The significance of natural killer cells in embryo implantation during in vitro fertilisation cycles

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“I wish it need not have happened in my time,” said Frodo.

“So do I,” said Gandalf, “and so do all who live to see such times. But that is not for them to decide.

All we have to decide is what to do with the time that is given us.”

JR tolkien The lord of the rings

“An absence of evidence does not mean evidence of absence

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Abbreviations

%-percentage

ANG2-angiopoietin 2

ANOVA-one way Analysis of Variance analysis

ART-Assisted reproduction technologies

AV-anchoring chorionic villi

BMI-body mass index

CD-cluster of differentiation

CGF-cochrane gynaecology and fertility Group

CL-corpus luteum

CRSO-cochrane central register of studies online

DAB-diaminobenzadine

DCs-dendritic cells

DCC-dextran coated charcoal

DGL-decidual granulated leukocytes

DMEM-dulbecco's modified eagle's medium

EnVT- endovascular cytotrophoblast cells

ELISA- enzyme linked immunosorbent assay

ET-embryo transfer

EVT-extra villous trophoblast

FC-flow cytometry

FCS-fetal calf serum

FER-frozen embryo replacement

FMO- flow minus one

FSH-follicle stimulating hormone

G-CSF-granulocyte colony stimulating factor

GnRH- gonadotrophin releasing hormone

GP-general practitioner

HBEGF-heparin binding epidermal growth factor

HCG-human chorionic gonadotrophin

HLA-human leukocyte antigens

HMG-human menopausal gonadotrophin

HRP-horseradish peroxidase

HRT-hormone replacement therapy

ICSI-Intracytoplasmic sperm injection

IFNγ- inteferon gamma

IGFBP-1- insulin like growth factor binding protein 1

IHC-immunohistochemistry

IL-interleukin

IU-international units

IU-intrauterine

IVD-in vitro diagnostic

IVF-in vitro fertilisation

IVIG-intravenous immune globulin

KIR-killer cell immunoglobulin like receptor

LH-leutinizing hormone

LIF-leukocyte inhibitory factor

LIT-lymphocyte immune therapy

MHC-major histocompatibility complexes

MMP-matrix metallo-proteinase

NHSSB-Nottingham Health Science Biobank

NK-natural killer

NKT-natural killer T cells

OHSS-ovarian hyper stimulation syndrome

OR-odds ratio

PB-peripheral blood

PMBC-peripheral blood mononuclear cells

pbNK-peripheral blood natural killer cells

PBS-phosphate buffered saline

PFA- paraformaldehyde

PGF-placental growth factor

RCT-randomised controlled trial

REC-research ethics committee

RIF-recurrent implantation failure

RR-relative risk

SC-subcutaneous

SD-standard deviation

TBS- Tri-buffered saline

Tc cells- cytotoxic CD8+ cells

Th-T helper

TNFα-tumour necrosis factor alpha

TVOR-transvaginal oocyte retrieval

TVS-transvaginal ultrasound scan

UK-united kingdom

uNK-uterine natural killer cells

VEGFC-vascular endothelial growth factor C

WHO-world health organisation

WOI-window of implantation

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Abstract

A delay in achieving pregnancy is a global challenge affecting 1 in 7 couples (Evers 2002). The wide accessibility of in vitro fertilisation(IVF) has revolutionized the treatment of infertility and led to the birth of 8 million babies worldwide (van Loendersloot, Repping et al. 2014, De Geyter, Calhaz-Jorge et al. 2018).

Despite this accomplishment, unsuccessful cycles and in particular implantation failure remain a challenging clinical diagnosis for patients and fertility specialists (Simon and Laufer 2012, Coughlan, Ledger et al. 2014). This has led to the introduction of numerous clinical tests aimed at testing the immune, anatomical and genetic components of implantation with very little scientific rationale (Kwak-Kim, Han et al. 2013).

The most widely available immunological investigation is the natural killer (NK) test (Tang, Alfirevic et al. 2011, Kwak-Kim, Han et al. 2013, Seshadri and Sunkara 2014). The interest in NK cells is supported, by the observation that they comprise around 70% of the immune cells in the late secretory phase and first trimester endometrium (Moffett-King 2002, Bulmer and Lash 2015). They may also have a specific function in orchestrating angiogenesis and vascular modelling of the early placenta (Quenby, Nik et al. 2009) along with tolerance of the hemi-allogenic fetal graft (Zhang, Dunk et al. 2016). Furthermore many cohort studies have shown NK cells are raised in women who have unsuccessful IVF treatment (Coulam and Goodman 2000, Tuckerman, Mariee et al. 2010, Sacks, Yang et al. 2012). The mechanism whereby this occurs and its significance is still poorly understood (Lash and Bulmer 2011).

NK testing involves measuring their numbers in either an endometrial biopsy and/or peripheral blood (PB) sample. There are currently no national guidelines on when this test should be offered and the reference range that constitutes an abnormal result (Tang, Alfirevic et al. 2011, Templer and Sacks 2016). Furthermore a range of immune therapies have emerged to treat raised NK results and promote implantation (Polanski, Barbosa et al. 2014). These interventions include oral steroids, intravenous immunoglobulin and intralipid (Alecsandru and Garcia-Velasco 2015, Nardo, El-Toukhy et al. 2015). There is uncertainty as to whether these potentially harmful drugs should be offered to patients when no clear relationship or reference ranges for abnormal NK levels exist (Rai, Sacks et al. 2005, Moffett and Shreeve 2015, Sacks 2015).

There are three main studies presented in this thesis with the aim of investigating the role of NK cells in IVF. One of the main strengths of this work is that women were recruited from a highly successful IVF clinic .The clinical pregnancy rates are around 10% higher than the national average (HFEA 2018). Therefore if IVF did not result in a pregnancy, then endometrial receptivity or adverse maternal immunology could be valid contributory factors.

The first study was a prospective analysis of 203 patients who had endometrial NK testing through immunohistochemistry (IHC) and subsequently embarked on IVF treatment. The main objective was to establish the prevalence of raised NK status in women undergoing IVF. This was coupled with a retrospective analysis of previous IVF and pregnancy history to identify features that were predictive of a raised result. The prevalence of raised NK status was high in this cohort of women (greater than 60%). This could be a true representation but is likely to be flawed due to timing the biopsy without LH testing and sample size. The only predictive demographic factors that showed statistical significance with raised NK status were age and embryo quality.

The second study involved the application of a comprehensive flow cytometry (FC) panel to paired PB and endometrial samples, taken from 42 women undergoing IVF treatment. This technique demonstrated a detailed analysis of NK cells and other immune cell populations; NKT, CD8+ and CD4+ which may have a role in implantation. A functional analysis of these populations with the measurement of inflammatory and angiogenic cytokines; perforin, granzyme, tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ) was also conducted. The main finding was that the expression of perforin and granzyme was noticeable in the PB samples as opposed to significant levels of TNFα and IFNγ in the endometrium.

Correlation of the panel with clinical outcomes revealed no differences in the immune cell populations present in the PB and endometrium. However the expression of PB NKT perforin, co- expression of perforin^{+}granzyme^{+} along with endometrial CD8+ TNFα^{+}IFNγ^{+} were statistically correlated with live birth. Furthermore a shift in the expression of PB perforin^{+}granzyme^{+} from a low percentage in those with a live birth to a higher value in patients with a negative pregnancy test was observed.

The third study was a large systematic review of 10 immune-therapies given in the context of unexplained implantation failure to determine if there was an improvement in live birth. This review highlighted that immune therapies were given without any risk assessment of side effects. Although there was some weak evidence to support the use of intralipid and heparin, the finding have to be interpreted with extreme caution.

Chapter 1

Background

Chapter 1 Background

1.1 Human Reproduction

1.1.1 Introduction

The process of human reproduction from fertilisation to the establishment of a successful pregnancy is a fascinating yet poorly understood phenomena.

The primary aim of human reproduction is to transfer genetic material through the creation of further offspring (Johnson 2012). The carriers of this genetic material are namely; the oocyte produced by females and spermatozoon by the male respectively.

A successful pregnancy commences with fertilisation which is defined as the union of a good quality oocyte ovum with a spermatozoon of equal genetic integrity (Hillier, Kitchener et al. 1996). Following fertilisation and early development, the embryo must undergo implantation into the endometrium. Successful invasion of the maternal circulation by the embryo is a crucial step that determines reproductive success (Cakmak and Taylor 2011). This process is multi-faceted and highly complex and the detailed mechanisms are yet to be fully defined (Guzeloglu-Kayisli, Basar et al. 2007, Fatemi and Popovic-Todorovic 2013, Gnainsky, Granot et al. 2015).

Furthermore these events are precisely orchestrated by molecular and cellular components driven by the embryo, the endometrium and the interaction between these two distinct entities (Simon, Martin et al. 2000). There is a vast array of hormonal, paracrine and autocrine

metabolites secreted by both the embryo and endometrial cells that allow for successful collaboration (Guzeloglu-Kayisli, Basar et al. 2007). Further the maternal immune system must recognise and develop tolerance to immunogenic embryo. This is also an intricate interplay of immune cells, their various markers and the endometrium.

1.1.2 **The female reproductive tract**

The female reproductive tract consists of the vagina, uterus, ovaries and fallopian tubes.

The uterus is a pear shaped muscular organ comprised of three regions, body, isthmus and cervix. Histologically it is composed of 3 major tissue types: epithelium and stroma collectively known as the endometrium, the muscular myometrium and the outer serosal layer (Stevens and Lowe 1997). The innermost layer, called the endometrium is a highly specialised mucus secreting epithelium (Glasser, Aplin et al. 2004) . It is constantly transforming in response to the cyclical ovarian hormones and is vital for reproductive function (Achache and Revel 2006) . The bulk of the uterus is comprised of the muscular layer known as the myometrium. The smooth muscle making up the myometrium grows rapidly during pregnancy and is essential for the propulsion of the fetus during childbirth (Snell 2007). The serosal layer is a thin layer of protective epithelial cells that encase the uterus.

The ovaries are almond shaped organs that have endocrine and reproductive functions. Each ovary contains the primary oocytes until their release or destruction. Each primary oocyte is surrounded by a

layer of protective granulosa cells and known as a follicle (Moore and Dalley 1999). Each month around 10-20 follicles begin to develop under the hormonal influence of the nervous and reproductive systems (Jones and Lopez 2013). Only one follicle is released from the ovary and this marks ovulation (Oktem and Oktay 2008). The remainder of the developed follicles undergo degeneration.

The two fallopian tubes are attached to the uterine wall and at their outermost have a fringe like ending called fimbriae. These ensure the ovum is directed from the ovary and into the tube (Padubidri and Daftary 2014). Fertilisation takes place in the fallopian tubes following which implantation and pregnancy continues in the female uterus (Moore and Dalley 1999).

1.1.2.1 *The anatomy of the human endometrium*

In order to describe how the endometrium transforms itself from a state of hostility to one of receptivity, an appreciation of its anatomy and physiology is required. The human endometrium is a dynamic and multi-layered organ that lies over the myometrium (Moore and Dalley 1999). It has two distinct zones; the upper functional layer (stratum functionalis) and the lower basal layer (stratum basalis). Underlying these zones is a thick connective tissue stromal layer (Stevens and Lowe 1997).

The functional layer consists of columnar epithelial cells that occupy two thirds of the endometrium with the basal layer comprising the remaining thickness (Critchley, Kelly et al. 2001). The basal layer remains

adherent to the myometrial tissue and acts as a foundation for the functional layer. It is not responsive to the cyclical hormonal influences of the cycle but instead contains numerous blood vessels known as spiral arterioles (Ferin, Jewelewicz et al. 1993). These change in length and morphology during the menstrual cycle (Heller 1994). It is also a rich source of stem cells which allow regeneration of the functional layer during menstrual cycle and pregnancy (Maruyama and Yoshimura 2008). The functional layer is a mucous membrane comprised of columnar epithelial and glandular cells. The epithelial layer is interspersed with glandular cells which extend from the surface in a tubular fashion right down to the stromal layer (Heffner and Schust 2010). These are highly responsive to the effects of oestrogen and progesterone .During menstruation the entire functional layer is shed and regenerated (Cooke, Spencer et al. 2013).

Beneath both layers of the endometrium is a thick stromal compartment. This is formed of connective tissue and becomes infiltrated by immune cells at the time of implantation. The stromal cells display increased proliferation and cell division in the first half of the menstrual cycle (Gellersen, Brosens et al. 2007). This activity declines after ovulation and there is a switch to an accumulation of fluid around the cells (oedema). The stromal cells also migrate to surround the spiral arterioles in the secretory phase of the cycle (Cooke, Spencer et al. 2013). This transformation of the stromal layer is known as decidualisation and dictates a successful pregnancy (Chan, Schwab et al. 2004, Brosens and Gellersen 2006).

It is the functional epithelial cells that are the first point of contact for the incoming blastocyst and the major mediators of implantation. However the stromal cells which are underlying the epithelial layer are also involved in coordinating molecular events (Jabbour, Kelly et al. 2006).

1.1.3 **The human menstrual cycle**

Female reproductive function and endometrial receptivity are governed by the menstrual cycle (figure 1.1). The human menstrual cycle involves close collaboration of the female nervous and reproductive systems (Silberstein and Merriam 2000). The cyclical pattern of hormonally controlled interactions are key to understanding the anatomical and functional changes that take place in the two end organs; the ovary and endometrium. Key events in the ovary can be classified into the follicular and luteal phase. Meanwhile the changes in the endometrium constitute the proliferative and secretory phase.

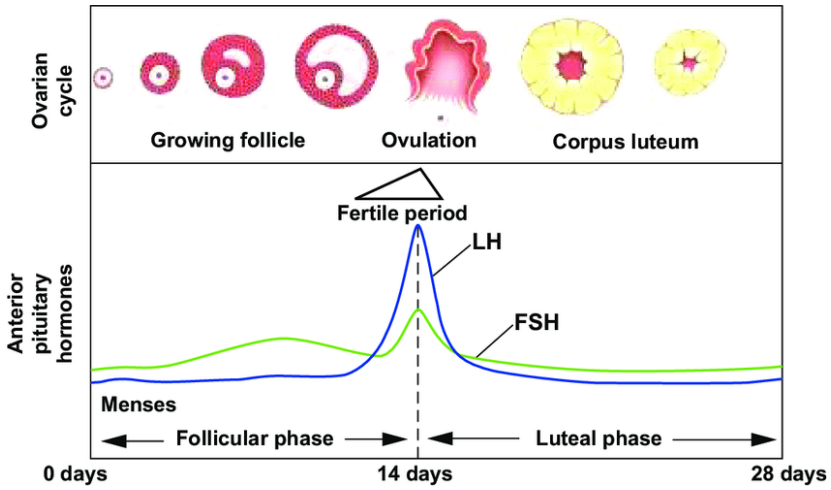


Figure 1-1- The human menstrual cycle

Image taken from (Koyama, Hagopian et al. 2013)

The cycle ranges between 25 to 32 days and begins with the breakdown and shedding of the endometrium (the menstrual bleed or

menses) (Johnson 2012). This is triggered by an absence of fertilisation and pregnancy. The first day of bleeding marks the start of the cycle and is termed day 1 (Hawkins and Matzuk 2008).

The initial proliferative (follicular) phase of the cycle ranging from day 1-14 involves the development and growth of ovarian follicles in preparation for ovulation .Under the influence of gonadotrophin hormones released by the anterior pituitary gland, namely follicle stimulating hormone (FSH) and leutinizing hormone (LH) , the ovarian follicles grow and mature (Oktem and Oktay 2008). This process is known as folliculogenesis , whereby a dominant follicle is selected to undergo ovulation with the premise that it will be fertilised by a sperm and form a viable embryo (Popat, Prodanov et al. 2008).

As the ovarian follicles grow in size, they release the female sex hormone oestrogen. Rising levels of oestrogen promotes regeneration of the endometrium through proliferation of its epithelium, stroma and vascular endothelium. This is referred to as the proliferative phase. During the late proliferative phase, the endometrium is filled with numerous developing glandular cells, immune cells and vasculature (Aplin 2000).

At around day 14 (mid cycle) a surge of FSH and LH causes ovulation (Hawkins and Matzuk 2008). The release of the ovum from the follicle leads to formation of the corpus luteum (CL). The CL is essentially the outer casing of the ruptured follicle and secretes the hormone progesterone. It is progesterone acts on the endometrium to undergo

further thickening in preparation for implantation (Glasser, Aplin et al. 2004). This stage in the cycle is known as the early secretory (luteal) phase (Padubidri and Daftary 2014). The glandular tissue within the endometrium becomes secretory and there is differentiation of the stromal cells. The leads to the endometrium becoming increasingly oedamatous, and is a critical step in achieving implantation (Aplin 2000).

During the mid-luteal phase which spans day 20-24 of the cycle, increasing levels of oestrogen and progesterone define the window of receptivity (WOI) (Ma, Song et al. 2003).This is when the endometrium is at its most conducive to implantation and subsequent pregnancy.

If a viable embryo does not approach the endometrium during the receptive window hormonal control is relinquished with degeneration of the CL and endometrium. The cycle is reset with further folliculogenesis and endometrial proliferation (Hawkins and Matzuk 2008).

Conversely, if there is successful implantation then the CL maintains its secretion of progesterone, there is release of HCG from the blastocyst which aids further development of the pregnancy (Loke and King 1995).

1.1.4 **Fertilisation**

Fertilisation occurs within the fallopian tube and comprises a series of well-crafted and coordinated events.

The process begins with the successful attachment of a single viable spermatozoon to the zona pellucida (thick outer layer of cells surrounding the ovum). Once attached there is release of digestive

enzymes from the head of the sperm known as the acrosome reaction (Heffner and Schust 2010). This enables the spermatozoon to penetrate and burrow its way through the zona pellucida. Upon entry into the oocyte, the cell membranes of the two gametes undergo fusion to become the Zygote. This confirms fertilisation and facilitates the completion of meiosis within the oocyte, thereby ensuring its genetic material is balanced. This crucial step also initiate a series of events that lead to both the oocyte and the zona pellucida becoming impenetrable to the entry of further sperm (Cooper 2012).

1.1.4.1 ***Embryo formation***

Once fertilised, the nuclei of the gametes enlarge and swell to develop into the male and female pro nuclei. The membranes surrounding both pronuclei disintegrate as replication of the maternal and paternal chromosomes ensues ready for numerous mitotic cell divisions (figure 1.3). Subsequent divisions lead to the embryo comprising of 6 to 12 cells by day 3 and 16-32 cells known as the morula by day 4 (Padubidri and Daftary 2014). Once the morula is fully formed it begins to migrate down the fallopian tube to enter the uterine cavity. There is also movement of the daughter cells to the centre of the morula and some to its periphery. Some of the dividing daughter cells known as blastomeres migrate to the centre of the morula to form the inner cell mass, which will subsequently become the fetus (Heffner and Schust 2010). The peripherally placed blastomeres form the trophectoderm which ultimately gives rise to the fetal component of the placenta (Norwitz, Schust et al. 2001).

By day 5 the osmotic pressure within the morula increases, there is a formation of a large fluid filled cavity and the embryo is now known as a blastocyst (Schoenwolf, Bleyl et al. 2012). There is further remodelling of the inner cell mass and trophoblast cells as this stage. The inner cell mass form a compact structure at one side of the cavity and the trophoblasts start to form a thin single epithelial layer around the blastocyst (Jones and Lopez 2013).

1.1.4.2 *Hatching*

In order for the blastocyst to fully adhere to the endometrium, it must detach itself from the zona pellucida (Cha, Sun et al. 2012). This process of hatching involves the release of enzymes by the blastocyst which creates an opening in the zona pellucida through which the embryo can exit. Once hatching is complete, the blastocyst is able to interact with the endometrial surface and start the implantation process (Dey, Lim et al. 2004).

1.2 Human implantation

Implantation is defined as the ability of the embryo to attach to the endometrium and establish into an ongoing pregnancy (Aplin 2000). It is a poorly understood phenomenon in human reproduction (Dey, Lim et al. 2004). Due to the ethical challenges of using human tissue, key events in the implantation process has been translated from experiments conducted on rodent uteri (Wang and Dey 2006). The IVF model in humans has expanded and extrapolated knowledge further (Boomsma and Macklon 2006).

It widely accepted that implantation can be divided into the following 4 stages (Enders and Schlafke 1967, Fatemi and Popovic-Todorovic 2013):-

- Rolling-The embryo rolls along the endometrium, orientating a particular position
- Apposition-Trophoblastic cells begin to adhere to the receptive endometrial epithelium and there is simultaneous shedding of the zona pellucida.
- Adhesion-The blastocyst anchors to the endometrial basal lamina and stromal extracellular matrix. At this stage the embryo is firmly connected with the endometrium and cannot become detached by flushing.
- Invasion-The embryo penetrates through the luminal epithelium and attaches itself to the stromal basal layer. Tiny projections of the uterine-epithelium known as pinopodes interlace with the outer layer of the blastocyst to create the initial adhesive effect (Usadi, Murray et al. 2003). Once an interface is established between the embryo and endometrium, the trophoblastic cells begin to form the placenta.

In order for successful implantation to occur, there needs to be a receptive endometrium, a normal and functional embryo at the blastocyst stage, as well as a timely yet well synchronised dialogue between the maternal and embryonic interface (Simon, Martin et al. 2000). The nature of the maternal-fetal interface along with the

chemically derived communication is critical in determining the success or failure of pregnancy (Duc-Goiran, Mignot et al. 1999).

The embryo arrives in the uterine cavity 3-4 day post ovulation(Croxatto, Ortiz et al. 1978). Initially it is relatively inactive due to being surrounded by the hard outer coating of the zona pellucida. This is shed 6 days post-ovulation after which, it is able to interact more freely with the endometrium (Buster, Bustillo et al. 1985). The hatching of the embryo should coincide with the WOI as this exposes the embryonic receptors to cytokines and cell signalling proteins present on the receptive endometrium (Tabibzadeh and Babaknia 1995).

Experiments in mice have determined that the blastocyst is naturally drawn to a crypt like area formed by the invagination of luminal epithelium (Carson, Bagchi et al. 2000). The process advances with the embryo displacing the luminal epithelium from the basal lamina thereby creating an easy passageway for the trophoblasts to invade the stromal layer (Parr and Parr 1989). The stromal cells present at the site of implantation simultaneously undergo extensive proliferation and differentiation into decidual cells (Psychoyos 1986, Quenby, Nik et al. 2009, Lash 2015). These highly specialised cells are needed to facilitate the development of the placenta (Yang, Zhou et al. 2019).

Although the process is not fully understood, implantation of the embryo involves internally driven signals that are recognised by the receptive endometrium. Conversely blastocyst development can only occur once it receives the external signals from the decidualised cells. This is

carried out by a variety of endometrial receptors, cytokines and signalling molecules (Guidice and Conti 2009).

As described a receptive endometrium is vital for normal embryo implantation. Architectural and biological changes occur during the menstrual cycle, which prepare the endometrium for successful interaction with the embryo (Stevens and Lowe 1997, Achache and Revel 2006).

Research over the last few years has determined that there are morphological changes that must occur in the endometrium to accept an embryo. However the functional changes that are under complex hormonal and molecular control are yet to be fully elucidated (Lessey 2011). Paradoxically a morphologically competent endometrium may be functionally incompetent in terms of achieving the golden state of receptivity. It is development of methods that assess the function of the endometrium around implantation that requires further work (Sharkey and Smith 2003, Diedrich, Fauser et al. 2007).

The attachment and invasion of the endometrium by the embryo does not appear to be a destructive event with the embryo digesting the multiple cell layers of the endometrium. Instead implantation from mouse models suggests that it is a synchronous process with the outer syncytial trophoblast layer developing projections that attach to the luminal epithelial cells(Carson, Bagchi et al. 2000, Wang and Dey 2006). This causes the luminal epithelial layer to spread apart thereby creating a tunnel for the embryo to enter the endometrial layers. The

embryo invades through the basement of the epithelial layer into the stromal layers where it seeks to gain access to the maternal circulation (Makrigiannakis, Karamouti et al. 2008).

1.2.1.1 *Placentation*

Once embryonic attachment and invasion has occurred, the embryo quickly increases its metabolic function so that it can survive fully below the luminal surface. At this stage the outer cyto-trophoblastic layer start to grow villi and invade the maternal vasculature (Glasser, Aplin et al. 2004). Evasion of the maternal immune system and deeper penetration of the extra villous trophoblast (EVT) layer into endometrium is controlled by a finely tuned array of molecular components and pathways (Moffett-King 2002, Male, Gardner et al. 2012).

1.2.1.2 *Trophoblastic invasion and modelling of the placental vasculature*

As the pregnancy develops the fetus does not come into direct contact with the maternal circulation (Manyonda 2006). The two compartments interact via the placenta resulting from an intimate relationship between the trophoblastic cells and decidua (Makrigiannakis, BenKhalifa et al. 2015).

Once implantation is nearing completion, the outer wall of embryo known as the trophoblast layer gives rise to the placenta through differentiating into two distinct tissue types (Harris, Smith et al. 2010). The mature placenta is characterised by a network of tree like

projections called villi bathed in blood from the maternal circulation (Chen, Liu et al. 2011).

The inner layer of the trophoblast cells differentiate into the cyto-trophoblastic cells which form the villi (Cartwright, Fraser et al. 2010). The outer layer is characterised by the loss of its lateral cell margins and forms the multinucleated syncytiotrophoblast (Glasser, Aplin et al. 2004, Cartwright, Fraser et al. 2010). Once the blastocyst becomes attached to the endometrium, individual trophoblast cells that comprise the cyto-trophoblast proliferate to form the villi. These cells are encased in the syncytiotrophoblastic layer which creates a pathway through the functional layer and into the endometrial stroma (Harris, Smith et al. 2010). Once fully penetrated into the decidualised stroma, the trophoblastic cells are able to erode the endometrial capillaries and ensure an adequate blood supply. This forms an interface with the maternal circulation, so that the transport of oxygen and protein is facilitated (Moffett and Colucci 2014). The remainder of the cells differentiate into the extravillous trophoblast layer (EVT). These have a dual role with some cells migrating and forming columns between the placenta and maternal decidua. These columns act as anchoring points so that the placental tissue is in intimate proximity to the maternal vasculature (Chen, Liu et al. 2011). It is at this interface that the developing placental tissue is exposed to the maternal immune cells. These include a small amount of B and T lymphocytes and numerous uNK cells (Loke and King 1995). A subset of EVT cells also invade the decidua along with the myometrium and facilitate remodelling of the

uterine spiral arteries. These are referred to as endovascular cytotrophoblast cells (EnVT) (Cartwright, Fraser et al. 2010). The extent and depth of infiltration of the trophoblastic cells determines whether a pregnancy will develop in the correct manner (Fraser, Whitley et al. 2012). This in turn is controlled by the balance of immune cells present at the time of implantation(von Rango 2008).

