilised and numbers that cleaved, $r= -.59$ $p=0.003$ and $r -.47$ $p=0.24$ respectively. PFNA, PFDA, HCB, p,p-DDE, PCB118 and PCB 180 had a significantly positive correlation with cleavage rate. B-HCH and p,p-DDE and PCB-118 had a significantly negative correlation with abnormal fertilisation rate.

Principal component analysis (PCA) was performed to reorganise the original variables into 5 new variables namely OCPs, PCBs, BDEs, HBCDD and polyfluoroalkyl agents for the 59 subjects. These were then correlated with the outcome variables, ovarian responsiveness, embryological response, and endocrine profile.

The results of the PCA demonstrated that if a patient had a high level of one congener of a group of chemicals (PFAA, PCB, OCP, HBCDD and BDE) they were more likely to have a higher level of other congeners within that group of chemicals. Spearman's rank correlation following the PCA demonstrated that HBCDD and BDE had no significant correlation with any of the outcome variables. However the OCPs had significant correlation with BMI ($p=0.05$), testosterone and FAI ($p=0.01$). PFAAs have a significant correlation with testosterone ($p=0.01$) and PCBs with FAI ($p=0.05$). Indicating these chemicals may interfere with the endocrine axis at an androgen level, however much larger studies would be needed to prove this possible link.

2.5 Discussion

2.5.1 Polyfluoroalkyl Acids (PFAAs)

The PFAAs are formed as a result of the degradation of polyfluorinated compounds used in many consumer products such as in the paper and textile industry. (Jensen et al. 2008). The three most abundant PFAAs are perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), and perfluorohexane sulfonic acid (PFHxS).

Olsen et al. (2007) estimated half-lives for PFOA, PFOS and PFHxS to be 3.8, 5.4, and 8.5 years, respectively. Fei et al. (2009) showed a possible relationship between exposure and PFOS, may reduce fecundity and result in increased time to pregnancy.

In this present study PFOS, PFOA, PFHxS and perfluorononanoic acid (PFNA) were detected in 100% of the serum samples and perfluorononanoic acid (PFDA) was detected in 76% of samples (51% PCOS, 49% control). There was a significant difference in PFOS ($p=0.03$), between the PCOS and control population. The PFAA found in the highest concentration was PFOS ($3.71 \pm 1.41\text{ng/ml}$), which was comparable with a recent study by Petro et al (2014) investigating PFAAs in women undergoing IVF. The levels of PFOS in this study were significantly lower than Pedro et al (2014).

The PFAAs had significant positive correlations with testosterone in the overall patient population and in both the control and PCOS subgroups. In the 59 patients overall, PFOS and PFDA had very significant positive correlations with testosterone ($r=0.411$, $p=0.001$, and $r=0.393$, $p=0.002$ respectively), with PFHxS, PFOA and PFNA having significant positive correlations at the $p=0.05$ level. In the control group PFOS and PFOA maintained a significant positive correlation ($p=0.02$ and 0.04 respectively) and in the PCOS group PFOS and PFDA maintained a significant positive correlation ($p=0.03$ and $P=0.02$ respectively).

There has been little work on PFAAs but animal studies have demonstrated that exposure to PFOS and PFOA may affect the integrate balance of sex hormones and pregnancy loss in animals (Case et al. 2001, Lau et al. 2006). Exposure of male rats to PFOA can reduce testosterone levels resulting in Leydig cell hyperplasia of the testis (Shi et al. 2007). Could PFAA exposure, particularly PFOS exposure in the human female ovary, affect the morphology of the theca cell, resulting in a disordered steroidogenesis, leading to an excessive androgen response in women

with PCOS? This study demonstrated that PCOS subjects had significantly higher levels of PFOS compared to controls, combined with its significant positive correlation with testosterone could there be a possible link between this EDA and PCOS.

2.5.2 Organochlorines

p,p'-dichlorodiphenyltrichloroethane (DDT)

DDT and its common metabolite p,p-DDE were detected in 75% and 100% of the serum samples respectively despite the pesticide being banned in the UK in 1984. It would make sense that p,p-DDE was detected in more samples as it is known that within the food chain DDT is broken down into its more stable metabolites p,p-DDE and p,p-DDD. p,p-DDE is a highly persistent compound with a half life of 13 years (Longnecker. 2005).

Indicating a higher level of chemical aid cleavage and lower levels increase the risk of abnormal fertilisation. It is important to remember that paternal gametes and their EDA exposure is not part of this study and with large numbers of statistical tests there is always a risk of finding a significant result but if this is clinically relevant further studies would need to be performed. Younglia et al (2004) have demonstrated that p,p-DDE could increase basal and FSH-stimulated granulosa cell aromatizing enzyme activity. The levels of p,p-DDE are higher in our PCOS population, maybe there is a possible link in the endocrine disrupting effects on p,p-DDE on the steroidogenic pathway of the PCOS women through receptor mediated changes in protein synthesis as discussed by Kelce et al. (1995).

Hexachlorobenzene (HCB)

Primate studies have demonstrated that HCB can suppress luteal progesterone that could lead to implantation failure (Foster et al. 1992), and germ cell loss (Farrell et al. 2003). In this study HCB demonstrated a strong positive correlation with cleavage rates but the relevance is unknown.

Polychlorinated Biphenyl Esters

Previous studies published approximately 10 years ago using American and Canadian subjects (Younglia et al. 2002 and De Felip et al. 2004) documented levels of PCBs significantly higher than this present study. The levels were significantly lower, $p=0.0001$, than a recent American study by Meeker et al (2009). Meeker et al. (2009) had collected samples for the study between 1994-1998 and 1993-2003 and themselves had documented on the declining concentrations throughout the time of the study. When we compared our results to a recent European study by Pedro et al (2014) the levels of all 4 detectable PCBs in this study were extremely significantly lower ($p=0.0001$), which demonstrates that the levels of these organochlorines that have only been banned since 2009 are dropping exponentially in the environment.

Regarding endocrine function all congeners of PCB had a significantly negative correlation with FAI ($p=0.05$) with a significantly positive correlation with SHBG ($p=0.05$). This would indicate higher levels of PCB with higher SHBG resulting in a lower FAI. Only PCB-153 and PCB-180 had a significantly negative correlation with FAI ($p=0.05$) but no other effect on endocrine function.

The significance of these findings this is not known. Numerous animal studies have shown that PCB congeners can affect the endocrine system of animals. However there is still no conclusive evidence of this effect in humans, regardless of fertility status. In vivo studies have demonstrated the most common PCB congeners (PCB-138, PCB-153 and PCB-180) like those in this study can affect both androgen and oestrogen receptors (Bonefeld-Jorgensen et al. 2001). Whether these chemicals are exerting any possible effect on the androgen receptor and interfering with the hormone homeostasis resulting in this possible relationship with FAI is difficult to say.

2.5.3 Organobromines

Polybrominated Fire Retardants

BDEs were banned in the European Union in 2009, however BDE-47, BDE-99, BDE-100 and BDE-153 remain the mayor PBDE congeners detected in humans and animals (Sjodin et al. 2003). This study again highlights their persistence in a population of women undergoing treatment for subfertility.

Of the 4 congeners detected only BDE-47 demonstrated any positive correlation with ovarian responsiveness with a positive correlation with antral follicle count, $p=0.03$. An animal study by Lilienthal et al (2006) demonstrated that when rats exposed to BDE-99 had reduced numbers of primordial/primary follicles at low doses of BDE-99 and decline of secondary follicles at high doses of BDE-99. BDE-153 demonstrated a significant positive correlation with FAI, $p=0.03$, no other correlations were demonstrated between the BDE congeners and embryological data.

On sub-group analysis only BDE-47 demonstrated a positive correlation with FAI, $p=0.04$ and a negative correlation with SHBG, $p=0.02$. In the PCOS group BDE-99 and BDE-47 had negative correlations with fertilisation rate ($p=0.04$) and top quality embryos ($p=0.04$) respectively. Again in the PCOS group BDE-47 and BDE-99 had significant positive correlations with insulin, $p=0.04$ and $p=0.001$ respectively, however only BDE-99 had a positive correlation with HOMA-IR, $p=0.02$.

Stoker et al (2005) have found anti-androgenic effects of BDE-47, BDE-99, BDE-100 and BDE-153 by the inhibition of binding of synthetic androgen to cytosolic androgen receptor. In this study we found certain BDE congeners had significant correlations with FAI. We know that androgens from the theca cells are not only responsible as a precursor for ovarian oestrogens but androgens also suppress progesterone secretion resulting within the follicles preventing premature luteinisation. Could this effect on the FAI, interrupt the androgen response from the

theca cells resulting in an excessive progesterone response resulting in preterm luteinisation and thus a disruption in normal ovarian function.

There are over 70,00 chemicals that may have potentially endocrine disrupting effects (Younglia et al. 2005). This study has only investigated the most common EDAs such as PCBs, DDT and BDE. It is well known that these chemicals are in the environment and bio-accumulate in the food chain due to their lipophilic nature resulting in possible toxic and endocrine disrupting capabilities. Studies have demonstrated associations with these EDAs and adverse reproductive effects and prolonged time to TTP, however there is no standard validated screening test to assess the potential exposure to EDAs and possible adverse reproductive outcomes (Daston et al. 2003).

We know from previous studies into sub-fertile women undergoing assisted reproductive treatment that levels of the most common EDAs are declining over time (Jarrell et al. 1993; Younglia et al. 2002; De Felip et al. 2004, Weiss et al. 2006; Meeker et al 2009). We compared our serum levels against the most recent study by Petro et al. (2014) who described their study group as matching the profile of the Western female population in terms of EDA contamination, this group being from Belgium. Petro et al (2014) highlighted that the serum levels in their study were comparable to other recent studies from other Western countries (Porta et al. 2011; Herrick et al. 2011; Kalantzi et al. 2011). In this present study levels of PCB-118, 138, 153 and 180 were significantly lower $p=0.001$ than in the recent study by Petro et al (2014). The levels of p-p-DDE in this study was three times lower 40.13 ± 60.00 vs. 119.9 ± 68.8 , $p=0.0001$, however there was no significant difference in p,p-DDT levels 3.72 ± 2.93 vs. 3.4 ± 2.0 , $p=0.65$. There was no significant difference in the fire retardant BDE 47, however BDE 99 was significantly lower in this recent study, $p=0.0001$. This gives further up to date evidence that EDA contamination is continuing to fall in the industrialised world due to banning of these persistent organo-pollutants.

2.6 Conclusion

This study demonstrates that as with other studies in the Western Hemisphere EDAs are present in the serum of women with and without PCOS undergoing IVF. Only PFOS exhibited a significantly higher concentration in the PCOS group. Interestingly the PFAAs demonstrated a significant positive correlation with testosterone in the overall patient population ($p=0.001$) and in both the control ($p=0.02$) and PCOS ($p=0.03$) subgroups. This relationship remained significant in the overall group following PCA when all PFAAs were grouped together. PFAAs may have an endocrine disrupting effect in the androgen pathway that effects both PCOS and non-PCOS women, but the higher levels of PFOS in the PCOS women may demonstrate that this population of women who already have an excess of androgen may have an inability to metabolise this group of chemicals making them more prone to the androgenic effects. However, further studies are needed to assess how PFAAs may affect the steroidogenic pathway.

The PFAAs, PCBs and p,p-DDE demonstrated significant positive correlations with cleavage rates ($p=0.04$, 0.01 , and 0.04 reselectively). Could these chemicals affect the division of cells in early embryo development, but the numbers of subjects in this pilot study make it difficult to make any firm conclusions. This study is limited in that it is a small pilot study to assess if endocrine disrupting chemicals are evident in women undergoing IVF and there is the risk of statistical errors in testing for multiple chemicals in such a small population of women. Further research in a much larger population is necessary to explore these possible links. Interestingly the study did give further information on the levels of contamination of these persistent EDAs within a Western population and further confirm that the levels continue to decline and would appear that the UK has much lower levels than other western industrialized nations.

CHAPTER THREE:

Vitamin D and Fertility: The effects of Vitamin D on the outcomes of controlled ovarian stimulation in patients with and without Polycystic Ovary Syndrome undergoing in vitro fertilisation.

3.1 Abstract.

Background: Studies have reported conflicting data as to whether or not Vitamin D may play a role in human reproduction. The objective of this study was to investigate what effect vitamin D had on parameters within an in vitro fertilisation (IVF) cycle in a population of infertile women with and without Polycystic Ovary Syndrome (PCOS).

Method: PCOS was diagnosed using the Rotterdam Consensus Criteria. Serum levels of Vitamin D [25(OH)D] were measured during the menstrual cycle prior to commencing IVF. 25(OH)D levels were compared against patient demographics and IVF cycle parameters between PCOS and non-PCOS groups.

Results: 59 women participated, 29 PCOS and 30 non-PCOS. 83% of the women had vitamin D insufficiency [25(OH)D < 50nmol/L]; there was no statistical significance in vitamin D levels between the groups ($p=0.12$). There was no significant difference in clinical pregnancy rates per IVF cycle in the non-PCOS group compared to the PCOS group (33% vs. 24%; $p=0.57$). There was no significant positive correlation ($p=0.03$) between vitamin D levels and fertilisation rates in women with PCOS.

Conclusion: This study further highlights that Vitamin D deficiency exists in British women with and without PCOS undergoing IVF, and that Vitamin D offers no added benefit to women with PCOS compared to women without PCOS undergoing IVF.

3.2 Introduction

Predicting the success of an in vitro fertilisation (IVF) cycle, can be difficult. This is dependant on multiple factors such as age, body mass index (BMI), ovarian reserve and endocrine status. Could the vitamin D [25(OH)D] status of women undergoing an IVF cycle be another predictor of outcome?

Vitamin D receptor (VDR) knockout mice have decreased aromatase activity in the ovary and impaired folliculogenesis (Kinuta et al. 2000; Yoshizawa et al. 1997), and vitamin D deficient mice have reduced fertility rates (Halloran and Deluca 1980). Women are borne with approximately 2 million primordial follicles; the majority of which will undergo atresia with only a few hundred being selected to undergo folliculogenesis. Vitamin D deficiency is associated with calcium deregulation, which contributes to the development of follicular arrest in PCOS patients, resulting in menstrual and fertility dysfunction. (Thomson et al. 2012)

PCOS is the most common cause of anovulatory infertility. Of these patients 30-40% have associated metabolic disturbances such as insulin resistance (IR) with compensatory hyperinsulaemia, impaired glucose tolerance and dyslipidaemia (Krul-Poel et al. 2013). These factors can have a crucial role in the response to gonadotrophins, as these patients have large indicies of ovarian reserve, ie the ovaries ability to produce ovums that can fertilise, resulting in a successful pregnancy, demonstrated by both their high anti-mullerian hormone (AMH) and antral follicles counts (AFC) resulting in a hyper-androgenic environment, and an increased risk of an inappropriate response, such as ovarian hyperstimulation syndrome (OHSS). OHSS is a complication specific to IVF, in which the ovarian response to the exogenous gonadotrophins exceeds the expected response resulting in a spectrum of sytoms ranging from mild to severe. Mild cases are usually self limiting with associated abdominal pain, bloating and ovarian enlargement <8cm, ranging to severe cases resulting in tense ascites, oliguria/anuria, thromboembolism and acute respiratory distress syndrome. The

release of inflammatory mediators such as VEGF, increases vascular permeability resulting in fluid loss into the third space (Braat et al. 2010), leading to ascites, pleural effusions and a prothrombotic state.

3.2.1 Vitamin D and Anti-Mullerian Hormone

There is a relationship between Vitamin D and AMH. AMH is a marker of ovarian reserve, important when deciding on how to induce superovulation in a patient undergoing IVF (Tal et al. 2015). AMH is expressed by the granulosa cells of the follicle and is involved in the recruitment of follicles from the primordial pool. Research has shown that environmental factors including vitamin D can alter its expression (Merhi et al. 2014). Wojtusik and Johnson. (2011) studied the effect of 1,25 hydroxyvitamin D3 regulated AMH mRNA in the granulosa cells of chickens and found enhancement of granulosa cell proliferation of 3-5mm and 6-8mm follicles incubated in 1,25 hydroxyvitamin D3.

AMH has a repressive effect inhibiting the loss of the oocyte pool by decreasing the recruitment of primordial follicles and slowing down growth that is followed by atresia and death. In women with PCOS, the high AMH reflects stasis in the oocyte pool, culminating in high numbers of pre-antral and antral follicles and the classic polycystic ovary morphology (Bhide et al. 2015). Optimal levels of vitamin D may have the ability to counteract the repressive effects of the AMH on the granulosa cells thus allowing the multiple follicles in the PCOS ovaries to mature. AMH like Vitamin D undergoes seasonal changes. Studies have shown that seasonal variation in AMH correlated with the changes to circulating Vitamin D. Patients supplemented with Vitamin D did not demonstrate seasonal changes in both the Vitamin D and AMH (Dennis et al. 2012).

3.2.2 Vitamin D, hyperandrogenaemia and Insulin Resistance

Hyperandrogenaemia is one of the diagnostic criteria of PCOS and can be measured by the free androgen index (FAI), which reflects the ratio of testosterone to sex

hormone binding globulin (SHBG). High circulating androgen can result in decreased follicular maturation despite the increased recruitment of primordial follicles into pre-antral follicles. Small observational studies have shown a positive association between vitamin D and SHBG (Hahn et al. 2006; Wehr et al. 2009) and a negative association between FAI and total testosterone. The relationship between SHBG and vitamin D was not significant once BMI was controlled for, suggesting the issue was due to obesity rather than Vitamin D.

Vitamin D may be associated with insulin resistance (IR) in the PCOS patient. Many associate IR with obesity in PCOS, however many lean women with PCOS have IR. A review article using regression analysis demonstrating that an increase in serum vitamin D levels was significantly associated with a reduction in HOMA-IR in PCOS and controls (Krul-Poel et al. 2013). However after multivariate regression analysis with vitamin D and BMI as independent variables, serum Vitamin D was no longer an independent predictor of IR in PCOS but remained significant for the control patients (Krul-Poel et al. 2013).

The literature remains limited regarding what effect does vitamin D have on the outcomes of a controlled ovarian hyperstimulation cycle. Most PCOS women are overweight or obese which is a major factor in the prediction of success in IVF. This makes it difficult to conclude if vitamin D deficiency contributes to the pathogenesis of PCOS independently from an elevated BMI. In this context we investigate if there was any correlation between these variables and Vitamin D and what effect Vitamin D levels may have in predicting the outcome of a clinical pregnancy within an IVF setting.

3.2.3 Measuring Vitamin D

There is consensus that Vitamin D should be measured from circulating 25(OH)D. However there are multiple assays that can measure 25(OH)D, including Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) chemoluminescence

immunoassays, enzyme immunoassay, competitive protein-binding assay, radioimmunoassay (RIA). With so many techniques, there are obvious inter assay variabilities in 25(OH)D levels.

Chromatography

The reference method for vitamin D analysis has been with the use of LC-MS/MS, which can measure vitamin D₂, vitamin D₃, and the D₃ epimer separately through calculation of the total vitamin D level (Hollis. 2010).

Immunoassay Techniques

Chemiluminescence measures total 25(OH)D and other hydroxylated vitamin D metabolites in human serum. This is a two step process. Firstly, 25(OH)D is dissociated from its binding protein and binds to the specific solid phase antibody, followed by the addition of vitamin D-isoluminol tracer; unbound material is removed with a wash cycle. In the second step, the reagents are added to initiate the chemiluminescent reaction. The light signal is detected by a photomultiplier as relative light units. This measurement is inversely proportional to the concentration of 25(OH)D (DiaSorin 2013).

Enzyme immunoassay uses homogenous enzyme-coupled vitamin D binding protein to measure total levels of 25(OH)D (i.e., the sum of D₃ and D₂). The vitamin D binding protein recognizes vitamin D₂ and D₃ equally and also recognizes the true-total level of 25(OH)D.

One study found that using a 25(OH)D deficiency cut off point at <50nmol/L, 57% of samples using chemiluminescence immunoassay were classified as deficient compared to 41% using LC-MS/MS, demonstrating that cut offs should be assay specific (Lai et al. 2012). Farrell (2012) compared 5 immunoassay techniques, an RIA technique and 2 LC-MS/MS methods in quantifying 25(OH)D. The immunoassay methods showed variability, and positive bias again overestimating Vitamin D

deficiency. The LC-MS/MS methods agreed well and the RIA had similar results to the LC-MS/MS. In this study a LC-MS/MS was used to analyse all samples for Vitamin D levels.

3.2.4 Measuring AMH and Antral Follicle Count

AMH and AFC have been seen as the best predictors of ovarian response to exogenous gonadotrophins. AFC requires the patient to undergo a transvaginal ultrasound scan between days 2 and 4 of their menstrual cycle. AFC is thus subject to inter and intra-operator variability. All AFC's are performed by trained ultrasonographers within the Hull IVF Unit. The accuracy of AMH and AFC is expressed as the area under the receiver operator curve (ROC), Figure 3.1. The review by Amanvermez and Tosun (2016) demonstrate that ROC curves for AMH and AFC are the most reliable tests to predict ovarian reserve.

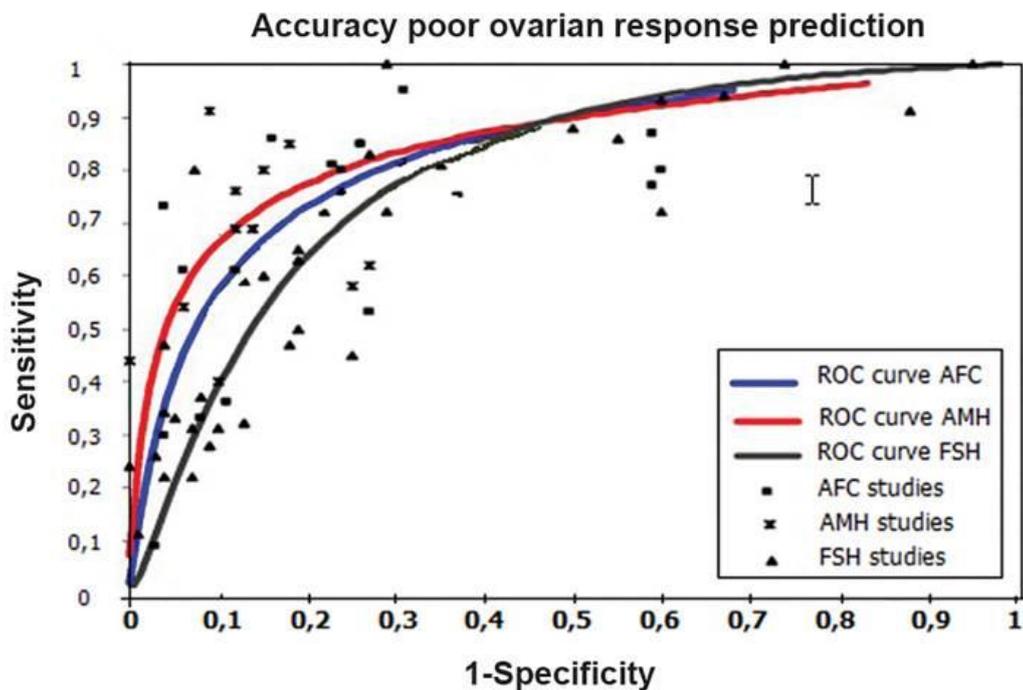


Figure 3.1. ROC curves of studies reporting on the performance of the AFC, AMH and basal FSH tests to predict poor ovarian response (Amanvermez and Tosun 2016). ROC; Receiver operating characteristic curve, AFC; Antral follicle count, AMH; Anti-mullerian hormone and FSH; Follicle stimulating hormone.

It was viewed that AMH and AFC were interchangeable as markers of ovarian response. A multicentre randomised control trial has demonstrated that standardised AMH assays are a much more accurate marker without the inter, intra operator variability that is what makes standardising AFC difficult. (Iliodromiti et al. 2014). A large review article that included over 300 papers concluded that AMH was the best available measure of ovarian reserve under a variety of clinical situations, such as infertility treatment (i.e. IVF), ovarian dysfunction (especially PCOS) and gonadotoxic cancer treatment or ovarian surgery (Broer et al. 2014). AMH helps clinicians to prescribe personalised dosing schedules for ovarian stimulation thereby improving the efficiency and safety of IVF.

There have been several commercially available assays for AMH: the Beckman Coulter's Immunotech assay and the Diagnostic Systems Lab (DSL) assay. These have been gradually replaced by Beckman Coulter's Gen II assay following a large multicentre evaluation was performed between the Gen II assay and the two commercial enzyme-linked immunosorbent assays: DSL and Immunotech (Wallace et al. 2011).

There had been problems with the Gen II. Assay. The original methodology resulted in potentially falsely high or low AMH values, apparently due to complement interference in the assay. There is no current standard for AMH measurement although the UK NEQAS has been conducting an international pilot scheme for the past 2 years (Syme et al. 2013).

A recent study compared a new Ansh Labs Ultra-Sensitive assay to the Gen II assay. The Ansh Labs Ultra-Sensitive assay performance characteristics are similar to the Gen II assay and may be suitable for clinical and epidemiological use. The enhanced sensitivity of the ultrasensitive assay enables measurement of low AMH concentrations. The authors stated that the results re-emphasize the need for an

AMH international standard (Welsh et al. 2014). We used the Beckman Coulter's Gen II assay throughout the study.

3.2.5 Measuring Free Androgen Index (FAI)

$$FAI = 100 \times \left(\frac{\text{Total Testosterone}}{SHBG} \right)$$

Testosterone

Testosterone is measured by stable isotope dilution chromatography-tandem mass spectrometry (ID/LC-MS/MS). This has taken over from the previous RIA or chemiluminescence as these sometimes resulted in inaccuracies in samples from women and children, leading to misdiagnosis and inappropriate treatment. Sensitivity and specificity of the ID/LC-MS/MS method offer advantages over immunoassay with a limit of detection at 0.05 nmol/L so it can accurately measure testosterone in women whose levels are typically low.

Sex Hormone Binding Globulin

Only very small amounts of androgens are in their active form approximately 1%. The majority of these hydrophobic hormones are carried in the circulation in an inactive state weakly bound to albumin or tightly bound to SHBG. Thus changes in the levels of SHBG will affect the level of free androgen in the circulation. The hyperinsulaemia associated with PCOS can lead to the reduction in SHBG and thus an increase in the circulating levels of active testosterone resulting in hyperandrogenism (Michelmore et al. 1999).

A study investigating the diagnostic value of calculated testosterone indices in the assessment of polycystic ovary syndrome, found FAI and bioavailable testosterone correlated significantly (all $p < 0.05$) with total testosterone, androstendione, LH/FSH ratio and DHEAS. FAI correlated significantly with hirsutism scores, ovarian volume and follicle count (Hahn et al. 2007). Quick tests such as the free androgen index

are now widely accepted, but only if the necessary assays have been validated. However the drawback is that SHBG can vary and unlike direct measurements of free testosterone, FAI is not a marker of hyperandrogenemia independent of obesity.

3.2.6 Measuring insulin resistance

The first work published on insulin resistance in PCOS was by Dunaif et al. (1989) who used euglycemic glucose clamps and demonstrated significant and substantial decreases in insulin-mediated glucose disposal in PCOS (Dunaif et al., 1989; Dunaif et al., 1992). This decrease (~35–40%) is of a similar magnitude to that seen in NIDDM. Meta-analysis has shown the risk of developing type-2 diabetes in PCOS, is 4.43 (OR, 95% CI 4.06-4.82) even after correcting for BMI (Moran et al. 2011). The result is hyperinsulinaemia, which affects other insulin sensitive tissues such as the theca cells of the ovary resulting in hyperandrogenism (Nestler et al. 1998).

Following the initial work by Dunaif et al. (1989) there remain many inconsistent definitions of IR and models to calculate it. This is not helped by the ever-changing diagnostic criteria of PCOS. IR measurement was not recommended by the 2003 Rotterdam joint ESHRE/ASRM consensus on PCOS possibly because this may result in women with milder forms of the disease not being diagnosed and receiving suitable medical treatment.

The euglycaemic-hyperinsulinaemic clamp is the gold standard for research-based assessment of IR (Greenfield et al. 1985; Stepto et al. 2013). This is an invasive test that involves two cannulas, one to infuse insulin and the other for glucose infusion. The plasma insulin concentration is acutely raised and maintained at 100 $\mu\text{U}/\text{ml}$ by a continuous infusion of insulin. The plasma glucose concentration is held constant at basal levels by a variable glucose infusion. When the steady state is achieved, the glucose infusion rate equals glucose uptake by all the tissues in the body and is thus

a measure of tissue insulin sensitivity. Mathematical models to calculate IR have been developed that correlate well with the euglycaemic clamp.

HOMA (Homeostasis Model Assessment) was developed as a method to quantify insulin resistance and beta-cell function, described under the name HOMA by Matthews et al. (1985).

$$HOMA-IR = \frac{(Fasting\ Insulin \times Fasting\ Glucose)}{25}$$

This model correlated well with estimates using the euglycaemic clamp method ($r = 0.88$), $P < 0.0001$ (Matthews et al. 1985). HOMA has been updated to a HOMA2 model (Rudenski et al. 1991). HOMA2 better reflected human physiology and was recalibrated to modern insulin assays.

The Quantitative Insulin Sensitivity Check Index (QUICKI) is a measure of insulin sensitivity that uses the fasting insulin and fasting glucose values (Altuntas et al. 2003) and is calculated as follows:

$$QUICKI = 1 / [\log(\text{insulin in uU/mL}) + \log(\text{glucose in mg/dL})].$$

QUICKI is the log of the HOMA-IR, measuring sensitivity as opposed to insulin resistance. This test has been correlated to measure insulin-moderated glucose disposal assessed by the glucose clamp technique, especially in patients with type 2 diabetes mellitus and/or those who are obese and corresponds with HOMA (Katz et al. 2000; Skrha et al., 2004).

3.3 Materials and Methods

3.3.1 Ethical Approval and Patient Recruitment

The Local Research Ethics Committee (LREC) Yorkshire & The Humber – Humber Bridge Ethics Committee in November 2013, approved the study. Women whom had been diagnosed with subfertility and requiring IVF or ICSI-IVF at the Hull IVF Unit from 2014 were invited to participate in the study, before commencing their IVF or ICSI-IVF cycle (Ethics number: 02/03043).

A patient information sheet was created for this study and was passed by the LREC, Yorkshire & The Humber – Humber Bridge Ethics Committee. This sheet was handed out at the monthly group session held at the East Riding Medical Education Centre within The Hull Royal Infirmary, for those patients who were about to commence a cycle of IVF. A member of the research team was present at the group session to give a short presentation regarding the study and ask questions. The patients could then contact the unit if they were willing to take part in the study. We attained most of our patient's through the group sessions and thus the majority of our patients were undergoing their first cycle of IVF.

We planned to collect 60 subjects for our study. 30 patients without PCOS undergoing IVF and 30 patients with PCOS using the revised 2003 criteria from the Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group (ESHRE/ASRM 2004), indicating PCOS to be present if any 2 out of 3 criteria are met

1. oligo and/or anovulation
2. clinical and/or biochemical signs of androgenism
3. polycystic ovaries (either 12 or more peripheral follicles or increased ovarian volume (greater than 10 cm³))

Diagnosis of PCOS can only be made when other aetiologies have been excluded (thyroid dysfunction, congenital adrenal hyperplasia, hyperprolactinaemia, androgen-secreting tumours and Cushing syndrome).

3.3.2 Sample collection

Women who expressed an interest to take part in the study were asked to attend the IVF unit at the time of their review appointment. This appointment occurred in the luteal phase of their menstrual cycle the cycle prior to commencing treatment. At this appointment the patients completed their Human Fertilisation and Embryology Authority (HFEA) consent forms, underwent their Mock embryo transfer and were instructed on the injection technique for their exogenous gonadotrophins.

The patients were invited to come fasted from midnight and to attend their appointment 30minutes early so informed written consent could be taken and fasting blood samples could be performed without affecting their down regulation appointment. The bloods were put on ice and transferred to the Michael White Diabetes Centre, Anlaby Road, Hull approximately 5 minutes' walk from the IVF unit. At this point the bloods were centrifuged at 3500g for 15 minutes and 5°C placed into aliquots and frozen at -80°C.

3.3.3 Biochemical Assays

The assays were processed at the biochemistry laboratory at Hull Royal Infirmary, Anlaby Road, Hull.

FSH:- Architect analyser (Abbott laboratories, Maidenhead, UK), using the manufacturer's recommended protocol.

Total Testosterone:- measured on an Architect analyzer (Abbott Laboratories, Maidenhead, UK), using the manufacturer's recommended protocol.

SHBG:- immunometric assay with fluorescence detection on a DPC Immulite 200 analyser (Euro/DPC, Llanberis UK), using the manufacturer's recommended protocol.

Serum Insulin:- competitive chemiluminescent immunoassay performed on the manufacturer's DPC Immulite 2000 analyzer (Euro/DPC, Llanberis, UK). The analytical sensitivity of the insulin assay was 2 μ U/ml, the coefficient of variation was 6%, and there was no stated cross-reactivity with proinsulin.

Plasma Glucose:- measured using a Synchron LX 20 analyzer (Beckman-Coulter), using the manufacturer's recommended protocol. The coefficient of variation for the assay was 1.2% at a mean glucose value of 5.3 mmol/liter during the study period.

Free Androgen Index (FAI):- this was calculated by dividing the Total cholesterol by SHBG, and then multiplying by a 100.

$$FAI = 100 \times \left(\frac{\text{Total Testosterone}}{SHBG} \right)$$

FAI has no units.

Insulin Resistance (IR). This is calculated using the homeostasis model assessment (HOMA). It was first described under the name HOMA by Matthews et al. in 1985 and a value of ≥ 2.5 is indicative of IR.

$$HOMA-IR = \frac{\text{Fasting Insulin} \times \text{Fasting Glucose}}{25}$$

25

Compared with the "gold" standard euglycemic clamp method for quantifying insulin resistance (Greenfield et al. 1981), quantification using Homeostasis Model

Assessment Insulin Resistance (HOMA-IR) is more convenient and has a good correlation with the euglycemic clamp (Tam et al. 2012).

3.3.4 The IVF Cycle

All patients underwent a standard IVF antagonist protocol. The patients commenced their rFSH stimulation on day 2 of their menstrual cycle using either Merional (Pharmasure) or Gonal-F (Merck Serono). A GnRH antagonist (Cetrotide: Merck Serono) was used to prevent a premature LH surge.

The patients underwent ultrasound scans from day 7 to observe the ovarian response to stimulation and were repeated every 48 hours. The scans were used to measure the diameters of the follicles thus observing response and follicle numbers. Final maturation was triggered when two or more leading follicles were \geq 18mm using either 5000-10000 IU human chorionic gonadotrophin (hCG, Pregnyl (Merck Sharp and Dohme) or 0.5mg Buserelin (Sanofi-Aventis, Frankfurt, Germany).

Oocyte retrieval was performed 36 hours later, using intravenous sedation. Luteal support was provided with micronised progesterone (Uterogestan; Besins Iscovesco Laboratories, Paris, France) at a dose of 600mg each night commencing on the day of oocyte retrieval. Depending whether or not the patient was undergoing standard IVF or ICSI, the embryologists inseminated the oocytes directly or the oocytes were stripped and had sperm injected 4 hours after retrieval.

The Hull IVF unit follow a single embryo transfer policy to reduce the risk of multiple births. Embryo transfers were performed on day 3 or ideally at day 5 (blastocyst) to give the best chance for implantation. The embryos were classified using standard criteria (Cutting et al. 2008) see 1.6.5. Transcervical embryo transfer was performed in the lithotomy position according to the plan documented at the time of the mock embryo transfer. The embryo transfer catheters used in this

period were Rocket Soft 18cm (Rocketmedical PLC, Washington, United Kingdom) and the Sydney IVF Embryo Transfer Set (Cook Medical, Limerick, Ireland). The embryos were suspended in 20-25uL of culture medium (Quinns Advantage. Blast, SAGE, USA).

A pregnancy test was performed 14 days following embryo transfer. If this was positive the patient would continue progesterone (Uterogestan; Besins Iscovesco Laboratories, Paris, France) for luteal support. They would attend the IVF unit for a trans-vaginal Ultrasound scan to assess, viability, how many fetuses were present and if the pregnancy was in the uterus. Clinical pregnancy was defined as a fetal heartbeat on a trans-vaginal ultrasound between cycle days 42 and 49.

3.3.5 Data analysis and Statistics

Statistical analysis was performed using SPSS (v22, Chicago, Illinois). Descriptive data is presented as mean \pm standard deviation (SD) for continuous data and n (%) for categorical data. A non-parametric Kolmogorov-Smirnov Test was used to test for normal distribution. T-tests were performed for normally distributed data and Mann Whitney tests were used to compare means if the data was not normally distributed. A p-value of <0.05 was considered to indicate statistical significance. Using the data from the study, which found in the control group a mean of 54.5 ± 14 , for 80% power and 5% significance, 32 patients in each arm were required in each group to detect a 10-point difference in Vitamin D.

3.4 Results

A total of 59 women were recruited into the study (Figure 3.1). There were 30 controls and 29 women with PCOS as diagnosed through the Rotterdam Consensus Criteria. The mean age of the controls and PCOS women were similar (32.6 ± 4.65 vs. 30.9 ± 4.76 , $p=0.14$ and 25.4 ± 3.58 vs. 26.03 ± 3.83 , $p=0.56$ respectively).

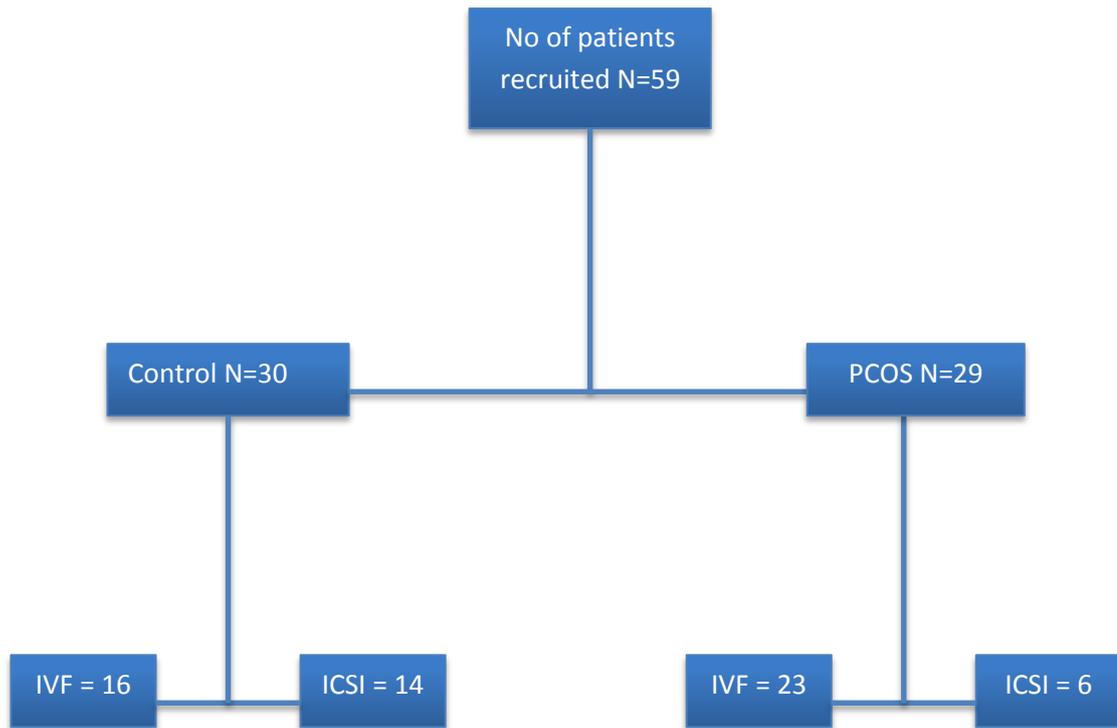


Figure 3.2: Flow chart demonstrating the breakdown of women recruited and treatment

Throughout the duration of the study only 1 cycle was abandoned due to the patient hyper stimulating at the time of follicle stimulation with exogenous gonadotropins (1.69%). 4 patients eggs failed to fertilise (6.78%) and 3 patients had their embryos frozen because of the risk of ovarian hyper stimulation syndrome (OHSS) occurring if the embryos were transferred (5.08%).

The mean demographic and biochemical results are presented in Table 3.1. There was a significant difference in ovarian reserve parameters (AMH and antral follicle count) between the control and PCOS groups (AMH 23.44 ± 13.29 ng/ml vs. 56.39 ± 14.23 ng/ml, $p=0.0001$; right ovary 8.57 ± 4.31 vs. 19.72 ± 9.95 , $p=0.0001$ and left ovary 8.67 ± 3.10 vs. 18.83 ± 10.77 , $p=0.0001$ respectively).

There was no significant difference in fasting insulin between the groups ($p=0.69$) and the mean HOMA-IR in the control and PCOS groups was similar (1.69 ± 1.00 vs. 1.97 ± 1.58 , $p=0.42$). However there was significant difference in androgen status

between the control and PCOS groups (testosterone 0.79 ± 0.36 nmol/L vs. 1.12 ± 0.52 nmol/L, $p=0.005$ and FAI 1.35 ± 0.57 vs. 4.21 ± 2.91 , $p=0.0001$). There was no difference in Vitamin D status between the two groups (46.16 ± 23.51 nmol/L vs. 54.02 ± 27.38 nmol/L, $p=0.24$, in controls and PCOS respectively).