During decidualisation, the endometrium becomes infiltrated with an abundance of maternal immune cells. These cells aggregate around the invading trophoblasts and have a role in establishing pregnancy and facilitating the immune response. The number of maternal immune cells changes as pregnancy progresses (Moffett-King 2002). The first trimester is dominated by uNK and activated macrophages but the numbers decline as the second trimester starts. They are at their lowest number by the time of full term pregnancy (Lash and Bulmer 2011).

uNK cells comprise 70% of the immune cells present at the time of implantation (Lee, Kim et al. 2015). They accumulate around the spiral arterioles/arteries and glands (Bulmer, Morrison et al. 1991). The exact function of these cells is unknown but it is hypothesised that they also act as regulators of EVT invasion and modelling of the placental vasculature (Fatemi and Popovic-Todorovic 2013). Human uNK cells produce a combination of important cytokines that mediate angiogenesis including vascular endothelial growth factor C (VEGFC),placental growth factor (PGF) and angiopoietin 2(ANG2) (Zhang, Chen et al. 2011).

Studies have shown that a lack of NK cells along with other essential cells such as activated macrophages and dendritic cell is associated with a poor outcome in the pregnancy (Manaseki and Searle 1989, Lash and Bulmer 2011). It is thought that in the absence of NK cells the trophoblast cells are unable to reach optimal endometrial vascularity and hence the pregnancy is not viable (Hanna, Goldman-Wohl et al. 2006).

NK cells have generated a lot of debate within the field of reproductive immunology (Rai, Sacks et al. 2005, Tang, Alfirevic et al. 2011, Moffett and Shreeve 2015). Researcher and IVF specialists have been fascinated by their predominant presence in the uterine decidua at the time of implantation (Le Bouteiller and Piccinni 2008). They have therefore hypothesised that they must play a key role in the outcome of a pregnancy (Tang, Alfirevic et al. 2011).

1.2.1.3 *Morphological changes within the endometrium during implantation*

For most of the menstrual cycle the endometrium is not conducive to embryo implantation (Bergh and Navot 1992, Cha, Sun et al. 2012). Numerous dynamic and functional changes have to occur within the cells in order to accommodate the attachment of a blastocyst .This transformation is referred to as endometrial receptivity (Lessey 2011). It is defined as the temporal and spatial changes that occur in the endometrium in order to facilitate successful implantation of the embryo (Giudice 1999). A receptive endometrium must undergo the acquisition

of adhesion ligands along with a loss of inhibitory components that will allow it to accommodate a successful pregnancy (Aplin 2000).

Endometrial receptivity is under strict hormonal control by oestrogen and progesterone (Mote, Balleine et al. 1999). The endometrium is initially primed by oestrogen in preparation for implantation. Rising levels of oestrogen through the first part of the menstrual cycle cause the endometrial cells to proliferate. Repair and proliferation of the epithelial, stromal and vascular endothelium lead to regeneration of the tissue (Oliver, Cowdrey et al. 1999). The glandular tissue also develops and assumes a tortuous morphology towards the end of the proliferative phase. It is however progesterone which is mainly secreted by the blastocyst that leads to differentiation and decidualisation of the cells.

1.2.1.4 *The endometrial stroma and decidualisation*

Decidualisation refers to the functional and morphological changes that occur within the endometrium once successful implantation has been successful (Christian, Mak et al. 2002, Ruan, Chen et al. 2014). In the human this occurs in the luteal or secretory phase of the menstrual cycle at around 10 days post ovulation (Arias-Stella 2002).

In the mouse, the stromal cells become rounded with the secretion of molecules such as prolactin and insulin like growth factor binding protein 1(IGFBP-1) to maintain the pregnancy (Lee, Jeong et al. 2007). Increased secretion of interleukin (IL)-15 by decidualised stromal cells promotes growth and differentiation of uterine natural killer (uNK) cells (Okada, Nakajima et al. 2004).The decidualised cells surround the

implantation site for a limited time span. This clustering facilitates expansion and development of the placental tissue (Oliver, Cowdrey et al. 1999).

Interestingly in the human, these changes occur within the endometrium irrespective of whether an embryo is present (Ramathal, Bagchi et al. 2010). The stromal cells proliferate and surround the spiral arterioles, with subsequent branching outwards across the whole endometrium if pregnancy continues (Ansell, Barlow et al. 1974, King 2000). In addition to this, nearby endometrial glands enlarge and the local uterine wall becomes more highly vascularised and oedematous (Bell 1990).Macroscopically the intracellular matrix framework of the endometrium also undergoes remodelling with an increased production of collagen, fibronectin and laminin (Dunn, Kelly et al. 2003). There is decreased activity of the matrix metalloproteinases (MMP-1 and MMP-3) hence promoting uterine vascularity further (Schatz, Krikun et al. 1997) .

As with implantation recent experiments indicate a pathway of communication between the embryo and decidualised cells (Teklenburg, Salker et al. 2010). In an attempt to understand the possible factors involved in establishing pregnancy, Teklenburg and colleagues (Teklenburg, Salker et al. 2010) cultured human blastocysts along with decidualised stromal cells. They noted that when good quality embryos were used, there were significant changes in the expression of molecular mediators. However when embryos of compromised quality were introduced to the stromal cells, there was a

reduction in many biomarkers of implantation including IL-1 and heparin binding epidermal growth factor (HB-EGF). Conversely when embryos were cultured in non decidualised cells, there was minimal expression of these markers. It can therefore be surmised that decidualised cells act as a filter for appropriate embryo development and competency. This is supported by the observation that inappropriate decidualisation is associated with early pregnancy loss and aberrant placental development.(Brosens, Pijnenborg et al. 2002, Norwitz 2006, Brosens, Parker et al. 2009).

Once the embryo has implanted, the trophoblast cells produce the hormone human chorionic gonadotrophin (hCG) (Jones and Lopez 2013). This supports the corpus luteum and thus maintains the supply of progesterone to the maternal-fetal unit and the highly vascularised endometrial state. The detection of hCG in the maternal serum occurs 8 days after ovulation and this is the principle used to confirm pregnancy (Cartwright, Victory et al. 1986).

If implantation does not occur as a result of the process being defective or out of reproductive choice, the endometrium undergoes shedding and this marks menstruation (Silberstein and Merriam 2000). This cycle of events starts again in anticipation for the next blastocyst to arrive.

1.2.1.5 ***The window of implantation (WOI)***

The embryo can only implant in the endometrium during a limited time interval known as the WOI (Strowitzki, Germeyer et al. 2006). The WOI is governed by appropriate morphological and functional changes that

occur in the endometrium including decidualisation (Navot, Scott et al. 1991).

It is hypothesised that the WOI spans for 5 days in total and coincides with days 20-24 of the menstrual cycle (Bergh and Navot 1992). This is usually 6 to 10 days post ovulation and in vivo studies looking at the first detection of a positive HCG in spontaneous pregnancies support this finding (Wilcox, Weinberg et al. 1988, Sharkey and Smith 2003).

It has been observed that a prolonged WOI beyond 5 days allows more embryos to implant irrespective of their quality demonstrating that it is the endometrium that is responsible for implantation (Tabibzadeh and Babaknia 1995). This lack of stringent embryo selection may be responsible for the implantation of incompetent embryos that are destined to result in biochemical or early pregnancy loss. Knowledge of the WOI is crucial for future studies as it will not only identify the optimal time for spontaneous conception but also assist in timing the correct day for embryo transfer in IVF cycles (Coughlan, Ledger et al. 2014).

1.3 Natural killer cells

NK cells are highly sophisticated leukocytes that change function and number according to tissue they penetrate (Zhang, Chen et al. 2011). They are versatile cells that can adapt to the immunological environment (Sun, Beilke et al. 2009). Their primary function is one of recognising and destroying viruses and tumours (Cerwenka and Lanier 2001).

NK cells are derived from the haematopoietic progenitor cells (HPC) in the bone marrow. They are present both in the endometrium termed uterine NK cells (uNK) and in the circulation termed peripheral blood NK cells (pbNK) (Seshadri and Sunkara 2014). They are identified immunologically by their expression of the CD56 surface marker (Robertson and Ritz 1990, Tang, Alfirevic et al. 2011). The CD56+ phenotype denotes a NK lineage which is part of a wider set of lymphocytes referred to as innate lymphoid cells (ILCs). This group of cells reside in the mucosal surfaces of the body and act as mediators of homeostatic control as well as defensive components of the immune system (Artis and Spits 2015).

Furthermore in peripheral blood there are two subtypes of NK cells present ,according to the extent of CD56 expression; 90% are CD56 ^{dim}, CD16+ and 10 % are CD56 ^{bright}, CD16-. These are phenotypically different from uNK (Dons'koi, Chernyshov et al. 2014), which are predominantly CD56 ^{bright} and CD16- with a small number being CD56 ^{dim} (Miko, Manfai et al. 2010). The uNK CD56 ^{bright} normally comprise 30% of the endometrial lymphocyte population but this increases at the time of pregnancy to around 70%.(Ashkar and Croy 2001).

Although both types of cells possess cytotoxic and nurturing properties, they are functionally distinct (Robson, Harris et al. 2012). uNK cells have a nurturing role in promoting early placenta development through the expression of cytokines and angiogenic growth factors (Rai, Sacks et al. 2005).Conversely pbNK possess cytotoxic qualities and have

more of a destructive role in protecting against pathogens and malignant cells (Moffett-King 2002).

PbNK levels are measured in blood samples through flow cytometry (FC) (Tuckerman, Mariee et al. 2010). UNK cells are measured using either FC or immune-histochemical staining of endometrial tissue (Santillan, Lozano et al. 2015). The levels of pbNK and uNK do not correlate and a high number of pbNK may not necessarily reflect the condition of the endometrium (Park, Lee et al. 2010).

1.3.1 The roles of uterine natural killer cells in implantation

The origin of uNK within the endometrium and decidua is debatable. On the one hand it is hypothesised that these cells arrive in the uterus as a result of trafficking from the peripheral circulation (Manaster, Mizrahi et al. 2008). Alternatively they may be formed from differentiation of precursors cells already present in the endometrium (Yagel 2009).

uNK are predominantly growth promoting but do possess weak cytotoxic potential (Kalkunte, Mselle et al. 2009) .However they do not demonstrate destructive properties towards the developing cytotrophoblast (El Costa, Tabiasco et al. 2009). Potential mechanisms for this phenomenon include the expression of MHC class-I molecules (HLA- G and E) on the NK cell surface receptors (i.e. KIR2DL4, CD94)(King, Allan et al. 2000). This pathway inhibits their ability to recognise their target cell. Furthermore genetic studies have indicated that in order for uNK cells to become activated, they must possess the ability to express KIRs (Chen, Han et al. 2010). These receptors specifically bind to fetal HLA-C antigens present on the trophoblastic cells (Sharkey, Gardner et al. 2008), thereby facilitating the vascular remodelling of the placenta and immune-tolerance (King, Hiby et al. 2000).

Women who exhibit an inhibitory version of the gene encoding for KIR show a higher incidence of pre-eclampsia through deactivation of the UNK cells (Hiby, Apps et al. 2010).

Although not fully elucidated, uNK cells play two important roles in implantation (Acar, Ustunel et al. 2011)

- Regulation of trophoblastic invasion
- Angiogenesis and spiral artery modelling

Both these crucial mechanisms are required for bringing about a state of endometrial receptivity.

Studies in mice lacking NK cells have shown a poorly developed decidua and reduction in fetal weights indicating that they may be associated with the decidualisation process (Ashkar, Di Santo et al. 2000, Barber and Pollard 2003, Chakraborty, Rumi et al. 2011). Animal models have also demonstrated that uNK cells change the depth and pattern of trophoblastic invasion(Chakraborty, Rumi et al. 2011). They maintain the balance of between superficial invasion and excessive penetration of the trophoblastic cells both of which can lead to defective placentation. It is this mechanism that leads to miscarriage and pregnancy related complications of pre-eclampsia.(Brosens, Pijnenborg et al. 2011).

Interestingly the production of growth factors and cytokines changes according to gestational age. Data suggests that uNK cells predominantly produce growth factors at 8-10 weeks of pregnancy with a decline as the first trimester is completed (Lash, Otun et al. 2006). Conversely after 12 weeks of pregnancy, uNK cells start to secrete cytokines which increase with gestation age (Lash, Schiessl et al. 2006). The reason for the change in activity from angiogenic growth

factor expression to that of cytokine secretion is largely unknown. It is thought that it could be a reflection of alterations in the proportion of UNK cells that undergo proliferation and differentiation (Li, Charnock-Jones et al. 2001).

1.3.1.1 *Uterine natural killer cells and trophoblastic invasion*

uNK cells have been seen as single cells or aggregates around the EVT, near endometrial glandular tissue and spiral arterioles (Song and Fraser 1995). This close intimacy with the developing trophoblast demonstrates their role in early placental development (Moffett-King 2002). In order for optimal trophoblastic invasion to occur, the muscle layer of the maternal blood vessels has to be destroyed so that the fetus is exposed to a supply of nutrients. The role of uNK cells in this process has been proposed via several mechanisms which are facilitated through cytokine IL15 and MMP 9 secretion (Lash and Bulmer 2011). In vitro experiments by Hanna et al (Hanna, Goldman-Wohl et al. 2006) have shown that uNK cells stimulated with the cytokine IL15 promoted invasion of cytotrophoblast cells. A similar experiment by Hu et al (Hu, Dutz et al. 2006) showed the opposite effect with no invasion mediated through interferon gamma.

1.3.1.2 *Angiogenesis and spiral artery remodelling*

Remodelling of the maternal vasculature especially the spiral arteries of endometrium is key to the success of a pregnancy (Robson, Harris et al. 2012). Dysfunction in this process has been associated with numerous pregnancy complications including pre-eclampsia, fetal growth restriction and recurrent miscarriage (Zhang, Chen et al. 2011).

A potential explanation for the pathogenesis of these conditions has been attributed to inadequate invasion of the spiral arteries by the EVT (Brosens, Pijnenborg et al. 2011). As described previously uNK cells aggregate around the spiral arteries in the late secretory and early decidual endometrium. They promote EVT invasion by attracting these cells to the vascular regions of the endometrium. Furthermore in vitro models using term placental tissue and non-pregnant myometrium show a clear separation of the vascular smooth muscle in the presence of UNK cells (Harris, Smith et al. 2010, Lash, Robson et al. 2010) These studies have also demonstrated that uNK cells taken at 12-14 weeks of gestation show a more pronounced effect on tissue modelling than those from the earlier phases of pregnancy. It is hypothesised that the growth factors Ang-2 and VEGFC are responsible for these mechanisms (Lash, Robson et al. 2010) .Looking more closely, uNK cells have been shown to stimulate the formation of new vessels in the spiral artery endothelial layer (Hanna, Goldman-Wohl et al. 2006).This is perhaps where their role in the development of smooth muscle within newly formed vessels may be a contributory factor in reproductive disorders (Tuckerman, Mariee et al. 2010).

1.3.2 **Implantation and other immune cell populations**

There are other immune cells; namely T cells present at the time of implantation that also play a significant role in the establishment of a pregnancy. Their main role is of immuno-tolerance and many work in conjunction with NK cells (Sargent, Borzychowski et al. 2007). This section has been included to describe the role of these immune cell population as they will be studied through flow cytometry.

The early pregnancy decidua is comprised of 10-20% T cells, of which CD8+ cell comprise 45-75% and CD4+ cells make up 30-45% (Taylor and Sasser 2017). A small proportion are NKT cells and there is a significant rise in all these cell types as gestation increases (Nancy and Erlebacher 2014).

1.3.2.1 ***NKT cells***

NKT cells are a unique sub population of T cells that express both CD56 and CD3 (Miko, Manfai et al. 2010). They comprise a small proportion of the peripheral blood lymphocytes and are highly cytotoxic (Borzychowski, Croy et al. 2005).They are able to launch a much more rapid immune response to viruses and infection than NK cells. However in early pregnancy they have an immune-tolerance role (Kronenberg and Gapin 2002) similar to that of NK cells.

1.3.2.2 ***CD8+ cells***

These cells form the largest population at fetal maternal interface and have a dual cytotoxic and immune-tolerance role (Perchellet, Jasti et al. 2013). Decidual CD8+ have been shown to produce perforin and

granzyme in early pregnancy but lose this role as in the later stages. The significance of this in determining a successful pregnancy is still unknown (Tilburgs, Schonkeren et al. 2010).

1.3.2.3 ***CD4+ cells***

CD4+ cells have a regulatory function and besides avoiding destructive autoimmunity, they are involved in graft tolerance (Kofod, Lindhard et al. 2017). They are therefore essential in protecting the semi allogenic conceptus from maternal rejection. The numbers of CD4+ cells have been noted to be low in women with a history of recurrent miscarriage (Arruvito, Sanz et al. 2007).

1.4 Implantation and the involvement of the maternal immune system

The ultimate function of the immune system during implantation is to provide immune-tolerance of the fetus and placenta as they invade the maternal circulation (Mor, Cardenas et al. 2011). During the later stages of the implantation process there is rapid migration of trophoblastic cells in to the endometrial layer. This is set in place so that the placenta is formed and nutrition from the maternal circulation is delivered to the growing fetus (Moffett-King 2002). There is essentially a transfer of metabolic control from the embryo with its temporary stores of energy to that of the more permanent and abundant source of nutrients from the maternal host. This feat is complex and is made possible by the host of molecular and immunological components (Loke and King 2000).

The role of the immune system in implantation is complex and mediated by a vast array of immune cells, receptors and molecular markers.

In order to understand how the maternal system is involved in implantation, the distribution of immune cells and how they change within the menstrual cycle is outlined here. The endometrium is a rich source of immune cells that play a significant role in menstruation and implantation (Lee, Kim et al. 2015). Figure 1.2 shows how the distribution of various immune components changes throughout the menstrual cycle.

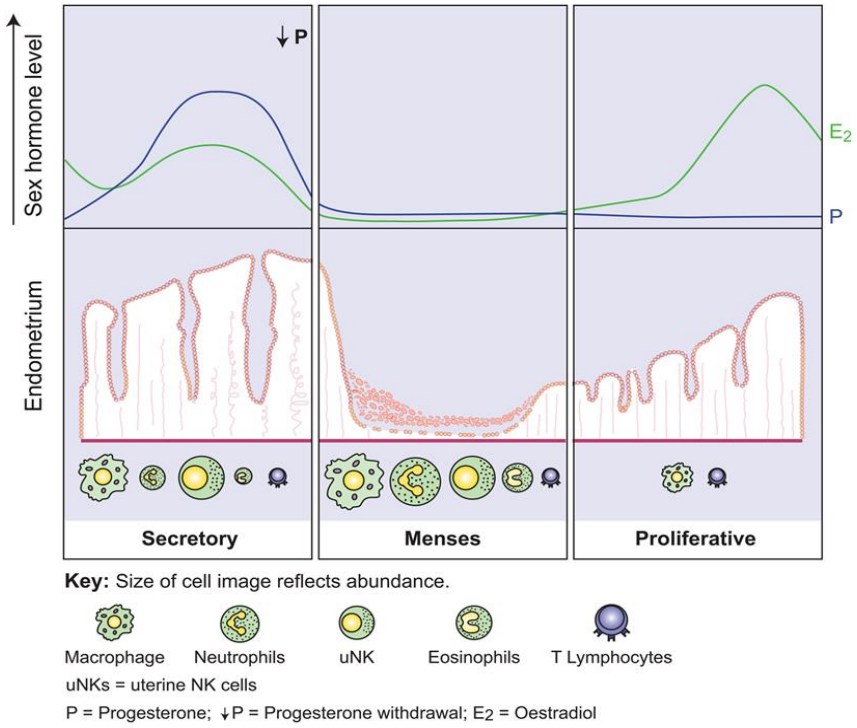


Figure 1-2- The distribution of immune cells in the human menstrual cycle

Image taken from (Maybin and Critchley 2015)

It can be seen that T cells remain fairly consistent during the menstrual cycle (Salamonsen and Woolley 1999). However uNK cells and macrophages show a cyclical increase in numbers with a peak in the secretory phase of the cycle (Salamonsen and Woolley 1999, King 2000, Lee, Lee et al. 2011). The rise in oestrogen seen at ovulation stimulates an influx of macrophages into the uterus (Salamonsen, Zhang et al. 2002). These cells remain close to the invading placental tissue, the glandular epithelium and uterine blood vessels. They have a variety of roles including defence against infections and tissue modelling (Schulke, Manconi et al. 2008). Once implantation is complete, decidual macrophages are thought to contribute to trophoblastic invasion and placental development through secretion of

growth factors and cytokines (Bonatz, Hansmann et al. 1992, Thiruchelvam, Dransfield et al. 2013).

NK cell levels vary within the menstrual cycle and are controlled by hormonal, immunological and genetic factors (Dosiou and Giudice 2005). Both pbNK and uNK levels vary throughout the menstrual cycle and change with pregnancy (Bulmer, Morrison et al. 1991). There is a dramatic increase in uNK cell numbers after ovulation with a significant number being present in the mid secretory phase (Lash, Robson et al. 2010, Lash, Robson et al. 2010, Bulmer and Lash 2015). This influx is triggered by progesterone and mediated through the decidualised stromal cells (Wilkens, Male et al. 2013). If successful implantation occurs then, there is a further increase in the density of uNK cells around the endometrial glands and vasculature (Lash, Schiessl et al. 2006, Quenby and Farquharson 2006). This infiltration of immune cells mediates implantation and invasion of the early placental tissue (Lash and Bulmer 2011, Robson, Harris et al. 2012). The first trimester is dominated by uNK cells and activated macrophages but the numbers decline as the second trimester starts. They are at their lowest number by the time of full term pregnancy (Lash and Bulmer 2011, Yang, Gilman-Sachs et al. 2019).

1.4.1 Theories describing the immunological components of implantation

Considerable changes occur in the maternal immune system in order for it to tolerate and nurture a developing fetus (Manyonda 2006). An area of much debate has been the issue of how the embryo and placental unit escapes rejection by the maternal immune system (Beer and Billingham 1976, Loke and King 1995, Bonney 2017). The fetus is semi-allogenic (partially foreign) to the maternal host. It is phenotypically and genetically different and therefore has the ability to evoke an immune response leading to implantation failure (Makrigiannakis, Petsas et al. 2011). There are various theories of how the fetus becomes fully accepted by the maternal immune system and described below.

1.4.1.1 *The theory of self-non-self discrimination*

It was originally though that the placenta was immunologically inert and therefore would be incapable of evoking an immune response (Billingham, Brent et al. 1953, Beer and Billingham 1976). It was also hypothesised that the maternal immune system was supressed in order to allow the fetus to develop without recognition and immunological destruction (Medawar 1953). This theory of self-non-self discrimination has been discounted as paternally derived antigens and responses have been seen in the maternal circulation at the time of fertilisation and implantation (Bonney 2017). Animal models have also shown an increase in miscarriage, low birth weight in offspring where immune responses are supressed (Clark 2014, Faas and De Vos 2017).

Furthermore clinical data is emerging to suggest that dysfunctional immune processes at the time of implantation is associated with preterm delivery, preeclampsia and fetal growth restriction (Brosens, Muter et al. 2017).

1.4.1.2 *The danger theory*

This was proposed as a counter argument to the self-non-self discrimination theory (Matzinger 2002). This key principle was the idea that the immune system is activated by tissue damage resulting in the release of dendritic cells (DCs). These cells recognise molecular, cellular and oxidative stress. The DCs in turn stimulates a T cell response to repair the damage and remove the causative pathogen. If there is minimal tissue damage, then the T cell response is not launched. This theory approaches immune responses as a reaction to “danger” signals as opposed to attacking “non-self” tissue. In the context of implantation, there has been criticism as DCs infiltrate the healthy endometrium and an overwhelming T cell response is not seen (Pradeu and Cooper 2012). This could be as a result of DCs being modulated by other cells in the endometrium or T cell response being supressed by a lack of a “danger” signal.

1.4.1.3 *The homeostatic model*

In contrast to the self-non-self discrimination theory, the homeostatic model proposes that an over-activation of the immune system is essential in order to allow a pregnancy to develop (Mor and Cardenas 2010). The exact details by which the fetus is able to avoid attack is still not entirely understood but the following mechanism has been

proposed and is widely accepted (Braud, Allan et al. 1998). In order for the maternal immune system to recognise and launch a response against the fetus, it must be presented with major histocompatibility complexes (MHC) which are molecules that appear on target cells (Chen, Liu et al. 2011). Several subtypes of these molecules exist and it has been established that the trophoblast population exposed to the maternal circulation lacks MHC type I and type II (Bulmer and Johnson 1985, Gurka and Rocklin 1987). The extravillous trophoblast (EVT) cells that invade the maternal uterine arteries express MHC type I but this is modified to allow immune-tolerance (Redman, McMichael et al. 1984) . All three MHC subtypes interact with 2 families of receptors known as the killer cell like immunoglobulin receptor (KIRs) and CD94/NKG2 (Perez-Villar, Melero et al. 1997, López-Botet, Llano et al. 2000) . Both these are expressed by NK and T cells (Lee, Llano et al. 1998). Binding of the MHC subtypes with the KIRs inhibits the cytotoxic activity of uNK cells and it is this pathway that allows the placenta to develop.

Deciphering this mechanism and the fact that uNK cells infiltrate the stromal layer at the time of implantation indicates that they are integral to maternal recognition of the fetal tissue (Moffett-King 2002).

1.4.1.4 *The Th1 and Th2 paradigm*

An alternative theory that describes the immunological events in pregnancy is that of the T helper (Th) 1-Th2 paradigm (Nakagawa, Kwak-Kim et al. 2015).

This theory is based in the concept that all the immune cells present in the endometrium and decidua at the time of implantation secrete a balance of pro-inflammatory and angiogenic cytokines (Gellersen, Brosens et al. 2007). The Th1 response consists of the expression of IL2, TNFα and IFNγ which are detrimental to pregnancy; hence anti-inflammatory. This has been observed in women who have a history of infertility (Kalu, Bhaskaran et al. 2008).

Conversely the Th2 response consists of a nurturing microenvironment with the release of IL4, IL5 and IL10. The predominance of one state over the other is determined by both embryonic and maternal factors (Ledee, Petitbarat et al. 2016). Uterine NK cells have been hypothesised to facilitate a Th2 response and in turn this environment leads to a reduction in their cytotoxic function (Sojka, Yang et al. 2018).

Recently the Th1/Th2 balance has been deemed an insufficient explanation as many of the immune cells involved secrete similar cytokines allowing them to exhibit dual function (Kofod, Lindhard et al. 2018). Equally a Th2 environment has also been reported in women with infertility (Chaouat 2007). Therefore categorising cells and responses into discrete entities is deemed to be basic.

1.4.1.5 *The pro-inflammatory-anti-inflammatory theory*

The current thinking has moved onto proposing that there is a well-orchestrated inflammatory response by both the innate and adaptive immune system to facilitate implantation (Robertson, Jin et al. 2016).

In order for pregnancy to ensue, dynamic changes occur in the local immune response of the female reproductive tract (Liu, Diao et al. 2017). These mechanisms are interwoven with cyclic immune changes that occur in response to hormonal changes over the course of every menstrual cycle (Clancy, Baerwald et al. 2013). In the mid-secretory phase of the cycle, an innate immune response characterised by the migration of a multitude of immune cells including macrophages, DCs and neutrophils into the endometrium is seen. This response is amplified if conception and implantation occur. There is also overarching exponential rise in uterine NK cells which appear to be the key facilitators of this process.

Cytotoxic NK cells release highly active molecules known as granzymes and perforins in order to carry their inflammatory function (Bulmer and Lash 2005). The perforin molecule bores a hole in the surface of the target cell thereby making it redundant (Bulmer, Morrison et al. 1991, Kwak‐Kim, Chung‐Bang et al. 2003). The granzymes are powerful digestive enzymes that enter the target cells and cause it to degenerate (Manyonda 2006). NK cells can also employ their destructive function through a protein called the fas-ligand (Zamai, Ahmad et al. 1998). This is expressed on the surface of the NK cell and interacts with a fas protein on the target cell. As the two proteins interlock it causes the

target cell to release proteases that induce apoptosis (Biron, Nguyen et al. 1999). The mechanisms by which NK cells recognise their target cell is yet to be fully elucidated but the widely accepted theory is one where NK cells possess two signals; The “kill signal” and the “do not kill signal” (Kwak‐Kim, Chung‐Bang et al. 2003). Both these functions are mediated through activating and inhibitory receptors on the surface of the NK cells. Furthermore NK cell activity is counteracted by inhibitory receptors specific for MHC-type 1.If there is an absence or deficiency of the MHC type 1 receptor on the target cell, then NK cells become disinhibited and instigate lysis.

UNK cells are a major source of both angiogenic growth factors and cytokines. These include tumour necrosis factor (TNFα),interleukin (IL) 10, granulocyte macrophage colony-stimulating factor, LIF and interferon-gamma (IFNγ) (Diedrich, Fauser et al. 2007). They also respond to cytokines particularly IL-15 and IL-12. These are also produced by monocytes and DCs which in turn induce NK cell proliferation (Lee, Kim et al. 2015). It is namely the production of TNFα and IFNγ by the NK cells that promote placental development (Jokhi, King et al. 1994). Invasion and remodelling of the maternal vasculature requires rapid breakdown of the extracellular matrix surrounding the blood vessels (Smith, Dunk et al. 2009). uNK cells have been shown to secrete protein digesting enzymes known as MMP-2 and 9 that promote trophoblastic invasion and development of the placental tissue (Naruse, Lash et al. 2009).

This initial overwhelming inflammatory response by the uNK cells is subsequently followed by activation of the adaptive immune compartment. This is leads to an over expression of T cell response and the generation of a specific class of immune cells known as regulatory T cells (Treg cells). Treg cells counteract the inflammatory response by exerting potent anti-inflammatory effects. This balance of immune activity is essential at the implantation site in order to supress inflammation, assist in vascular remodelling, and curtail rejection of the conceptus tissue (Nederlof, Meuleman et al. 2017).

Recently a consensus has been reached within the field that no single theory can explain the immunological events of implantation and early pregnancy (Hyde and Schust 2016). The process is likely to be more nuanced than once described (Bonney 2017, Yang, Gilman-Sachs et al. 2019).

1.5 Infertility, assisted reproduction and uterine natural killer cells

The life changing phenomenon of conceiving and delivering a healthy baby is a challenging area of reproductive medicine. In humans the chance of attaining a pregnancy is around 15% within each menstrual cycle and is related to age of the female partner (Evers 2002).

The World health organisation (WHO) defines infertility as:-

“A disease of the reproductive system in which a pregnancy is not achieved despite 12 months of regular unprotected sexual intercourse” (Gnoth, Godehardt et al. 2005).

Globally it is estimated that around 15% of couples experience fertility problems (Homan, Davies et al. 2007). This same trend is seen in the United Kingdom (UK) with 1 in every 7 couples classified as infertile (HFEA 2009, HFEA 2012). Infertility not only causes emotional distress for the patients through stigmatisation, but poses implications on the healthcare system and society as a whole (Seibel and Taymor 1982, NICE 2013).

In the UK, couples are usually investigated by their general practitioner (GP) and referred to a specialist fertility clinic. The basic tests include an assessment of ovulation, tubal function and sperm parameters (Balen and Rutherford 2007).

Once a diagnosis is established treatment options include:-

1. Medical treatment- The use of medicines or drugs to correct ovulation disorders

2. Surgical treatment- The use of surgical techniques to treat tubal damage /endometriosis or correct an abnormality of the uterus

3. Assisted Reproduction technologies (ART):- The process of creating embryos outside of the body and transferring them into uterine cavity. The most common technique of in virto fertilisation (IVF) involves mixing the collected ovums from the female partner with sperm from the male partner under laboratory conditions. If there are anticipated fertilisation problem such as poor sperm numbers or quality, then a more specialised form of IVF known as Intracytoplasmic sperm injection (ICSI) is performed. This involves the micromanipulation of a single sperm with direct injection in to the oocyte (Belva, Henriet et al. 2007).

1.5.1 **The success and challenges of IVF**

It is now estimated that over 8 million babies have been born through IVF worldwide (De Geyter, Calhaz-Jorge et al. 2018).

Advances in ART have been facilitated through a clearer understanding of the events that lead to a successful pregnancy and the factors contributing to infertility. As a result, the success rates of IVF defined as achieving the birth of term healthy baby has steadily improved over the last decade from 11% in 1991 to 22% in 2016 (HFEA 2012, HFEA 2018).

Despite this encouraging trend, fertility specialists and researchers are in agreement that success rates could be better (Boomsma and Macklon 2006). The major barrier for most IVF cycles are a high occurrence of chromosomal defects and poor development seen in the

embryos created through culture (van Montfoort, Fiddelers et al. 2006). Despite this, even when high quality embryos are created, the implantation rates remain at around 25-35% (De los Santos, Mercader et al. 2003). High rates of implantation failure and early pregnancy loss in IVF is frustrating for both the fertility specialist and patients. This coupled with the widespread practice of transferring a single embryo into the uterus has led to IVF clinics searching for strategies that will increase the chance of successful implantation (Thurin, Hausken et al. 2004). Interestingly over 30% of IVF cycles in the UK are started due to unexplained causes of infertility (HFEA 2012, HFEA 2018).This is where both partners are healthy and have no reproductive dysfunction. Therefore research into identifying the molecular, genetic and immunological factors that underlie infertility and IVF outcome is needed (Cakmak and Taylor 2011). Furthermore a reliable marker of optimal endometrial receptivity needs to be determined so that the correct timing of embryo transfer can be defined (Lessey 2011).

1.5.2 **The IVF process**

During IVF the events that normally occur in the fallopian tube (fertilisation) and uterus (implantation) are circumvented. Treatment is initiated with the intention of overcoming identified barriers that may be contributing the infertility faced by the couple. The processes that occur naturally are mimicked and optimised in the laboratory setting. Eggss and sperm are fertilised in a culture dish (in vitro) outside the body. The embryos that result are then artificially transferred in the uterine cavity (figure 1.3).

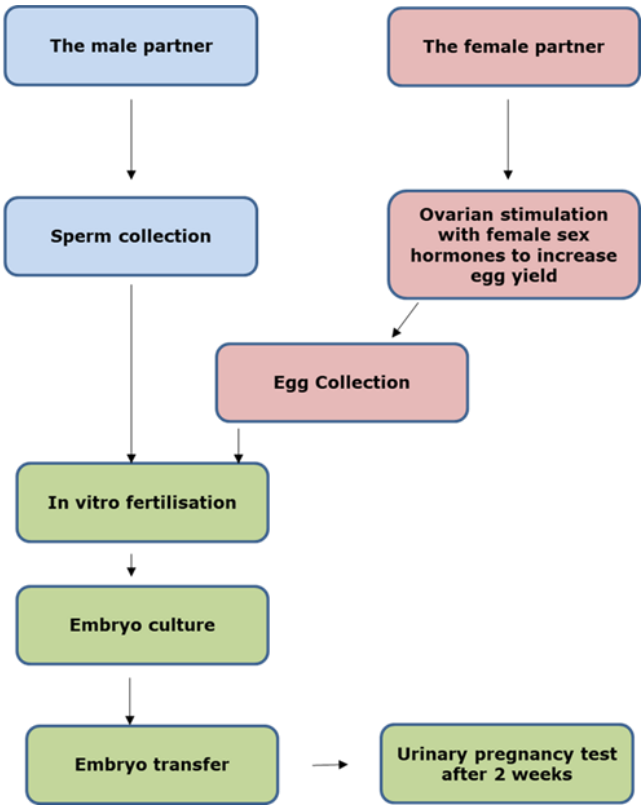


Figure 1-3- The process of IVF

The IVF population has provided the best insight into all aspects of the implantation (Rinehart 2007). The process itself optimises the fertilisation and development of the embryo and therefore reduces the impact of embryonic factors. Physically transferring the embryo into the uterine cavity also eliminates the anatomical factors that may hinder the path of the embryo. As a result of overcoming these factors it is hypothesised that implantation rates should be close to 90%. However in reality the success of IVF is still around 30%-40% even when high quality embryos are created (Voullaire, Wilton et al. 2002).

This alludes to the fact that the interplay between the embryo and endometrium is still not fully understood. Furthermore the state of endometrial receptivity must determine the success of both spontaneous and IVF pregnancies.

1.5.3 Endometrial receptivity and unsuccessful IVF cycles

The process of ovarian stimulation, egg collection and embryo culture are largely understood (Mitri, Nayot et al. 2016). Several developments in fertility drugs and culture techniques have led to an optimisation in the number and quality of embryos that are transferred in IVF cycles (Cutting 2018). In contrast the same improvements have not been observed in endometrial receptivity and its defective nature is seen as an important cause of the low embryo implantation rates seen in IVF cycles (Lessey 2011). Furthermore experience from donor egg cycles where the embryos are created from good gametes show higher implantation rates (Diedrich, Fauser et al. 2007). This has been attributed to the fact that patients undergoing IVF with donor egg ovums are given oestrogen and progesterone sequentially to optimise their endometrium for embryo transfer. High doses of FSH/LH needed for ovarian stimulation are not required and this negates the detrimental effect on the endometrium (Fatemi and Popovic-Todorovic 2013).

1.5.3.1 *Implantation failure in IVF cycles*

Implantation failure is defined the inability of the embryo to reach a stage where there is a visible intrauterine sac discernible on an ultrasound scan (Coughlan, Ledger et al. 2014). In the IVF setting the appearance of an intrauterine gestation sac is usually confirmed by ultrasound around 3 weeks after egg collection. A clearer understanding of the mechanisms that govern implantation may allow clinicians to treat infertility, prevent early pregnancy loss and improve success rates of IVF.

Unsuccessful implantation can be differentiated into maternal and embryonic factors. Dysfunctional endometrial receptivity is responsible for 60-70% of implantation failures whereas the remaining cases are caused by poor embryo quality (Ledee-Bataille, Lapree-Delage et al. 2002).

Defects in embryo morphology and development usually have a genetic basis (Das and Holzer 2012). Assessment of maternal factors contributing to unsuccessful implantation is more complex. These include age related poor egg quality (Ferraretti, La Marca et al. 2011) and structural abnormalities of the uterus such as a congenital septum (Raga, Bauset et al. 1997), polyps or fibroids within the cavity (Taylor and Gomel 2008, Bosteels, Weyers et al. 2010). Looking more widely blood clotting disorders such as antiphospholipid syndrome (Qublan, Eid et al. 2006) and immunological conditions of which NK testing is a component have also been implicated but not entirely proven (Stephenson and Fluker 2000).

1.5.4 **Recurrent implantation failure (RIF)**

RIF is a condition specific to those pregnancies conceived through ART. It covers a spectrum of disease ranging from infertility to the loss of pregnancy at various gestations.

Although definitions of RIF vary (Polanski, Baumgarten et al. 2014), the most widely accepted is one where there is no pregnancy achieved after a minimum of 3 cycles of fresh/frozen IVF and where at least 4 good quality embryos have been replaced (Coughlan, Ledger et al.

2014). The prevalence of RIF is largely unknown but studies have estimated it to occur in around 10% of IVF cycles (Margalioth, Ben-Chetrit et al. 2006). The prevalence increases with each unsuccessful attempt (Roberts and Stylianou 2012).

The causes of RIF are multifactorial. They can be divided into embryo related and maternal factors (see figure 1.4)

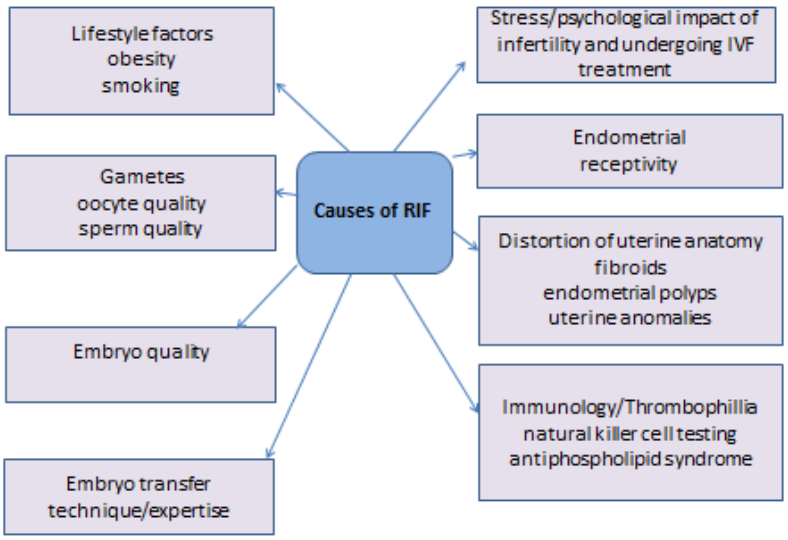


Figure 1-4- The causes of RIF

RIF is a perplexing and frustrating clinical outcome for both patients and clinicians. The emphasis clinicians place on the available tests and the extent to which they investigate women with RIF is highly variable (Kwak-Kim, Han et al. 2013). Part of the reason for this variation in patient care is attributable to the fact that there is no clear or robust evidence to advocate any of the investigations that are offered (Clark, Coulam et al. 2006). Furthermore once diagnosed, there are very few treatments available with debatable proven efficacy. The two most recent systematic reviews investigating the role of NK cells in

reproductive disorders have both concluded good quality studies seeking to clarify the diagnosis and treatment of RIF are much needed (Tang, Alfirevic et al. 2011, Seshadri and Sunkara 2014).

1.5.5 NK testing in the context of RIF

Patients who present with RIF and how uNK cells may play a role is an important area of research as changes that occur within the endometrium and how NK cells facilitate implantation is currently poorly defined. Furthermore patients with RIF are offered NK testing without any robust guidance or treatment options. This is where this research will assist in clarifying the need for testing and treatment.

uNK cells are the most abundant immune cell present in the endometrium at the time of embryo implantation. This has led to the assumption that they must be involved in the normal and dysfunctional aspects of the process (Moffett-King 2002).

It was work performed by Beer et al (Beer, Kwak et al. 1996) that first cited that women with RIF and miscarriage have a higher percentage of peripheral NK cells. They noted that levels below 12% were conducive to pregnancy and, in their cohort of patients no pregnancies were seen at levels above 18%. Following this initial conclusion, several cohort studies have described an association of NK cells with RIF in both the blood and endometrial samples (Lachapelle, Miron et al. 1996, Coulam and Roussev 2003, Tuckerman, Mariee et al. 2010).The results of these studies have yielded mixed results with regards pregnancy outcome. The lack of conclusive evidence is multifactorial with researchers using

differing populations and analysis methods-immunohistochemistry versus flow cytometry (Tang, Alfirevic et al. 2011). Furthermore the reference ranges used within the studies are variable with no uniformly accepted parameters (Toth, Würfel et al. 2011). Despite this heterogeneity within the studies, currently the most widely accepted uNK cell levels is defined as less than 5% of the endometrial stromal cells (Quenby et al 2005). In the context of pbNK cells, normal levels vary in the literature from less than 12% to 18% (Seshadri and Sunkara 2014).

As with most uncertain areas in clinical practice, there is a huge degree of heterogeneity within the studies with varied numbers, availability of a control group and definitions of what is classified as RIF (Makrigiannakis, Petsas et al. 2011). The methods used to establish NK cell status are flawed with investigators measuring different phenotypes as well using a wide variety of reference ranges and techniques (Tang, Alfirevic et al. 2011). The clinical rationale for NK testing is based on the notion that raised NK cells directly “attack” the fetus and hence prevent pregnancy from developing normally (Hill, Polgar et al. 1995). However there is a paucity of evidence to show that NK cells directly interact with the fetus (Moffett and Colucci 2014). Their function is mediated through the trophoblastic and decidual cells only. The two largest systematic reviews (Tang, Alfirevic et al. 2011, Seshadri and Sunkara 2014) also showed that women with raised NK cells had poorer reproductive outcomes both in terms of RIF and miscarriage. However they both

concluded that NK testing should only be reserved for research purposes until the evidence base becomes more robust.

The mechanism by which NK cells contribute to poor outcomes in women with infertility is largely unknown (Bulmer and Lash 2015). It was initially hypothesised that uNK cells caused RIF and miscarriage through their cytotoxic activity against the implanting embryo (Hill, Polgar et al. 1995). However this theory has since been discounted as there is no evidence to support that NK cells are detrimental to the early trophoblast cells (Moffett and Colucci 2014).

Other authors have surmised that it is not the abundance of NK cells that are detrimental but the imbalance between the dim and bright ratio of CD56+ cells within the uterine environment that plays a significant role (Matteo, Greco et al. 2007, Manaster, Mizrahi et al. 2008). It has been proposed that women with reproductive failure have a higher proportion of CD56 ^{dim} cells that are the predominant type in the blood. These are not naturally present in the endometrium and exhibit stronger cytotoxic activity, hence preventing implantation (Matteo, Greco et al. 2007). They also alter the NK subtype ratio in the uterine environment and it may be the imbalance between fewer CD56+ ^{bright} cells that leads to implantation failure (Makrigiannakis, Petsas et al. 2011).

Another emerging theory is that of uNK cells altering the blood flow to the developing fetus (Quenby, Nik et al. 2009). It is known that implantation and development of the placenta occurs under hypoxic conditions of 2-3% oxygen (Yedwab, Paz et al. 1976). As a result of this

the syncytiotrophoblast undergoes oxidative hypoxic damage in this environment, facilitating its ability to adapt to higher oxygen levels as pregnancy progresses (Hung, Skepper et al. 2001). Studies looking at this phenomenon in the context of recurrent miscarriage have found that there is inappropriate blood flow to the developing fetus in women with a poor pregnancy outcome (Hustin, Jauniaux et al. 1990, Hempstock, Jauniaux et al. 2003). There seems to advanced endometrial blood vessel development in women who miscarry spontaneously (Goswamy, Williams et al. 1988). This has been correlated with the percentage of uNK cells present in mid secretory endometrium (Le Bouteiller and Tabiasco 2006). It is therefore proposed that a high level of uNK activity causes a more pronounced effect of EVT invasion and spiral artery remodelling (Quenby, Nik et al. 2009). This is detrimental to the embryo as increased blood flow at the implantation site compromises the hypoxic environment that is conducive to pregnancy viability. Whilst a trend in the numbers of uNK cells is emerging in the context of infertility and pregnancy loss, little is known about alterations in the function of these cells (Moffett-King 2002).

Despite these uncertainties, NK testing is widely offered to women who have 2-3 cycles of unsuccessful IVF treatment and therefore deemed to have RIF (Kwak-Kim, Han et al. 2013). It is also offered in the context of recurrent miscarriage (Tang, Alfirevic et al. 2013). It remains uncertain as to whether they are the cause or effect of reproductive dysfunction (Wold and Arici 2005). There is also much debate in the literature as to

whether the evidence is robust enough to support the wide spread testing that is apparent (Rai, Sacks et al. 2005, Tuckerman, Mariee et al. 2010, Robertson, Jin et al. 2016). Some experts (Moffett and Shreeve 2015, Sacks 2015) argue that although challenges exist with testing, in reality most patients want to know their immune status. They also agree that a standardised method of NK cell testing should be introduced (Chen, Mariee et al. 2017) and large adequately powered randomised controlled trial (RCT) conducted looking at whether women with raised NK cells will benefit from immunotherapy (Boomsma and Macklon 2008). Nevertheless a whole industry has emerged within the field of IVF that offer testing and non- evidence based treatment aimed at normalising raised NK cells. This includes the use of Immunoglobulin (IVIG) infusions, oral steroids and intralipid aimed at supressing detrimental levels of NK cells.

Oral steroids such as prednisolone are used in other areas of medicine to supress immune responses such as inflammation and allergy (Urbanowicz, Lamb et al. 2010). It has been shown that prednisolone treatment in the non-pregnant uterus leads to a reduction in uNK cell numbers as well blood vessel development (Quenby 2005). There are currently very few trials that have looked at the effects of prednisolone on implantation failure. The majority of studies have small numbers with flawed methodology making it difficult to draw any robust conclusions. Clarification of the role of NK cells in implantation and the most effective method of treating abnormal levels is much needed (Seshadri and Sunkara 2014).

1.6 NK testing techniques

NK Testing is widely offered and utilised by patients in IVF and recurrent miscarriage clinics. In recent years a debate has started within the literature both scientifically and clinically as to the relevance of NK cells testing in the context of the implantation process and success of pregnancy (Moffett and Shreeve 2015). Traditionally pbNK cells are measured using flow cytometry whilst uNK cells are measured with both flow cytometry and immunohistochemistry (IHC).

1.6.1 Flow cytometry

Flow cytometry can be used to measure subtypes of cells and their activation state. The tissue has to be processed immediately on collection and therefore this is ideal for testing blood but not endometrium.

The principles of flow cytometry involve isolating the cells of interest and placing them in a liquid suspension. The cells are passed through a laser beam or light source and separated according to their size and how they scatter light. NK analysis involves the use of fluorescent dyes called flourochromes that are attached to antibodies. These antibodies are specific for the antigen present on the NK cells such as CD56 and/or CD16. The antibodies will attach to the different subtypes and then the numbers are calculated by a detection device that measures the degree of illumination by the attached flourochrome. Figure 1.5 shows how flow cytometry is conducted.

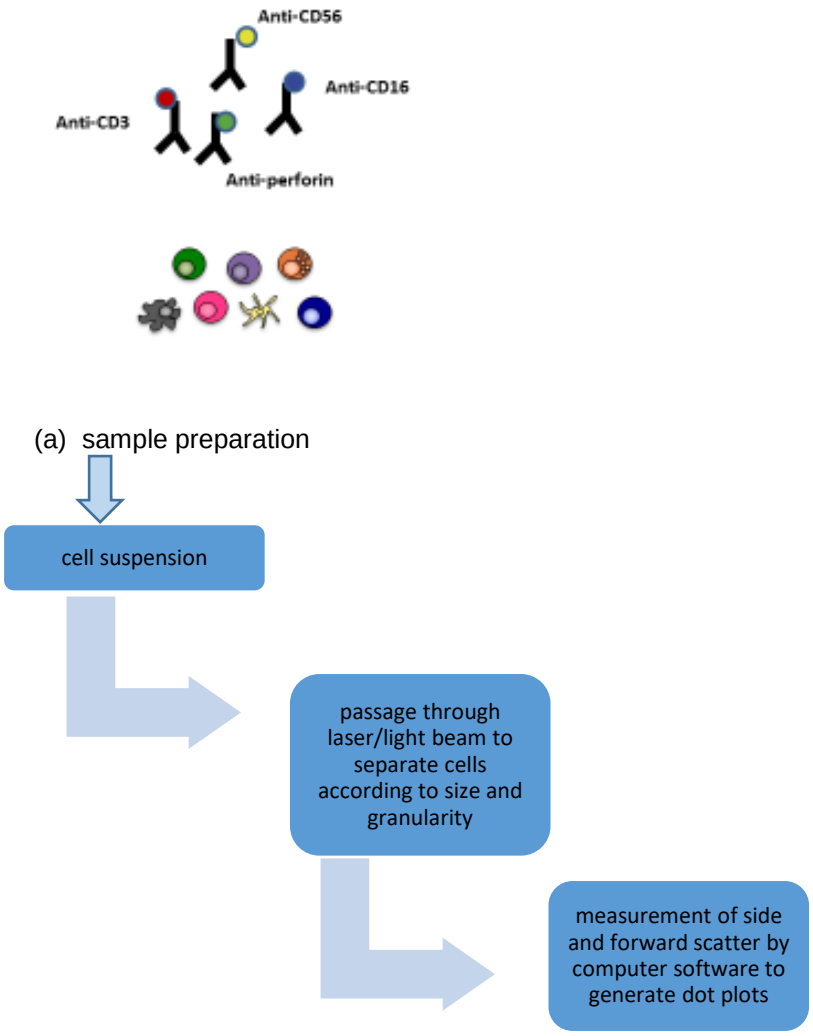


Figure 1-5- The stages of flow cytometry

The degree of illumination obtained from flow cytometry are presented as dot plots where the cells of interest are present or absent. This allows the % numbers of cells to be measured and functional activity to be assessed. The demarcation of the quadrants is dependent of the gating strategy which is turn dependent on positive and negative control. Figure 1.6 shows a dot plot for the expression of perforin and granzyme by NK cells as a worked example. The cells are separated into 4 quadrants on a grid representing their absence or presence.