Table 3.1 Mean Demographics and biochemical data

	Control (n=30)	PCOS (n=29)	
	Mean data (\pm S.D)	Mean data (\pm S.D)	p-value
Age (years)	32.6 ± 4.65	30.93 ± 4.76	0.14
BMI (kg/m^2)	25.47 ± 3.58	26.03 ± 3.83	0.56
AMH (pmol/l)	23.44 ± 13.29	56.39 ± 14.23	0.0001 ^{***}
Antral follicle count			
Right	8.57 ± 4.31	19.72 ± 9.95	0.0001 ^{***}
Left	8.67 ± 3.10	18.83 ± 10.77	0.0001 ^{***}
Fasting Insulin (mIU/ml)	7.68 ± 4.01	8.13 ± 4.69	0.69
Fasting Glucose (mmol/L)	4.81 ± 0.35	4.49 ± 0.82	0.06
HOMA-IR	1.69 ± 1.00	1.97 ± 1.58	0.97
SHBG (nmol/l)	114.23 ± 80.86	60.45 ± 51.47	0.004 ^{**}
Testosterone (nmol/L)	0.79 ± 0.36	1.12 ± 0.52	0.005 ^{**}
Free Androgen Index	1.35 ± 0.57	4.21 ± 2.91	0.0001 ^{***}
Vitamin D (nmol/L)	46.16 ± 23.51	54.02 ± 27.38	0.24

(* $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$)

To exclude the seasonal variation in vitamin D status, similar quality of controls and PCOS women were recruited during each month. However In June and July we had recruited all controls and at this point only had our remaining PCOS patients to collect (Table 3.2).

Table 3.2 Patient recruitment per month

Month	Control (n=30)	PCOS (N=29)
January	8	6
February	5	4
March	7	4
April	5	4
May	5	5
June	0	5
July	0	1

3.4.1 The IVF cycle and embryological Data

There was no difference in endometrial thickness between the Control and PCOS groups at the time of ovum retrieval ($10.31\text{mm} \pm 1.78$ vs. $10.72\text{mm} \pm 2.06$, $p=0.42$ respectively, Table 3.3). As expected the PCOS group had a significantly greater numbers of follicles aspirated and eggs retrieved compared to the controls (15.96 ± 5.30 vs. 11.47 ± 5.11 , $p=0.002$ and 11.29 ± 5.02 vs. 8.47 ± 5.08 , $p=0.04$ respectively). Excluding those patients that had to freeze embryos there was no significant difference ($p=0.68$) in clinical pregnancy outcome between groups: Control (35%) and PCOS (29%).

Table 3.3 Outcome data for stimulated ovarian cycle for Control and PCOS groups.

	Control (N=30) Mean (\pm S.D.)	PCOS (N=28) Mean (\pm S.D.)	p-value
Endometrial thickness at OR (mm)	10.31 \pm 1.78	10.72 \pm 2.06	0.42
Follicles aspirated	11.47 \pm 5.11	15.96 \pm 5.30	0.002**
Eggs Retrieved	8.47 \pm 5.08	11.29 \pm 5.02	0.04*
Fertilisation	4.82 \pm 2.65	8.43 \pm 3.87	0.004**
Fertilisation rate (%)	65.5 \pm 27.2	74.76 \pm 20.36	0.19
Cleavage	4.68 \pm 2.72	7.26 \pm 4.40	0.01*
Cleavage rate (%)	94.1 \pm 19.1	82.64 \pm 30.86	0.35
G3D3	3.00 \pm 2.29	4.17 \pm 3.47	0.16
Blastocyst	1.46 \pm 1.77	2.91 \pm 3.01	0.05
PDT	11	10	1.00
Clinical Pregnancy	10	7	0.57

(*p<0.01, **p<0.001, ***p<0.0001)

3.4.2 Vitamin D

- Deficiency refers to levels less than 50 nmol/L.
- Vitamin D insufficiency refers to levels between 50 – 75 nmol/L.
- Vitamin D sufficiency refers to levels above 75 nmol/L.

The following table 3.4 shows various categories – 3 groups as per above, deficient (<50), insufficient (50-75) and sufficiency (>75).

Table 3.4 Breakdown of Vitamin D categories and patient groups

		Group					
		Control		PCOS		Total	
		Count	Column N %	Count	Column N %	Count	Column N %
Vit.D Group	<50	17	56.7%	12	41.4%	29	49.2%
	50-75	10	33.3%	10	34.5%	20	33.9%
	>75	3	10.0%	7	24.1%	10	16.9%
Vit. D 50	<50	17	56.7%	12	41.4%	29	49.2%
	>=50	13	43.3%	17	58.6%	30	50.8%
Vit. D 75	<75	27	90.0%	22	75.9%	49	83.1%
	>=75	3	10.0%	7	24.1%	10	16.9%

Overall for the three categories there was no significant difference between the control and PCOS groups ($p=0.30$). 57% (17/30) of the controls were vitamin D deficient compared to 41% (12/29) of the PCOS women ($p=0.24$). 33% (10/30) of the controls were vitamin D deficient compared to 35% (10/29) of the PCOS women ($p=0.93$). 10% (3/30) of the controls were sufficient compared to 24% (7/29) of the PCOS women ($p=0.15$).

3.4.3 Fertilisation

The mean follicle count for the PCOS women was significantly higher compared to the controls, $p=0.001$, and the mean oocyte number was again significantly higher in the PCOS women, $p=0.04$ (Table 3.3). There was no significant difference between fertilisation and cleavage rates for the control and PCOS groups $p=0.19$ and $p=0.35$ respectively (Table 3.3). One woman had ovarian hyper-stimulation and four failed to fertilise. These women were not excluded from the final analysis of follicle count, but they were excluded from the fertilisation data.

There was no significant correlation with Vitamin D levels in the PCOS women and fertilisation rates after ovarian stimulation ($p<0.03$, $r = 0.44$) (Figure 3.2). The line of best fit showed a trend that increasing vitamin D levels may have a positive effect on fertilisation. When the failed to fertilise patients were omitted, a significant

positive correlation ($p=0.03$) existed between vitamin D concentrations and fertilisation rates in PCOS women, however the numbers are small $n=23$. There was no significant relationship between vitamin D levels in the PCOS patients and AMH and antral follicle count. Other factors that may affect oocyte quality include insulin and androgen, which are indicative of PCOS. No correlation was found between insulin resistance as measured by HOMA ($p=0.51$) and testosterone levels ($p=0.27$). There was a significantly negative correlation between SHBG and Vitamin D however after adjusting for BMI, SHBG is not significantly associated with Vitamin D.

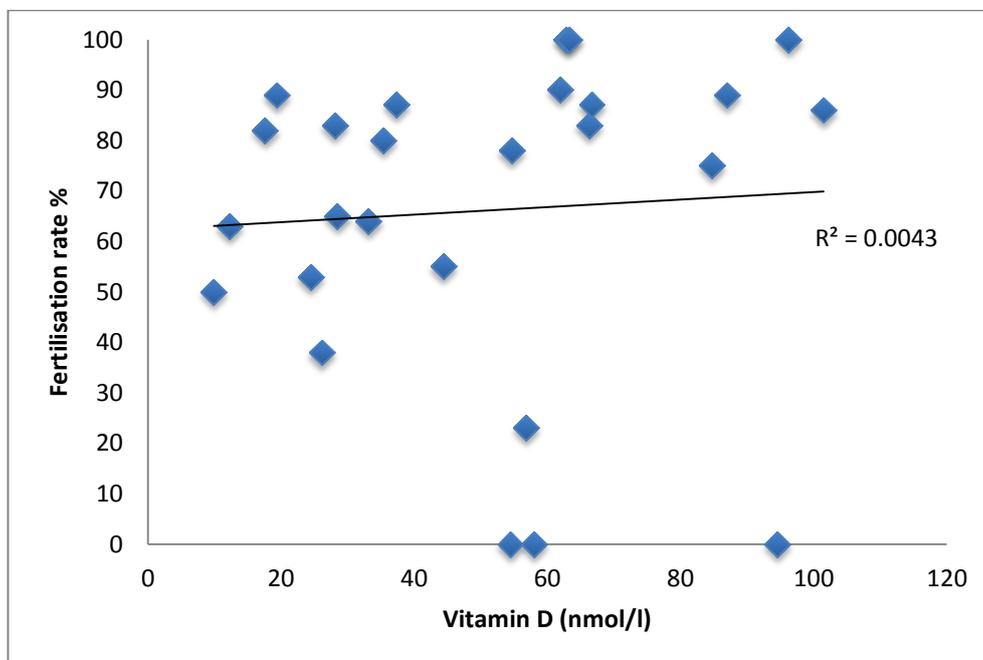


Figure 3.3 Scatter plot with line of best fit demonstrating no correlation between Vitamin D levels and fertilization rates in PCOS group ($p=0.98$)

No correlation was demonstrated between vitamin D levels in the control group and fertilisation rates after ovarian stimulation ($p<0.97$, $r = 0.006$) (Figure 3.3). There was no significant relationship between vitamin D levels in the control group, AMH and antral follicle count. There was no correlation between AMH, Insulin and androgen, in the non-PCOS patients

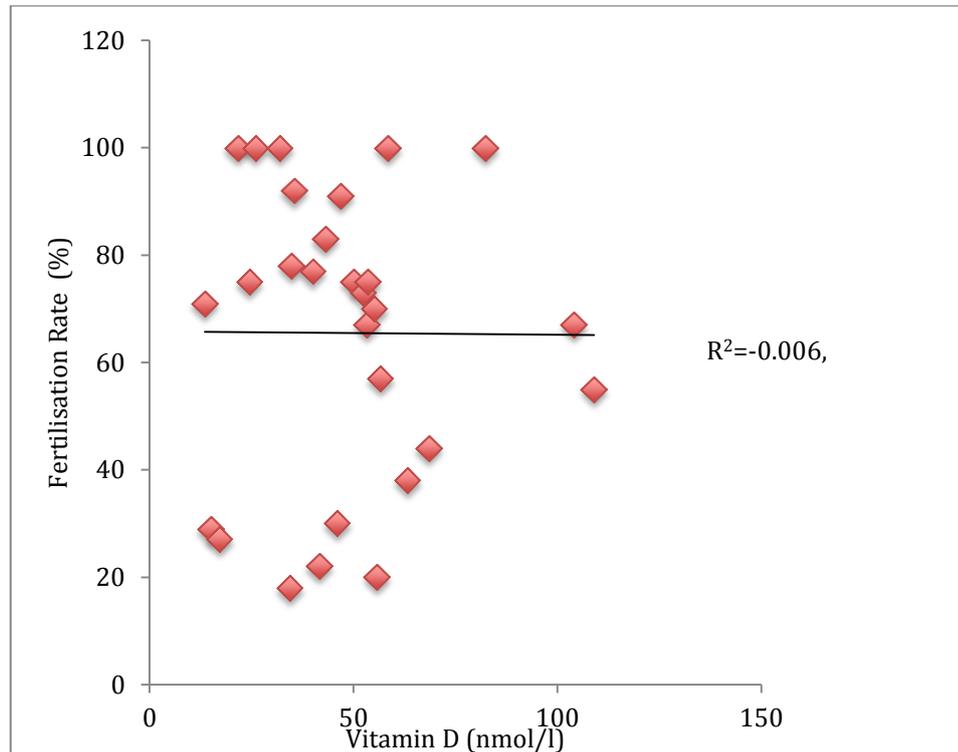


Figure 3.4 Scatter plot with line of best fit demonstrating no correlation between Vitamin D levels and fertilization rates in non-PCOS group

3.5 Discussion

This study has demonstrated that there is no correlation ($p=0.98$) between the levels of Vitamin D and fertilization rates in PCOS patients. There was no correlation demonstrated in the control group. However there appears to be a linear trend in the PCOS group demonstrating a possible positive relationship between Vitamin D and fertilisation in the PCOS population. A significant positive correlation ($p=0.03$) was demonstrated between vitamin D and fertilisation rates in PCOS women, when the failed to fertilisation patients were omitted. Thus the higher the 25(OH)D concentration the greater chance for the eggs to fertilise. This may be due to $1,25(OH)_2D_3$ having an effect on AMH and FSH gene expression. Research in mice has shown that Vitamin D decreased AMH receptor Type-II (AMHR-II) and FSH-receptor (FSH-R) gene expression (Durlinger et al 1999). This allows the follicles to become less dependent on FSH and more dependent on LH, resulting in maturation, and in a spontaneous cycle ovulation. The increased levels of vitamin D

possibly allow those ovum within the stimulated follicles to reach a more mature stage prior to ovum retrieval. As a result these more mature eggs have much better capability of achieving fertilisation with the spermatozoa.

Many studies have been published describing the outcomes of PCOS women undergoing IVF. These women typically produce large numbers of oocytes, often of poorer quality. This results in lower fertilisation and implantation rates. (Heijnen et al. 2006; Sahu et al. 2008). Studies have suggested that the impaired oocyte maturation and resultant embryonic developmental competence in PCOS women is possibly due to the abnormal endocrine/paracrine and the environment within the follicle at the time of folliculogenesis (Franks et al. 2002; Wood et al. 2007).

A possible relationship may exist between Vitamin D and fertilisation in PCOS women. Is there another pathway in these patients possibly as a result of androgen status or insulin resistance that results in large numbers of these embryos failing, or is it merely due to ovum immaturity. The results demonstrate that eggs from the patients with higher levels of Vitamin D are surviving, but large numbers of eggs of those patients with low Vitamin D levels dying, resulting in larger failure rate per cycle. Thus does Vitamin D activate intra and/or extra ovarian factors that may aid fertilisation.

Vitamin D may act within the ovary to allow maturation of the oocytes via the AMH-II receptor. Vitamin D may also act on other Intra-ovarian factors that are involved in folliculogenesis, such as Epidermal growth factor (EGF). We know within PCOS women that EGF levels are higher which may affect antral follicle growth, resulting in follicular arrest in this cohort of patients (Artini et al., 2007). Other proteins include vascular endothelial growth factor and cytokines such as interleukins and TNF α . It is known these factors are elevated in PCOS, with studies demonstrating poor oocyte quality and decreased fertilisation and pregnancy rates (Artini et al., 2008, Gallinelli et al., 2003). This study demonstrated a weak

correlation ($p=0.04$) between increasing Vitamin D levels and AMH. However after adjusting for BMI this was not significant ($p=0.09$).

Extra-ovarian factors that may affect oocyte quality include Insulin and androgens that are indicative of PCOS. No correlation was found between Insulin resistance ($p=0.51$) and testosterone ($p=0.27$) in this study, which would agree, with some studies and not with others (Ngo et al., 2011; Hahn et al., 2006 respectively). However there was a significant negative correlation between SHBG and Vitamin D however after adjusting for BMI, SHBG is not significantly associated with Vitamin D indicating the relationship was due to BMI and not Vitamin D status. For women with PCOS on metformin therapy, Kos et al. (2012) demonstrated that metformin does not have any negative affect on treatment of vitamin D deficiency and vitamin D deficiency itself is not a concern for those patients treated with metformin.

This study also demonstrated that as a cohort vitamin D levels were surprisingly low and that the controls had lower levels of 25(OH)D compared to the PCOS group, although not statistically significant (46.16 ± 23.51 vs. 54.02 ± 27.38 , $p=0.24$) Within the control group 17 (56.6%) patients were vitamin D deficient i.e. having 25(OH)D levels less than 50nmol/l. These results differ from previous studies that found Vitamin D deficiency to be more common in PCOS subjects (Mahmoudi et al. 2010; Li et al. 2011).

Mahmoudi et al. (2010) also demonstrated that overweight/obese women with PCOS has significantly decreased levels of 25(OH)D compared to normal weight ($BMI < 25 \text{ kg/m}^2$) women with PCOS. When sub-analysis was performed within this study, overweight PCOS women ($BMI \geq 25 \text{ kg/m}^2$) had significantly lower 25(OH)D levels compared to normal weight PCOS ($BMI \leq 25 \text{ kg/m}^2$) patients (43.48 ± 25.39 vs. 66.99 ± 24.74 ; $p=0.02$). This effect was not seen within the control group ($p=0.35$). The main source (80-90%) of Vitamin D is from UV-B radiation with the rest attained from the diet or supplementation. This would indicate that the study

population is not receiving appropriate exposure to the sun, and possible dietary insufficiency such as not enough fish and vegetables dietary sources of Vitamin D. We attempted to exclude seasonal variation by recruiting similar numbers of patients each month but PCOS patients represented a smaller group of patients within the IVF unit and we recruited the last 5 patients in July which may account for this discrepancy.

Vitamin D levels were low across both groups because the majority of these cycles occurred during the winter and spring months. It is well known that Vitamin D levels demonstrate seasonal variation with greater levels in the summer months. Secondly living in the northern hemisphere results in large degrees of contrast in solar luminosity. Studies have shown reduced conception rates in northern countries in winter and higher conception rates during the summer (Riojansky et al. 1992). This group also demonstrated reduction in ovulation rates and the receptivity of the endometrium in the dark winters in northern countries (Riojansky et al. 2000).

However a large retrospect study of 7368 IVF cycles conducted in Switzerland was unable to identify seasonal variation in fertilisation, pregnancy and implantation rates in women undergoing IVF (Wunder et al. 2005). A recent large prospective study of 385 patients demonstrated that vitamin D deficiency results in significantly lower pregnancy rates in womes undergoing single blastocyst transfer (Polyzos et al. 2014). The numbers in this present study are too small to make that comparison without resulting in a Type 2 error. However Polyzos et al. (2014) documented that despite a significant difference in Vitamin D levels ($P < 0.01$) across different seasons, seasonality itself did not significantly affect clinical pregnancy rates ($p = 0.17$), which would agree with Wunder et al. (2005).

Polyzos et al. (2014) is the largest prospective study to date that demonstrates that vitamin D deficiency results in significantly lower pregnancy rates within the IVF

setting. Ozkan et al. (2010) studied 84 patients undergoing IVF and found women with higher levels of vitamin D in serum and follicular fluid were significantly more likely to achieve a clinical pregnancy. Interestingly there was a high correlation between the serum and follicular fluid vitamin D levels ($r=0.94$) suggesting follicular fluid levels are reflective of body stores. Research by Agic et al. (2007) demonstrated Vitamin D receptor is expressed within the ovary. Follicular fluid Vitamin D levels may facilitate progesterone and estradiol production aiding the process of oocyte development and implantation. It has been demonstrated that $1,25(\text{OH})_2\text{D}_3$ in vivo stimulated progesterone production by 13% and estradiol production by 9% indicating a role in steroidogenesis of the gonadotropins (Parikh et al., 2010). However, a smaller study of 82 patients undergoing IVF found no significant associations between serum and follicular fluid 25(OH)D levels and clinical pregnancy rates (Aleyasin et al. 2011).

Only one study to date has found an inverse relationship between Vitamin D status and reproductive outcomes. In 101 intracytoplasmic sperm injection (ICSI) patients, those with sufficient levels of 25(OH)D had poorer embryo quality and had a significantly lower pregnancy rate compared to patients with insufficient and deficient 25(OH)D. However this was a small study and there was no mention on how many embryos were transferred in each group.

3.5.1 Vitamin D in pregnancy

It is important to discuss that, despite a proportion of these women achieving a clinical pregnancy, a large number were either deficient or insufficient in Vitamin D. In June of 2014 the RCOG published a scientific Impact Paper No.43 which concerned vitamin D in Pregnancy. Within this it states, the 2012 recommendation from the UK Chief Medical Officers and NICE guidance state that all pregnant and breastfeeding women should be informed about the importance of vitamin D and should take 10 micrograms of vitamin D supplements daily (Chief medical Officers for the United Kingdom. 2012; NICE, 2008). An American study has highlighted that

approximately 2 in every 3 pregnant women have suboptimal levels of Vitamin D, which is higher in African Americans (Looker et al. 2008). Obese pregnant women ($BMI \geq 30 \text{ kg/m}^2$) have higher levels of vitamin deficiency compared to normal weight pregnant women ($BMI \leq 25 \text{ kg/m}^2$) 61% vs. 36% respectively (Bodnar et al. 2007a).

Vitamin D deficiency amongst pregnant women has been associated with an increased risk of pre-eclampsia (Baker et al. 2010; Bodnar et al. 2007b). These women with levels of $25(\text{OH})\text{D} < 50 \text{ nmol/l}$ were associated with a 5-fold increased risk of severe pre-eclampsia (Baker et al., 2010). A large meta-analysis has reported a strong association with vitamin D and pre-eclampsia also found a greater association with bacterial vaginosis that can increase the risk of miscarriage and pre-term delivery (Aghajafari et al. 2013).