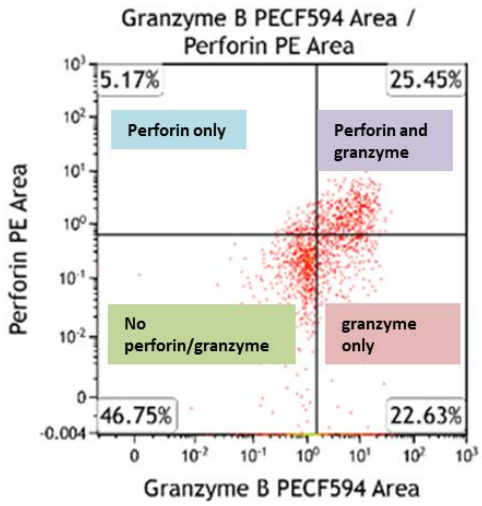


Figure 1-6- The interpretation of flow cytometry dot plots

The dot plot appears as a grid chart with 4 quadrants. The 2 quadrants on X axis represent the presence/absence of the marker plotted horizontally e.g. granzyme. Similarly the 2 quadrant along the y axis outline the presence/absence of the marker plotted vertically e.g perforin. The quadrant in the bottom left hand side invariably depicts cells that express none of the measured marker.

Sampling the endometrium or blood for FC involves taking a large sample and this can be invasive to patients. Conversely immunochemical staining can be carried out on small biopsies which do not require immediate assessment. They can be fixed in wax paraffin and processed at a later date without losing the integrity of the tissue.

NK cell testing varies from clinic and laboratory and there is there is no consensus regarding reference ranges and which patients should be offered the test. One of the biggest challenges faced by researchers and clinicians is the observation that NK cell numbers have no bearing on their activity. Therefore assessing number and their function require different tests and that is where FC has an advantage over IHC.

1.6.2 **Immunohistochemistry testing of uNK cells**

Immunohistochemistry (IHC) has traditionally been used to stain and assess NK cell levels in the endometrium.

This technique involves staining the selected tissue for the CD56 antigen using a highly selective primary antibody, and subsequently counting those cells taking up the stain down the microscope. The results are often reported as the percentage of stained cells in relation to the total number of endometrial stromal cells (Santillan, Lozano et al. 2015).

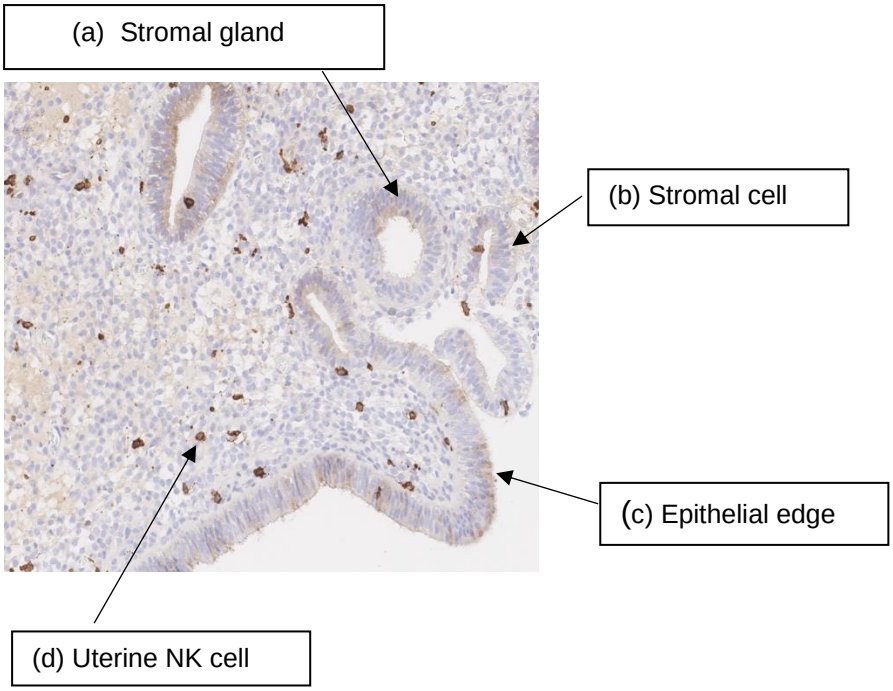


Figure 1-7- Computerised microscopic image produced by IHC staining of the endometrium

This figure shows the stromal glands which are generally avoided when selecting a section for analysis. This normally includes the epithelial edge (c), with the primary and secondary antibody reaction leading to staining the NK cells brown (d). The addition of haematoxylline will complete the staining and highlight the stromal cells blue (b).

1.6.2.1 *Advantages and pitfalls of IHC*

Although IHC is a quick, easy and widely available technique, it cannot be performed on peripheral blood. Staining the cells using IHC can also yield limited information as not all the subtypes of the CD56 antigen are tested. It is also very subjective and operator dependent as counting cells is prone to error. The endometrium itself can be difficult to assess as there can be clumping of NK cells in the glandular tissue. The inclusion of these hidden pockets of cells within the overall count can give completely different results.

1.6.2.2 *Factors affecting NK levels by IHC testing*

There are numerous factors that have been cited to affect the NK levels. The most significant of these is the timing of the test as NK cells

fluctuate throughout the menstrual cycle. They are also influenced by maternal age, BMI and to some extent inter observer variability.

1.6.2.2.1 Endometrial biopsy taking and uNK status

There is evidence to suggest that the process of taking the endometrial biopsy for assessment of uNK is beneficial and can affect these cells in the endometrium (El-Toukhy, Sunkara et al. 2012) .This medical procedure known as endometrial scratching has also been associated with encouraging angiogenesis (Gnainsky, Granot et al. 2010) as well the release of pro-inflammatory cytokines such as TNFα, IL15 and IL11 (Nastri, Ferriani et al. 2013). There is evidence to suggest that it improves implantation (Potdar, Gelbaya et al. 2012) and live birth in RIF patients (Vitagliano, Di Spiezio Sardo et al. 2018).

1.6.2.2.2 BMI and uNK status

High BMI (>30) or obesity has been associated with early pregnancy loss such as miscarriage as well as obstetric complications of gestational diabetes, pre-eclampsia and need for operative delivery (Athukorala, Rumbold et al. 2010).

Observational studies have shown that women with high BMI have impaired or low levels of NK cells in the endometrium (Perdu, Castellana et al. 2016, Jung, Park et al. 2018). This has a detrimental effect on both the development of the placenta and the global gene expression within the uterine environment.(Muktabhant, Lumbiganon et al. 2012, Lee, Kim et al. 2016).

1.6.2.2.3 **Age and uNK status**

Studies looking at the epidemiology of uNK demonstrate that levels fall as humans age(Solana, Alonso et al. 1999). This has been linked to the age related increase in inflammatory and malignant disease (Hazeldine and Lord 2013, Naumova, Pawelec et al. 2016).

1.6.2.2.4 **Timing of testing and the influence of the menstrual cycle**

Analysis of uNK levels is highly influenced by the day of the menstrual cycle on which the biopsy is taken (Liu, Mariee et al. 2014). In humans, the proportion of uNK cells increase abundantly 6-7 days following ovulation reaching a peak level by 20 weeks of gestation, after which they gradually regress until term (Lima, Zhang et al. 2014). The mechanisms whereby uNK cells infiltrate the endometrium have been extrapolated from animal studies in mice. These suggest that uNK cells arise from a distinct tissue-specific lineage derived from tissue-resident NK cell precursors (Sojka, Plougastel-Douglas et al. 2014). Conversely they may also appear due to an influx or tissue-homing potential of peripheral NK cells into the uterus. This notion is supported by bone marrow transfer experiments in mice showing that NK cells homing to the uterus repairs uterine arterial defects observed in NK cell–deficient mice (Ashkar and Croy 2001, Zhang, Yamada et al. 2009).The menstrual cycle is under hormonal control, which primarily mediates changes in the architecture and function of the endometrium. Therefore sampling at different times in the cycle will give variable NK cell counts. Results are also affected by presence of oedema in the endometrial biopsy as well the site of sampling and depth of tissue taken (Moffett,

Regan et al. 2004). The majority of clinics sample in the luteal phase, which can be confirmed by the LH surge +7-9 days. In the decidualisation window, studies have shown that there is a dramatic increase in the number of NK cells (5 to 40% of stromal cells) over 10 days (Tuckerman, Mariee et al. 2010).

1.6.2.2.5 uNK status and inter observer variability

There is controversy over the exact reference range of what constitutes an abnormal level. Some studies have defined this as 5%(Quenby, Kalumbi et al. 2005, Kuroda, Venkatakrishnan et al. 2013) whilst others have set it at 13.9% (Wong, Archer et al. 2014). There have been recent efforts in the literature currently to standardise testing and inter-observer variability (Mariee, Tuckerman et al. 2012, Lash, Bulmer et al. 2016, Chen, Mariee et al. 2017). The ideal clinical test would therefore be interpreted according to a reference range for each day for the menstrual cycle. It is therefore imperative that laboratories create a reference range for each day. There could lead to a discrepancy where patients have been labelled as having high NK cells when in reality this may have a normal level for the time in that cycle. After the initial surge in uNK numbers there is very little change in the luteal phase (Koopman, Kopcow et al. 2003). Therefore if a change has been detected between women with poor pregnancy outcomes and normal controls, this may be due to a delayed or accelerated response of the endometrium as a whole as opposed to pathological levels in one group over the other (Russell, Sacks et al. 2013).

Deviations in the hypothalamic/pituitary/ovarian (HPO) axis will affect all aspects of the menstrual cycle including the numbers of uNK at various stages (Kosova, Stephenson et al. 2015). Mariee et al (Mariee, Tuckerman et al. 2012) also showed a 30% discrepancy between uNK cell numbers when sampling the endometrium in two consecutive menstrual cycles. Although the sample size of 10 women was small, there was wide variation in the results. The author concluded that the difference in results was due to biological variability as opposed to intra-observer variability. Therefore selecting the best test for NK analysis as well the right timing needs to be determined.

1.7 Treatment options for patients with recurrent implantation failure

In recent years a wide range of immune therapies has been introduced to treat patients with unsuccessful IVF, recurrent implantation failure (RIF) and recurrent miscarriage without much regard for their safety profile (Moffett and Shreeve 2015).

The rationale for the use of immune therapies to address inadequate uterine receptivity and improve implantation rates has been derived from animal models, human in vitro systems and observational data in women with recurrent implantation failures following IVF.

1.7.1 Immunotherapies for RIF patients

The most commonly used immune therapies cited in the literature for women with a history of failed implantation include:-

- glucocorticoids,
- intravenous immune globulin (IVIG)
- lymphocyte immune therapy (LIT)
- administration of peripheral blood mononuclear cells (PBMC)
- Leukaemia inhibitory factor (LIF)
- growth colony stimulating factor (G-CSF)
- anti-TNFα therapy
- Intralipid

The main effects of these agents is to modulate the immune system and facilitate implantation. Glucocorticoids exert a range of positive effects, such as suppression of uterine NK cells and stimulation of hCG

secretion, as well as promotion of trophoblast proliferation and invasion (Michael and Papageorghiou 2008). Similarly, IVIG has been used for early reproductive failure in a variety of regimens,(Clark, Coulam et al. 2006) LIT is administered in the form of allogenic immunisation with paternal lymphocytes (Carp, Toder et al. 1990, Günther, Alkatout et al. 2018). PBs have been administered into the uterine cavity, in the form of a combination of cultured and freshly isolated PB, before embryo transfer (Nakayama, Fujiwara et al. 2002, Yoshioka, Fujiwara et al. 2006). Although, heparin is an anticoagulant, it is used in the context of RIF as it has some immunomodulatory effects (Nelson and Greer 2008).

1.7.2 **Treatment options for raised NK cells**

Several of the treatments described above have been proposed to either supress the detrimental high NK cell numbers or correct the imbalance between the different subtypes. These include agents such as intravenous IVIG, intraplipid and varying courses of oral steroids.

A recent systematic review looking at therapeutic interventions aimed at treating raised peripheral NK cells and their effects on reproductive outcomes concluded that there was a lack of evidence to support the use of treatment (Polanski, Barbosa et al. 2014). The authors found 3 small studies, 2 of which were case-control in their design (Winger, Reed et al. 2011, Moraru, Carbone et al. 2012) and a randomised controlled trial (Alhalabi, Samawi et al. 2011).

The studies by Moraru and Winger (Winger, Reed et al. 2011, Moraru, Carbone et al. 2012) looked at the effects on IVIG on women with recurrent miscarriage and implantation failure. IVIG is thought to work by reducing NK cell numbers (Maddur, Vani et al. 2011) and promoting an immune system shift towards the Th2 response. This is conducive to pregnancy and also facilitates a reduction in fetal graft rejection. Both studies showed an increase in the clinical pregnancy and live birth rate in women who had IVIG compared to controls. However the studies were observational and heterogeneous in their selection of the study population. The duration and dosage of IVIG was different in both populations as were the methods used to assess NK cell numbers and activity. The patients in the study by Winger et al (Winger, Reed et al. 2011)received steroids as well as heparin alongside their IVIG. It is therefore difficult to tell which agent was beneficial in promoting pregnancy.

Alhalabi (Alhalabi, Samawi et al. 2011) looked at the administration of 20mg of oral prednisolone to women undergoing ICSI treatment. The authors found that the clinical pregnancy rate in women with high pbNK cells was 48% in prednisolone treated group as opposed to 29% in the non-treated group. The study had a RCT design but lacked a placebo group and the duration of steroid use was not specified. The reviewers concluded that adequately powered RCT studies with valid patient selection and consistent NK cell testing was required.

1.7.2.1 *The use of oral steroids as a treatment option in women with raised NK cell levels*

Prednisolone is a synthetic glucocorticoid that is used to treat inflammatory conditions such as asthma and connective tissue disorders (Parrillo and Fauci 1979) .Once bound to its receptor on the target cell, it is able to inhibit the synthesis of many inflammatory cytokines and adhesion molecules (Waller and Sampson 2013).

Experiments in vitro and in vivo have shown that oral prednisolone has the ability to inhibit the cytotoxic properties of pbNK cells (Callewaert, Moudgil et al. 1991). This is thought to be mediated through suppression of granzyme activity (Zhou, Olsen et al. 1997). Further evidence from vitro studies show that uNK cells have a receptor that binds to glucocorticoids (Thum, Bhaskaran et al. 2008). Although not entirely proven, it is also thought that prednisolone suppresses NK cell numbers and activity through their inhibitory effect on cytokine production (Vitale, Chiossone et al. 2004). They may also have a role in controlling the migration of the cytotoxic peripheral NK cells into the uterine environment which can be detrimental to implantation (Xu, Makris et al. 2005).

The latest Cochrane review (Boomsma, Keay et al. 2012) has shown no clear evidence that administration of steroids around the time of implantation in IVF cycles improves the clinical outcome .However this review excluded women with a history of RIF.

Dan et al (Dan, Wei et al. 2015) have recently looked at the efficacy of oral steroids in women undergoing ICSI. In their meta-analysis of 2 RCTs (Ubaldi, Rienzi et al. 2002, Duvan, Ozmen et al. 2006) the authors concluded that there was no improvement in pregnancy or implantation rates with oral steroids. Although both these studies had a good study design with double blinding and placebo controlled randomisation, they differed in their patient selection and steroid regimen. Ubaldi et al (Ubaldi, Rienzi et al. 2002) randomised 313 patients with a history of 3 or less ICSI cycles to a 4 week course of oral prednisolone (10mg) on the day of ovarian stimulation .They found no difference in the pregnancy and the implantation rates between the control and treatment groups (50.0 and 23.3% versus 49.0 and 23.6% respectively). Conversely Duvan et al (Duvan, Ozmen et al. 2006) randomised 200 women undergoing their first cycle of ICSI into 4 groups. The groups were given either aspirin, oral prednisolone, the two interventions together or placebo based on randomisation carried out on the day of embryo transfer. This study also found no difference in pregnancy and implantation rate between the 4 groups.

Although there is sparse data to support the use of steroid on uNK cells in the context of IVF or RIF, there are some trends that can be drawn from studies conducted in women with recurrent miscarriage. (Quenby and Farquharson 2006) studied 75 women with a history of recurrent miscarriage and 18 controls with raised uNK cells. They administered a 3 week course of oral prednisolone from day 1-21 in the menstrual cycle and repeated the uNK cell biopsy in the mid secretory phase of

the same cycle. The median NK cell level fell from 14% to 9% which was clinically significant.

Gomaa et al (Gomaa, Elkholy et al. 2014) randomized 160 women with a history of recurrent miscarriage to oral prednisolone, heparin and aspirin or a combination of placebo, heparin and aspirin. They concluded that the combination of the three active agents was beneficial in the continuation of pregnancy beyond 20 weeks gestation (70.3% of women remained pregnant whilst 29.7 % miscarried). This is in comparison to the 9.2% in the placebo group having a successful outcome while 90.8% miscarried. There was also a reduction in CD16 positive NK cells in the group that had steroid therapy and therefore this may be the mechanism whereby steroids exert their nurturing effects.

A feasibility study in the context of recurrent miscarriage where 40 women with raised uNK levels were randomised to a course of oral steroids or placebo at 4-5 weeks gestation found a live birth rate of 60% in the treatment group (Tang, Alfirevic et al. 2013) .The authors also looked a patient acceptance of the treatment and testing. They found that women were willing to participate and were motivated to be randomised despite knowing they had a raised uNK level. There were no pregnancy related complications or adverse side effects to the women.

Discussion and conclusions from the literature show a paucity of studies looking at effects of raised uNK cells in the context of women with RIF (Boomsma and Macklon 2006). Hence a well-designed RCT aimed at

administering oral prednisolone to women with RIF is clinically and academically needed.

1.8 Conclusion

Implantation involves close collaboration between the embryo and endometrium. Its success depends on the competency of the embryo and timely receptivity of the endometrium (Navot, Scott et al. 1991).This intricate process is facilitated by multiple mediators including anatomical, hormonal and immunological factors.

Disorganisation at any stage of embryo development and/or endometrial receptivity can lead to reproductive failure as well as placental and fetal growth dysfunction.

Much of the detailed pathways that control implantation are poorly understood, however researchers are in agreement that it is endometrial receptivity that holds the key to understanding female infertility and its treatment (Guzeloglu-Kayisli, Basar et al. 2007).

The role of NK cells in successful implantation and endometrial receptivity needs further clarification. The widespread availability of IVF has led to many couples conceiving. However implantation rate remain low despite advances in technique and experience. RIF and unsuccessful IVF cycles remains a challenge for patients and their fertility specialists. The role of NK cells in RIF needs to be fully evaluated. This is where this research will help to clarify whether uterine NK testing is a suitable screening test for those with unsuccessful IVF treatment and which therapeutic modality is effective in its treatment.

1.9 Aims and Objectives

As result of comprehensively reviewing the literature the research questions in this thesis are:-

1. What is the prevalence of raised NK cells by immunohistochemistry (IHC) in women undergoing IVF?
2. Are there any predictive demographic features such as previous IVF history that influence raised IHC NK status and subsequently live birth?
3. Does the application of a comprehensive flow cytometry (FC) panel provide a more detailed analysis of NK phenotype and function in those undergoing IVF treatment?
4. Are there other immune cell populations such as NKT and cytotoxic T cells that influence pregnancy outcome in IVF?
5. Are there any cytokines produced by NK and other immune cell populations that specifically influence the success of pregnancy?
6. How does IHC and FC compare to one another and are they as equally predictive of live birth through IVF?
7. After systematically appraising the current literature which immune therapy is effective in women undergoing IVF following a history of RIF?

1.10 Hypotheses

There are 7 hypotheses in this thesis

1. The prevalence of raised NK status will be greater than 20% in women undergoing IVF treatment (Chapter 3)
2. An increase in the number of previous unsuccessful IVF cycles will lead to a higher prevalence of raised NK status (Chapter 3)
3. A raised NK status will be associated with a reduced chance of live birth (Chapter 3)
4. The application of a flow cytometry (FC) panel will lead to the identification of other immune cell populations and provide a functional aspect to testing (Chapter 4 and 5)
5. The FC panel will reveal immune cell populations or cytokines that are associated with IVF success (Chapter 5)
6. Stratification according to IHC NK status will reveal further trends in the FC data (Chapter 6)
7. A systematic review of the literature will support the use of oral steroid treatment as an immune therapy for RIF (Chapter 7)

Chapter 2

Materials and Methods

Chapter 2 Materials and Methods

2.1 Introduction

This Chapter describes the laboratory methods used in this thesis.

2.2 Ethical approval

This research was supported and approved by the Human Tissue Authority-Nottingham Health Science Biobank (NHSSB) sited within the Nottingham University Hospital NHS Trust. NHSSB is ethically approved by the North West-Greater Manchester Central Research Ethics Committee (REC) reference: 15/NW/0685. All patient gave written, informed consent.

2.2.1 Setting

Patients undergoing both fresh and frozen in vitro fertilisation (IVF) cycles at NURTURE fertility, Nottingham, UK were approached from June 2016 to October 2017 at different stages of their fertility history. Some participants were having their first cycle of treatment whilst others were presenting after multiple unsuccessful cycles.

One of the main strengths of this work was that women were recruited from a highly successful IVF clinic .The live birth and clinical pregnancy rates at NURTURE fertility are around 10% higher than the national average (HFEA 2018).(table 2.1).

Table 2-1- A comparison of clinical pregnancy rates between NURTURE fertility and the national average from 2015-2016

Live birth rate (%per embryo transferred)-fresh IVF cycles		
Age group (yrs)	National average	NURTURE fertility
All ages	21	32
Under 38	27	39
38 and over	11	17
Clinical pregnancy rate (% per embryo transferred)		
fresh IVF cycles		
Age group (yrs)	National average	NURTURE fertility
All ages	24	34
Under 35	38	47
35-37	33	47
38-39	25	35
40-42	19	29
frozen IVF cycles		
All ages	25	33
35-37	33	61

The higher success rates are attributable to stringent laboratory processes leading to the creation of optimal quality embryos and their transfer. Therefore it is reasonable to assume that if IVF did not result in a successful pregnancy, then endometrial receptivity or adverse maternal immunology could be valid contributory factors.

2.2.2 **Sample collection**

All patients were given written information about endometrial scratching and NK biopsy at their medical consultation by an IVF specialist. In order to ensure that the NK biopsy result was available before the embryo transfer, patients were advised to attend the unit one month before undergoing IVF treatment.

When patients attended for their endometrial scratching, verbal information about the blood sample and flow cytometry (FC) assessment was given. If they agreed to participate, a written consent form was completed. An endometrial sample was taken for NK testing

through immunohistochemistry (IHC) and FC analysis. A venous peripheral blood (PB) sample was also taken so that there were paired samples of blood and endometrium for the FC analysis.

2.2.2.1 *Endometrial scratching and biopsy collection*

The procedure was carried out in the mid luteal phase of the menstrual cycle (day 19-24) with a pipelle de cornier ® (CCD, Paris) sampler. The cervix was visualised using a cusco's speculum. The pipelle sampler was introduced into the uterus until resistance from the fundus was felt. The inner piston was withdrawn to generate negative pressure. The device was subsequently rotated through 360 degrees to allow equal coverage of the endometrial cavity. It was also moved in and out of the cavity for a maximum of 30 seconds. This was performed to allow the pipelle device to fill with endometrial tissue.

Occasionally, in order to gain entry into the endometrial cavity a stabilising and traction device (vulsellum) was applied to the anterior lip of the cervix. If entry with the pipelle biopsy taker could not be gained (due to a tight/narrow cervical os or acute cervico-uterine angle), then a malleable embryo transfer catheter was used (Cook, Medical, UK) as an alternative device. In such cases, the catheter was introduced up to the fundus. The inner stylet was removed and a 10 ml syringe was attached to the catheter and to generate negative pressure through pulling the plunger. The catheter was moved in and out of the cavity in a similar manner to the pipelle.

The collected endometrial tissue was then divided into two parts. Samples used for IHC analysis were placed into 4% paraformaldehyde (PFA) and fixed for 24-48hours.

The remaining tissue sample was placed into 8mls of 10% dextran coated charcoal (DCC) medium in order to preserve the cell integrity until separation. The preparation of the 10% DCC medium is outlined in section 2.2.3.1.1. This tissue was subsequently used for FC analysis. The samples were processed immediately for FC analysis and within 48 hours for IHC staining

The patients continued with IVF treatment and the results of both immuno-histochemical and FC assessment were collated with the pregnancy outcome.

2.2.2.2 *Collection of peripheral blood sample for FC analysis*

A 20ml sample of venous blood was also taken at the same time as the biopsy.

2.2.3 **Immunohistochemical tissue preparation and staining**

The translational research team in the histopathology department in the Queens Medical Centre, Nottingham performed the tissue preparation and immune histochemical staining.

The endometrial sample was fixed in formalin for 24-48 hours and embedded into paraffin wax blocks to retain the tissue architecture and integrity for staining. Sections measuring 3µm were cut using a microtome and placed in Xylene to remove the wax. The tissue was rehydrated with 3% hydrogen peroxide in methanol and Tri-buffered

saline (TBS) for 20 minutes. The sections were mounted on to glass slides and placed in an oven at 72 °C for 1 hour to ensure tissue adherence and removal of any residual paraffin wax. The slides were subsequently placed into the Ventana ® Benchmark Ultra slide staining system for 3 hours to complete the CD56 staining (1µl of anti-CD56 clone 123C3 mouse monoclonal primary antibody Roche®) and counter stained with Mayer's haemotoxylline. Although automated, the Ventana staining system uses the principles of enzymatic digestion involving the enzyme; horseradish peroxidase (HRP) and the substrate; diaminobenzadine (DAB). The oxidative reaction created by these two chemical entities causes a colour change in the tissue sample. This change indicates attachment of the primary (CD56) antibody to the protein complex expressed on the cells of interest.

The primary antibody is normally matched to identify a protein present on the cells of interest i.e. CD56 in the case of NK cells. A secondary antibody with an enzyme such as HRP conjugated to the Fc domain is subsequently added and will in turn attach itself to the primary antibody. The substrate namely DAB will be added to the tissue sample and HRP will immediately catalyse an oxidative reaction, resulting in the generation of a golden brown compound. The appearance of this stain will highlight the cells of interest. These are counter stained with another dye such as haematoxyllin to create contrast between the tissue stained using IHC and the non-coloured regions. This final step leads to clearer visualisation and counting of the cells of interest.

2.2.3.1 ***Slide analysis***

Currently there is no universally agreed method of counting uNK cells (Tang, Alfirevic et al. 2011). Historically, these levels have been expressed as either mean number of uNK cells, percentage of total stromal cells or a percentage of CD45+cells(Seshadri and Sunkara 2014).

The gold standard method for counting uNK cells involves performing a manual count using a computer software programme. This method is not only reproducible but reduces observer variation (Mariee, Tuckerman et al. 2012) .