Maternal vitamin D deficiency has been associated with an elevated risk of gestational diabetes (GTD) and concentrations within the serum are significantly lower in those women with GTD (Maghbooli et al. 2008). There has also been an association between small for gestational age (Bodnar et al. 2010), reduced bone density within the fetus (Javaid et al. 2006), resulting childhood rickets (Wagner and Greer. 2008) and asthma (Camargo et al. 2007)

3.5.2 Vitamin D Supplementation

The results from this study highlight that Vitamin D insufficiency is a problem of both PCOS and non-PCOS populations of women. The results of observational studies indicate that Vitamin D deficiency has effects on reproduction and pregnancy, however large randomised control trials are required. Supplementation is a cheap and effective treatment that may have an effect on improving reproductive capability and reducing pregnancy complications. It is necessary to account for two factors when commencing patients on Vitamin D supplementation. Primarily the dosage of the supplementation and secondly the absorption relative to the BMI of the patient, as obesity may contribute to low circulating Vitamin D

levels by essentially trapping Vitamin D within the adipose tissue. CMACE/RCOG Joint Guideline: Management of Women with Obesity in Pregnancy (2010) advises health care professionals to counsel patients with a BMI $\geq 30\text{kg/m}^2$ to take 10micrograms Vitamin D supplementation daily during pregnancy and while breastfeeding (CMACE/RCOG. 2010).

The Chief medical Officer recommends 3 categories of Vitamin D supplementation (Chief medical Officers for the United Kingdom. 2012):

1. **In General**, 10micrograms (400units) daily for all pregnant women
2. **High risk patients** i.e. those with increased skin pigmentation, reduced exposure to sunlight or those who are socially excluded or obese) advised to take 1000 units (25micrograms) daily. It is important to remember many hirsute PCOS patients tend to hide from public exposure and are obese.
3. **Treatment:** For those women who are Vitamin D deficient ($<25\text{nmol/l}$). Commence a 4 - 6 week course, using either cholecalciferol 20,000IU per week or ergocalciferol 10,000 IU twice weekly, followed by standard supplementation.

The opinion of the RCOG Scientific Impact Paper (RCOG. June 2014) is that treating Vitamin D deficient women with Vitamin D supplementation is safe and recommended for all women who are pregnant or breastfeeding. It would be of benefit for all women undergoing subfertility treatment to have their Vitamin D levels checked as this may require simple advice regarding general supplementation to be commenced prior to starting treatment with a dose of Vitamin D to correct a deficiency if necessary.

This study has demonstrated that a correlation exists between fertilization rates and Vitamin D levels in PCOS patients undergoing exogenous gonadotrophin ovarian stimulation through IVF. This is important because if Vitamin D is having a theoretical effect by decreased AMH receptor Type-II (AMHR-II) gene expression, enabling granulosa cell proliferation, then it is important to optimize Vitamin D

levels in this subgroup of women prior to commencing IVF. The study also highlighted that, within the population as a whole only 10 (16.9%) had sufficient levels of 25(OH)D.

It is important to remember that the primordial follicle enters the growing follicle pool approximately 4 months prior to ovulation (Gougeon 1986). Folic acid supplementation was commenced in 1992 to reduce neural tube defects the Department of Health advised all women considering to conceive, those who were planning a pregnancy, or who might become pregnant, should consume more folate rich foods and take a daily supplement of 400 µg of folic acid continued for up to 12 weeks after conception (Department of Health. 1992). Introducing Vitamin D in subfertile women with PCOS may help to reduce AMHR-II gene expression, enabling the follicles to mature and have a greater chance of successful fertilization. This would also help reduce the effects of maternal pre-eclampsia and fetal complications such as IUGR. Within the context of supplementation, PCOS patients have a greater likelihood for being overweight or obese and women with high BMI are deficient in essential micronutrients (Nicklas et al. 2001). As stated, recent guidance advises patients with a BMI $\geq 30\text{kg/m}^2$ to take 10micrograms Vitamin D and high dose folic acid (5mg) daily before and during pregnancy based on need and relative deficiency (CMACE/RCOG 2010).

However to validate the preliminary results of a possible link between Vitamin D and fertilisation in PCOS patients requires a much multi-center larger study for confirmation. Furthermore research would be required into what effect BMI has on the bioavailability of 25(OH)D. Is Vitamin D insufficiency as a consequence of obesity, or is obesity as a consequence of Vitamin D insufficiency?

Furthermore this study is comparable with previous studies that PCOS patients typically produced more oocytes, which are often of poorer quality. This results in lower fertilisation, cleavage and implantation rates. More research is required into

what effect vitamin D has on the developmental competence and maturation of the oocytes of PCOS women. Research has shown that the VDR is within the endometrium. Unfortunately this study was unable to investigate what, if any effect, Vitamin D has on the VDR within the endometrium, but this maybe an important factor required to aid the process of implantation. In approximately 70% of IVF cycles implantation is the rate-limiting step in many IVF cycles (Coughlan et al. 2014).

3.6 Conclusion

In conclusion our results have highlighted that there appears to be a **no correlation** between Vitamin D levels and fertilization rates in PCOS women undergoing IVF, but a possible trend may exist demonstrating higher levels of Vitamin D levels may improve fertilisation rates in PCOS women but larger studies are needed to prove this The exact physiological reason for this is unknown but this may be due to the altered expression of AMHR-II. Within the study Vitamin D deficiency/insufficiency appears to be endemic in our population of patients. There is no conclusive evidence regarding what effects Vitamin D has on AMH, however Vitamin D supplementation is cheap and safe and may be an important factor to optimize fertilisation, reproductive and on-going pregnancy success with both women with and without PCOS.

CHAPTER FOUR:

The Effect of Endometrial Scratch on In vitro Fertilisation.

4.1 Abstract

Background: Endometrial scratching using devices such as a biopsy pipelle, curette, or hysteroscope causes physical injury to the endometrium, which could potentially improve implantation rates. It is believed the endometrial injury results in a significant release of cytokines, growth factors and interleukins, inducing decidualisation and increasing receptivity of the endometrium creating a more favorable environment for implantation. There is no consensus on the timing of endometrial scratch with respect to menstrual cycle, the degree of injury required or the frequency with which the procedure needs to be performed. The objective of this observational study was to assess the effectiveness of endometrial scratch on outcomes of in vitro fertilization (IVF).

Method: This observational study compared IVF outcomes in women undergoing IVF/ICSI (intra-cytoplasmic sperm injection) cycles with and without an endometrial scratch using a biopsy catheter (Pipelle de Cornier). All patients in 2014 having fresh IVF/ICSI cycles were offered an endometrial scratch after the Mock-ET on their first IVF cycle. These results were then compared to the 2013 results in which all the treatment cycle parameters were the same with the exception of an endometrial scratch.

Results: 301 patients underwent an endometrial scratching in 2014 and this was compared with 321 patients in 2013. There was no significant difference in patient demographics between the two groups for age, body mass index (BMI), anti-mullerian hormone (AMH), endometrial thickness and quality of embryo replaced. 359 patients had their first cycle of IVF, endometrial scratching showed no added benefit to a Mock-ET with no significant difference in pregnancy rates 67/174

(38.5%) v.s. 76/185 (41.3%), $p = 0.66$. Of the 161 patients undergoing second cycles, the women who underwent endometrial scratch were found to have significantly lower clinical pregnancy rates compared to women who didn't have an endometrial scratch 18/74 (24.3%) vs. 35/87 (40.22%), $p = 0.04$. For the 102 patients with 3 or more cycles of IVF, the endometrial scratching had no added benefit to no treatment, 19/53 (35.8%) vs. 21/49 (42.8%), $p = 0.54$. There was also no effect on clinical pregnancy rates when logistic regression analysis was performed, to compare for confounding variables such as age and BMI.

Conclusion: This study suggests an endometrial scratch offers no additional benefit in women undergoing first IVF cycle with respect to pregnancy rates. On subsequent IVF cycles the endometrial scratching appears to have significantly worsened outcomes in the second cycle and no added benefit for those patients with recurrent implantation failure, for which the procedure has been proposed to have a benefit.

4.2 Introduction

Implantation remains the main rate-limiting step in in vitro fertilization (IVF) with only 25-40% of embryos implanting successfully (Ferrara et al. 2002; de los Santos et al. 2003; Coughlan et al. 2014;). The most recent data on IVF outcomes according to *European Society of Human Reproduction and Embryology (ESHRE)*, show an implantation rate of only 32% for fresh embryo transfers, resulting in a clinical pregnancy (Ferraretti et al. 2013). This is thought to be due to a combination of factors such as poor uterine receptivity, embryo developmental potential and interaction of the embryo with the endometrium (Diedrich et al. 2007). It is proposed that uterine receptivity is responsible for two thirds of implantation failures and embryo-derived factors for the remainder (Simon et al. 1998; Ledee-Bataille et al. 2002).

ESHRE define recurrent implantation failure (RIF) as a failure to achieve pregnancy after ≥ 3 unsuccessful transfers of high quality embryos, or the transfer of ≥ 10 embryos in total during multiple IVF cycles (Thornhill et al. 2005). With the move to limit multiple gestations by single embryo transfer, the definition of RIF is being refined. Thus, Coughlan et al. (2014) define RIF as the failure to achieve a clinical pregnancy after transfer of at least four good-quality embryos, in a minimum of three fresh or frozen cycles, in a woman under the age of 40 years.

Basash et al. (2003) originally described the endometrial scratch as local injury to the endometrium with a biopsy catheter to increase the incidence of implantation. It is thought that endometrial scratching induces inflammation within the endometrium aiding implantation. Specifically, this inflammatory effect assists in the up regulation of cytokines, chemokine and growth factors, inducing decidualisation thereby increasing receptivity of the endometrium and creating a more favorable environment for implantation (Gnainsky et al. 2010, Dunn et al. 2003; Kelly et al. 2001). Gnainsky et al. (2010) demonstrated elevated levels of pro-inflammatory cytokines, $\text{TNF}\alpha$, growth regulated oncogene- α and macrophage

inflammatory protein 1B (MIP-1B) in a day 21 biopsy and found a positive correlation between the levels of inflammatory cells and pregnancy outcome. Kalma et al (2009) reported that endometrial scratching induced an increase in the expression of 183 genes related to endometrial receptivity. Almog et al. (2010) hypothesized that local injury increased endometrial receptivity by modulating the expression of a variety of genes.

Following the results published by Barash et al. (2003) it is thought that endometrial scratching may improve clinical pregnancy rates in women with RIF. A number of non-randomised and randomised control trials (RCTs) have claimed to demonstrate significant improvements in clinical pregnancy rates following an endometrial scratch in women with RIF (Raziel et al. 2007; Narvekar et al. 2009; Karimzadeh et al. 2009). However these studies had small sample sizes and large variations in methodology making the comparison of the procedures difficult. Nevertheless, recent systematic reviews and meta-analyses have been published using the results of these trials, as well as unpublished work, concluding that endometrial scratching is of benefit to women with RIF undergoing IVF (El-Toukhy et al. 2012; Nastri et al. 2012). By contrast, two recent RCTs, which included 336 women, have demonstrated that endometrial scratching is of no benefit to an unselected population of women undergoing IVF (Baum et al. 2012; Yeung et al. 2014).

Study Objective

The objective of this observational study was to assess whether an endometrial scratch was more effective in causing injury sufficient to aid implantation, compared to a Mock-Embryo Transfer (Mock-ET), in a first cycle of IVF and no intervention in second and subsequent cycles of IVF.

4.3 Materials and Methods

4.3.1 Ethical Approval and Patients Recruitment

The Hull IVF unit ethical committee approved the study in January 2014. The Hull IVF unit was piloting the effects of endometrial scratching (ES) to all women diagnosed with subfertility and requiring a fresh cycle of IVF or ICSI, during the luteal phase of their menstrual cycle, the cycle before commencing their IVF/ICSI treatment. Endometrial scratching was offered routinely to all indicated patients in our Unit. We did this as an observational study rather than a randomised controlled study since it was felt unethical to withhold treatment that was a routinely offered in our Unit.

All patients who were about to commence a cycle of IVF attended a group session held at the East Riding Medical Education Centre within Hull Royal Infirmary, UK. A short presentation about ES was given and what the possible complications involved in the procedure included. They were sent further information regarding the ES with their introductory pack, which included their Human Fertility and Embryology Authority (HFEA) consent forms and all relevant information regarding their IVF cycle within the Hull IVF unit. A signed informed consent was obtained before the procedure was performed. All women undergoing fresh IVF/ICSI cycles who had consented to having an ES were included in the study. This included women undergoing their first cycle of IVF and those patients whom had multiple unsuccessful cycles of IVF. We excluded those patients who were undergoing cycles of IVF with donor gametes to reduce confounding variables.

4.3.2 Recruitment

Women attended the IVF unit for their review appointment prior to commencing their IVF treatment. They were advised to have 400 mg of Ibuprofen or 1g of paracetamol, one hour prior to the procedure, since an ES can be uncomfortable and elicit menstrual cramping. They were also advised to bring a vaginal pad as they could experience some minor bleeding afterwards.

The review appointment was scheduled in the luteal phase of their menstrual cycle prior to commencing their IVF. During this appointment the patients completed their HFEA consent forms, and were instructed on how to inject their exogenous gonadotrophins. They also signed a consent form to have endometrial scratching. The consent form detailed the risks and benefits of the procedure and that they were not pregnant as the endometrial scratching could disrupt an ongoing pregnancy. A pregnancy test was not performed prior to the procedure. During the review appointment all patients undergoing their first cycle of IVF have a Mock Embryo Transfer (Mock-ET).

4.3.3 Mock-ET Technique

During a mock transfer, no embryo was transferred. The procedure was performed with the patient awake and requires no analgesia. The patient was placed in the lithotomy position and a transvaginal ultrasound was performed to visualize whether the uterus was anteverted or retroverted.

Anteverted uterus is when the long axis of the body of the uterus is bent forward on the long axis of the vagina (Figure 4.1), occurring in 50% of women (Daftary and Chakravarti 2011)



Figure 4.1 Transvaginal Ultrasound of an anteverted uterus.

Retroverted uterus is the opposite of an anteverted uterus, where the body of the uterus is bent backward on the long axis of the vagina (Figure 4.2), occurring in 25% of women. The rest have an axial uterus when the long axis of the body of the uterus is at an equal plane at the level of the vagina.



Figure 4.2 Transvaginal Ultrasound of a retroverted uterus.

A Cusco speculum was introduced into the vagina and opened to allow direct visualization of the external os of the cervix. The external os was cleaned with a dry swab and then a Mock-ET catheter (Figure 4.3) is passed through the cervix into the uterus. This demonstrates how easy or difficult it is to pass the catheter through the external os, the cervical canal and internal os into the uterine cavity, and at this point a different Mock-ET catheter may be required to complete the procedure. In some instances if the catheter cannot be passed through the cervical canal, then a cervical dilatation is performed under local anesthetic.



Figure 4.3 Mock ET-Catheter

Once the catheter was passed through the cervical canal it was advanced until it makes contact with the uterine fundus and the measurement of the depth of the uterine cavity was taken using the graduated measuring scale on the side of the Mock-ET catheter. 20mm is then subtracted from this total length and this is the optimum point in the lower third of the uterine cavity to place the embryo. The whole procedure takes no more than about 10 to 15 minutes and the patients was warned that they might experience some light bleeding for a few days post procedure.

4.3.4 Endometrial Scratching

This is performed straight after the Mock-ET in patients undergoing their first cycle of IVF or as an independent procedure for those patients' undergoing subsequent cycles of IVF.

Following the insertion of the Cusco speculum and preparation of the cervix a Pipelle de Cornier (Prodimed, Neuilly-en-Thelle, France) endometrial biopsy catheter was passed through the cervical canal into the uterine cavity to the level of the uterine fundus. The inner sheath of the pipelle is withdrawn to create negative pressure and the pipelle is moved up and down the endometrial cavity several times and rotated gently at the same time (Figure 4.4). This movement causes the scratch (injury) to the endometrial cavity.

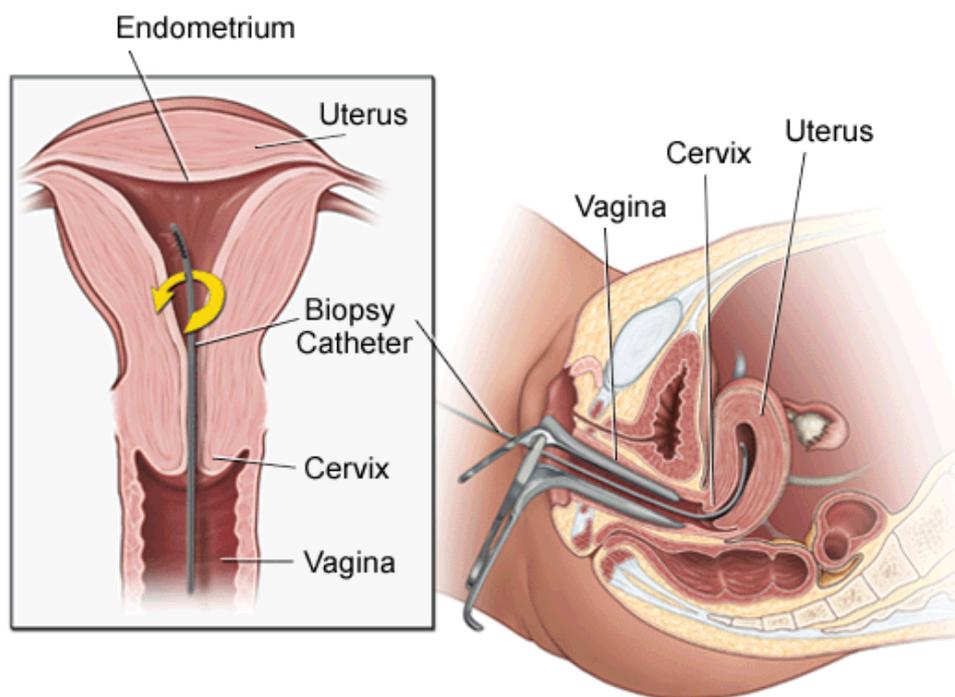


Figure 4.4 Sagittal Image of pelvis demonstrating Endometrial Scratching. Krames Staywell. 780 Township Road, Yardley, PA 19067,267-685-2500

4.3.5 The IVF Cycle

All patients in 2014 underwent an antagonist IVF protocol. This protocol was the same as those patients in 2013, the only new variable being the endometrial scratch. The dose of exogenous gonadotropin was based on the women's AMH and age. The patients commenced their rFSH stimulation on day 2 of their menstrual

cycle using either Merional (Pharmasure) or Gonal-F (Merck Serono). A GnRH antagonist (Cetrotide: Merck Serono) was used to prevent a premature LH surge.

The patients underwent ultrasound scans from day 7 to observe the ovarian response to stimulation and they were repeated every 48 hours. The scans were used to measure the diameters of the follicles thus observing response and follicle numbers. The hCG trigger Buserelin (Sanofi-Aventis, Frankfurt, Germany) or Pregnyl (Merck Sharp and Dohme) was administered when the leading follicle was $\geq 18\text{mm}$.

Oocyte retrieval was performed 36 hours later. This was carried out under sedation, and the follicles were aspirated under ultrasound guidance. Luteal support was provided with micronised progesterone (Uterogestan; Besins Iscovesco Laboratories, Paris, France) at a dose of 600mg, each night commencing on the day of oocyte retrieval. The embryologists at this point, according to whether the patient was undergoing IVF or ICSI-IVF, inseminated the oocytes directly or the oocytes were stripped and had sperm injected 4 hours after retrieval.

The Hull IVF unit follows a single embryo transfer policy to reduce the risk of multiple births. Embryo transfers were performed on day 3 or ideally at day 5 (blastocyst) to give the best chance for implantation. The embryos were classified using standard criteria (Cutting et al. 2008) at the cleavage stage and for blastocyst stage. A good quality day 3 embryo is defined as having the correct number of cells corresponding to the day of development and a good quality blastocyst is defined by the degree of expansion and the quality of the inner cell mass. (Cutting et al. 2008).

Transcervical embryo transfer was performed in the lithotomy position, according to the plan documented at the mock embryo transfer. The embryo transfer catheters used in this period were Rocket Soft, 18cm (Rocketmedical PLC, Washington, United Kingdom) and the Sydney IVF Embryo Transfer Set (Cook

Medical, Limerick, Ireland). The embryos were suspended in 20-25uL of culture medium (Quinns Advantage. Blast, SAGE, USA).

A pregnancy test was performed 14 days following ET. If this was positive the patient would continue the progesterone (Uterogestan; Besins Iscovesco Laboratories, Paris, France) for luteal support. They attend the IVF unit for a trans-vaginal Ultrasound scan to ensure that the pregnancy was intrauterine, viable, and how many fetuses were present. Clinical pregnancy was defined as a fetal heartbeat on a trans-vaginal ultrasound between cycle days 42 and 49.

4.3.6 Data analysis and Statistics

Descriptive data is presented as mean \pm SD for continuous data and n (%) for categorical data. T-tests or Mann Whitney tests were used to compare 2013 cycles and 2014 cycles for continuous data and Chi-square tests for categorical data. Logistic regression analysis was used to compare clinical outcome, while adjusting for potentially confounding variables. All analyses were undertaken on SPSS (v22, Chigaco, Illinios). A p-value of <0.05 was considered to indicate statistical significance.

4.4 Results

301 women had an endometrial scratching (ES) performed in fresh IVF/ICSI cycles in 2014 compared to 321 who had no ES performed in 2013 (Table 4.1). There was no significant difference in age, BMI and AMH when we compared the No-ES and ES groups at the start of the IVF cycle. There was no significant difference in duration of subfertility between the two groups (3.00 ± 1.09 and 3.01 ± 0.98 ; $p= 1.00$). Male factor was the most common cause of subfertility in each group 34.89% in No-ES and 37.89% in ES, $p=0.48$, followed by unexplained 21.80% in No-ES and 22.58% in ES, $p= 0.31$. Within each group there was approximately 55-60% of patients undergoing their first cycle ($p= 0.96$), 25-30% undergoing their 2nd cycle ($p=0.53$) and 15-20% undergoing 3 or more cycles of IVF ($p=0.50$), with no significant

difference between each group. There were no infections or periods of prolonged bleeding following an endometrial scratch.

Table 4.1 Descriptive demographics for the No-ES and the ES groups.

	No-ES (n=321)	ES (n= 301)	P-value
	2013	2014	
Age	33.55 (4.74)	33.99 (4.76)	0.25
BMI (kg/m²)	24.99 (3.66)	24.56 (4.17)	0.19
AMH (pmol/l)	21.11 (18.91)	20.24 (15.50)	0.54
Duration of Subfertility	3.00 (1.09)	3.01 (0.98)	1.00
Causes of Subfertility			
Male	112 (34.89%)	114 (37.89%)	0.48
Anovulatory	30 (9.35%)	36 (11.96%)	0.04*
Endometriosis	22 (6.85%)	17 (5.65%)	0.65
Tubal	37 (11.52%)	28 (9.30%)	0.44
Unexplained	70 (21.80%)	77 (25.58%)	0.31
Mixed	50 (15.57%)	29 (9.64%)	0.04*
Number of Previous IVF cycles			
0	185 (57.6%)	174 (57.7%)	0.96
1	87 (27.1%)	74 (24.7%)	0.53
2 or more	49 (15.3%)	53 (17.6%)	0.50

Data expressed as mean (Standard deviation) or number (percentage) as appropriate. * P < 0.05 Indicates significant difference.

There was no difference in the types of assisted conception technique performed (Table 4.2). In 2013, 207 patients had IVF compared to 178 in 2014; p=0.19, and in 2013, 99 had ICSI compared to 114 in 2014; p=0.08. There was no difference in endometrial thickness measured at the time of ovum retrieval 10.61 ± 2.20 mm in No-ES and 10.41 ± 2.37 mm in ES, p=0.31. There was no significant difference in the mean follicle count between the years with 12.68 ± 6.20 in No-ES and 11.70 ± 5.25

in ES, $p=0.10$. However there was a significant reduction in numbers of oocytes collected in ES, 8.40 ± 3.92 compared to 9.28 ± 4.56 in No-ES, $p=0.03$. Despite the difference in oocytes collected there was no significant difference in high quality embryos at day 3, 3.80 ± 2.89 vs. 3.36 ± 2.51 ; $p=0.05$, although the trend was close to significance. Table 4.2 illustrates there was no difference in numbers of embryos transferred and transfer day between the two groups with the majority of embryos transferred being day 5 (70.1% in 2013 and 64.5% in 2014; $p=0.15$).

Table 4.2 Cycle characteristics of the No-ES and ES groups.

	No-ES (2013)	ES (2014)	P-value
Assisted Reproduction			
IVF	207	178	0.18
ICSI	99	114	0.08
IVF/ICSI split	15	9	0.83
Endometrium at OR (mm)	10.61 (2.20)	10.41 (2.37)	0.31
Number of follicles	12.68 (6.20)	11.70 (5.25)	0.10
Oocytes collected	9.28 (4.56)	8.40 (3.92)	0.03*
G3D3 embryos	3.80 (2.89)	3.36 (2.51)	0.05
No of embryos transferred	1.22 (0.02)	1.29 (0.03)	0.91
Day of embryo transfer			
2	25 (7.8%)	32 (10.6%)	0.41
3	69(21.5%)	75 (24.9%)	0.34
5	227(70.7%)	194(64.5%)	0.15

(OR; Ovum retrieval, G3D3; Top quality embryos at day three). Data expressed as median (Standard deviation) or number (percentage) as appropriate. * $P < 0.05$ Indicates significant difference.

There was no difference in positive pregnancy tests between those women whom had an ES compared to No-ES (42.9% vs. 46.1%, $p=0.42$), Table 4.3. At the time of ultrasound scan (USS) to confirm a clinical pregnancy, there was no significant difference in those women whom had an ES and No-ES (34.9% vs. 41.43%, $p=0.11$). In those pregnancies where a fetal heartbeat was detected, there was no significance difference between the ES group and No-ES (33.56% vs. 38.94%, $p=0.18$).

Table 4.3 Pregnancy outcomes of No ES and ES groups

Pregnancy outcomes	No-ES (n=321)	ES (n=301)	P-value
Positive pregnancy test (PDT)	148 (46.1%)	129 (42.9%)	0.42
Clinical pregnancy	133 (41.43%)	105 (34.9%)	0.10
Fetal Heart Beat	125 (38.94%)	101 (33.56%)	0.18
Singleton	118 (36.76%)	95 (31.56%)	0.18
Twins	7 (2.18%)	6 (2%)	1.00

4.4.1 Subgroup analysis

Since the only significant change within the two study years was to perform an endometrial scratch its effect on varying treatment cycles were studied for the following reasons:

- 1) Was an endometrial scratching any more effective than a Mock-ET that is performed in all women undergoing their first cycle of IVF-ICSI
- 2) Previous studies investigating endometrial scratching had demonstrated a benefit if performed in the second cycle of IVF

- 3) Endometrial scratching was designated for patients with recurrent implantation problems, i.e. patients having undergone two or more failed fresh cycles of IVF/ICSI.

4.4.2 Mock-ET vs. a Mock-ET and Endometrial Scratch

This group included all patients who were undergoing their first cycle of IVF. Within this sub-group all patients undergoing their 1st cycle of IVF in 2013 only had a mock-ET compared to those patients in 2014 that had a Mock-ET followed by an ES (Table 4.4). There were 359 patients within this subgroup (185 Mock-ET only vs. 174 Mock-ET and ES, P=0.65). The age of the women and their BMI (32.88 ± 4.82 vs. 33.11 ± 4.71 , $p=0.65$ and $25.04 \text{ kg/m}^2 \pm 3.73$ vs. $24.25 \text{ kg/m}^2 \pm 4.39$, $p=0.08$, respectively) in the groups was similar.

During the IVF/ICSI cycle the endometrial thickness of the ES group was significantly thinner than the Mock-ET only group ($10.18 \text{ mm} \pm 3.08$ vs. $10.78 \text{ mm} \pm 2.12$, $p=0.04$). There was no significant difference in the oocytes recovered between the 2 groups (9.28 ± 4.64 vs. 8.38 ± 4.01 , $p=0.05$ respectively), however the quality of the oocytes was significantly poorer in the ES group (3.09 ± 2.64 vs. 3.83 ± 2.84 , $p=0.01$). There was no difference in the numbers of embryos transferred between the 2 groups ($p=0.46$) and the day of transfer ($p=0.17$). Clinical pregnancy rates were similar between the 2 groups 41.62% vs 39.00%, $p=0.67$.

Table 4.4 Mock-ET vs a Mock-ET and Endometrial Scratch Demographics.

	Mock-ET (N=185)	Mock-ET and ES (N=174)	p- value
Age	32.88 (4.81)	33.11 (4.71)	0.65
BMI (kg/m ²)	25.04 (3.73)	24.25 (4.39)	0.08
AMH (pmol/l)	22.02 (20.94)	20.08 (15.84)	0.33
Endometrium at OR (mm)	10.78 (2.12)	10.18 (3.08)	0.04*
Eggs recovered	9.28 (4.64)	8.38 (4.01)	0.05
G3D3	3.83 (2.84)	3.09 (2.64)	0.01*
Transfer day	4.36 (1.08)	4.20 (1.09)	0.17
Number transferred	1.16 (0.03)	1.19 (0.03)	0.46
PDT	85 (46.0%)	83 (47.70%)	0.75
Clinical Pregnancy	77 (41.62%)	68 (39.00%)	0.67

Data expressed as median (Standard deviation) or number (percentage) as appropriate. * P < 0.05 Indicates significant difference.

The results from a forward stepwise regression model demonstrated that ES is not a significant independent predictor variable of clinical pregnancy, after controlling for the following factors: female age, BMI, endometrial thickness, top quality embryos, and blastocysts.

4.4.3 No additional uterine intervention vs. an ES in 2nd cycle IVF/ICSI

Previous studies recruited patients with only one previous cycle of IVF for an endometrial scratch. A further sub-group analysis on those patients with only one previous cycle of IVF was performed to see if an ES had any effect on those patients having one previous cycle of IVF (Table 4.5). There were 161 patients within this subgroup (87 having no intervention vs. 74 having ES, p=0.52). The age of the women and their BMI (34.54 ± 4.34 vs. 34.77 ± 4.50 , p=0.75 and $25.22 \text{ kg/m}^2 \pm 3.55$ vs. $24.78 \text{ kg/m}^2 \pm 3.74$, p=0.44, respectively) in the groups was similar.

Table 4.5 No Intervention vs. ES in 2nd cycles of IVF/ICSI.

	2 nd cycle no treatment (n=87)	ES (n= 74)	p-value
Age	34.54 (4.34)	34.77 (4.50)	0.75
BMI (kg/m ²)	25.22 (3.55)	24.78 (3.74)	0.44
AMH (pmol/l)	18.59 (14.50)	19.93 (15.62)	0.58
Endometrium at OR (mm)	10.19 (2.53)	9.84 (2.75)	0.41
Eggs recovered	8.73 (4.05)	7.78 (4.16)	0.15
G3D3	3.09 (2.53)	2.66 (2.46)	0.43
Transfer day	4.29 (1.05)	4.22 (1.24)	0.70
Number transferred	1.30 (0.05)	1.32 (0.05)	0.79
PDT	40 (46.0%)	23 (31.1%)	0.07
Clinical Pregnancy	35 (40.22%)	18 (24.3%)	0.04*

Data expressed as median (Standard deviation) or number (percentage) as appropriate. * P < 0.05 Indicates significant difference

During the IVF/ICSI cycle the endometrial thickness and number of oocytes collected was similar within each group (10.19 mm \pm 2.53 vs. 9.84 mm \pm 2.75, p=0.41 and 8.73 \pm 4.75 vs. 7.78 \pm 4.16, p=0.43 respectively). There was no difference in the numbers of embryos transferred between the 2 groups (p=0.46) and the day of transfer (p=0.17). The difference between positive pregnancy rates between the 2 groups did not quite make statistical significance (46% vs. 31.1%, p=0.07). However the ES group had significantly fewer clinical pregnancies (24.3% vs. 40.22%, p=0.04) compared to the no intervention group.

The results from a forward stepwise regression model demonstrated that ES is not a significant independent predictor of clinical pregnancy compared to no intervention, after controlling for the following factors: female age, BMI, endometrial thickness, top quality embryos, blastocysts in patients undergoing a 2nd cycle of IVF.

4.4.4 No uterine intervention vs. 3 or more cycles of IVF

This group contains the patients who have had recurrent unsuccessful IVF/ICSI cycles and have undergone an ES. They have been compared to a similar group of patients from 2013 that had no intervention. The demographic of each group can be seen in Table 4.6. There were 102 patients within this subgroup (49 having no intervention vs. 53 having ES, $p=0.45$). The age of the women and their BMI (34.5 ± 4.73 vs. 35.64 ± 4.66 , $p=0.23$ and $24.48 \text{ kg/m}^2 \pm 3.55$ vs. $25.29 \text{ kg/m}^2 \pm 3.92$, $p=0.32$, respectively) in both groups were similar.

Table 4.6 No intervention vs. 3 or more cycles of IVF/ICSI.

	≥ 3 cycles no treatment (n=49)	ES (n= 53)	p-value
Age	34.5 (4.73)	35.64 (4.66)	0.23
BMI (kg/m^2)	24.48 (3.55)	25.29 (3.92)	0.32
AMH (pmol/l)	20.75 (16.91)	20.59 (15.42)	0.96
Endometrium at OR (mm)	10.44 (2.40)	9.40 (3.81)	0.13
Eggs recovered	10.51 (4.89)	8.32 (3.68)	0.01 *
G3D3	4.04 (3.74)	3.03 (2.29)	0.10
Transfer day	4.25 (1.10)	4.20 (1.15)	0.82
Number transferred	1.33 (0.476)	1.56 (0.5)	0.02 *
PDT	22 (44.90%)	22 (41.5%)	0.84
Clinical Pregnancy	21 (42.86%)	19 (35.8%)	0.54

Data expressed as median (Standard deviation) or number (percentage) as appropriate. * $P < 0.05$ Indicates significant difference

During the IVF/ICSI cycle the endometrial thickness was similar within each group ($10.44 \text{ mm} \pm 2.40$ vs. $9.40 \text{ mm} \pm 2.75$, $p=0.11$). However significantly fewer oocytes were collected in the ES group (8.32 ± 3.68 vs. 10.51 ± 4.89 , $p=0.01$) despite similar AMH ($20.59 \text{ pmol/l} \pm 15.42$ vs. $20.75 \text{ pmol/l} \pm 16.91$, $p=0.96$) compared to the no intervention group. There was similar quality of embryos between the 2 groups

(4.04 ± 3.74 vs. 3.03 ± 2.29 , $p=0.10$). There was no difference between clinical pregnancy rates on USS between the no intervention and ES groups (42.86% vs. 35.8%, $p=0.54$).

4.5 Discussion

The results of this study would indicate that performing an endometrial scratch by inducing injury to the endometrium in the preceding menstrual cycle had no effect on pregnancy rates in the general population of women undergoing IVF. Subgroup analysis demonstrated that there was no significant improvement in clinical pregnancy rates in women undergoing their first IVF cycle and women having 3 or more cycles of IVF, but a significantly lower clinical pregnancy rate was detected in those women undergoing their second cycle of IVF.

It is thought that endometrial scratching induces inflammation to the endometrium aiding implantation. This inflammatory effect aids in the up regulation of cytokines, chemokine and growth factors inducing decidualisation and increasing receptivity of the endometrium creating a more favorable environment for implantation (Dunn et al. 2003; Kelly et al. 2001; Yoshinaga 2008). Kalma et al. (2009) demonstrated that endometrial scratching induced an increase in the expression of 183 genes related to endometrial receptivity. Almog et al. (2010) hypothesized that local injury increases endometrial receptivity by modulating the expression of a variety of genes.

There are few studies, which take an unselected group of patients and perform an endometrial scratch. We observed the effects of endometrial scratching on 301 women, who accepted the offer of an endometrial scratch throughout 2014 and compared them to 321 patients having fresh cycles in 2013 having no endometrial scratch. There was no difference in patient demographics and numbers of previous IVF cycles. There was no significant difference in clinical pregnancy rates in those women who had endometrial scratching (34.9% vs. 41.43%, $p=0.11$), despite 30

more clinical pregnancies in the no scratching group. Yeung et al. (2014) published similar findings, in an unselected population who demonstrated that endometrial scratching did not result in significant improvement in clinical pregnancy rates ($p=0.55$). Nastri et al. (2013) randomised 158 unselected women and demonstrated significantly greater clinical pregnancies within the endometrial scratch group compared to controls (49.4% vs. 29.1%, $P = 0.01$). Interestingly this study like ours reported no difference in endometrial thickness between the two groups in this unselected population. However, despite powering the study to 324 to allow for exclusions the study, was stopped after interim analysis, which showed a significant benefit of the intervention. This leads to an underpowered study with interim data that may have overestimated the treatment effect resulting in bias.

4.5.1 Mock-ET vs. Mock-ET and Endometrial Scratching

Subgroups analysis was performed within this cohort to help answer other questions that studies had yet to answer. This is the first study to compare Mock-ET and endometrial scratching in women undergoing their first IVF cycle. In 2013 women undergoing a first cycle of IVF had a Mock-ET only, designed to measure the uterine cavity. It appeared to cause sufficient injury and/or stimulation to aid the process of implantation and that an endometrial scratching added no further benefit in clinical pregnancy rates, 41.08% (Mock-ET) vs. 38.5% (Mock-ET and Scratch), $p=0.69$. There was no difference in demographic characteristics between the Mock-ET only and Mock-ET and Endometrial scratching groups.

The most interesting and important finding was that those patients who had undergone an endometrial scratching following their Mock-ET had significantly thinner endometrium compared to the those patients who only had a Mock-ET (10.18 ± 3.08 vs. 10.78 ± 2.12 , $p=0.04$). No previous studies have published findings to state that an endometrial scratching can have a negative effect on endometrial growth. It is known that thinner endometrium $<7\text{mm}$ can itself had a negative effect on implantation success. (Casper 2011).

Yeung et al. (2014) performed a sub-group analysis of women undergoing 1st cycles of IVF, they included 209 (69.7%) subjects undergoing their first cycle of IVF and there was no significant difference in pregnancy rates ($p=0.67$), between women with or without an endometrial scratch. Our study included 359 patients undergoing their first cycle of IVF (185 Mock-ET only vs. 174 Mock-ET and scratch, $P=0.65$). Despite our study being observational, Yeung et al. (2014) stated their study was adequately powered with 300 patients. It is felt there are adequate numbers of patients within this observational study to clearly state and agree with those of Yeung et al. (2014), that endometrial scratching has no added benefit to those patients undergoing their first cycle of IVF and in this case may even have detrimental effect on the development of the endometrium.

Karimzade et al. (2010) performed a RCT of 156 women, comparing scratching on the day of OR with no intervention in women undergoing their first cycle of IVF. The clinical pregnancy rate was significantly lower in the endometrial scratching group ($p<0.05$). The author's observed endometrial scratching had a negative effect on clinical and ongoing pregnancy rates, possibly by affecting the environment within the uterus to allow implantation to take place. This study demonstrates that endometrial scratching in the same cycle as an embryo transfer is detrimental to patients undergoing their IVF.

Previous studies have reported that endometrial scratching improved clinical pregnancy and/or live birth rates (Narvekar et al. 2009; Karimzadeh et al. 2009). Which in some studies was as much as a 2-fold increase (Barash et al (2003); Raziel et al. 2007; Zhou et al. 2008). However, these studies tended to include patients who had previously had only one failed cycle of IVF whilst others such as Karimzadeh et al. (2009) included those with 2 or more failed cycles of IVF. This study performed further sub-group analysis on patients undergoing second cycles of IVF and 3 or more cycles of IVF in order to compare these findings with the published literature.

4.5.2 Second cycle of IVF/ICSI: Endometrial Scratching vs. no uterine intervention

74 patients undergoing their 2nd cycle of IVF had an endometrial scratching and this was compared to 87 in 2013 ($p=0.52$). There were no differences in subject's age ($p=0.75$), BMI ($p=0.44$), AMH ($p=0.58$), endometrial thickness at OR ($p=0.41$), and quality of eggs ($p=0.43$). However the endometrial scratching group had significantly fewer clinical pregnancies (24.3% vs. 40.22%, $p=0.04$) compared to the group with no intervention.

The results highlight that in this group of patients endometrial scratching is of no added benefit and may in fact be detrimental to their chances of achieving a clinical pregnancy. It is important to remember that having one failed cycle does not fit the criteria of recurrent implantation failure. Previous studies have included patients into their studies with only one failed cycle, and have demonstrated significant improvements in clinical pregnancy rates (Barash et al. 2003; Navekar et al. 2009). However the protocols differed greatly, both in terms of the number of biopsies performed, timing of biopsies and patients demographics, so it is difficult to make any direct comparison.