In this thesis the cell counting method of choice was performed with computer assistance. The number of CD56 positive cells were counted in 3 random non-overlapping microscopic fields at a magnification of 20 x. This was also performed independently by two operators to eliminate inter-observer variability.

The cell numbers were expressed as a percentage relative to the total stromal cells with a normal range of less than 5%. Some of the results in the study population were equal or more than 100%. This is because the measurement is technically a ratio and has been reported to be a validated method in the literature by several studies (Bulmer, Morrison et al. 1991, Tuckerman, Mariee et al. 2010, Santillan, Lozano et al. 2015)

2.2.3.2 *Image acquisition and analysis*

Images were acquired using panoramic SCAN (3D HISTECH Ltd, Hungary). A further software package known as Panoramic Viewer (v.1.15.2, 3D HISTECH Ltd, Hungary) was used to randomly select 3 to 5 images from each sample using 20 x magnification.

The following guidance and criteria were adhered to when selecting the region for analysis:-

- The number of glands and blood vessels in the image were minimised
- The epithelial edges of the tissue were included

Once the image was selected, the screenshot was saved as a .tiff file and labelled with the study and image number.

2.2.4 **Flow cytometry tissue separation and staining**

There are well established protocols for the separation of both peripheral blood (Urbanowicz, Lamb et al. 2009, Wang, Urbanowicz et al. 2013) and endometrial tissue (Salker, Christian et al. 2011, Kuroda, Venkatakrishnan et al. 2013) in order to extract the lymphocytes for flow cytometry (Chan, Schwab et al. 2004, Masuda, Katoh et al. 2016). The methodology from these experiments was adapted to separate the cells for staining through flow cytometry.

2.2.4.1 ***Separation of endometrial tissue for flow cytometry***

The endometrial sample was prepared and processed on the same day as the collection, in order to prevent degradation of the immune cell numbers and function. This protocol primarily uses both mechanical and enzymatic digestion to separate out the endometrial cell types.

The endometrial biopsy was poured into a 90mm petri dish after decanting and discarding the excess 10% DCC medium into a sterile universal tube. The tissue was mechanically and chemically digested. Initially the sample was broken down using a disposable sterile scalpel for 5 minutes. Fast tapping movements with the scalpel were used along with rotation of the petri dish. This ensured that equal breakdown of the tissue and exposed a larger surface area for digestion.

The digestion medium comprised of pre-warmed (37°C), 110µl Collagenase® Sigma (50mg/ml), 110µl DNase ®Roche (10mg/ml) in 10mls of 1% fetal calf serum (FCS) and Dulbecco's modified eagles medium ® (DMEM) (without phenol red) Sigma. This was added to the

degraded endometrial sample using a 0.2µl sterile syringe filter. Stray sample tissue was collected by gently rotating the petri dish and aspirating the solution up and down using a 10ml serological pipette.

The sample solution was transferred into a T25 flask and vigorous aspiration with the pipette was continued to ensure further disruption of the tissue. The flask was shaken vigorously and incubated in a 37°C incubator with 5% CO2 for one hour. The flask was taken out of the incubator and shaken in a similar manner at 20 minute intervals during the hour incubation period. Following this, the sample was pipetted into a 50ml falcon tube containing 10ml of warmed (37°C) 10% DCC medium to deactivate the collagenase. The sample was then centrifuged for 5 minutes at 215xg. Following this the supernatant was aspirated and discarded. The pellet was re-suspended in 13mls of 10% DCC medium and transferred to a T75 flask. The flask was incubated at 37°C with 5% CO2 with the medium being removed and replaced after 1-2 hours. This ensured that red blood cells and epithelial cells were removed appropriately, (after 24 hours the uNK cells will have proliferated and become adherent to the bottom of the flask). After 24 hours the medium was removed again so that any glandular cells, red cells and debris could be discarded. The adherent cells were gently washed by adding 13mls of 10% dextran coated charcoal stripped fetal calf serum (DCC) medium, removing it and replacing it with the same volume. The cells were returned to the incubator until 90% confluence was achieved. The medium was changed every 48hours until the desired confluence was apparent. Once 90% confluence was reached

the uNK cells were washed with trypsin to lift them from the site of adherence and placed them in cell solution. This solution was then ready for antibody staining and FC analysis.

2.2.4.1.1 Preparation of the 10% DCC medium

The 10% DCC medium was prepared in advance using aseptic technique in a class II safety cabinet. It was made by adding 50mls of premade DCC stripped fetal calf serum (FCS) (outlined in sub section 2.2.3.1.2), 5mls of 1% penicillin/streptomycin (Invitrogen, 15240), 5mls of 200mM L-glutamine (Fisher, 25030-024), 100µl of Insulin (Sigma,91077C) and 5µl β-oestradiol (Sigma,E2758) to 50mls of DMEM with phenol red. This initial mixture was added to 450mls of DMEM with phenol red using a sterile syringe filter (0.2µl). The culture medium was then stored at 4°C until usage.

2.2.4.1.2 Preparation of DCC stripped fetal calf serum

This was prepared prior to the endometrial biopsy collection and separation. The FCS contains endogenous steroid hormones which can affect the activity of the other reagents. Therefore dextran coated charcoal is added to remove impurities. A serological pipette was used to measure 250mls of heat inactivated FCS (Sigma F2442) which was placed into 2 sterile schott flasks. Two separate 60mls pots were each filled with 62.5mg of charcoal (Sigma C9157). This was repeated with 625mg of Dextran (Sigma, 31390) being placed into 2 separate 60ml pots. 20mls of FCS from one of the schott flasks was added to one set of charcoal and dextran pots. This was repeated with FCS taken from the second flask and adding it to the remaining two 60ml pots. All four

pots were inverted gently to ensure that the charcoal and dextran were equally dissolved in FCS. These were then added back to their respective FCS flask and placed in a sterile water bath at 56°C for 2 hours. The containers were inverted every 30 minutes. After incubation, 50mls of medium was then decanted into 10 large falcon tubes. The tubes were then centrifuged for 30 minutes at 4000xg. The supernatant from was removed from each tubes and filtered using a sterile 0.45µm syringe filter into a 500ml Schott Duran® bottle. The filtered supernatant was then filtered again using a Nalgene filter attached to a vacuum pump. The resulting medium was aliquoted into 50ml falcon tubes and stored at -20°C.

2.2.4.2 *Isolation of peripheral blood mononuclear cells*

The PB sample was diluted in a 2:1 ratio with RPMI 1640 ®medium (Sigma R8758). This solution was then layered on histopaque-1077® (Sigma 10771) to form a density gradient and centrifuged to separate out the lymphocyte population. The PB layer was removed from the surface of the histopaque-1077® and placed in a sterile tube. Precautions were taken to remove as little serum and histopaque-1077® as possible in order to prevent contamination of the lymphocyte population.

The cells were mixed with 20mls of RPMI 1640 ®medium (Sigma R8758) and centrifuged for 10 minutes. The supernatant was removed and the remaining cell pellet was re-suspended in 10mls of RPMI 1640 ®medium (Sigma R8758). This solution was then used for staining. It

was noted that this protocol led to minimal cell damage and clear staining.

2.2.4.3 *Cell counting and washing*

The purified cells from both samples were counted on a haemocytometer. The cell suspensions were made up to a concentration of 2 million cells/ml with RPMI 1640 ®medium (Sigma R8758) and divided equally into FACS tubes.

2.2.4.4 *Cell purification and staining*

The tubes were subsequently mixed with 20µl of red cell lysis buffer ®Thermo Fisher scientific to purify the lymphocytes and washed thrice with phosphate buffered albumin (PBA). Prior to antibody staining the cells were incubated with 10µl of human anti serum for 15 minutes to block the Fc region and prevent aberrant binding.

The purified cells were then stained with the antibody markers comprising the panel outlined in tables 2.1-2 with volumes ranging from 0.025µl to 5µl. The samples were fixed in 3% formaldehyde and permeabilised with 8% Saponin ® (Sigma SAE0073) for 2 hours to allow for intracellular staining of CD3, CD8, perforin, granzyme, tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ). The stained cells were fixed with 0.5% formaldehyde covered with aluminium foil and refrigerated at 4°C until analysis. The samples were analysed on the MoFlo® Astrios ™ Beckmann-Coulter flow cytometer within 7 days of staining.

2.2.5 **The flow cytometry panel**

A current and validated panel was adapted for use in endometrial tissue (Costanzo, Creegan et al. 2015). This panel had primarily been applied and optimised using frozen peripheral blood samples in human immunodeficiency virus (HIV) patients. This protocol included many of the cell populations that were of interest in the context of implantation and this formed the basis of the staining and gating strategies for the panel used in this study.

A wide variety of immune cells have been identified to work in synchronisation with NK cells in order to facilitate endometrial receptivity and immune-tolerance.

The CD markers used in this panel included CD56/CD16/CD3/CD8 along with perforin, granzyme TNFα and IFNγ. They were specifically chosen to delineate NK, NKT, CD8+ and CD4+ cells. Perforin and granzyme was added to measure the cytotoxic properties of these cells. Furthermore TNFα and IFNγ were included to assess the angiogenic function.

The following tables outlines the panel in detail (tables 2.2-2.3).

Table 2-2- The antibody markers used in the flow cytometry panel

Antibody Marker	Clone Number and Company	Function
Live/dead staining	Live/Dead™Aqua Fixable Green dead cell stain kit Thermofisher Scientific Product number L23101	marker expressed by the proportion of live lymphocytes
CD56	CD56 PECY™7 Mouse Anti-Human Clone NCAM 16.2 BD Biosciences Product number 335826	antibody marker expressed by NK,NKT and cytotoxic CD8+(Tc) cells
CD16	Pacific Blue™ Mouse Anti-Human CD16 Clone 3G8 BD Biosciences Product number 558122	antibody marker used to differentiate NK cells originating from the blood or endometrium
CD3	APC-CY™7 Mouse Anti-Human CD3 Clone SP34-2 BD Biosciences Product number 557757	antibody marker expressed by NKT cells
CD8	Brilliant Violet 785™ Mouse Anti-human CD8a Clone RPA-T8 BioLegend Product number 301046	antibody marker expressed by Tc cells
CD14/CD19	CD19 Mouse Anti-Human (TRI-COLOR®) Clone SJ25-C1 life technologies Product number MHCD1906 CD14 Mouse Anti-Human (TRI-COLOR®) Clone TüK4 life technologies Product number MHCD1406	antibody marker expressed by monocytes and B cells
CD45+	CD45 APC Mouse Anti-Human Clone J33 Beckman Coulter Product number IM273U	antibody surface marker expressed by lymphocytes
Anti-EpCAM	Anti-EpCAM PerCP-Cy™5.5 Mouse Anti-human Clone EBA 1 Product number 347199	marker expressed by epithelial cells
CD56/CD16/CD3 in vitro diagnostic test (IVD)	CD56-PE Mouse Anti-Human Clone N901 (NKH-1) CD16-FITC Mouse Anti-Human Clone 3G8 CD3-ECD Mouse Anti-Human Clone UCHT1 Beckman Coulter Product number A07728	mixture of 3 key antibody markers that can be used to differentiate between NK and NKT cells

Table 2-3 The cytokines used in the flow cytometry panel

Antibody marker	Clone number and company	Function
TNFα/ IFNγ	TNFα Brilliant Violet 650™ Mouse Anti-human Clone MAb11 BioLegend Product number 502938 IFNγ Brilliant Violet 711™ Mouse Anti-human Clone 4S.B3 BioLegend Product number 502540	cytokines released by angiogenic cells to promote vascular growth and mediate inflammation
Perforin/Granzyme	Perforin PE Mouse Anti-Human Clone Delta G9 eBioscience™ Product number 12-9994-42 Granzyme B PE-CF594 Mouse Anti-Human Clone GB11 BD Biosciences product number 562462	cytokines released by cytotoxic NK cells to destroy pathogens and cause inflammatory changes

2.2.5.1 ***Developing the gating strategy***

The interpretation of FC plots is described in Chapter 1.

A traditional gating strategy was used to define the immune cell populations as described by (Costanzo, Creegan et al. 2015). The principle was to apply logical gates to purify and identify the NK (CD56+CD16+CD3-), NKT (CD56+CD16+CD3+) and Tc cells (CD56+CD8+CD3-) populations.

The initial gating defined all the cells present in the sample (figure 2.1A) by plotting side scatter against forward scatter. The single cells were then selected and termed as singlets (figure 2.1B).The proportion of live cells were plotted and gated as the cells of interest (figure 2.1C).

In order to remove the B cells and monocytes, cells positive for CD14 and CD19 were excluded (figure 2.1D). Various gates for CD56, CD16, CD3 and CD8 were applied to define the immune cell populations.

The CD56+CD16+ were classified as NK cells (figure 2.1E).The CD56+ and CD16 negative were seen as the NK ^{bright} and those possessing CD16 were classified as the dim. The NKT cells were defined as CD56+CD3+ and CD16- (figure 2.1F). The Tc cells were then filtered out by plotting CD3 against CD8 (figure 2.1G). The CD4+ cells were defined from this plot as those that were CD56+CD3+CD8-.

Furthermore, for each of the cell types, the perforin and granzyme expression was then plotted to give an estimation of the cytotoxic properties (figures 2.1H-J). The TNFα and IFNγ expression was

estimated in a similar manner as the panel developed and the gating strategy is presented in figure 2.2.

The same gating strategy was applied to the PB sample (figure 2.3A-J). The immune cell populations and cytokine expression were compared between the two paired samples to check if there were differences and this is discussed fully in Chapter 4.

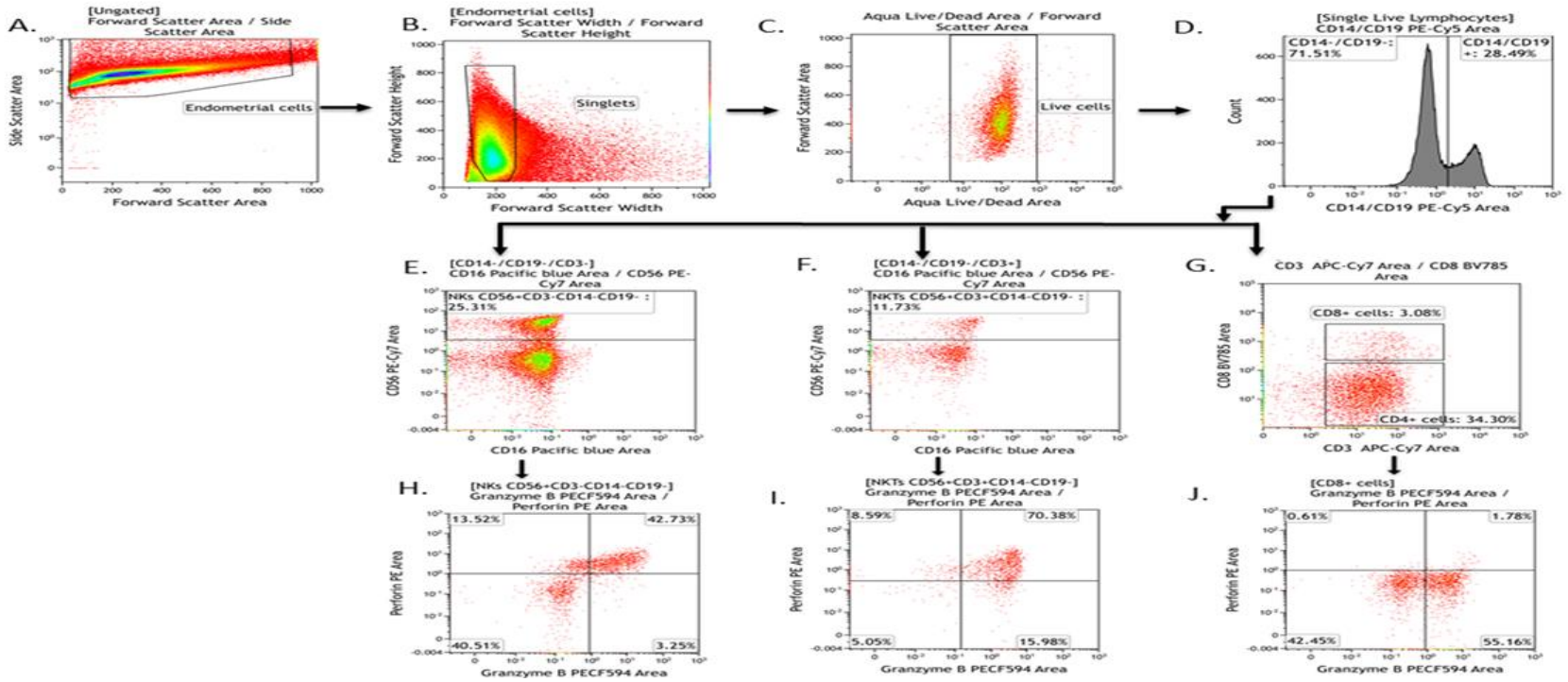


Figure 2-1- The endometrial gating strategy

The initial gating defined all the cells present in the sample (figure 2.1A) by plotting side scatter against forward scatter. The single cells were then selected and termed as singlets (figure 2.1B).The proportion of live cells were plotted and gated as the cells of interest (figure 2.1C). In order to remove the B cells and monocytes, cells positive for CD14 and CD19 were excluded (figure 4.1D). Various gates for CD56, CD16, CD3 and CD8 were applied to define the immune cell populations. The CD56 and CD16 positive cells were classified as NK cells (figure 2.1E). The NKT cells were defined as CD56+/CD3+ and CD16- (figure 2.1F). The CD8+cells were then filtered out by plotting CD3 against CD8 (figure 2.1G). The CD4+ cells were defined from this plot as those that were CD56+CD3+ and CD8-.The perforin and granzyme expression was plotted for each population ;NK cell (figures 2.1H), NKT cells (figure 2.1I and CD8+cells(figure 2.1J).

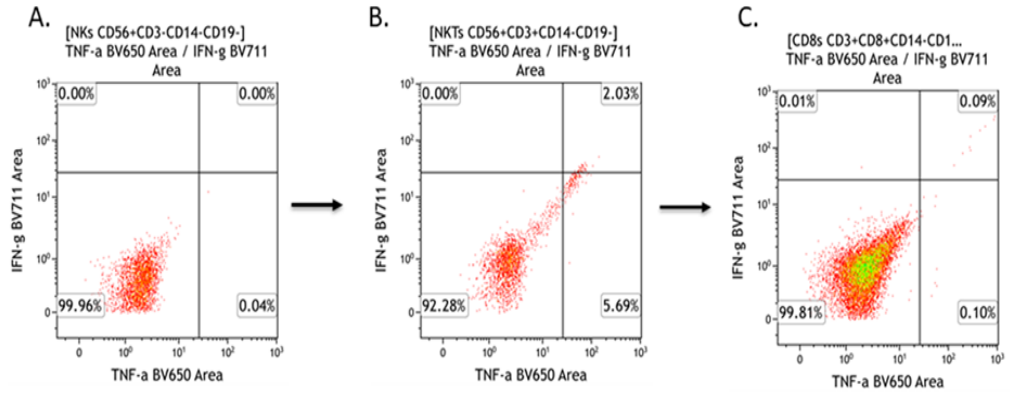


Figure 2-2- The gating strategy for TNFα and IFNγ

The TNFα and IFNγ expression was plotted for the NK (figure 2.2A), NKT (figure 2.2B) and CD8+ cells (figure 2.2C).

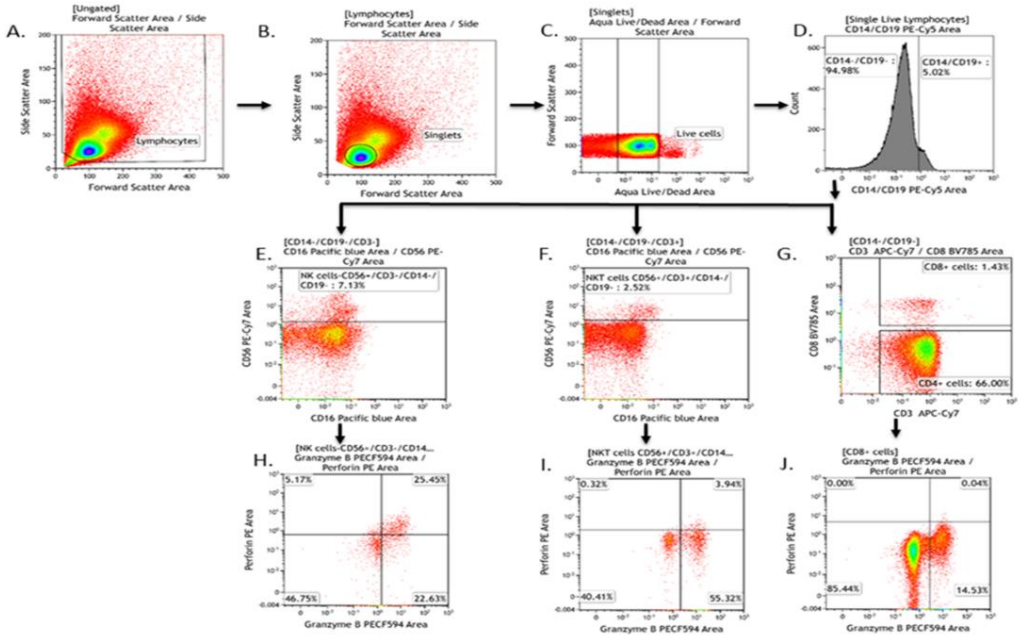


Figure 2-3- The gating strategy for the PB population

The initial gating defined all the cells present in the sample (figure 2.3A) by plotting side scatter against forward scatter. The single cells were then selected and termed as singlets (figure 2.3B).The proportion of live cells were plotted and gated as the cells of interest (figure 2.3C).In order to remove the B cells and monocytes, cells positive for CD14 and CD19 were excluded (figure 2.3D). Various gates for CD56, CD16, CD3 and CD8 were applied to define the immune cell populations. The CD56 and CD16 positive cells were classified as NK cells (figure 2.3E). The NKT cells were defined as CD56+CD3+ and CD16- (figure 2.3F). The CD8+ cells were then filtered out by plotting CD3 against CD8 (figure 2.3G). The CD4+ cells were defined from this plot as those that were CD56+CD3+ and CD8-.The perforin and granzyme were determined for each immune cell population; NK (figure 2.3H),NKT (figure 2.3G) and CD8+ cells (figure 2.3J).

2.2.5.2 *Quality control measures and methodological strengths*

A robust gating strategy was created to correctly identify all the immune cell populations by using a series of positive and negative controls. This was achieved by equally dividing the peripherally sourced lymphocytes into FC tubes. A “single colour” control tube with lymphocytes and an individual antibody marker e.g. CD56 was aliquoted. This was followed by a set of flow minus one (FMO) control tubes where all except one of the antibody markers were added. This was subsequently repeated for the full panel. This systematic omission of each marker and single colour controls then allowed for accurate placement of the gating strategy in the fully stained samples (figures 2.4A-B).

Figure 2.4A displays the single colour control with just the CD56 marker present. Figure 2.4B shows the same plot for the FMO with no CD56 present.

The single colour and FMO controls were used to clearly set the boundaries or gating for where the cells of interest were truly present or absent. This is required in FC as all fluorochromes emit a spectrum of light frequencies that naturally overlap a phenomenon known as spectral overlay. This spill over is more evident as the number of fluorochromes increases and hence may give an inaccurate assessment of the entity of interest. Figure 2.5 displays the principle of spectral overlay and shows the overlap between two commonly used fluorochromes (FITC and PE).

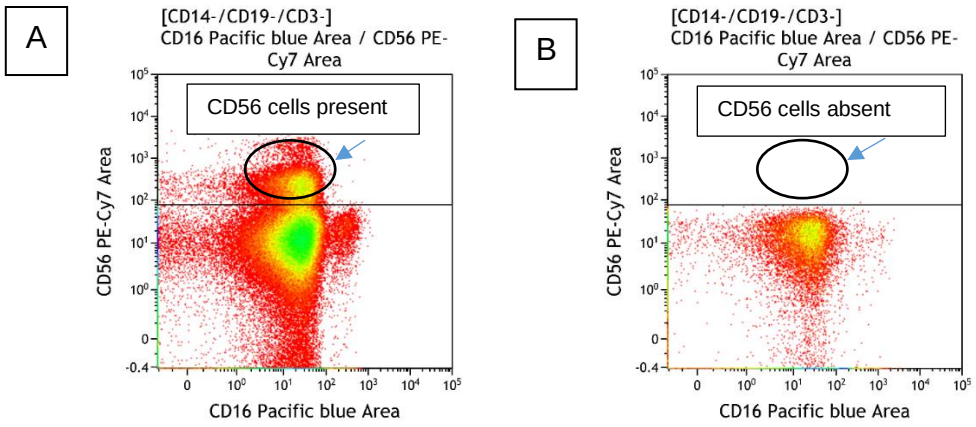


Figure 2-4- The use of positive and negative controls to define the correct gating strategy

Figure 2.4A displays the single colour control with just the CD56 marker present. Figure 2.4B shows the same plot for the FMO with no CD56 present. Both controls were used to delineate the gate for the fully stained samples.

The principle of spectral overlay. The bold blue arrow highlights the overlap of two fluorochromes. This is an area where the cells of interest may be not be identified correctly

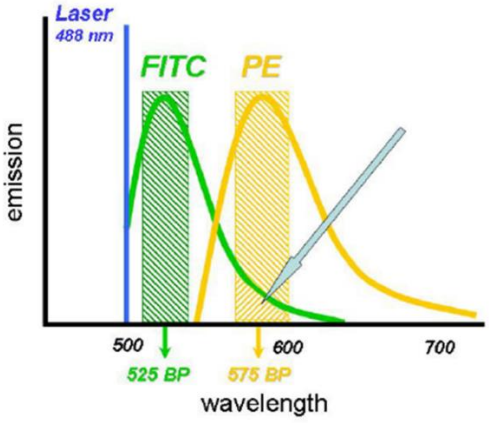


Figure 2-5- The principles of spectral overlay in FC

Taken from http://flowbook.denovosoftware.com/chapter-5-immunofluorescence-and-colour-compensation

2.2.6 **Validation of the panel and experimental challenges**

2.2.6.1 ***Purification of the endometrial cells***

Endometrial tissue is comprised of glandular, stromal tissue and blood vessels. The led to the introduction of various steps in the endometrial cell isolation protocol in order to optimise staining.

The purification of the endometrial cells was initially challenging as preliminary experiments revealed that cell culture and especially media changes were leading to poor staining (figure 2.6).