Yeung et al. (2014) subgroup analysis agreed with this study that in those women with repeated failed cycles of IVF the endometrial scratching group demonstrated reduced ongoing pregnancy rates. However the numbers in this study were small, with only 81 patients having undergone repeated cycles of IVF. With a pregnancy rate of 22.5% in the scratching group and 43.9% in the non-scratching group $p=0.05$. In this present study, of 161 patients undergoing second cycles, a much stronger argument that endometrial scratching is of no benefit and can be detrimental in this sub-group of patients.

It is known that approximately 70% of IVF cycles will be unsuccessful, but in this particular subgroup there may have been other reasons why implantation has not been successful. Thus, the decision to offer an invasive treatment such as an

endometrial scratching, maybe unnecessary in this group of women. In today's environment in which patients want to try anything that may improve their chances of taking a baby home, clinicians have to be very selective in what treatments are offered to provide a safe and evidence-based approach.

4.5.3 Three or more cycles of IVF/ICSI: Endometrial Scratching vs. no uterine intervention

This specific sub-group is key to demonstrating whether endometrial-scratching is of benefit in those patients with recurrent failed cycles of IVF, i.e. three or more failed transferred cycles. This study included 102 patients (53 ES, 49 no treatment). This represented 16.4% of our total cohort. The numbers of patients in this cohort was greater than previous studies by Barash et al. (2003), Raziel et al. (2007), Zhou et al. (2008) (n= 45, n=63 and n=60 respectively) and equivocal to other RCTs by Narveker et al. (2009) n=100 and Karimzadeh et al. (2009) n=115.

In our study there was no difference in age and BMI between the groups. There was no difference in markers of ovarian reserve (AMH, AFC) and endometrial thickness was similar between each group. There was however significantly fewer eggs collected in the endometrial scratching group (8.32 ± 3.68 vs. 10.51 ± 4.89 , $p=0.01$) despite similar AMH (20.59 ± 15.42 vs. 20.75 ± 16.91 , $p=0.96$) compared to the no intervention group. There was similar number of good quality embryos between the 2 groups (4.04 ± 3.74 vs. 3.03 ± 2.29 , $p=0.10$). There was no difference between clinical pregnancy rates between the no intervention and ES groups (42.86% vs. 35.8%, $p=0.544$).

These results demonstrate that endometrial-scratching adds no further benefit to those patients with recurrent implantation failure trying to achieve a successful pregnancy. The numbers in this study are comparable to those in the literature. The results would agree with recent studies by Baum et al. (2012) and Dain et al. (2014)

that endometrial scratching performed in the luteal phase the cycle before IVF, has no effect on clinical pregnancy rates.

In this study, in contrast to the previous studies that stated endometrial scratching had significant improvement in pregnancy rates, several important factors need to be taken into account. In several of the previous studies the numbers of embryos transferred varied greatly with the majority of them transferring 3 or more embryos. For example in the studies of Raziel et al. (2007) and Navekar et al. (2010) the numbers of embryo transferred were 3.3 ± 0.9 and 3.18 ± 0.5 respectively. In contrast one study followed a single embryo transfer policy to reduce the risk of high parity pregnancies. There was a significant difference in the numbers of embryos transferred between the no intervention and endometrial scratching group with the later receiving significantly more embryos (1.33 ± 0.746 vs. 1.55 ± 0.5 , $p=0.02$). However, despite this there was no affect on clinical pregnancy rates. Yeung et al. (2014) despite having only 27 subjects, having ≥ 3 previous cycles they again transferred no more than 2 embryos maximum and there was no difference in clinical pregnancy rates, though the authors stated the numbers were too small to draw any reliable conclusions.

The next issue to discuss is the number of endometrial biopsies and timing of the procedure. Is this the critical step necessary to achieve implantation and subsequently a clinical pregnancy? Karimzade et al. (2010) is the only RCT to report that performing endometrial scratching at the time of OR, during the transfer cycle, has a negative effect on implantation most likely by disrupting the receptive endometrium. Gnainsky et al. (2010) discovered elevated levels of pro-inflammatory cytokines, $\text{TNF}\alpha$, growth regulated oncogene- α and macrophage inflammatory protein 1B (MIP-1B) in a day 21 biopsy and demonstrated a positive correlation between the levels of these cells and pregnancy outcome.

Barash et al. (2003) performed biopsies on days 8,12,21, and 26 of the cycle prior to treatment on 134 subjects. Raziel et al. (2007) performed the biopsies on days 14 and 19 on 120 subjects. Both groups demonstrated significant improvement in clinical pregnancy rates, twofold by Barash et al. (2003) and 30% vs. 12% by Raziel et al. (2007). However these were not RCT's and high numbers of embryos were transferred. A recent RCT by Baum et al. (2012) performed the scratching on days 9-12 and 21-24 on the cycle prior to commencing treatment and found no improvement in clinical pregnancy rates between the scratching and no treatment groups. This study contained patients with high numbers of failed cycles (8.5 scratching group and 8.8 in the no intervention group) making it of much better quality.

The recent RCTs by Karimzadeh et al. (2009) and Navekar et al. (2010) who only performed one endometrial scratch during the luteal phase demonstrated significantly higher pregnancy rates for the endometrial scratching group. However the patient groups had 2.5 and 2.3 mean number of failed cycles respectively and high numbers of embryos were transferred. Karimzadeh et al. (2009) lost 9 patients to follow-up, and excluded 13 patients due to poor embryo quality, thus affecting true patient representation in the study and causing bias. These results demonstrate that there is no consensus on optimal timing and number of procedures, but from the literature at this present time the studies differ greatly in so many parameters that no firm conclusion can be made.

Systematic reviews and meta-analysis cite the benefit of endometrial scratching the cycle prior to commencing a fresh IVF cycle (El-Toukhy et al. 2012; Nastri et al., 2012, Potdar et al. 2012). Yet it is important to note that one of these meta-analysis contained only 4 randomised control trials (Nastri et al. 2012) another only 2 RCT's (Potdar et al. 2012), both of which were included in the meta-analysis by El-Toukhy et al. (2012) who also included another 6 non-randomised or unpublished studies. The reviews mainly based their results on the RCTs by Karimzadeh et al. (2009) and

Navekar et al. (2010). From the literature review it is clear there were severe methodological differences in the studies by Karimzadeh et al. (2009) and Navekar et al. (2010) including differing numbers of embryos replaced and the inclusion of patients who did not have recurrent implantation failure. Podkar et al. (2012) included hysteroscopic scratching, but in view of the results of the TROPHY trial, this systemic review is of no value as it has been proved hysteroscopy has no effect on clinical pregnancy rates (El-Toukhy et al. ESHRE 2014). Thus these systematic reviews, despite their claims, add little further support to the intervention due to the inclusion of poorly controlled and, in some cases, unpublished work.

4.6 Conclusion

This study demonstrates that an endometrial scratching using a pipelle is of no added benefit compared to a Mock-ET in those women undergoing their first cycle or as an independent procedure in 2nd and subsequent cycles. This is in keeping with the recent TROPHY trial that demonstrated that hysteroscopy during the cycle prior to commencing IVF treatment added no benefit (El-Toukhy et al. ESHRE 2014).

The endometrium is a unique tissue that has the ability to regenerate every month to allow the possible implantation of an embryo. There are many studies that have demonstrated that there is very limited time during which implantation can occur which has been labeled the “window of implantation.” The seminal paper by Gnainsky et al. (2010) that demonstrated that an inflammatory response in the subsequent cycle resulting in increased pregnancy rates, was a small study and has not been repeated on a large scale involving different clinics.

It is clear that the results of this observational study highlight that they need to be validated in a large multicentre trial controlling for variables such as cycle number, numbers of embryos transferred etc, not terminated early because of interim analysis (Nastri et al. 2013). It is important to remember that no interventional procedure is not without risk. In this study no complications such as excessive

bleeding, acute pelvic infection or uterine perforation occurred. However many women complained that the procedure was very painful and on occasions it did require cervical dilation that itself is an uncomfortable procedure.

The finding that ES could even have a detrimental effect was highlighted within two of our sub-groups. In those women undergoing their first cycles of IVF, the ES group developed significantly thinner endometrium compared to the Mock-ET only group. This procedure may have inadvertently either affected the physiology of the endometrium, resulting in this change, or created such an inflammatory affect that the endometrium has become toxic to the embryo.

This theory could be applied to women undergoing their 2nd cycle of IVF, in which women having an endometrial scratch had clinical pregnancy rates significantly less than no intervention. There is the potential that scratching has affected the implantation potential of the endometrium. For those women in whom the procedure was designed to aid implantation no effect was observed. This demonstrates that our knowledge of the process of implantation remains limited, and physiological studies on the endometrium are needed prior to performing a procedure which at best has no added benefit but may in fact harm a woman's chances of achieving a pregnancy. Benyamini et al. (2005) stated, "Infertility is a complex disorder with significant medical, psychological and economic aspects." Its potential should not be complicated by giving patients false hopes by providing procedures with little scientific background, which the patient may be required to pay for.

In conclusion our results demonstrate that an endometrial scratch is of **no added** benefit in women undergoing assisted reproduction. The Mock-ET provides information regarding the ease/difficulty of transfer and is unlikely to cause any major disruption to the uterine cavity, such as hysteroscopy. Despite an endometrial scratching being a relatively quick and easy procedure to perform there is no evidence from this study to show it improves clinical pregnancy rate.

CHAPTER FIVE:

Intralipid and IVF “Does Intralipid infusions during an IVF cycle affect the population of Natural killer cells in women with recurrent implantation failure.”

5.1 Abstract

Background: Studies have demonstrated that women with recurrent implantation failure (RIF) may have an underlying immunological response affecting the implantation process. These women appear to have elevated numbers of peripheral Natural Killer (NK) cells that are cytotoxic to the implanting embryo. However there is some evidence to suggest that intralipid, composed of non-esterified fatty acids, can dampen this effect. The objective of this study was to investigate the effect of intralipid on the NK-cell population of women with RIF compared to women undergoing their first cycle of IVF.

Method: Women with RIF (≥ 2 cycles of IVF or ≥ 3 miscarriages) received intralipid during the stimulation phase, at the time of embryo transfer and after a positive pregnancy test. Bloods were taken for NK-cells before each infusion. Controls had blood samples for NK-cells at the same time in the IVF cycle the study subjects were receiving intralipid.

Results: 14 women participated, 10 controls and 4 receiving intralipid. There was no difference in patient demographics between each group. The control group had significantly higher peripheral NK-cell count prior to commencing the IVF cycle. The intralipid infusions had no effect in reducing peripheral NK-cell count in those women with recurrent implantation failure $p=0.86$. The control group demonstrated significant reduction in peripheral NK-cell count between the Mock-ET and the stimulation phase of the IVF cycle, $p=0.049$ however this did not meet

significance when measured from the time of Mock-ET to elicitation of a positive pregnancy test ($p = 0.05$).

Conclusion: Intralipid infusions had no effect on the peripheral NK-cell population in women with recurrent implantation failure undergoing IVF and should not be offered to women with RIF.

5.2 Introduction

The success rates of IVF has improved over time, implantation failure remains a major issue. One school of thought is that immunological factors including Natural Killer (NK) cells may play a role in infertility and implantation failure. The NK cells consist of peripheral NK-cells and uterine NK-cells. These cells express the surface antigen CD56, however the majority of uterine NK-cells are CD56⁺ and CD16⁻ and peripheral NK-cells are CD56⁺ and CD16⁺, making them both phenotypically and functionally different (Lash et al. 2010; Kwak et al. 1995).

Studies have shown that women with recurrent implantation failure (RIF) have significantly higher numbers of peripheral NK-cells compared to normal fertile controls (Kwak et al. 1995; Matsubayashi et al. 2001; Thum et al. 2008). These peripheral NK-cells have cytotoxic activity suggesting they could play a role in pregnancy failure. However uterine NK-cells are thought to have a possible protective effect. They are the most abundant leucocyte population in the endometrium at the time of implantation and early pregnancy (Lash et al. 2010). It is unclear whether this is due to different surface antigens, or they are different functional cells (see section 1.6.3).

Intralipid infusions have been shown to be effective in suppressing the number and activity of NK-cells, implicated in the pathogenesis of RIF (Roussev et al. 2007; Roussev et al. 2008; Coulam & Acacia 2012). Studies have shown that intralipid infusion may be able to have an effect on the immune system by suppressing pro-inflammatory mediators, namely NK-cells (see section 1.6.3) (Granato et al. 2000; Mayer et al. 2003).

As a consequence, in some centres intralipid infusions are routinely given to women who are undergoing IVF therapy where there have been recurrent implantation failures. However, there is little published work on the effect of intralipid on immune modulation using more up to date methodologies to

investigate this. We hypothesize that the mechanism of these intralipid infusions given routinely to help address implantation failure is due to modulation of NK-cells, that in turn may impact on endothelial function.

Study objective

A prospective study to investigate whether Intralipid infusions had any effect on NK-cells in women with RIF undergoing a fresh cycle of IVF compared to control women undergoing their first cycle of IVF with no treatment. Due to the small numbers of patients undergoing IVF with RIF it was felt that over the duration period of the study, insufficient subjects would be recruited, thus a control group of women undergoing their first cycle of IVF had been included to study the phased effect of an IVF cycle on NK cells.

5.3 Materials and Methods

5.3.1 Ethical Approval

Favorable ethical approval for the original version of the study was given on the 9th of January 2014. LREC reference 13/YH/0395, IRIS project ID 139126. Unfortunately just prior to recruiting patients the research and development (R&D) department wanted clarification by the Medical Health Regulatory Authority (MHRA) as to whether intralipid was an investigational medicine product. On the 26th of April following discussion with the MHRA their assessor felt it was a Clinical Trial of an Investigational Product (CTIMP) because the investigators were using intralipid as a therapeutic intervention. Leeds East ethical approval was given on 14th of August 2014, REC reference: 14/YH/1082, EndraCT number 2014-002842-30. Interestingly at the time of the meeting the panel felt that the study did not meet CTIMP approval in the outcome letter they stated, "The Committee disagreed with the MHRA and did not consider this application to be a CTIMP because it was using standard treatment."

Recruitment commenced in the October of 2014. The study was progressing well at this point. Unfortunately the President of the Royal College of Obstetricians and Gynaecologists (RCOG) sent a letter to all members of the RCOG regarding intralipid infusion as part of IVF treatment. Within the letter it discussed how Public Health England had become aware of three women whom had developed severe sepsis as a result of the infusion of 20% intralipid. It was believed to be as a result of poor practice in the administration leading to product contamination. It was felt by the research team that this would involve several changes to the study:

- 1) All patient information sheets and consent forms would have to be amended via REC and MHRA; to inform patients there was a risk of sepsis in light of this new information.
- 2) REC and MHRA themselves would have to be informed regarding this new information.

Due to this the study team made the decision to stop the study on grounds of patient safety.

5.3.2 Patient recruitment

i) Intralipid subjects were recruited from the Infertility clinics at Hull Royal Infirmary and the Hull IVF unit. To be deemed eligible for the Intralipid arm of the study the following criteria had to be met.

Inclusion Criteria: Age 20-45, BMI \leq 30, History of recurrent implantation failure (2 or more IVF cycles) and /or History of recurrent miscarriages (3 or more miscarriages)

Exclusion Criteria: Allergy/hypersensitivity to soya oil, allergy to eggs, allergies to peanuts/products Patients with diabetes, renal or liver insufficiency, acute or chronic infections. Patients with

systemic inflammatory diseases, age < 20, age > 45, known Immunological disease, uterine abnormality

Patients were given an information sheet about the study, and given as much time as they wished to consider participating in the study. If they decided to participate, they contacted the IVF unit and were consented by the study doctor at their baseline appointment, after having been given a further opportunity to ask questions prior to written consent.

ii) Control subjects consisted of patients undergoing their first cycle of IVF. They had baseline bloods like the intralipid group. At the point in the IVF cycle that the intervention group is receiving the intralipid, the control group had a blood sample for NK cells to allow comparison. They had to meet the following criteria prior to inclusion into the study.

Inclusion Criteria: Age 20-45, BMI \leq 30, 1st cycle of IVF

Exclusion Criteria: Allergy/hypersensitivity to soya oil, allergy to eggs, allergies to peanuts/products, Patients with diabetes, renal or liver insufficiency, acute or chronic infections. Patients with systemic inflammatory diseases, Age > 20, age > 45, known Immunological disease, uterine abnormality.

5.3.3 Study Protocol

i) Intralipid Group

Intralipid therapy procedure: Each patient will have three infusions of 100ml 20% Intralipid over 1 hour in the mid-stimulation phase of IVF, at the time of embryo transfer and if a pregnancy test was positive, as per normal clinical practice.

Visit 1 (Mock embryo transfer appointment)

- Obtaining consent for
 1. Baseline bloods and the additional blood samples at the beginning of the routine infusion,

2. Collect a sample of follicular fluid at ovum retrieval that is normally discarded once the egg has been retrieved.

- Mock endometrial transfer as per normal practice

Visit 2 (Mid stimulation phase)

- Cannulation of patient
- Blood samples will be taken for the studies of NK cells.
- Commencement of the Intralipid infusion, 20% (100 ml).
- Intralipid is given over one hour and observations for blood pressure, temperature and pulse are taken every 5 minutes for the first 15minutes and then every 15 minutes if the vitals are stable, until the infusion is over.
- The patient will be asked to wait/recover for 10-15 minutes after the infusion, as per normal practice. A final set of observations will be made before discharge.

Visit 3 (Ovum Retrieval)

- A sample of follicular fluid will be collected at ovum retrieval. This fluid is normally discarded once the egg has been retrieved.

Visit 4 (Embryo Transfer)

- Visit 4 procedure will be the same as visit 2 except that the patients will have their Intralipid infusion within 24 hours of embryo transfer.

Visit 5 (Following positive pregnancy test)

- Visit 5 will be the same as visit 2.

Table 5.1 Schedule of visits for intralipid group

	Visit 1 Mock -ET	Visit 2 Stimulation Phase	Visit 3 Ovum retrieval	Visit 4 Embryo Transfer	Visit 5 ±ve pregnancy test
Informed Consent	<u>√</u>				
Medical History	<u>√</u>				
Body mass Index	<u>√</u>				
Blood Sampling	<u>√</u>				
Intralipid Infusion		<u>√</u>		<u>√</u>	<u>√</u>
BP,Pulse, Temperature		<u>√</u>		<u>√</u>	<u>√</u>
Blood sample pre and post intralipid		<u>√</u>		<u>√</u>	<u>√</u>
Follicular Fluid Aspiration			<u>√</u>		

ii) Control Group

Each patient will have a blood sample taken at the same time in their IVF treatment cycle as the intralipid group, i.e. in the mid-stimulation phase of IVF, at the time of embryo transfer and if a positive pregnancy test.

Visit 1 (Mock embryo transfer appointment)

- Obtaining consent for
 1. Baseline bloods and the additional blood samples in the mid-stimulation phase of IVF, at the time of embryo transfer and after a positive pregnancy test.
 2. Collect a sample of follicular fluid at ovum retrieval that is normally discarded once the egg has been retrieved.
- Mock endometrial transfer as per normal practice

Visit 2 (Mid stimulation phase)

- Blood sample will be taken for NK-cells.

Visit 3 (Ovum Retrieval)

- A sample of follicular fluid will be collected at ovum retrieval that is normally discarded once the egg has been retrieved.

Visit 4 (Embryo Transfer)

- Visit 4 procedure will be the same as visit 2.

Visit 5 (Following positive pregnancy test)

- Visit 5 will be the same as visit 2.

Table 5.2. Schedule of visits for control group

	<u>Visit 1</u>	<u>Visit 2</u>	<u>Visit 3</u>	<u>Visit 4</u>	<u>Visit 5</u>
Informed Consent	<u>√</u>				
Medical History	<u>√</u>				
Body mass Index	<u>√</u>				
Blood Sampling	<u>√</u>	<u>√</u>		<u>√</u>	<u>√</u>
Follicular Fluid Aspiration			<u>√</u>		

5.3.4 Laboratory Methods

Blood Samples

1. Blood was collected into a BD Vacutainer CPT (Sodium Heparin) using standard venepuncture and mixed by 8-10 gentle inversions.
2. After collection the tube was stored upright at room temperature until centrifugation. All blood samples were centrifuged within 2 hours of collection.
3. The tube was centrifuged at 1500 Relative Centrifugal Force (RCF) for 20 min at 21°C.
4. After centrifugation, mononuclear cells appear in cloudy layer (white) immediately below the plasma layer (yellow). A pasteur pipette was placed directly into tube to aspirate the cloudy layer (See figure 5.1).

The tube was kept upright so that plasma layer was not disturbed or aspirated.