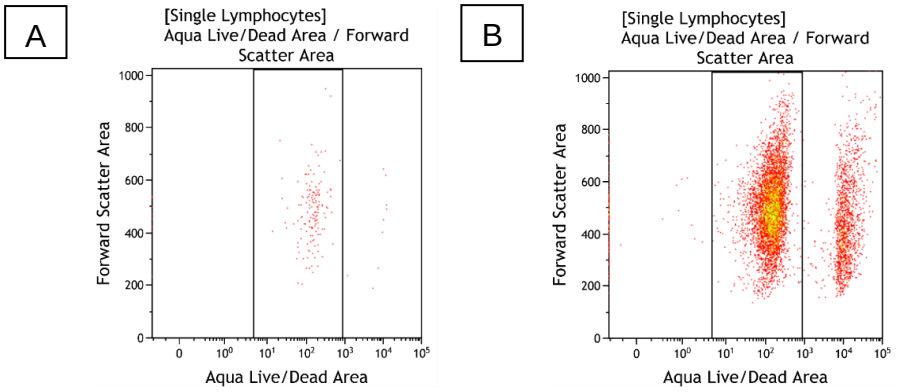


Figure 2-6- Dot plots of staining for live lymphocytes in the endometrium

The dot plots show the staining with cell culture (figure 2.6A) compared to staining the tissue immediately after separation (figure 2.6B).

The cell yield when the cells were cultured showed very poor staining (figure 2.6A).However this improved with staining the cells immediately after separation (figure 2.6B).

The reasons for this were two-fold. The cells were not culturing well and were being washed away with the changing of culture medium. The addition of trypsin which is used to lift the cell from the culture flask was also leading to cell death. Therefore a modification of staining the cells immediately after separation was made.

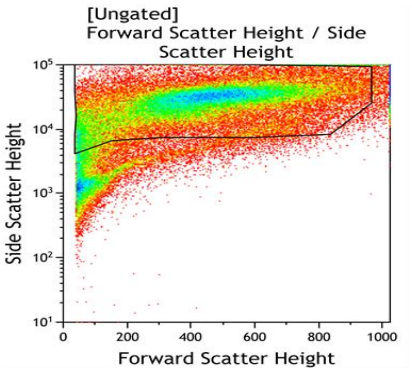
Although the mechanical and enzymatic digestion was sufficient to separate out the lymphocytes, it was noted that the samples contained a high proportion of red blood cells which delayed the sample processing through the flow cytometer. In order to address this, 20µl of red cell lysis buffer ®Thermo Fisher scientific was added to eliminate red blood cells. An epithelial cell marker; EpCam was added to further separate out the lymphocytes but this did not stain well and was not included into the final protocol.

2.2.6.2 ***Endometrial cell gating techniques***

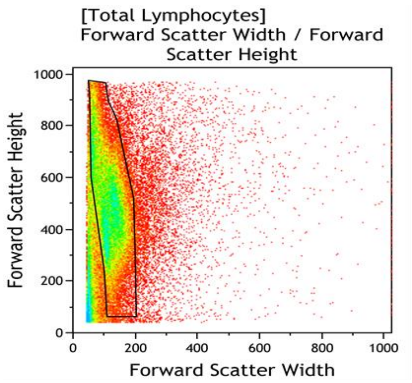
The gating strategies used to identify the correct population of endometrial cells was performed in three ways as the panel evolved. Initially a large and tight gate was drawn around the cells to include all the potential lymphocytes. However this was dependent on personal judgment. This was then adapted with the addition of CD45+ to the latter 25 samples, which made the gating more accurate as only lymphocytes express this marker. The tight gating strategy of drawing around the cells was still performed in these samples to compare concordances and correlations.

All the gating strategies were initiated with the forward scatter and side scatter plot to demonstrate all the endometrial lymphocytes in the sample (figure 2.7A). The endometrial cells were identified in the first 16 samples by estimation and this was defined as the large gating strategy (figure 2.7B). The gating was subsequently made more accurate and this was termed as the tight gating strategy (figure 2.7C). The large and tight gating strategies both involved manually drawing around the cells

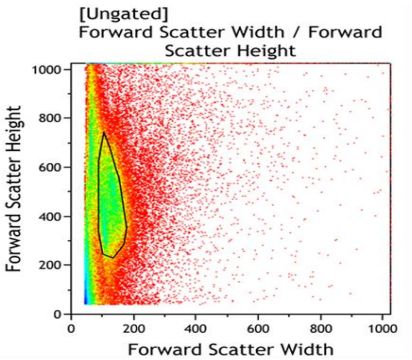
of interest. The addition of CD45+ was introduced into the latter 25 samples to identify the lymphocytes (Figure 2.8A and 2.8B).This allowed the cell populations to be identified with more accuracy.



A. Total endometrial lymphocytes



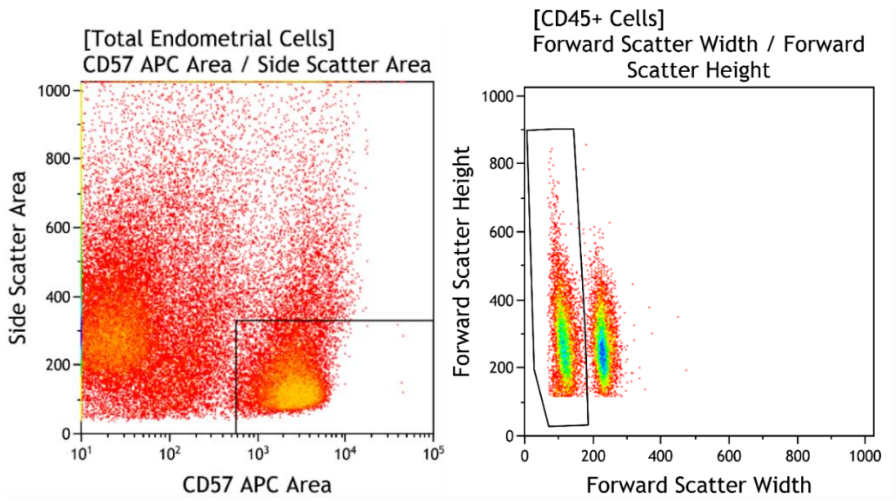
B. Large gating strategy



C. Tight gating strategy

Figure 2-7- Endometrial cell gating techniques

The forward and side scatter were plotted to show the total endometrial cells present in the sample (2.7A). The large gate was drawn manually as an estimation of where the endometrial lymphocytes were present (Figure 2.7B). This was then made more stringent by drawing a tighter gate (2.7C).



A. total endometrial lymphocytes

B. total endometrial lymphocytes with CD45+ gating

Figure 2-8- The endometrial gating strategy with the addition of CD45+

The forward and side scatter plot was initially drawn against the CD45+ (CD57 APC area channel) to show the total endometrial lymphocytes (figure 2.8A). The lymphocytes were then gated with CD45+ cells to reveal a more distinct population (figure 2.8B).

2.2.6.3 *Prevention of antibody cleavage*

The addition of collagenase was needed to purify the endometrial cells. It was noted that this was causing cleavage of some of the key CD markers, namely CD16. This phenomenon has been documented in the literature for both endometrial (Abuzakouk, Feighery et al. 1996) and other tissues (Chen, Chen et al. 2014).

Searching the literature revealed that the addition of FCS could prevent this (Lonergan, Carolan et al. 1996, Fukui, Fujii et al. 1999, Fukui, Kwak-Kim et al. 2008) . A protease inhibitor was also added but this made no difference. Therefore the final protocol added FCS only.

2.2.6.4 *Compensation issues*

Applying an extensive antibody panel yielded compensation challenges. The manufacturer guidance advised the addition of either 2.5-5µl of each flourochrome to the cells. However due to the numerous antibody markers being studied, this led to compensation issues with spectral overlay. This was evident for the CD3 and CD4 channel. A series of dilutions were made to reduce this without compromising the staining (figure 2.9). The composite plot shows that as the concentration of the CD3 antibody marker is diluted from 0.5 to 0.1µl, the delineation between the positive and negative CD3 cells is clearer.

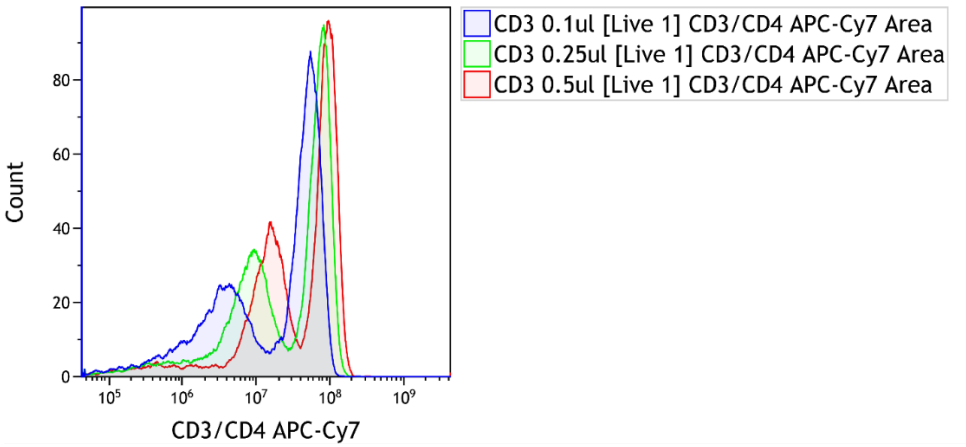


Figure 2-9- Composite plot of CD3+ dilutions

2.2.6.5 *The addition of CD56/CD16/CD3 in vitro diagnostic preparation*

In order to improve the efficiency of the panel, 14 of the latter PB samples had the addition of primary antibodies (CD56, CD16 and CD3) as an in vitro diagnostic (IVD) test preparation. The CD56+, NK and NKT populations could then be identified. Figure 2.10 shows the gating strategy. The total population of cells was identified through the forward scatter and side scatter (figure 2.10A). The NK and NKT cells were identified by plotting CD56 against CD16 (figure 2.10B) and CD3 (figure 2.10C). This can be compared to the populations identified through the individual staining for NK (figure 2.10D) and NKT cells (figure 2.10E) with the percentage of cells being similar.

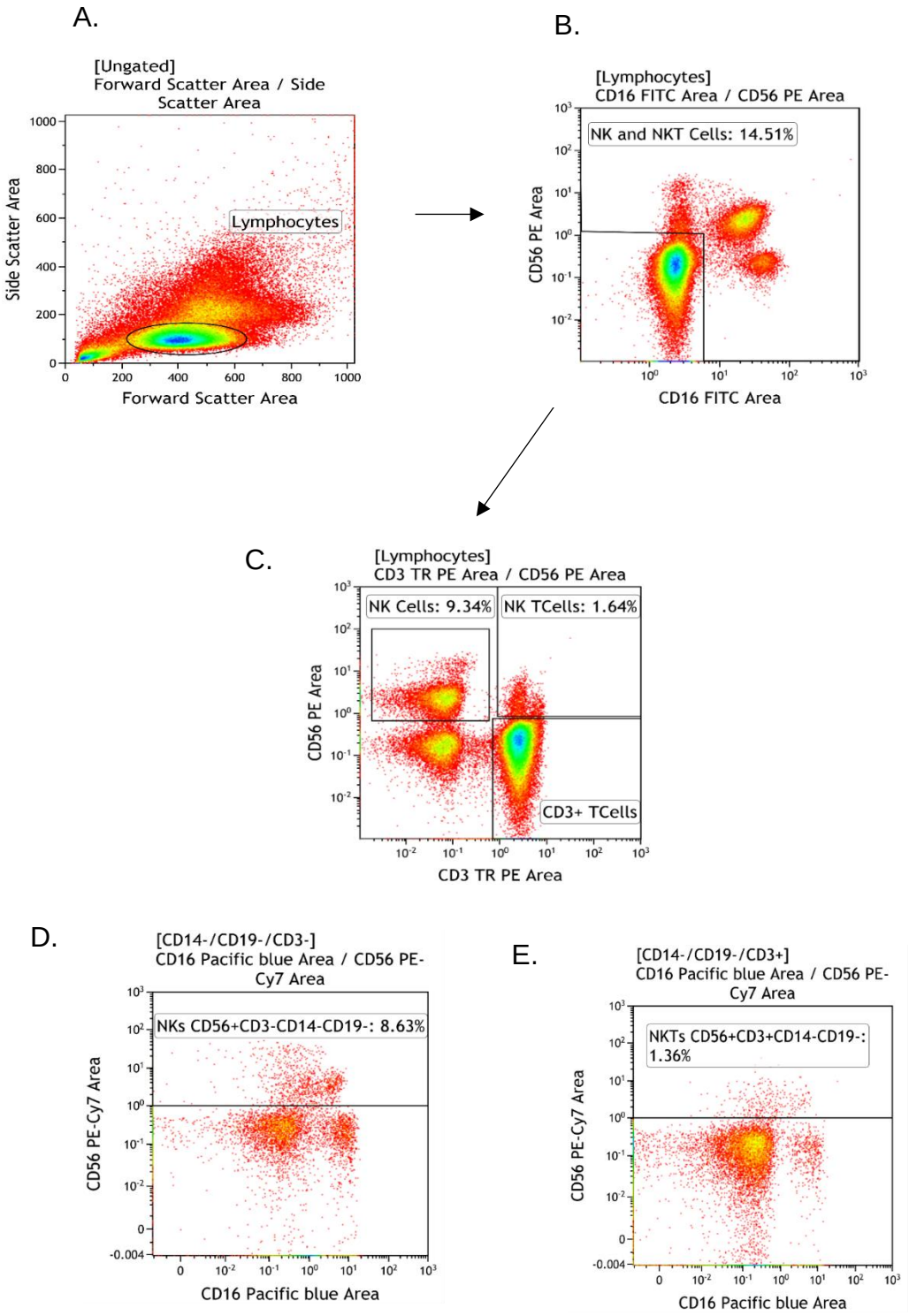


Figure 2-10 -The gating strategy with the CD56/CD16 and CD3 in vitro diagnostic test

The total lymphocytes in the PB sample were identified with the forward and side scatter plot (figure 2.10A). The CD56 and CD16 area was plotted to identify the NK and NKT cells (figure 2.10B). The CD56 and CD3 were also plotted to further identify the cells of interest (figure 2.10C).This plot is compared to the individual staining with CD56 and CD16 to show the NK (figure 2.10D) and NKT cells (figure 2.10E) respectively.

2.2.7 **In vitro fertilisation treatment**

After sample collection and analysis, all the study participants embarked on IVF treatment.

2.2.7.1 ***Fresh IVF cycles-long and short protocol***

The majority of patients had a fresh IVF cycle in which the ovaries were initially down regulated and subsequently stimulated to promote follicular development. The ovums were collected and fertilised in the same treatment cycle.

The standard long protocol at NURTURE fertility involves starting a course of pituitary down regulation medication (gonadotrophin releasing hormone) GnRH agonist, buserlin, Aventis Pharma, Kent, UK) seven days prior to the expected date of the next menstrual period. This is administered by a thrice daily nasal spray or daily subcutaneous injections. Therefore for the majority of patients who have a 28 day cycle, treatment is commenced on day 21. The patients informed the unit on day 1 of their menstrual cycle and down regulation was commenced at the appropriate time.

The patients experienced a withdrawal bleed within two weeks of commencing downregulation. Once bleeding commenced, they were instructed to attend the IVF unit for a Transvaginal Ultrasound Scan (TVS) and serum oestradiol level to confirm downregulation had occurred. This was noted when the scan findings revealed an endometrial thickness of <5mm, inactive ovaries with follicles less than 10mm in size and the oestradiol level was <300 pmol/l.

Once downregulation was confirmed, a period of ovarian stimulation was commenced. This involved daily subcutaneous injection of either Human Menopausal Gonadotrophin (HMG, Menopur, Ferring UK) or recombinant Follicle Stimulating Hormone (FSH) (Gonal-F, Merck Serono, UK). The dose was individualised to each patient and adjusted according to the rate and size of follicular growth. The duration of ovarian stimulation ranged between 12-14 days.

From day 9 of stimulation, patients were monitored for their ovarian response; a process known as follicular tracking. This was assessed through a combination of transvaginal scans and measuring serum oestradiol levels.

Conversely patients who were at risk of over-response and ovarian hyper stimulation syndrome (OHSS); a serious condition that can be life threatening were started on an antagonist short protocol. This did not involve any down regulation and patients commenced HMG or Gonal-F on day 2 of their menstrual cycle. Follicular tracking was commenced on day 7 and when the leading follicle developed to 1.4mm a subcutaneous injection of GnRH antagonist cetrorlexis (Cetrotide, Merck Serono, UK) was given. The aim of this was to control follicular growth and prevent super-ovulation.

The follow up regimen was the same for those on long and the short protocol. Once patients met the criteria for ovum retrieval which was defined as >3 leading follicles of >1.8mm, 10,000IU of pregnyl was

administered to trigger oocyte maturation. Transvaginal oocyte retrieval (TVOR) was planned 36 hours after the pregnyl injection.

The TVOR was performed in an operating theatre under sedation. The oocytes were retrieved by inserting a transvaginal ultrasound guided needle into each follicle and collecting the fluid. The follicular fluid was assessed by a trained embryologist using microscopy to identify oocytes. The sperm sample being used for treatment was also prepared by the embryology team prior to TVOR.

All patients undergoing IVF, intracytoplasmic sperm injection (ICSI) and frozen embryo replacement (FER) treatment were advised to self-administer progesterone pessaries (cyclogest, Alpharma, UK) 400mg twice daily starting 2 days after TVOR or confirmation of adequate endometrial thickness in the case of FER. These were continued until 9-10 weeks of pregnancy

2.2.7.2 ***Frozen embryo replacement cycle***

In FER cycles, the patients underwent a period of down regulation similar to the fresh IVF/ICSI cycle. However once down regulation was confirmed with the TVS and oestradiol levels, a 2 week course of oral progynova was commenced. This is a form of hormone replacement therapy (HRT) aimed at thickening the endometrium. Adequate thickening of the endometrium to 8mm was checked by TVS at the completion of the HRT course. Once this was confirmed, the patient was instructed to start their progesterone pessaries. The embryos are thawed and transferred in the same manner as a fresh IVF cycle.

2.2.7.3 ***Natural cycle IVF***

In some women with a low number of predicted or collected ovums in a previous cycle, the option of natural cycle IVF was offered. This involves giving no hormonal stimulation medication and collecting the ovum from follicles that develop naturally. Patients undergoing this type treatment were scanned transvaginally from day 5 of their menstrual cycle. The serum oestrogen and LH levels were also measured to monitor follicular development and ovulation. The follicular development was checked every 2-3 days. At each scan, the ovarian follicles were measured and when the mean diameter is 1.5mm was above, a subcutaneous injection of GnRH antagonist cetrorlexis (Cetrotide, Merck Serono, UK) was given to prevent ovulation. An injection of 5000IU of pregnyl was administered to trigger oocyte maturation. Transvaginal oocyte retrieval (TVOR) was planned 38 hours after the pregnyl injection in the same manner as stimulated IVF cycles.

2.2.7.4 ***Ovum donation cycles***

Women who were recipients of ovum donation underwent a period of down regulation similar to those having stimulated and frozen embryo replacement cycles. Once down regulated, a 2 week course of oral oestrogen tablets was commenced. Adequate thickening of the endometrium to 8mm was checked by TVS at the completion of the HRT course. Once this was confirmed, the patient was instructed to start their progesterone pessaries. The embryos created through donor ovums and their partners sperm and transferred in the same manner as an IVF/ICSI cycle.

2.2.7.5 *IVF laboratory procedures*

Conventional IVF or ICSI treatment was selected for each patient according to sperm sample parameters. If the cause of infertility was male factor or the sperm parameters were below the normal range then ICSI was used. The ovums were mixed or injected with the sperm depending on which assisted conception technique was required. A fertilisation check was performed the following day and the embryos were cultured for up to five days; blastocyst stage. If the number of high quality embryos at the day 2 stage was less than 4, then a single or double embryo transfer was performed.

The number of embryos transferred was planned according to the patient's age and embryo quality. The embryo quality was graded using a validated system (Richardson, Brearley et al. 2015) whereby the embryologist would assess the morphology of each embryo under microscopic examination outlined in earlier literature (Dokras, Sargent et al. 1993, Gardner, Surrey et al. 2004). Table 2.3 outlines the classification systems for day 2, 3 and 5 embryos. The embryos at day 2-3 were given a grade from 1-4 depending on the severity of fragmentation seen. Fragmentation within an embryo is defined as cytoplasmic debris that arises as result of cellular division. Although the mechanisms are poorly understood, fragmentation of more than 10% of the embryo volume is deemed to be detrimental for pregnancy .Day 5 embryos were classified as A-C with the compacting and cavitating grade given for poor quality embryos (table 2.4).

The embryology team performed a culture media change at day 2 or 3 and if the embryos were not satisfactorily developing then an embryo transfer was planned at this stage. The majority of patients were envisaged to have an elective single embryo transfer at day 5. If the grade of embryo was low quality or the patient was over the age of 37 then a double embryo transfer was undertaken.

Table 2-4- The morphological classification of embryo quality at day 2-3

Embryo grade	Morphological features	
	Cell shape	Percentage of fragmentation
1	Regular cells	Less than 5
1-2	Regular cells	5-10
2	Regular cells	10-20
2-3	Regular cells	20-30
3	Irregular cells	30-50
4	Irregular cells	Greater than 50

Table 2-5- The morphological classification of embryo quality at day 5

Embryo grade	Morphological feature
A	Expanded blastocyst with thinning of the zona pellucida. A clear inner cell mass and cohesive trophectoderm layer identified
B	Full Blastocyst with a clear inner cell mass and cohesive trophectoderm. However the zona pellucida does not show signs of thinning and the blastocyst has not yet fully expanded.
C	Blastocyst with a small inner cell mass and/or irregular trophectoderm cells. Small area of degeneration/a single excluded cell or a single large vacuole may be present in the inner cell mass.
Cavitating	Evidence of any cavity forming within the embryo with no clear inner cell mass or trophectoderm layer being visible
Compacting	Evidence of any signs of compaction between cells of the embryo

2.2.7.6 ***Embryo transfer***

On the day of embryo transfer (2, 3 or 5 days after TVOR), the patients were asked to arrive at the unit with a full urinary bladder. They were placed in the lithotomy position and the embryo(s) were transferred into the uterine cavity under abdominal ultrasound guidance. The embryo(s) were loaded into a wallace catheter between 2 small air bubbles. The catheter was then attached to a 1ml syringe of embryo culture medium. The sonolucent tip of the catheter was subsequently introduced into the cervical canal. This was performed after visualising the cervix with a cusco's speculum and cleaning the mucus away with a cotton bud. The tip was slowly advanced into the uterine cavity until it was estimated to be around 2 centimetres from the fundus. The fluid in the syringe was injected by the embryologist and the embryo(s) were transferred and the presence of the air bubble were noted on the abdominal scan. The catheter was then checked by the embryologist to ensure that the embryo(s) were not retained in the tubing or tip. If there was resistance felt in the cervical canal or a previous transfer had been noted to be difficult, then a malleable catheter with a rigid stylet was used to negotiate the cervix. This was introduced up to uterine-cervical junction and the stylet was removed. The soft wallace catheter was subsequently fed through the malleable into the uterine cavity as described above.

2.2.7.7 ***Confirmation of cycle success and pregnancy***

In order to confirm pregnancy patients were instructed to perform a urinary pregnancy test 2 weeks post embryo transfer. If the pregnancy test was negative, the cycle was deemed unsuccessful and a clinical review booked to discuss further management. If the pregnancy test was initially positive and become negative through urine testing or peripheral blood within 28 days this was defined as a biochemical pregnancy.

If the pregnancy test was positive the patients continued the medication that has been allocated to them and had a TVS at 6-7 weeks gestation to confirm and date a clinical pregnancy.

Clinical pregnancy was confirmed by visualisation of an appropriately sized intrauterine gestation sac (s) with a yolk sac (s), fetal pole (s) and heartbeat (s). All these features should be present at 6-7 weeks of gestation. The scans were performed by a trained sonographer using the Voluson E8 machine with a 5-9MHz and 9-12MHz transvaginal probe.

A miscarriage was defined as the involuntary loss of a pregnancy before 24 completed weeks. In the first trimester the absence of a fetal heart or an appropriately size intrauterine gestation sac at TVS were used to diagnose a miscarriage. This may be associated clinical symptoms of vaginal bleeding and or abdominal pain.

A live birth was defined as the delivery of a live fetus beyond 24 weeks gestation.

If a patient was started on oral prednisolone due to a raised NK level, they were instructed to discontinue this if the pregnancy test was negative or the viability scan confirmed a miscarriage. Immediate discontinuation was not advised as stopping steroid medication abruptly can cause unpleasant side effects. Therefore patients advised to undergo a reducing regimen of halving the dosage to 10mg once daily for a week and then 5mg for the subsequent week. After completion of a week of 5mg, the medication was discontinued altogether.

This same reducing regimen was also advised to those who had a viable pregnancy on scan. They were advised to continue the medication at 20mg once daily up to 10 completed weeks of pregnancy. In the 11th week, they were instructed to halve this to 10mg daily for one week. At 12 weeks of pregnancy, this was reduced to 5mg daily and then stopped after one week.

All the pregnancy outcomes were recorded including negative pregnancy test, biochemical pregnancy, miscarriage and live birth.

Chapter 3

The prevalence and assessment of uterine NK cells by Immunohistochemistry in women undergoing IVF

Chapter 3 The prevalence and assessment of NK cells by Immunohistochemistry in women undergoing IVF

3.1 Introduction

In this chapter, the most commonly used technique of immunohistochemistry (IHC) to assess uterine natural killer (uNK) status is analysed by studying it's the clinical relevance.

The relationship between uNK and subsequent pregnancy is illustrated through prospectively studying a cohort of 203 women who opted to have testing and subsequently embarked on IVF treatment.

This area of scientific enquiry were deemed to be clinically relevant and would address some of the deficiencies in the literature highlighted in the preceding chapter.

Remarkably, although testing is widespread in those undergoing IVF, there is currently very sparse data to define the prevalence of raised uNK levels measured by IHC (Chen, Mariee et al. 2017). This was initially evaluated.

Sub group analyses looking at number of previous IVF cycles, cause of infertility, previous pregnancy success both through IVF and spontaneously were performed to determine whether these factors were predictive of a positive NK result in the endometrium.

The current evidence presented in the literature is centred on raised NK status in women undergoing IVF being associated with a detrimental pregnancy outcome. This could be either due to NK cells being

cytotoxic to the embryo endometrium complex or causing aberrant placentation. Their exact function is unclear but due to their close proximity to the early invading trophoblast coupled with a noticeable rise in early pregnancy, they must facilitate implantation in some way.

The hypothesis of whether a raised uNK was predictive of a live birth through IVF could not be fully tested in this study. This was because the conventional treatment in the IVF clinic where these women were studied, was to give 20mg of oral prednisolone daily, a day before embryo transfer to all the patients that tested positive. The administration of oral steroids may have influenced the outcome of the cycle and hence live birth rates in those testing positive and negative for NK levels could not be accurately compared.

One of the main observations found in this study was that patients who were IHC positive for uNK were more likely to have a live birth. This association may have been confounded with prednisolone treatment correcting a perceived imbalance. In order to overcome this discrepancy, a separate subgroup analysis was performed in women who had raised NK levels to see if a range of uNK severity was predictive of live birth. The data was further explored to analyse if previous spontaneous pregnancy or IVF success were predictive of high NK status.

A sub group of women who had IHC testing who then went on to have profiling with FC will be described in Chapter 4.

3.2 Aims

There were three main aims of this study:-

- Determining the prevalence of raised uNK cells in an IVF population
- Identifying demographic factors that were predictive of a raised uNK result
- Demonstrating whether a statistically significant relationship exists between NK testing and IVF related live birth

Data collection

The sample collection and preparation is outlined in Chapter 2.

The age, BMI, timing of the biopsy was collected for all patient. The cause of infertility, spontaneous pregnancy and IVF history was summarised and recorded. The details of the subsequent IVF cycle such as the type of treatment e.g. fresh or frozen, embryo quality and pregnancy outcome were also collected prospectively.

3.2.1 Statistical Analysis

The data was analysed using the SPSS statistical package version 25 (Statistical Package for Social Sciences; IBM, IL, USA).

The majority of the data was categorical and cross tabs and chi square testing was performed to check if there was any statistically significant differences in factors that may influence live birth or NK levels.

Univariate logistic regression analyses with crude odds ratios (OR) and 95% confidence intervals (CI) were conducted for the main outcome of raised NK levels, in relation to each of following variables in turn; female

age, cause of infertility, embryo quality, previous IVF and spontaneous pregnancy history. A multiple regression of those factors that were found to be significant in the univariate analysis was performed, to determine which variables were independent predictors of NK status.

As mentioned in the methods section, the women who were NK cell positive all given oral prednisolone. Therefore a logistic regression to determine if NK positivity influenced live birth could not be conducted, but amongst the positive women, we explored the effect of the severity of NK levels on live birth.

In the 125 women that were positive, the population was stratified into low, medium, high and very high levels of NK positivity according to clinical parameters .A univariate logistic regression was then performed in this subgroup to check if this new NK variable was significantly associated with live birth.

A P value<0.05 was deemed to be statistically significant.

3.3 Results

3.3.1 Population demographics

There were 203 patients included in the final analysis. Two women conceived spontaneously after undergoing the biopsy and were not included in any analysis involving IVF outcome data. They were however included in analysis for demographic factors affecting NK status.

Figure 3.1 shows the IVF outcomes of patients through according to their NK status. The results are comparable for each group.

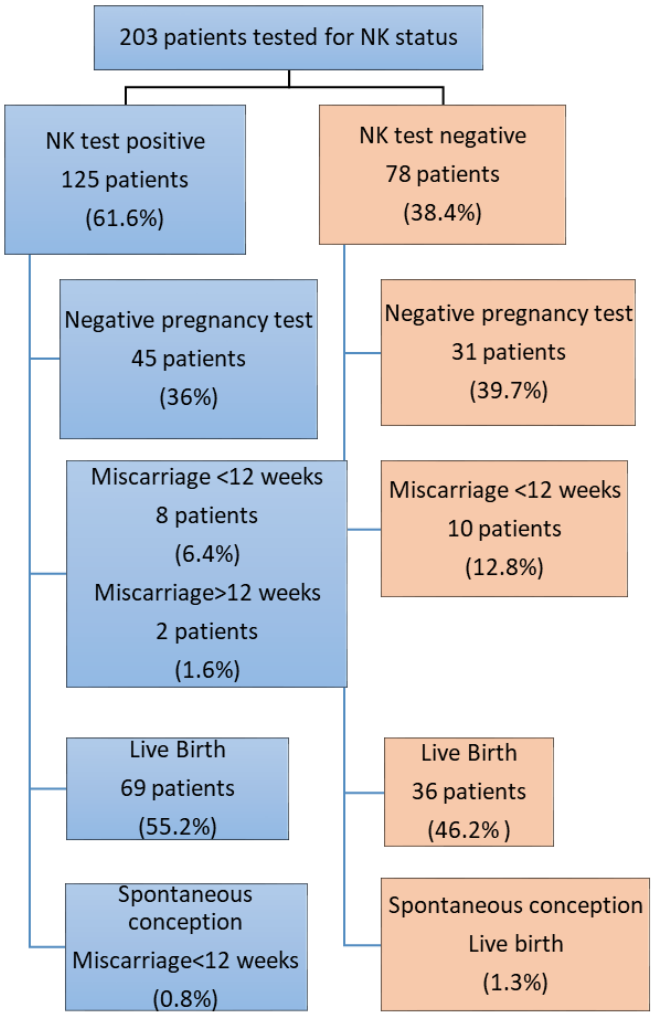


Figure 3-1- The IVF outcomes of the study population

3.3.1.1 *General population characteristics*

The age of the patients ranged from 24-44 years with a mean age of 35.12 ± 4.02 years. All patient has a BMI below 36.5 (mean 23.3 ± 6.31) The number of previous IVF cycles ranged from 0-7 and the mean number was 1.32 ± 1.21.The causes of infertility varied with the most common being male factor (38.9%).

The majority of patients had fresh IVF cycles (71.6%) with day 5 good quality embryos (81.8%).

Table 3-1- The study population demographics

Demographic factor	**N=203 (Mean±SD)**	
age (yrs)	35.12±4.02	
BMI (weight/height^2)	23.03±6.31	
previous number of IVF cycles	1.32±1.21	
cause of infertility	N=203 (%)	
unexplained	47 (23.2)	
male	79 (38.9)	
tubal	22 (10.8)	
other	17 (8.4)	
multifactorial	38 (18.7)	
IVF treatment demographics*	N=201 (%)	
type of IVF treatment	Fresh	144 (71.6)
	Frozen	57 (28.4)
day of embryo transfer	Day 2	22 (10.8)
	Day 3	13 (6.4)
	Day 5	166 (81.8)
number of embryos transferred	Single	60 (29.9)
	Double	140 (69.7)
	Triple	1 (0.50)
endometrial thickness at embryo transfer (mm)	10.87±2.56	

*2 patients excluded from IVF treatment demographics as conceived spontaneously and did not have embryo transfer

3.3.1.2 *Uterine NK status*

38.4% (n=78) of patients had a negative NK result whereas 61.6% (n=125) were positive. 6 patients had a NK result of at least 100%. These were also included in the positive group.

Table 3.1 shows the demographic characteristics of the population studied according to NK cell status.

There were no major differences in the patient demographic apart from a higher proportion of patients having male factor infertility in those that were NK positive

Table 3-2- The patient demographics according to IHC status

Demographic factor		**NK negative (N=78) Mean±SD**	**NK positive (N=125) Mean±SD**
age (yrs)		35.58 ±3.91	34.84 ±4.08
BMI (weight/height^2)		23.64±6.15	23.09 ±6.42
previous number of IVF cycles		1.35±1.07	1.30±1.30
Cause of Infertility		NK negative N=78 (%)	NK positive N=125 (%)
unexplained		21 (44.7)	26 (55.3)
male		24 (30.4)	55 (69.6)
tubal		12 (54.5)	10 (45.5)
other cause		7 (41.2)	10 (58.8)
multifactorial		14 (36.8)	24 (63.2)
IVF treatment demographics*		NK negative N=77 (%)	NK positive N=124 (%)
type of IVF treatment	Fresh	54 (37.5)	90 (62.5)
	Frozen	23 (40.4)	34 (59.6)
day of embryo transfer	Day 2	7 (31.8)	15 (68.2)
	Day 3	5 (38.5)	8 (61.5)
	Day 5	65 (39.2)	101 (60.8)
number of embryos transferred	Single	21 (35)	39 (65)
	Double	56 (40)	84 (60)
	Triple	0 (0)	1 (100)
endometrial thickness at embryo transfer –mm (Mean±SD)		11.04±2.42	10.77±2.65

*2 patients excluded from IVF data due to spontaneous conception

3.3.2 **Prevalence of raised NK status in women undergoing IVF**

The first objective of this study was to define the prevalence of raised NK cells in women undergoing IVF treatment.

The prevalence of raised uNK status in the total population was 61.6% (CI 54.9-68.3).The prevalence of normal NK cells was 38.4% (CI 31.7-45.1).

The next sections in this chapter will present the prevalence of raised uNK according to age, cause of infertility, previous IVF and spontaneous history. Although rarely discussed in the literature the prevalence of raised uNK result are also presented according to the embryo quality generated in the subsequent IVF cycle. Statistical differences through chi square tests and logistic regression are also displayed for NK status and the above variables (tables 3.3-6).

3.3.2.1 ***Female age and raised NK status***

Figure 3.2 shows the age group distribution of the patient population.

The majority of patients were aged between 35-39 years (46.8%).

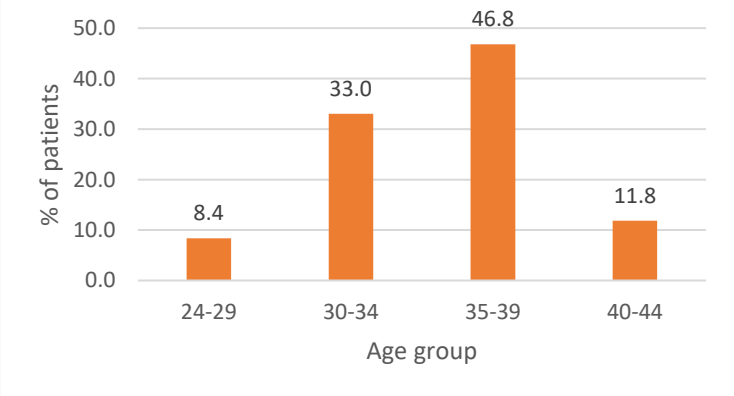


Figure 3-2- The age distribution of the study population

Surprisingly the prevalence of raised NK cells was the highest in the youngest patients aged 24-29 (70.6% CI 48.9-92.3) (table 3.3) and this comprised the smallest group of patients (8.4%).The prevalence decreased with increasing patient age with those aged between 40-44 having a prevalence of 41.7% (CI 22.1-61.3).

The logistic regression showed that age was not overall statistically significant (P value-0.14). However there was a trend of older patients being much less likely to have high NK results which was statistically significant (P trend-0.04).

Table 3-3- NK status, age and cause of infertility

Variable	Groups	Number of NK positive patients/total number of patients	Percentage of NK positive patients (prevalence)	Crude OR (95% CI)	P value
age group (yrs)	24-29	12/17	70.6	1	0.14 P trend-0.04*
	30-34	45/67	67.2	0.85 (0.27-2.72)	
	35-39	58/95	61.1	0.65 (0.21-2.01)	
	40-44	10/24	41.7	0.30 (0.08-1.12)	
cause of infertility	Unexplained	26/47	55.3	1	0.24
	Male	55/79	69.6	1.85 (0.88-3.91)	
	Tubal	10/22	45.5	0.67 (0.24-1.86)	
	Other	10/17	58.8	1.15 (0.38-3.55)	
	Multifactorial	24/38	63.2	1.39 (0.58-3.32)	

3.3.2.2 *Cause of infertility and raised NK status*

The most popular cause of infertility in this population was male factor (figure 3.3).

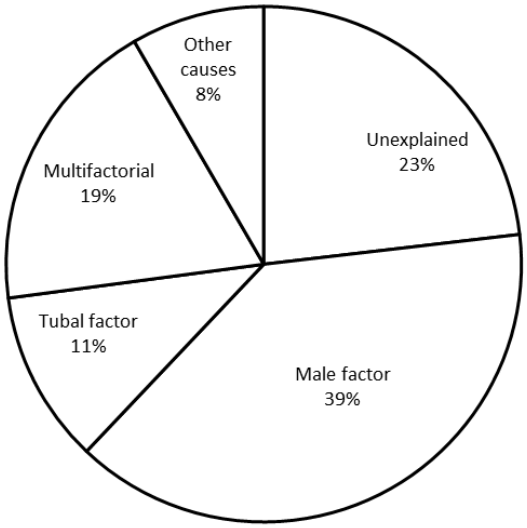


Figure 3-3- The cause of infertility in the study population

This was defined as abnormal sperm parameters according to the WHO criteria that is used in most fertility clinics (Patel, Leong et al. 2017). This may have skewed the data as the female partners did not have any significant gynaecological history and majority were likely to have a good IVF prognosis. The other causes compromised of 17 patients who has either PCOS or endometriosis. The multifactorial group included couples with at least one cause of infertility, which in the majority of cases involved both male and female factors.

The prevalence of raised uNK was the highest in those with male factor (69.6% CI 59.5-79.7) and lowest in those with tubal factor (45.4% CI 24.7-66.3). The patients with no perceived fertility cause i.e. unexplained had a prevalence of over 50% (55.5% CI 41.1-69.5) (table

3.3).The logistic regression also revealed male factor to be the most common cause of infertility in the NK positive group crude OR 1.85 (CI 0.88-3.91). However this was not statistically significant (P value -0.24).

3.3.2.3 *Previous IVF history and raised NK status*

The highest % of patients were those who had 1 previous unsuccessful IVF cycle or no treatment. Only 6.9 % of patients had 3 more or unsuccessful cycles and 13.3 % of patients had a history of live birth through IVF (figure 3.4).

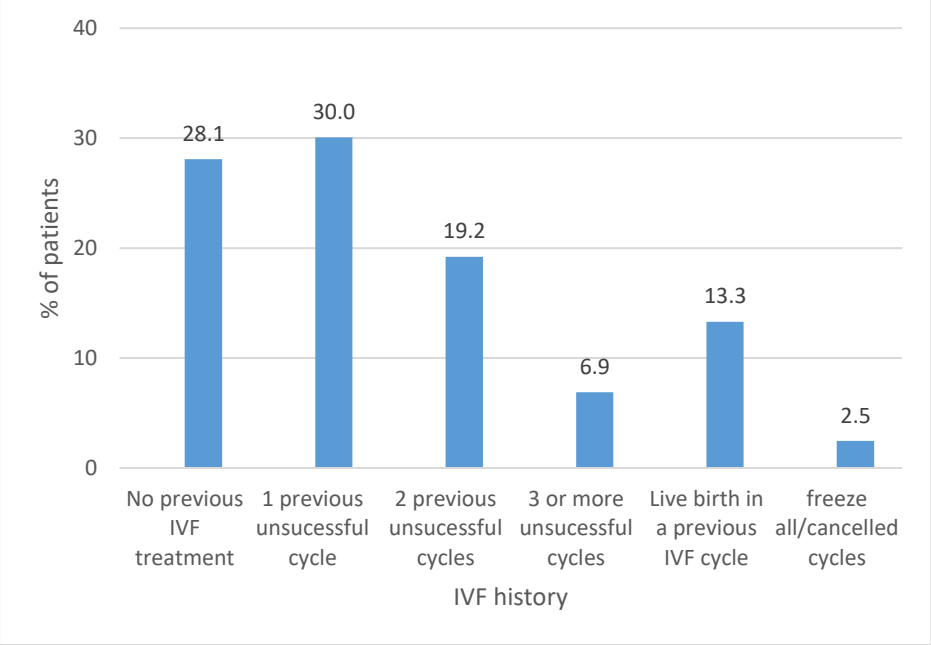


Figure 3-4- IVF history in the study population

The prevalence of raised NK cells was highest in those who has 3 or more unsuccessful cycle (71.4% CI 47.8-95.1) and the lowest in those with 2 previous unsuccessful cycles (43.6% 28.0-59.1).Surprisingly the prevalence in women with no previous IVF treatment was also high 66.7% (CI 54.4-78.9).The prevalence was 55.6% (CI 36.8-74.3) in patients who had a previous live birth through IVF.

The logistic regression revealed that patients were less likely to be NK positive if they had 2 previous unsuccessful cycles; crude OR 0.39 (0.17-0.89) or a previous live birth through IVF; crude OR 0.73 (0.30-1.79) (table 3.4).

However they were more likely to be NK positive if they had 1 previous unsuccessful IVF; crude OR 1.03 (0.48-2.21) or more than 3 previous unsuccessful IVF treatment; crude OR 1.25 (0.35-4.51). Overall both these associations were not statistically significant (P=0.12).

Table 3-4- Raised NK status and previous IVF history

Variable	Groups	Number of NK positive patients/total number of patients	Percentage of NK positive patients	Crude OR (95% CI)	P value
previous IVF History	no previous treatment	38/57	66.7	1	0.12
	1 previous unsuccessful cycle	41/61	67.2	1.03 (0.48-2.21)	
	2 previous unsuccessful cycles	17/39	43.6	0.39 (0.17-0.89)	
	at least 3 previous unsuccessful cycles	10/14	71.4	1.25 (0.35-4.51)	
	live birth in a previous IVF cycle	15/27	55.6	0.73 (0.30-1.79)	

3.3.2.4 *Previous spontaneous pregnancy history and raised NK status*

71 (35%) patients had a previous history of spontaneous pregnancy.

The prevalence of raised uNK status in those who had achieved a previous spontaneous pregnancy (secondary infertility) was 70.4%

(CI -59.8-81) versus 56.8% (CI 48.4-65.3) for those with a history of no pregnancies (primary).

Although the prevalence in the termination of pregnancy group was the highest (85.7% CI 59.8-111.6), there were only a total of 7 patients in this group making the findings questionable and this was reflected in the wide confidence interval. The next highest prevalence was seen in the previous miscarriage and ectopic pregnancy group (73.5% CI 61.1-85.9). The prevalence of those who had a previous live birth was the lowest (53.3% CI 28.1-78.5) which was comparable to those who has a live birth through IVF (55.6% CI 36.8-74.3).

The logistic regression showed borderline statistical significance for raised uNK results in those having a previous spontaneous pregnancy (crude OR 1.81 (CI 0.98-3.35)) with a P value of 0.057 (table 3.5).

Stratifying the population in various pregnancy outcomes showed that those who had a previous termination of pregnancy; crude OR 4.56 (0.53-38.95) and miscarriage; crude OR 2.11 (1.02-4.33) were more likely to be NK positive. Those who a live birth; crude OR 0.87 (0.30-2.54); were less likely to be NK positive However these associations were not statistically significant (P value 0.096).

Table 3-5- Previous spontaneous pregnancy history and raised NK status

Variable	Groups	Number of NK positive patients/ total number of patients	Percentage of NK positive patients	Crude OR (95% CI)	P value
previous spontaneous pregnancy	No previous pregnancies	75/132	56.8	1	0.057*
	History of previous pregnancy	50/71	70.4	1.81 (0.98-3.35)	
outcome of previous spontaneous history	No pregnancies	75/132	56.8	1	0.096
	Miscarriage/ ectopic	36/49	73.5	2.11 (1.02-4.33)	
	Termination of pregnancy	6/7	85.7	4.56 (0.53-38.95)	
	Live birth	8/15	53.3	0.87 (0.30-2.54)	

3.3.2.5 *Embryo quality and raised NK status*

Embryo quality and NK cells have seldom been investigated in the literature. Embryos were graded by the embryology team and this was stratified according to the number and grade to create equal groups. The mixed quality group was used to denote embryos that were a combination of high and low quality. The “other” group contained embryos that were single low quality and the one patient who had a triple low quality transfer.

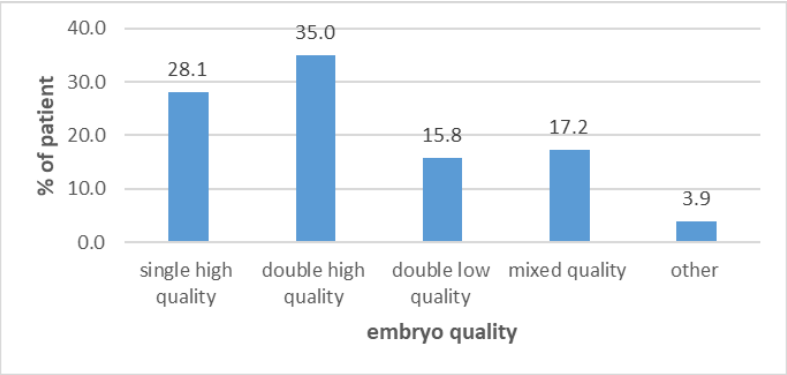


Figure 3-5- The distribution of embryo quality in the patient population

The majority of patient had a high quality single (28.1%) or double (35%) embryo transfer. Interestingly, the double low quality group had the highest prevalence of raised NK results (71.9% CI 56.3-87.5). Conversely the double high quality (54.9% CI 43.3-66.5) and mixed quality embryo (60% CI 43.8-76.2) groups had the lowest prevalence of NK positive results (table 3.6). The cross tab revealed no significant differences in NK status amongst the group of embryo quality (P value-0.55).

Table 3-6- NK status and subsequent embryo quality

Variable	Groups	Number of NK positive patients/total number of patients	Percentage of NK positive patients	Crude OR (95% CI)	P value
embryo quality in the subsequent IVF cycle	single high quality	37/57	64.9	1	0.55
	double high quality	39/71	54.9	0.66 (0.32-1.35)	
	double low quality	23/32	71.9	1.38 (0.54-3.55)	
	mixed quality	21/35	60	0.81 (0.34-1.93)	
	other quality*	5/8	62.5	0.90 (0.20-4.17)	

3.3.3 IHC testing and prediction of live birth

The other main hypothesis in this study was to test if NK status is predictive of live birth through IVF treatment. As already described in the introduction, this was not possible for the whole patient population (203 patients) as the women who tested positive had a therapeutic intervention. Whilst these women could not be compared to the negative NK cohort, a sub-group analysis looking at a positive result to see if there was an association with live birth was performed.

There were 105 patient who had a live birth and 69 (65.7%) had raised NK status. The 124 patients who tested positive for uNK status were divided into 4 severity groups (table 3.7).

Table 3-7- The classification of NK severity

NK severity	**Low (5-9.9%)**
	Medium (10-19.9%)
	High (20-49.9%)
	Very high (>50%)

These arbitrary values were set according to data from previous studies where raised uNK cells varied from 5% (Quenby, Kalumbi et al. 2005) to 13.9% (Liu, Mariee et al. 2014). Splitting the NK result of the whole population also revealed the 5th centile to be 1.85% and 90th centile as 45.4% and therefore confirming the reference ranges. The category of high NK cell severity was therefore classified as over 20% and very high as greater than 50%.

3.3.3.1 *Differences in NK cell status and live birth*

The prevalence of live birth was 46.8% in the NK negative group and 55.5% in those testing positive. The logistic regression revealed a crude OR of 1.43 (0.81-2.53) for the positive group but this was not statistically significant (P value-0.22).

3.3.3.2 *Differences in raised NK status severity and live birth*

The 124 patients with raised NK status was stratified according to the severity of the result as outlined in sub section 3.3.3. This revealed those with low-high severity being very similar in prevalence. However those with a NK cell level greater than 50% had a lower live birth rate; crude OR 0.72 (0.24-2.19) but this was not statistically significant (P value 0.87).(table 3.8).

Table 3-8- NK status severity and live birth

Variable	Groups	Number of patient who had a live birth/total number of patients	Percentage of patients who had a live birth	Crude OR (95% CI)	P value
NK status	negative	36/77	46.8	1	0.22
	positive	69/124	55.6	1.43 (0.81-2.53)	
NK severity	low (5-9.9%)	27/49	55.1	1	0.87
	medium (10-19.9%)	21/36	58.3	1.14 (0.48-2.72)	
	high (20-49.9%)	13/22	59.1	1.18 (0.43-3.26)	
	very high (>50%)	8/17	47.1	0.72 (0.24-2.19)	

3.3.3.3 *Raised NK status, age and live birth*

The same logistic regression model was applied to the 124 patients who tested positive to see if there were any statistical significant predictors of live birth in this group.

Table 3.9 shows an age related decline in live birth in those that were NK positive. Although this was not statistically significant overall, there was a strong association with age P trend-0.02.

3.3.3.4 *Raised NK status, cause of infertility and live birth*

The univariate analysis for this variable revealed that there was a near equal association with live birth regardless of the cause of infertility. This was not statistically significant with a P value of 0.94. The crude OR ranged from 1.40 (CI 0.54-3.61) for male factor and 1.62 (0.37-7.20) for both tubal and other causes of infertility (table 3.9).

Table 3-9- Raised NK status, live birth, age and cause of infertility

Variable	Groups	Number patients with a live birth/total number of patients	Percentage of patients who had a live birth	Crude OR (95% CI)	P value
age group (yrs)	24-29	9/12	75	1	0.12 P trend-0.02
	30-34	28/45	62.2	0.55 (0.13-2.32)	
	35-39	29/57	50.9	0.35 (0.085-1.41)	
	40-44	3/10	30	0.14 (0.022-0.94)	
cause of infertility	unexplained	12/25	48	1	0.94
	male	31/55	56.4	1.40 (0.54-3.61)	
	tubal	6/10	60	1.62 (0.37-7.20)	
	other	6/10	60	1.62 (0.37-7.20)	
	multifactorial	14/24	58.3	1.52 (0.49-4.69)	

3.3.3.5 *Raised NK status, previous spontaneous pregnancy history and live birth*

This revealed that those with a previous history of spontaneous pregnancy and raised uNK status were more likely to have a live birth; crude OR 1.11 (CI 0.54-2.29).However this was not statistically significant P value -0.79.

There was also an observation of those with a previous history of an ectopic or early miscarriage having a higher likelihood of live birth; crude OR; 1.40 (CI 0.62-3.20) as opposed to those that had previous successful live birth being lower; crude OR; 0.83 (0.19-3.57). This trend was also not statistically significant P value=0.56 (table 3.10).

Table 3-10- Raised NK status, livebirth and previous spontaneous pregnancy

Variable	Groups	Number patients with a live birth/total number of patients	Percentage of patients who had a live birth	Crude OR (95% CI)	P value
previous spontaneous pregnancy	no previous pregnancies	41/75	54.7	1	0.79
	history of previous pregnancy	28/49	57.1	1.11 (0.54-2.29)	
outcome of previous spontaneous history	no pregnancies	41/75	54.7	1	0.56
	miscarriage/ectopic	22/35	62.9	1.40 (0.62-3.20)	
	termination of pregnancy	2/6	33.3	0.42 (0.07-2.40)	
	live birth	4/8	50	0.83 (0.19-3.57)	

3.3.3.6 *Raised NK status and embryo quality in the subsequent IVF cycle*

This revealed a similar trend to age with the likelihood of live birth declining as the embryo quality declined (table 3.11). Although this not was statistically significant overall, there was borderline significance in the declining trend (P value=0.27, P trend-0.057).