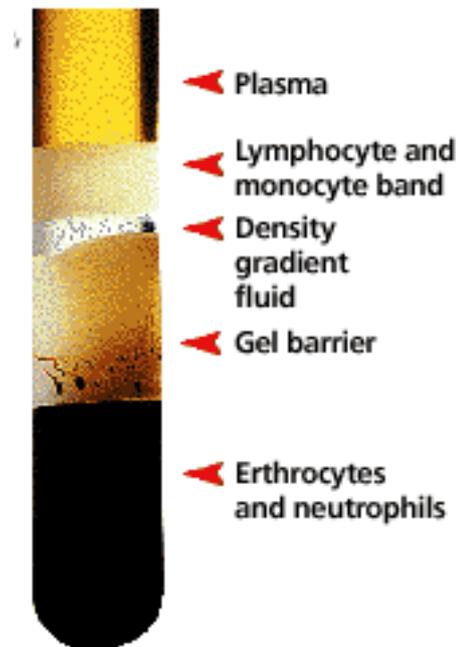


Figure 5.1 . BD Vacutainer[®] CPT[™] Cell Preparation Tube with Sodium Heparin. For the separation of Mononuclear cells from whole blood.

5. The cloudy layer was transferred to a fresh 15 mL Falcon tube, and 10 mL phosphate buffered saline (PBS) added, and then mixed by inversion.
6. The tube was then centrifuged at 400 RCF for 10 min at 21°C.
7. After centrifugation the PBS was poured off and the cell pellet was suspended in foetal calf serum/dimethyl sulfoxide (DMSO) (9/1 v/v), and frozen at -80°C overnight before transferring to liquid nitrogen for medium-term storage

Staining Protocol for Blood Samples

1. The samples were defrosted and PBS was added. The tube was then centrifuged at 400 RCF for 10 min at 21°C.
2. After centrifugation, the PBS was poured off and the pelleted cells re-suspended in 5 mL PBS/ bovine serum albumin (BSA).
3. Centrifuge tube at 400 RCF for 10 min at 21°C.

4. After centrifugation, the PBS/BSA was poured off and the pelleted cells re-suspended in 1 mL PBS/BSA.
5. 200 μ L of re-suspended cells was pipelleted into 5 appropriately labeled FACS tubes containing the following antibodies:

Table 5.3 Staining protocol for blood samples

Tube	CD3-APC	CD16-FITC	CD56-PE
Cells	N/A	N/A	N/A
CD3-	N/A	5 μ L	20 μ L
CD16-	20 μ L	N/A	20 μ L
CD56-	20 μ L	5 μ L	N/A
Sample	20 μ L	5 μ L	20 μ L

Cells:- this test-tube contains no antibodies and is analysed to check gating.

CD3-/CD16-/CD56-; are fluorescence minus one (FMO) controls and contain all but one of the antibodies.

Sample, contains all antibodies.

6. The re-suspended cells were mixed with antibodies by vortexing at low speed for 2-3 seconds and then incubated for 20 min at room temperature in the dark.
7. After incubation, 1 mL PBS/BSA was added to the tubes and centrifuged at 400 RCF for 10 min at 21°C.
8. After centrifugation, the PBS/BSA was poured off, and the cells re-suspend in 200 μ L PBS/BSA, and analyzed by flow cytometry.

Samples should be analysed using the NK Cells template on the BD Accuri C6 software. Appropriate compensation values have been applied to this template.

5.3.5 The IVF Cycle

All patients underwent a standard IVF antagonist protocol. The patients commenced their rFSH stimulation on day 2 of their menstrual cycle using either Merional (Pharmasure) or Gonal-F (Merck Serono). A GnRH antagonist (Cetrotide: Merck Serono) was used to prevent a premature LH surge.

The patients underwent transvaginal ultrasound scans from day 7 to observe the ovarian response to stimulation, and were repeated every 48 hours. The scans were used to observe response to stimulation and to determine when the patient was ready for ovum retrieval. Final maturation was triggered when two or more leading follicles were $\geq 18\text{mm}$ using either 5000-10000 IU human chorionic gonadotrophin (hCG, Pregnyl (Merck Sharp and Dohme) or 0.5mg Buserelin (Sanofi-Aventis, Frankfurt, Germany).

Oocyte retrieval was performed 36 hours later. This was carried out under sedation, the follicles were aspirated under ultrasound guidance. Luteal support was provided with micronised progesterone (Uterogestan; Besins Iscovesco Laboratories, Paris, France) at a dose of 600mg each night commencing on the day of oocyte retrieval. The embryologists depending on whether the patient was undergoing IVF or ICSI, inseminated the oocytes directly or the oocytes were stripped and had sperm injected 4 hours after retrieval.

The Hull IVF unit followed a single embryo transfer policy to reduce the risk of multiple births. Embryo transfers were performed on day 3 or ideally at day 5 (blastocyst) to give the best chance for implantation. The embryos were classified using standard criteria (Cutting et al. 2008).

A pregnancy test was performed 14 days following oocyte retrieval. If this was positive the patient would continue progesterone (Uterogestan; Besins Iscovesco Laboratories, Paris, France) for luteal support. 21 days after a positive pregnancy

test all women would have an USS to ensure location, viability, and whether a single or multiple pregnancy. Clinical pregnancy was defined as a fetal heartbeat on a trans-vaginal ultrasound between cycle days 42 and 49.

5.3.6 Data analysis and Statistics

Statistical analysis was performed using SPSS (v22, Chicago, Illinois). Descriptive data has been presented as mean \pm SD for continuous data and n (%) for categorical data. T-tests or Mann Whitney tests were used to compare means, as the data was not normally distributed. A p-value of <0.05 was considered to indicate statistical significance

5.4 Results

10 women were recruited as controls and 4 women underwent intralipid infusions. There was no significant difference in age, BMI and AMH when we compared the two groups prior to commencing the IVF cycle (Table 5.4). There was no difference in the days of stimulation between groups and other IVF parameters such as the endometrial thickness, numbers of eggs recovered and embryo quality. Four (40%) of the controls and 2(50%) of the intralipid patients achieved a clinical pregnancy but the numbers are too small to be of any clinical significance. None of the women developed any adverse events including wound site infections or sepsis.

Table 5.4 Descriptive demographics for intralipid and control group. Mean and SD

	Intralipid treatment (n=4) Mean±S.D	Control (n= 10) Mean±S.D	p- value
Age	31.50 ± 1.73	31.90 ± 3.57	0.83
BMI (kg/m ²)	25.10 ± 1.30	26.19 ± 2.98	0.50
AMH (pmol/l)	36.75 ± 13.03	27.80 ± 12.20	0.25
Days of Stimulation	10.50 ± 1.00	11.90 ± 1.66	0.15
Endometrium (mm)	10.09 ± 1.39	10.42 ± 2.21	0.79
Follicles Aspirated	16.50 ± 5.07	9.89 ± 6.62	0.10
Eggs retrieved	12.75 ± 4.92	7.33 ± 4.53	0.07
Cleavage rate	8.15 ± 3.59	5.43 ± 2.82	0.17
G3D3	6.25 ± 2.50	4.57 ± 2.82	0.33
Fetal heart beat	2 (50%)	4 (40.0%)	

The control group had a significantly higher mean average NK-cell count compared to the intralipid group at the Mock-ET appointment, the menstrual cycle prior to commencing IVF, $p=0.005$ (Table 5.5). However as the patients progressed through their IVF cycles there was no significant difference between NK cell counts between either group at the time of ET and pregnancy test however the numbers of women were small 4 and 2 for the control and intralipid groups respectively.

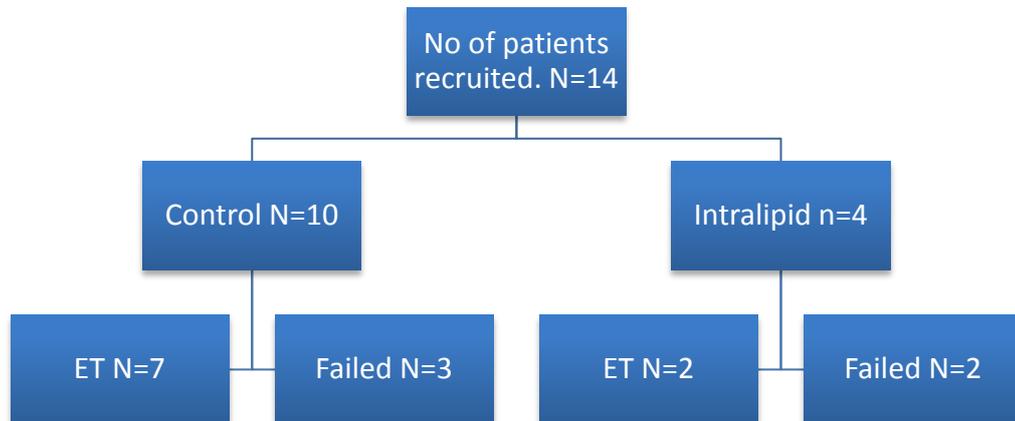


Figure 5.2: Flow chart demonstrating the breakdown of women recruited and treatment

Table 5.5 Mean CD56⁺CD16⁺ NK cell count intralipid and control.

	Intralipid	Control	p-value
	Mean (SD)	Mean (SD)	
Mock-ET appointment	26.16 (14.75)	51.45 (11.37)	0.005*
Embryo transfer	23.86 (18.03)	38.86 (13.47)	0.12
PDT	24.00 (8.04)	33.97 (14.75)	0.49

* P < 0.05 Indicates significant difference

Tables 5.6, 5.7, and 5.8 demonstrate the CD56⁺ NK-cells during the IVF cycle from each group. Within the intralipid group there was no significant decrease in the peripheral NK-cell (CD56⁺CD16⁺) population whilst this group of women went through their IVF cycle. However in the control group there was a significant

reduction in the CD56⁺CD16⁺ population between the Mock-ET appointment and the time of embryo transfer $p=0.049$. Between embryo transfer and positive pregnancy test there was no further significant reduction in CD56⁺CD16⁺ cell numbers, $p=0.59$, in the control group. When we look at the CD56⁺CD16⁺ population in the controls from the time of Mock-ET to positive pregnancy test, despite there being a fall in this cell population overall, it does not quite make significance, $p=0.054$.

Table 5.6 Mean \pm (S.D.) of CD56+ cells at Mock-ET and ET for each group.

No of Patients	Intralipid			Control		
	4	2		10	7	
	Mock-ET	ET	P-value	Mock-ET	ET	P value
(CD56+CD16-)	3.87 (1.74)	2.00 (0.96)	0.31	6.10 (3.59)	5.01 (3.35)	0.57
(CD56+CD16+)	26.16 (14.75)	23.86 (18.03)	0.85	51.45 (11.37)	38.86 (13.47)	0.049 [*]

* P < 0.05 Indicates significant difference

Table 5.7 Mean \pm (S.D.) of CD56+ cells at ET and PDT for each group.

No of Patients	Intralipid			Control		
	4	2		7	4	
	ET	PDT	P-value	ET	PDT	P-value
(CD56+CD16-)	2.00 (0.96)	2.02 (1.02)	0.98	5.01 (3.35)	6.73 (3.34)	0.43
(CD56+CD16+)	23.86 (18.03)	24.00 (8.04)	0.99	38.86 (13.47)	33.97 (14.75)	0.59

Table 5.8 Mean \pm (S.D.) of CD56+ cells at Mock-ET and PDT for each group.

	Intralipid			Control		
No of Patients	4	2		10	4	
	Mock-ET	PDT	P-value	Mock-ET	PDT	P-value
(CD56+CD16-)	3.87 (1.74)	2.02 (1.02)	0.25	6.10 (3.59)	6.73 (3.34)	0.78
(CD56+CD16+)	26.16 (14.75)	24.00 (8.04)	0.86	51.45 (11.37)	33.97 (14.75)	0.054

5.5 Discussion

From the present study it is difficult to make any firm conclusions due to the small numbers of patients involved. However we noted a number of things within this small patient group. Interestingly our control population had a higher level of CD56⁺CD16⁺ peripheral NK-cells compared to the intralipid group. Despite studies by Coulam et al. (1995) and Kwak et al. (1995) on women with RIF demonstrating significantly higher levels of NK-cell activity and Matsubayashi et al. (2001) who examined the NK-cell number of 94 infertile women with significantly higher NK-cell activity of the infertile women compared to controls (40.2% \pm 14.7 vs. 31.5% \pm 11.9, P<0.0001). This was not associated with age and infertile duration.

Secondly the intralipid infusion had no significant effect on reducing the CD56⁺CD16⁺ cell population in our subjects with RIF. This would agree with Almond et al. (1985) who investigated the impact on intralipid on NK-cells directly and concluded that intravenous infusion of 20% intralipid had no effect on NK-cell activity within the conditions of their study. Rousev et al. (2007) reported that intralipid along with intravenous immunoglobulin (IVIg) and soluble human leukocyte antigen-G suppressed NK-cell activity. A small study by Ndukwe et al. (2011) also suggested that intralipid may improve implantation rates however this was a very small uncontrolled study. The mechanisms behind this proposed

immunomodulatory role of Intralipid is yet to be elucidated by immunologists/haematologists, before it has even been considered to be used in patients with RIF.

5.5.1 Lipid and follicular fluid

It is unclear when in the developmental stage that lipid accumulation takes place but McKeegan and Sturmeay. (2012) suggest the concentration of lipids within an oocyte is ever changing and is dependent on its environment. Whilst the precise mechanism of the formation of the fluid in an ovarian follicle is not known, it is widely accepted that in general terms, the chemical profile of the follicular fluid reflects the plasma (Rogers and Irvine-Rogers 2010). This is true for lipid compounds. Valckx et al. (2012) measured the metabolic composition of follicular fluid in women undergoing ART, and found that the levels of cholesterol, HDL cholesterol, non-esterified fatty acids (NEFA) and apolipoprotein A1 present in the follicle were directly correlated to plasma concentrations with similar results by Robker et al. (2009), who reported, notably, that levels of triglycerides were elevated in the follicular fluid of overweight women. Valckx et al. (2012) could not find a significant association between fertility treatment outcome and NEFA concentrations. Elevated NEFAs (Leroy et al. 2005; Jungheim et al. 2011; Van Hoeck et al. 2011) have been linked to impaired cumulus oocyte complex morphology and oocyte developmental competence.

Yang et al. (2012) exposed mouse oocytes to follicular fluids of differing lipid content from women attending IVF. They found that the hyperlipidaemia of obesity correlated directly with the environment of the ovarian follicle i.e. increasing BMI increasing NEFA levels in the follicular fluid. The mouse cumulus oocyte complex maturation in the high lipid follicular fluid resulted in enhanced lipid accumulation and expression of lipid droplet protein. This resulted in the release of endoplasmic stress markers and impaired maturation. This would agree with human studies (Jungheim et al. 2011) that the NEFA content of the follicular fluid is associated with

poor cumulus oocyte complex morphology and that the treatment of bovine cumulus oocyte complex with increasing doses of specific fatty acids impairs oocyte maturation and embryo development (Van Hoeck et al. 2011; Leroy et al. 2005).

There is a growing body of literature that has considered the impact of fatty acids on fertility and the effect of health and viability of the embryo, both during early development and the consequences for the health of the offspring (Leroy et al. 2012 and McKeegan & Sturmey 2012). This is important as during pre-implantation development, the early embryo activates its genome and undergoes major epigenetic remodeling, during which time, epigenetic marks, such as base methylation and histone methylation/acetylation are permanently imparted to the cells of the embryo. As a consequence, any intervention that potentially alters metabolic activity of early embryos in the periconceptual period could have a profound impact on later development and persist in the resultant offspring. Specifically, an infusion of intralipid provides a source of “essential fatty acids”. The detailed composition of the lipid fraction of the product suggests that soybean oil contains 10% palmitic acid, 5% stearic acid and 45% oleic acid with the remainder largely comprising linolenic acid and linoleic acid (Carpenter et al. 1975).

5.5.2 Intralipid effects on the developing oocyte and embryo.

Yang et al. (2012) reported that lipid-rich follicular fluid is associated with reduced oocyte maturation. In particular, they showed that oocytes exposed to lipid-enriched follicular fluid had elevated levels of endoplasmic reticulum stress. Data from animal models have shown that, when cultured in the presence of high fat, oocytes and embryos increase their intracellular triglyceride (Sturmey et al. 2009, McKeegan and Sturmey 2012); changes that persist into the embryo (Ferguson and Leese 2006). Bols et al. (2013) recently showed that when bovine oocytes are exposed to an environment containing high, but physiologically relevant levels of non-esterified fatty acids (NEFA), oleic acid, stearic acid and palmitic acid (those that are most abundant in intralipid), subsequent embryo viability is profoundly

reduced. Furthermore, exposure to high NEFA during oocyte maturation leads to changes in the expression of a number of key genes and dramatically modifies the metabolic activity of the resulting blastocysts particularly with respect to glucose (Van Hoeck et al. 2013). This is important, since a defining characteristic of mammalian embryo development is a sudden, sharp increase in the consumption of glucose as they reach the blastocyst stage. This is the case for blastocysts of cows, pigs, sheep, mice and human and seems to represent a conserved metabolic strategy (Smith and Sturmey 2013). However, cattle blastocysts produced from oocytes exposed to high NEFA during maturation, have a significantly diminished capacity for glucose consumption (Van Hoeck et al. 2011).

Thus to finish there are important implications to consider when making the decision to commence intralipid on a women with recurrent implantation failure for a possible dampening effect on the immune system to enable implantation despite little good quality research to offer this treatment. There is a large body of data that demonstrates that the plasma composition of lipid profiles directly influences the composition of follicular fluid and exposure of oocytes to elevated lipids, particularly oleic, palmitic and stearic acid that is present in intralipid, negatively impacts embryo viability and phenotype. Thus there are potentially lipotoxic effects of giving intralipid during the period of follicular recruitment within an IVF cycle. There is conflicting evidence that oral prednisolone may be given as a first line in women with recurrent implantation failure. In Fertility 2015 ACE/BFS conference, Professor Quenby a leading expert in natural killer cells, stated that diagnosing patients by their NK cell status could only be successful if appropriately designed RCTs were able to determine if treatments such as endometrial scratching, steroids, intralipid were successful.

5.6 Conclusion

In conclusion our results have highlighted that intralipid has **no effect** on NK-cells in women with recurrent implantation failure compared to those on there first cycle

of IVF. We cannot make firm conclusions due to the small numbers of women in this pilot study. However due to the evidence that giving high dose of oleic, palmitic and stearic acid at the time of follicle recruitment could have toxic effects on the oocyte, combined the fact that there has been little conclusive good quality research and recent cases of sepsis during administration of intralipid it would be best not to offer this treatment to women with recurrent implantation failure. The Human Fertility and Embryo Authority (HFEA) states: "There is little scientific evidence to show that these treatments are beneficial."

Chapter 6: General Conclusion

The IVF cycle has enabled scientists to study the effects of exogenous gonadotropins on women, in both the response of the growing follicle, the physiology of the rapidly dividing embryo and the interaction of the embryo and endometrium in the process of implantation.

The dominant hormones of the female reproductive system are oestrogen and progesterone that act on the ovaries and endometrium via paracrine and autocrine mechanisms and feedback through both positive and negative feedback to the pituitary affecting the release of FSH and LH. During the follicular phase of the menstrual cycle, FSH induces the proliferation of granulosa cells within the developing follicles of the ovary. LH induced androgen production from the ovarian thecal cells result in a dominant pre-ovulatory follicle. Throughout this time oestrogen enables growth and proliferation within the endometrium Progesterone dominates the luteal phase with less emphasis on growth but on the production of a decidualised endometrium, receptive for implantation.

Within this process is a complex interaction of growth factors acting, within the ovary and endometrium. Some of these growth factors include androgens and insulin that act on the primordial and early pre-antral follicles that are independent of the endocrine control. Other factors that may affect folliculogenesis include nutritional deficiency and man made endocrine disrupting chemicals.

A common endocrine condition of women namely PCOS allows scientists the opportunity to study in detail what possible effects nutritional deficiency and synthetic endocrine disrupting agents can have on this unique patient group. These women classically have menstrual irregularities, hyperandrogenaemia, and anovulation. They usually respond poorly to exogenous gonadotropins either by producing large quantities of immature eggs, with lower fertilization and

implantation rates or they fail to respond to stimulation. Pregnancies within this group result in higher miscarriage rates, increased fetal anomaly rates and growth-restricted babies.

Unfortunately clinical pregnancy rates remain low within assisted reproductive technologies such as IVF/ICSI and implantation appears to be one of the resounding problems. In this thesis, I examined the effects of some of the important environmental, physical and nutritional factors affecting in vitro fertilisation.

In the first study I looked into the effect of endocrine disrupting chemicals on IVF. The possibility that certain man persistent chemicals may have endocrine disrupting effects that could affect fertility has been a controversial area within the last few decades. These EDAs persist in the environment for decades, and can interfere with the normal endocrine balance responsible for the maintenance of homeostasis, and ultimately causing adverse effects to not only this organism but also its progeny. This has resulted in the ban of the use of certain chemicals such as pesticides in large-scale agriculture and chemicals such as organochlorides in the production of many consumer items. This prospective pilot was designed to observe whether common endocrine disrupting chemicals were present in the serum of a cohort of British women with and without PCOS undergoing IVF and to observe if any correlation was present between these EDAs, pregnancy rates and other independent variables such as endocrine, stimulation and embryological factors.

59 women were recruited to the study, of which 29 had PCOS and 30 without PCOS. Each woman participated in one IVF cycle. The levels of EDAs within the serum were measured by HPLC-MS/MS. The levels of EDAs in the serum were comparable in each group with only PFOS that exhibited a significant difference between the PCOS and control group. There was no correlation between the levels of contamination with the EDAs and pregnancy in either the PCOS and control groups.

Principal component analysis reorganized the EDAs into 5 common variables (OCP, PCB, PBDE, HBCDD, and Polyfluoralkyl). This demonstrated that if a subject had a high level of one congener within a group of chemicals they were more likely to have higher levels of other congeners within that group. Correlation following principal component analysis demonstrated no correlation with HBCDD and polyfluoralkyl congeners and assisted productive, embryological and endocrinological outcomes. OCPs had significant correlation with FAI, PCBs with cleavage rate and testosterone. PBDE had significant correlation with BMI, FAI and testosterone, but the relevance of this uncertain.

The results from chapter 2 support the findings (section 1.4.5 and 1.4.6) that EDA contamination is continuing to fall in the industrialised world due to banning of these persistent organo-pollutants. In this present study levels of the most congeners of PCBs, BDEs and p,p-DDE were significantly lower compared to the most recent within this patient group (Petro et al. 2014). The results demonstrate that the EDAs are present in such small quantities it is difficult to ascertain whether this could have any effect on the reproductive processes. The data regarding possible significant correlations between the EDAs, endocrine profile of the PCOS women and variables within the IVF cycle such as ovarian responsiveness and embryological response need to be interpreted with caution due to the small numbers of women recruited into this pilot study. However this study agrees with the literature regarding the presence of these EDAs within the serum, but there effect on endocrine and reproductive processes is difficult to ascertain.

The use of EDAs to assess within the serum is a good marker to assess long-term contamination status of an individual however its relevance within an IVF cycle remains unknown. A large multi-centre study would need to be performed to validate any findings in this small pilot study, to allow for all confounding factors such as age, occupation, ethnicity, geography and dietary status. We could also examine different bodily fluids such as follicular fluid, semen and adipose tissue

that may add further evidence as to possible endocrine disrupting effects within the IVF setting.

In the second study we looked into the relationship of vitamin D, which is mainly derived by exposure of skin to sunlight on IVF. It is well known that Vitamin D is lower in women with PCOS and obesity can affect the bioavailability of Vitamin D. A prospective pilot study was designed to observe the effect of Vitamin D on outcomes of an IVF cycle with exogenous gonadotrophins on women with and without PCOS.

Over a 6-month period 59 women were recruited into the study. Their Vitamin D levels were analysed using LC-MS/MS. The subject's ovarian reserve was measured using both AMH and antral follicle count, androgen status by free androgen status and insulin resistance using HOMA-IR. Throughout the IVF cycle follicles numbers, eggs retrieved, fertilization, cleavage rates were taken and these were correlated with the Vitamin D in both the PCOS and non-PCOS women.

The findings demonstrated that there was no significant difference in age and BMI between the 2 groups. Serum Vitamin D levels between the PCOS and non-PCOS subjects were non-significant. As expected the PCOS subjects had significantly higher markers of ovarian reserve androgen status than the non-PCOS subjects but there was no significant difference in insulin resistance as measured by HOMA-IR.

There were significantly higher fertilization and cleavage rates in the PCOS group, however there was no difference in embryo quality and clinical pregnancy rates between the 2 groups. The serum Vitamin D within the PCOS group demonstrated a significant positive correlation with fertilization rates that was not evident within the non-PCOS women. No correlation was demonstrated between the Vitamin D levels AMH, FAI and HOMA-IR in either the PCOS and non-PCOS women.

When sub-analysis was performed within this study, overweight PCOS women (BMI $\geq 25\text{kg/m}^2$) had significantly lower 25(OH)D levels compared to normal weight PCOS (BMI $\leq 25\text{kg/m}^2$) patients (43.48 ± 25.39 vs. 66.99 ± 24.74 ; $p=0.02$). This was effect not seen within the control group without PCOS ($p=0.35$).

These results support the hypothesis that Vitamin D may have a positive effect in women with PCOS. This is the first study to report an association with Vitamin D and fertilisation rates within this cohort of women that was not reciprocated in the non-PCOS controls. Regarding IR and FAI as discussed in sections 1.5.6 and 1.5.7, the literature remains conflicting regarding as to what effect Vitamin D has on these variables, usually because adjustments have not been made for BMI which is intrinsic to both androgen and insulin status in these women, however the women in this study were not obese. It is clear that there are benefits for Vitamin D as it is recommended for women with large BMI to take in pregnancy (CMACE/RCOG, 2010). Large clinical trials are warranted to assess the effect of Vitamin D status and reproductive outcomes in PCOS women.

The next study looked into the effects of endometrial scratching on in vitro fertilisation. Implantation remains the rate-limiting step in vitro fertilization with approximately 25-40% of embryos implanting successfully. Recent steps in assisted conception have greatly reduced the risks of OHSS and improvements in embryo culture media has greatly improved embryo development both in fresh and frozen cycles. However the secrets have yet to be unlocked in understanding in depth the process of implantation of the embryo into a receptive endometrium. Unfortunately some women fail to achieve implantation despite numerous IVF attempts resulting in the term recurrent implantation. It has been suggested that trauma to the endometrium in the preceding cycle can elicit an inflammatory response that may aid in the process of implantation in women with recurrent implantation failure. An observational study was designed to observe the

effectiveness of endometrial scratch on outcomes of IVF in an unselected group of women attempting IVF.

301 women had an endometrial scratch performed over a 1 calendar year period and the results of these cycles were compared to the preceding calendar year of 321 patients. All patients having fresh IVF/ICSI cycles were offered an endometrial scratch after the Mock-ET on their first IVF cycle or as an independent on second and subsequent cycles. These results were then compared to the preceding years results in which all the treatment cycle parameters were the same with the exception for an endometrial scratch. All descriptive parameters such as age, BMI, markers for ovarian reserve and all IVF cycle parameters such as follicles numbers, endometrial thickness at ovum retrieval, eggs retrieved, fertilization, cleavage rates and pregnancy rates were prospectively collected and compared against the preceding year.

There was no significant difference in patient demographics between the two groups for age, BMI, AMH, endometrial thickness and quality of embryo replaced. Endometrial scratching in the first cycle of IVF showed no significant difference in pregnancy rates compared to a mock-ET. Women who had an endometrial scratch during their second cycle of IVF had significantly lower clinical pregnancy rates compared to women without an endometrial scratch. For women with 3 or more cycles of IVF, the endometrial scratching offered no added benefit. There was also no effect on clinical pregnancy rates when logical regression analysis was performed, to compare for confounding variables such as age and BMI.

These findings demonstrate that endometrial scratching offers no benefit in improving pregnancy rates. This study reciprocates data from the most recent RCT (Yeung et al. 2014) in section 1.8.3, that endometrial scratch has no effect on first cycles of IVF, and is of no more benefit compared to a mock-ET used to assess the uterine cavity. Women commencing their second cycle of IVF resulted in

significantly lower clinical pregnancy rates however these results may require caution when being interpreted due to the sample size and being an observational study. The endometrial scratch resulted in no significant difference in pregnancy rates to women with recurrent implantation failure, which contraindicated the literature in section 1.8.3. However until a large randomized multicentre trial is performed these results may serve as caution against the widespread use of this invasive procedure.

In the final study I examined the immunological factors effecting IVF. It has been suggested that immunological causes may be an underlying factor for some women with recurrent implantation failure. It is thought that a heightened immunological reaction attacks the implanting embryo resulting in failure to implant. One theory is that NK-cells are cytotoxic to the implanting embryo. NK-cells are a type of granular lymphocyte and comprise approximately 15% of the lymphocytic population and in humans they usually express the surface markers CD16 and CD56. NK-cells have the ability to recognize stressed cells in the absence of antibodies and major histocompatibility complex, allowing for a much faster immune reaction. Although the possible relationship between NK-cells and reproductive failure is controversial as it has been used to guide immunological treatments (Rai et al. 2005) despite no clear consensus on what is an abnormal concentration of NK-cells. It has been suggested that intralipid has the ability to reduce NK-cell numbers, but the effects on clinical pregnancy rates is unknown.

4 women with recurrent implantation and 10 women undertaking their first cycle of IVF were recruited into the study. Intralipid infusions were given during the stimulation phase of the IVF cycle, within 24 hours of embryo transfer and if a positive pregnancy was achieved. NK-cells were separated from whole blood and stained. Flow cytometry was performed to calculate NK-cell concentration within the venous blood samples.

There was no difference in patient demographics between each group. The control group had significantly higher NK-cell count prior to commencing the IVF cycle. The intralipid infusions had no effect in reducing the NK-cell count in those women with recurrent implantation failure. Interestingly the control group demonstrated a significant reduction in NK-cell count between the Mock-ET visit and the stimulation phase of the IVF cycle, however this did not meet significance when NK-cells were measured from the time of Mock-ET to elicitation of a positive pregnancy test.

It is difficult to make any firm conclusions from Chapter 5, as the study was limited by a small sample size and closing the study prematurely due to advice released from the RCOG. However it appeared that intralipid had no effect on NK cells on those participants in this study and thus does not support the use of intralipid as a treatment modality in women with recurrent implantation. Despite some small studies stating that intralipid reduces NK-cell numbers in women with recurrent implantation failure (section 1.8.6) there is little clinical data to support its use. Whilst this study was being undertaken the RCOG with NHS England issued a statement advising against the use of intralipid due to the risk of sepsis. There is also evidence from animal studies that the non-esterified fatty acids that comprise intralipid may be lipotoxic to the developing ovum (Yang et al. 2012). Before any further studies are conducted into the effects of intralipid therapy on women with recurrent implantation failure, large trials are required to establish both, what is a high level of NK-cells within the population, and what exactly is the proposed immunomodulatory role of Intralipid, before it has even been considered to be used in patients with RIF.

In conclusion, this thesis was undertaken to generate answers to hypothesis as to what effect environmental, physical and nutritional factors may have on an IVF. Chapter 2 enabled further insight into contamination status of a population of women with and without PCOS with persistent organopollutants. We have demonstrated these chemicals appear to have little endocrine disrupting effect on

ovarian response, embryological and metabolic factors such as insulin resistance, hyperandrogenaemia in both PCOS and controls. This generates further hypothesis as to whether or not these chemicals have any significant effect on infertility and is there really an environmental aspect to the etiology of PCOS.

Chapter 3 has demonstrated a possible link between Vitamin D and fertilization rates in PCOS. The findings bear some consistency between Vitamin D levels and pregnancy rates and further emphasizes the importance of optimizing all modifiable variables in order to improve reproductive success. However this hypothesis needs to be explored further by studying this possible effect in a large trial however as previous studies have stipulated seasonal variation should not play a factor in fertilisation rates (Wunder et al. 2005).

Chapters 4 and 5 have demonstrated that adjunctive treatments offer no greater chances of achieving successful implantation. It is clear from the endometrial scratch pilot study that the treatment has no benefit and may even be detrimental against the chances of achieving successful implantation. This hypothesis requires a large multi-centred RCT to see if this procedure is of any benefit to a sub-population of women undergoing IVF. The intralipid pilot study generates multiple hypothesis, in particular what is a high level of NK-cells and is this dependent on age, ethnicity, BMI and what effect does intralipid have on the immune system.

6.1 Reflection

Overall my MD has been a very positive experience. Throughout this time I have exponentially heightened my clinical knowledge regarding subfertility and treating the subfertile couple. I have learnt invaluable skills on writing protocols, attending ethics committees and completing a research project.

On reflection I would have liked to have tackled a few of the studies differently. I think if the circumstances had allowed I would have performed a RCT for the endometrial scratch study to rule out the confounding factors that is experienced in an observational study and directly compared scratch and no scratch as opposed to scratch and Mock-ET vs Mock-ET as again this is another confounding variable.

The vitamin D study required a larger cohort and thus I should have tried to collect patients over a longer period of time, which could have excluded seasonal variability and had all PCOS subjects off metformin to reduce any confounding effects. I managed to recruit all PCOS patients over a 6-7 month period and again it dependent on what patients are attending the unit at any given period of time.

Unfortunately in the Intralipid study large periods of time were lost due to trying to achieve HMRA ethical approval and attending copious ethical committees. The 6-8 months that were lost resulted in the inability to recruit subjects and commence the study, unfortunately as the study got under way the RCOG released information against the use of Intralipid. I attempted to investigate the endometrium for NK cells however due to the large numbers of red blood cells I struggled to get clear levels of NK cells despite trying to strain these cells from the samples. I feel that I may have needed more laboratory experience in order to process these samples as the NK cells cumulate in the basal layer of the endometrium not in the secretory endometrium, thus the samples collected via the pipelle had all layers of the

endometrium resulting in a difficulty in getting a true representation combined by the red blood cells due to the trauma of the procedure.

Future Research

Endometrial scratch remains a topic of much debate within the IVF community, with several large multicentred RCT ongoing at present. Despite these clinical trials I feel there is much needed research on the underlying physiology on exactly what effect the endometrial scratch has on the endometrium and how exactly this improves the mechanism of implantation.

Secondly in which population if any should endometrial scratch be prescribed? A large multicentred trial needs to look at specific populations specifically, women undergoing a fresh cycle of IVF with embryo transfer, and women with recurrent unsuccessful fresh cycles of IVF. There is also the possibility to look at other populations that endometrial scratch may benefit, such as those women with unexplained infertility who are attempting to conceive naturally, women with recurrent miscarriage who are trying to conceive naturally, and women on ovulation induction protocols.

The Vitamin D study offers different opportunities. Vitamin D clearly offers many health benefits and research is pointing to obvious effects within the fertility setting. This current study has demonstrated an association with Vitamin D and fertilisation in PCOS women. A large cohort study could offer the opportunity to investigate the effects of Vitamin D on the differing phenotypes of PCOS, there would be the opportunity to perform sub-group analysis on the effects of BMI as this can affect the absorption of Vitamin D. There is no obvious effect of metformin on Vitamin D however it is important to omit this confounding variable on future studies.

Endocrine disrupting chemicals are present within the environment, however as demonstrated in this study the concentrations within the serum are incredibly low and continue to decline in view of legislation banning their use. Work is required to see what effect these chemicals have within the micro-environment within the developing follicle and do they affect the endocrine and paracrine processes of the androgen pathway. Studies have shown that supraphysiological doses of these chemicals have adverse effects, but what effects if any do the levels measured in this study have on the developing follicle.

I feel that intralipid has little proven effect on treating women with recurrent implantation failure as a result of possible underlying immunological cause. In the context of future research until there is clear understanding as to what effect intralipid has on NK cells in vivo, it should be omitted to patients in view of the risks of sepsis and immunosuppression.

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ABBREVIATIONS

AEPCOS	Androgen Excess and PCOS Society
AFC	Antral Follicle Count
AGEs	Advanced glycation end-products
AMH	Anti Mullerian Hormone
AMHR-II	AMH receptor II
AR	Androgen Receptor
ART	Assisted Reproductive Technology
ASRM	American Society For Reproductive Medicine
ATSDR	Agency for Toxic Substances and Disease Registry
BDE	Polybrominated diphenyl ether
BMI	Body Mass Index
BPA	Bisphenol A
BSA	Bovine Serum Albumin
CAM	Cell Adhesion Molecule
C18	18 Carbon
C19	19Carbon
C21	21 Carbon
CE	Collision Energy
CDC	Centers for Disease control and Prevention
CMACE	Centre for Maternal and Child Enquiries

COX 1	Cyclooxygenase 1
COX2	Cyclooxygenase 2
CTIMP	Clinical Trial of an Investigational Product
D2	Ergocalciferol
D3	Cholecalciferol
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DDE	Dichlorobischlorophenylethylene
DE	Declustering potential
DEHP	Di-(2-ethylhexyl) phthalate
DHEA	Dehydroepiandroestrone
DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DMSO	Dimethyl Sulfoxide
DSL	Diagnostic Systems Lab assay
E1	Oestrone
E2	Oestradiol
E3	Oestriol
EDA	Endocrine Disrupting Agent
ERα	Oestrogen Receptor Alpha
ERβ	Oestrogen Receptor Beta

ES	Endometrial Scratching
ESCF	European Scientific Committee on Food
ESHRE	European Society Of Human Reproduction And Endocrinology
ESI	Electrospray ionization
ET	Embryo Transfer
FAI	Free Androgen Index
FDA	Food Standards Agency
FET	Frozen Embryo Transfer
FF	Follicular Fluid
FSH	Follicle Stimulating Hormone
GDF-9	Growth Differentiation Factor 9
GNRH	Gonadotrophin Releasing Hormone
GTD	Gestational Diabetes
GTT	Glucose Tolerance Test
HCG	Human Chorionic Gonadotrophin
HEGF	Heparin-binding EGF
α-HBCDD	α -hexabromocyclododexane
β-HBCDD	β -hexabromocyclododexane
γ-HBCDD	γ -hexabromocyclododexane
HCB	hexachlorobenzene

γ-HCH	γ -hexachlorocyclohexane
α-HCH	α -hexachlorocyclohexane
β-HCH	β -hexachlorocyclohexane
HFEA	Human Fertilisation Embryo Authority
HOMA	Homeostasis Model Assessment
HOMA-IR	Homeostasis Model Assessment Insulin Resistance
HPLC-MS/MS	Liquid chromatography–mass spectrometry
ICSI	Intracytoplasmic Sperm Injection
IDI	Information Dependent Acquisition
IGF-1	Insulin Like Growth Factor-1
IGF-2	Insulin Like Growth Factor-2
IL-	Interleukin -
IFN-γ	interferon-gamma
IR	Insulin Resistance
IVF	In Vitro Fertilisation
IVIG	Intravenous Immunoglobulin
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LIF	Leukaemia Inhibiting Factor
LH	Luteinising Hormone
LOR	Limit of reporting
LREC	Local Research Ethics Committee

MEHP	Monoethylhexyl phthalate
MHC	Major Histocompatibility Complex
MHRA	Medical Health Regulatory Authority
MIP-1B	Macrophage Inflammatory Protein 1B
Mock-ET	Mock Embryo Transfer
MS	Mass Spectrometer
MUC1	Mucin 1
MRM	Multiple Reaction Monitoring
NEFA	Non-esterified fatty acids
NICE	National Institute Clinical Excellence
NK-Cell	Natural Killer Cell
non-CTIMP	non-Clinical Trial of an Investigational Product
NSAIDs	Non-Steroidal Anti-inflammatory Drugs
OCP	organochlorine pesticides
OHSS	Ovarian Hyperstimulation Syndrome
OPN	Osteopontin
P-A	Progesterone receptor A
P-B	Progesterone Receptor B
P13K	Phosphoinositide 3-kinase
PBDE	Polybrominated diphenyl ether
PBS	Phosphate Buffered Saline

PCB	Polychlorinated biphenyls
PCOS	Polycystic Ovarian Syndrome
PFBA	Perfluorobutanoic acid
PFPeA	Perfluoropentanoic acid
PFHxA	Perfluorohexanoic acid
PFHpA	Perfluoroheptanoic acid
PFOA	Perfluorooctanoic acid
PFNA	Perfluorononanoic acid
PFDA	Perfluorodecanoic acid
PFUnDA	Perfluoroundecanoic acid
PFDoDA	Perfluorododecanoic acid
PFBuS	Perfluorobutanesulfonate
PFAA	Polyfluoroalkyl agent
POP	Persistent Organic Pollutant
PVC	Polyvinyl chloride
QUICKI	Quantitative Insulin Sensitivity Check Index
R&D	Research and Development
RCOG	Royal College of Obstetricians and Gynaecologists
RCT	Randomised Control Trial
RCF	Relative Centrifugal Force
RIA	Radioimmunoassay

RIF	Recurrent Implantation Failure
RNA	Ribonucleic Acid
RPM	Revolutions per minute
rFSH	recombinant Follicle Stimulating Hormone
SCPOP	Stockholm Convention on Persistent Organopollutants
SHBG	Sex Hormone Binding Globulin
sRAGE	soluble receptor for AGEs
TEM	Temperature
TGFB	Transforming Growth Factor-B
TNF-α	Tumour Necrosis Factor α
TTP	Time to Pregnancy
uNK	Uterine Natural Killer cells
VDR	Vitamin D Receptor
WHO	World Health Organization
WOI	Window of Implantation