Those with a double embryo transfer of high quality embryos were the most likely to have a live birth; crude OR 1.09 (CI 0.43-2.76) as opposed to those with mixed quality having the lowest chance; crude OR 0.38 (CI 0.12-1.13). This however was not statistically significant (P=0.27).

Table 3-11- Raised NK status, live birth and embryo quality

Variable	Groups	Number patients with a live birth/total number of patients	Percentage of patients who had a live birth	Crude OR (95% CI)	P value
embryo quality in the subsequent IVF cycle	single high quality	23/37	62.2	1	0.27 P_{trend} 0.057
	double high quality	25/39	64.1	1.09 (0.43-2.76)	
	double low quality	11/23	47.8	0.56 (0.19-1.60)	
	mixed quality	8/21	38.1	0.38 (0.12-1.13)	
	other quality*	2/4	50	0.61 (0.08-4.82)	

3.4 Discussion

The first hypothesis in this chapter was that of determining the prevalence of raised uNK status in women undergoing IVF treatment.

The prevalence of raised uNK status was noted to be 61.6% (CI 54.9-68.3).in the IVF population. Furthermore the prevalence in women with no previous IVF treatment (primary infertility) was also high 66.7% (CI 54.4-78.9).

One of the main strengths of this work is that women were recruited from a highly successful IVF centre. The clinical pregnancy rates are 2-3 fold higher than the national average e.g. 65% for women aged under 35 versus 29% respectively (HFEA 2018). This is mainly due to stringent laboratory processes leading to the creation of optimal quality embryos and their transfer. Therefore if IVF did not result in a pregnancy, then endometrial receptivity or adverse maternal immunology could be contributory factors.

Conversely the main weakness in this study and the literature at large is that the prevalence of raised uNK cells in normal fertile controls had not been fully established (Seshadri and Sunkara 2014). Therefore a baseline value of healthy controls is not available for comparison (Laird, Mariee et al. 2011). However a recent retrospective study with 215 women undergoing IHC testing (Chen, Mariee et al. 2017) found the prevalence of raised uNK status amongst healthy fertile controls to be 3.5% and those with RIF to be around 29.4%. A raised uNK cell status was defined as 4.5% and the study was conducted using robust

methodology with the endometrial biopsies being taken at 7 to 9 days after the LH (ovulation) surge.

The correct timing of the NK biopsy which is under the influence of the menstrual cycle has been cited as one of the main determinants of obtaining accurate results (Lash, Bulmer et al. 2016). This further is complicated by NK cells in the uterus varying from menstrual cycle to cycle (Mariee, Tuckerman et al. 2012).

In this study the prevalence of raised uNK in those with RIF (three or more unsuccessful cycles) was also high (71.4% CI 47.8-95.1). This is supported by previous literature (Ledee-Bataille, Dubanchet et al. 2004, Thum, Bhaskaran et al. 2004) However this was more than double than the results stated by (Chen, Mariee et al. 2017). This variability may be explained by the fact that the biopsies were not timed according to the LH surge and were offered between day 19-24 of the menstrual cycle. This may have led to a high number of false positives in the overall patient population. Looking at the literature, studies where endometrial biopsies were taken without LH timing showed a range of 3.9-52.8%(Laird, Mariee et al. 2011). NK levels are also much higher prior to menstruation (Salamonsen and Lathbury 2000).

The higher prevalence of raised uNK status in women with male factor infertility was interesting (69.6% CI 59.5-79.7) as these patients had no gynaecological issues and were free of autoimmune disease. They could be perceived as healthy controls. Furthermore patients with no fertility cause i.e. unexplained also had a prevalence of over 50%

(55.5% CI 41.1-69.5). NK testing on the whole is not widely available in most fertility clinics and very little data being available for this group. This may be a new finding and certainly needs corroborating with a functional assessment of this immune dysfunction.

The retrospective analysis of stratifying the patients into their previous IVF history revealed that the prevalence was highest in those who has 3 or more unsuccessful cycle (71.4% CI 47.8-95.1) as already stated. The prevalence was the lowest in those with 2 previous unsuccessful cycles (43.6% 28.0-59.1). This was lower than those with no previous treatment 66.7% (CI 54.4-78.9) and one previous cycle (67.2% CI 55.4-79.0). Patients who had a previous live birth through IVF had a slightly lower prevalence that these two groups (55.6% CI 36.8-74.3). Looking at the live birth rate in those that had a raised uNK status according to the number of previous IVF cycles, it was also highest in those with 2 previous unsuccessful treatment cycles 64.7%. The lowest live birth rate was seen in those that had a previous live birth through IVF (44.4%). These differences in live birth could be explained by the fact that prednisolone was given to all women with a raised uNK status. This may have been beneficial to those that had not achieved a pregnancy previously with perceived correction of immunological dysfunction. However those who had previously been pregnant may not have needed this “correction” and in fact medical treatment was not advantageous to these women.

The differences in uNK status in these may be due to the notion of immune “priming” where patients who have had more than one attempt

with IVF and have an activation in their immune cell pathways or correction of raised uNK cells. There is some evidence to suggest that the high oestrogenic state that occurs as a result of ovarian stimulation in IVF can reduce the proportion of cytotoxic CD56 ^{dim} NK cells (Lukassen, Joosten et al. 2004).The same phenomenon may explain the lowered prevalence in those that achieved a live birth. The other contributing factor may have been the beneficial effects from the biopsy itself i.e. the binary advantage of endometrial scratching which is known to improve live birth in women undergoing IVF (Vitagliano, Di Spiezio Sardo et al. 2018).

The subgroup analysis of previous spontaneous pregnancy revealed a high prevalence of raised uNK status in those who had a previous spontaneous miscarriage (73.5% CI 61.1-85.9). This was similar to those with RIF. Patients with no previous pregnancies and spontaneous live birth had the lowest prevalence of 56.8 (CI 48.4-65.3) and 53.3 (28.1-78.5) respectively. This trend is supported by the literature as women with a history of recurrent miscarriage and RIF exhibit raised uNK status (Tang, Alfirevic et al. 2011, Kuon, Weber et al. 2017, Ledee, Prat-Ellenberg et al. 2017).

The univariate analysis revealed an association with age. Although there were no statistical differences between the age groups (P value 0.12) there with a decline in raised uNK status as age increased (P $_{trend}$-0.04). Similarly the association of raised uNK status and live birth was not statistically significant overall, there was a strong association with age (P trend-0.02). It is well documented that age is an independent

factor for IVF success due to decline in ovum quality (Spandorfer, Chung et al. 2000). There was a similar trend with the likelihood of live birth declining as the embryo quality declined. Although this not was statistically significant overall, there was borderline significance in the declining trend (P value=0.27, P trend-0.057).

It is known that the immune cell function declines with age. Although the focus of literature has been on raised NK cells, the significance of low NK cells is unknown. They may also be detrimental to pregnancy outcome and contribute to the age related decline in fertility.

There was a similar trend seen in the prevalence data for raised NK results and embryo quality in the subsequent IVF cycle. Although not statistically significant, those that had 2 good quality embryos replaced, had the lowest prevalence of raised NK status. The reason for this could have been two-fold. The patients who had a good prognosis in terms of embryo quality may have had a normal NK status. The other plausible explanation may have been the corrective potential of IVF to lower NK cytotoxicity as already discussed, which led to an improvement in embryo quality.

The prevalence of live birth was 46.8% in the NK negative group and 55.5% in those testing positive. The logistic regression revealed a crude OR of 1.43 (0.81-2.53) for the positive group but this was not statistically significant (P value-0.22). In this study there were no differences in livebirth with low-high severity in raised uNK. However those with a NK cell level greater than 50% had a lower live birth rate,

even though these patients were given steroid treatment; crude OR 0.72 (0.24-2.19) but this was not statistically significant (P value 0.87).

A separate sub group analysis was performed for the women who tested IHC positive as they were given steroid treatment. It is debatable to whether steroid treatment is beneficial for those that have raised uNK (Polanski, Barbosa et al. 2014). However there is some emerging evidence from recent studies in patients with IVF failure and RIF that have shown an improvement in live birth through both oral (Ledee, Prat-Ellenberg et al. 2018) and intrauterine steroid administration (Zhang, Huang et al. 2017).

It is known that NK cells have a receptor for endogenous steroids and normal implantation is initially facilitated by progesterone derived cortisol (Robertson, Jin et al. 2016). There may have been group of women in this cohort that displayed an impaired immune response in whom exogenous steroid support was beneficial. However the exact mechanism and clinical test need to identify these patients is still unknown.

3.4.1 Limitations of the study and further work

The main limitation of this work was that although the methodology was delivered to a high standard in a good IVF centre, the biopsies were not timed according to the LH surge. This may have led to the extremely high prevalence seen in patients with male factor and unexplained infertility.

As already discussed in this chapter 1, uNK status is influenced by hormonal, menstrual cycle variability, pathogenic and laboratory interpretation. Therefore to gauge an accurate assessment of whether immune cells are implicated in pregnancy, a functional aspect of study is required.

One of the biggest confounder in this study was that all the women who tested positive for NK levels were given oral prednisolone. Stratifying the population into the severity of uNK positive status did not reveal a statistically significant association or trend with live birth. This may have been because of low study numbers or a lack of association as cited by earlier work (Ubaldi, Rienzi et al. 2002, Boomsma, Keay et al. 2012) .

Whilst this is reflective of clinical practice, the direction of future work would be to design a randomised controlled trial whereby NK positive and negative patients are allocated to prednisolone treatment. The subgroup analyses would then establish whether prednisolone is an effective intervention for negative and positive patients. The practical consideration of such a trial would be the large number of participants

required but such a study is not only urgently needed but would be acceptable to patients (Tang, Alfirevic et al. 2013).

3.4.2 **Conclusion**

IHC assessment of uNK status is currently one of the most commonly used methods of assessing this dynamic and multifunctional immune cell population.

In this study of 203 patients, although the prevalence of raised uNK status was high for those with RIF and a history of miscarriage, there were only statistically significant associations found for age in terms of predicting a positive result.

Therefore IHC has to be complimented with another technique such as flow cytometry to determine if uNK are significant in implantation and pregnancy success.

Chapter 4

A comparison of immunohistochemistry and flow cytometry in women undergoing IVF

Chapter 4 A comparison of immunohistochemistry and flow cytometry in women undergoing IVF

4.1 Introduction

After determining that IHC gives a limited perspective of NK analysis in the context of in vitro fertilisation (IVF), the focus of this chapter is to explore if flow cytometry (FC) provides detailed information on the function of these cells and their immunological counterparts.

The purpose of introducing such a panel is to expand upon the literature that supports FC is a useful tool for measuring NK cells (Manaster, Mizrahi et al. 2008, Santillan, Lozano et al. 2015, Jiang, Yan et al. 2017). One of the main advantages of such panels is that immune cells populations are measured alongside their functional components e.g. cytokine expression.

The results of a prospective study of 42 patients undergoing IVF where an extensive FC panel aimed at assessing NK cells and various markers was applied to paired endometrial and peripheral blood samples are presented.

The main aim of this work was to demonstrate the use of FC in women undergoing IVF that goes beyond determining CD56+ cell numbers only; which is one of the main pitfalls of IHC. The key objective was to decipher the significance and function of other immune cells including NKT, CD8+ (Tc) and CD4+ cells which may be relevant in determining pregnancy outcome. Furthermore the expression of cytokines such as perforin, granzyme, tumour necrosis factor alpha (TNFα) and inteferon

gamma (IFNγ) expression was evaluated as these may also play a significant part in implantation.

4.2 Methods

These are fully described in Chapter 2.

Patients undergoing both fresh and frozen IVF cycle at Nurture fertility IVF clinic, Nottingham, UK between; June 2016-October 2017 were approached at different stages of their fertility history. A written consent form was completed by all patients recruited to the study.

An endometrial biopsy was taken in the mid luteal phase of the menstrual cycle (day 19-24) with a pipelle de cornier ®sampler. A 20ml sample of venous blood was also taken. Both samples were processed immediately for flow cytometric (FC) analysis and within 48 hours for IHC. A raised IHC test was defined as a NK percentage of 5% and above, via computerised slide analysis. The flow cytometry data analysis was carried out using the Kaluza ®version 1.5a flow analysis software package.

The patients continued with IVF treatment and the results of both IHC and FC assessment were collated. The pregnancy outcomes of the subsequent IVF cycle were also studied and this is discussed in detail in Chapter 5.

Furthermore stratifying the same patient group into prednisolone treated (positive NK result) versus no treatment and correlating this to the FC results is presented in Chapter 6.

4.2.1 Statistical analysis

Statistical analysis of the flow cytometry data was conducted using the graph pad prism® version 7.05 package. The normality of the data was checked by constructing histograms of paired differences in the outcome variable. This revealed that the data was normally distributed.

Clinical data including age, BMI, cause of infertility and previous IVF history for all the patients. This was analysed using the SPSS version 25 (Statistical Package for Social Sciences; IBM, IL, USA). A paired student t test was used to assess any difference in the mean % of cells between the IHC and FC samples (PB and endometrial). This was then repeated for the comparison of cytokine expression between the different immune cell populations in the FC samples.

A P value<0.05 was deemed to be statistically significant.

4.3 Results

The panel was originally applied to 47 patients. However 5 patients were excluded from the final analysis. 3 of these patients displayed weak staining rendering the results unreliable. One of the patients has their IVF treatment in another clinic and the remaining patient did not have the full panel applied. Data was analysed for the remaining 42 patients and is presented here.

4.3.1 **Patient demographics and characteristics**

Patient characteristics such as age, BMI, previous IVF history were collected and are shown in Table 4.1.

Table 4-1- The study population demographics

Demographic factor	**N=42 Mean±SD**
age (yrs)	35.43±4.59
body mass index (BMI)	24.45±4.38
previous number of IVF cycles	2.05±3.11
Cause of infertility	N=42 (%)
unexplained	4 (9.5)
male	19 (45.2)
tubal	4 (9.5)
other	6 (14.3)
multifactorial	9 (21.4)
NK status with IHC testing	N=42 (%)
negative	21 (50)
positive	13 (31)
inadequate result	2 (4.8)
declined biopsy	6 (14.3)

N=number of patients

(%)-percentage of patients

SD-standard deviation

The age of the patients ranged from 21-42 years. The number of previous IVF cycles had a wide range between no previous treatment (10 patients) to one patient having had 19 previous IVF cycles. Over half of the patients (57%) had either one or two previous unsuccessful IVF cycles. The majority of patients also had male factor infertility (45.2%).

6 patients had cycles that were either cancelled or did not yield embryos for transfer. 3 of these patients were cancelled due to inadequate response to medication, 1 patient had a failed fertilisation and 1 patient had a freeze all cycle due a thin endometrium. The

remaining patient had NK testing through IHC and participated in the sample collection for FC assessment but did not return for IVF treatment. However these patients were included in the final analysis and labelled as no embryos transferred.

Table 4.2 displays the patient demographics stratified into the results obtained for NK testing by IHC.

Table 4-2- IHC NK status and study population demographics

Demographic Factor	**Total N=42 Mean±SD**	**NK negative N=21 Mean±SD**	**NK positive N=13 Mean ±SD**	**Inadequate NK result N=2 Mean ±SD**	**Declined biopsy N=6 Mean±SD**
age (yrs)	35.43±4.59	34.71±4.86	35.85±4.14	37.5±4.95	36.33±5.16
body mass index (BMI)	24.45±4.38	25.5±5.06	23.76±3.56	21.75±1.63	23.41±4.16
previous number of IVF cycles	2.05±3.11	1.38±1.16	1.69±1.70	1.00±0.00	5.5±7.06
Cause of Infertility	N=42	NK negative (%)	NK positive (%)	Inadequate NK (%)	Declined biopsy (%)
unexplained	4	1 (25)	2 (50)	0 (0)	1 (25)
male	19	11 (57.9)	4 (21.1)	2 (10.5)	2 (10.5)
tubal	4	1 (25)	3 (75)	0 (0)	0 (0)
other	6	5 (83.3)	1 (16.7)	0 (0)	0 (0)
multifactorial	9	3 (33.3)	3 (33.3)	0 (0)	3 (33.3)

Half (50%-21 patients) of the patients tested negative and 31% (13 patients) were positive for NK testing. There were 6 patients who declined IHC testing but all opted to participate in the study and had FC analysis. Two patients underwent biopsy but had an inadequate result with IHC. There were no differences in the baseline characteristics of the two patient groups. The 13 patient who tested positive for NK cells by IHC were treated with 20mg of oral prednisolone in the same regimen outlined in chapter 3.

4.3.1.1 *Development of the FC panel and sample collection*

During the data collection period, the FC panel changed with the addition of additional markers. The original panel included the markers that identified the 4 immune cell populations along with measuring their expression of perforin and granzyme. The panel was then expanded to include TNFα and IFNγ as the compensation and experience was gained. Table 4.3 shows the tissue samples taken and FC panel applied.

Table 4-3- The FC panel applied and tissue types collected

Type of sample taken	Full panel with perforin and granzyme N=23	Full panel perforin, granzyme, TNFα and INFγ N=5	Full panel with all 4 cytokines and in vitro diagnostic test (CD56,CD16 and CD3) N=14
paired samples blood and endometrium	20	5	14
endometrium only	2	0	0
peripheral blood (PB) only	1	0	0

N=total number of patients tested

The first 20 patients with paired peripheral blood (PB) and endometrial samples had all 4 cell populations assessed along with perforin and granzyme. The 3 patients with either a blood or endometrial sample only also had this panel of markers applied.

The remaining 19 patients had perforin, granzyme, TNFα and IFNγ added. In an attempt to streamline the panel further 14 of these

samples has the full panel with all 4 cytokines and an in vitro diagnostic (IVD) test of CD56, CD16 and CD3. The IVD test contained 3 markers that could accurately identify the NK and NKT cells. These samples were analysed separately to check if the IVD was as reliable as the individual markers.

4.3.1.2 *Endometrial cell gating strategies*

The gating strategies used to identify the correct population of endometrial cells was performed in three ways as the panel evolved.

Initially a “large” gate encompassing all cells was used. The percentage of NK cells were therefore identified by gating for CD56+cells. This was strongly dependent on not only the number of NK cells but also the total endometrial cell population. Therefore an improved strategy was sought. The first step was to identify lymphocytes based on scatter and a “tight” gate emerged. However this was also prone to error. Therefore the final strategy included the addition of CD45+which is a lymphocyte marker. This approach was applied to the latter 25 samples, which made the gating more accurate. The tight gating strategy of drawing around the cells was still performed in these samples to compare concordances and correlations.

Table 4.4 displays the gating strategy used and types of tissues tested.

Table 4-4- The gating strategies used to identify the endometrial cells

Type of sample	**Tight gate N=42**	**CD45 gating N=25**
paired samples	39	25
endometrium only	2	0
PB only	1	0

4.3.2 **Comparison of the IHC and FC panel**

The results in the remainder of this chapter are presented as a series of experiments that initially display the differences in the two techniques of IHC and FC. NK testing through IHC was determined by assessing the cells that stained for CD56+only. This is displayed along with the CD56+ cells identified through FC with the application of the different endometrial gating strategies (Experiments 1-3).

4.3.2.1 ***Experiment 1- IHC CD56+ and FC CD56+/NK cell analysis using the tight gating strategy***

The IHC CD56+ (NK) result was compared to the FC CD56+ and NK population. The endometrial FC populations were identified by drawing a tight gate around the endometrial lymphocytes.

Figure 4.1 displays that although the IHC CD56+ results were higher, there was no significant correlation between the two testing techniques.

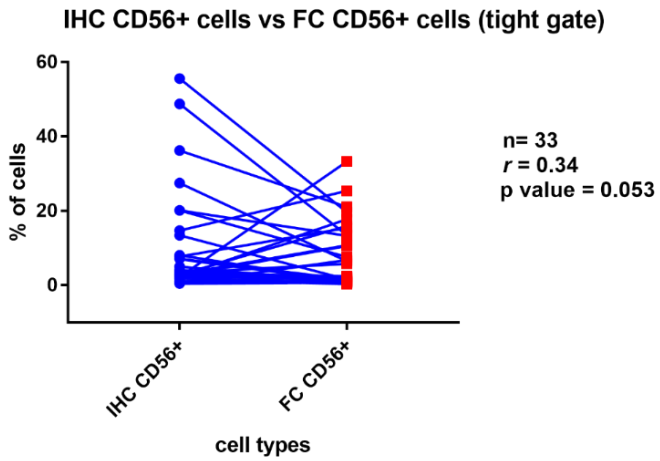


Figure 4-1- Comparison of IHC CD56+ with the FC panel CD56+ cells

4.3.2.2 *Experiment 2-IHC and FC CD56+/NK cell analysis using the large gating strategy*

Figure 4.2 shows the same analysis between IHC NK analysis and the FC results in samples where the large gating strategy was applied. The large gating strategy was used in the earlier 13 samples and involved drawing around the cells of interest which included all cell types making it prone to error.

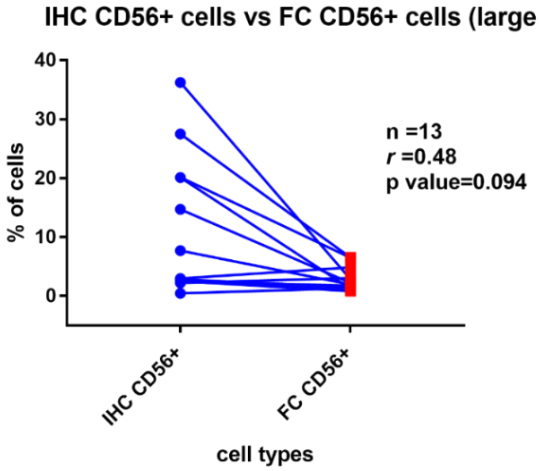


Figure 4-2- Comparison of IHC CD56+ with the FC panel CD56+cells

The IHC result was higher than the cells identified with FC. However there was no significant correlation between the two techniques.

4.3.2.3 *Experiment 3-IHC testing and FC CD56+ cell analysis using the CD45+ gating strategy*

Figure 4.3 shows the difference between the cells when CD45+ was added to the endometrial samples (N=20).

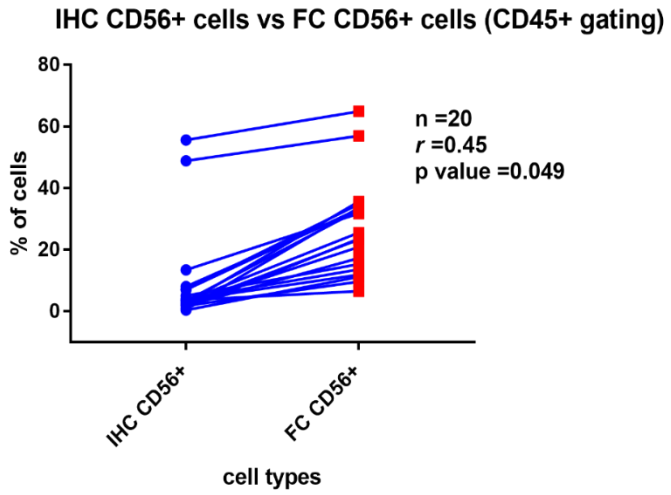


Figure 4-3- Comparison of IHC CD56+ with the FC panel CD45+/CD56+

The addition of the CD45+ led to the two techniques being weakly correlated (r=0.45, p value= 0.049). This can be seen in figure 4.3 as there is less spread of the values.

4.3.3 **Comparison of IHC and FC peripheral blood CD56+ cells**

Figure 4.4 shows the IHC CD56+ plotted against the peripheral blood (PB) CD56+ cells identified through FC. The two cell types were not correlated (p value = 0.284).

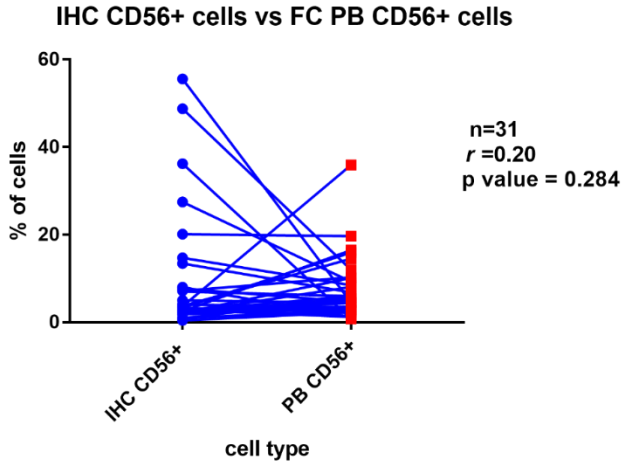


Figure 4-4- Comparison of IHC NK with the FC panel PB CD56+ and NK cells.

4.3.4 Comparison of FC analysis of the PB and endometrial immune cell populations

The remainder of this chapter will compare the different immune cell populations between the paired PB and endometrial samples.

4.3.4.1 *The PB immune cell populations*

Figure 4.5 shows the percentage (%) of the 5 different immune cell populations in the 39 PB samples.

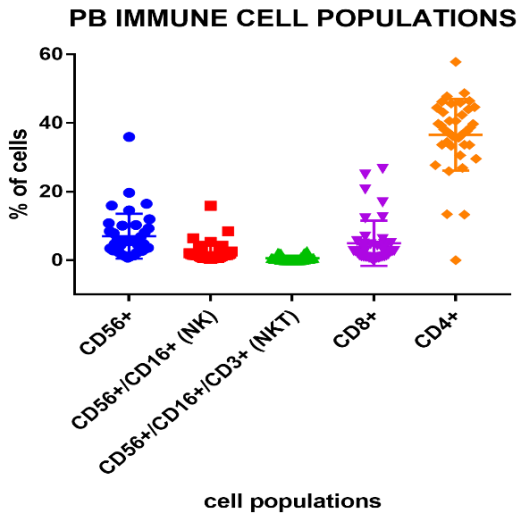


Figure 4-5- Mean percentage (%) of PB immune cell populations derived from the FC analysis

The populations varied in the PB samples with NKT being the lowest (mean %- 0.59±0.75) and the CD4+ cells the highest (mean %- 36.59±10.43).

The NK cell differed significantly to all the populations apart from the CD8+ cells. The NKT and CD4+ cells were statistically different from all cell populations. There were also differences between the CD8+ and CD4+ cells.

4.3.4.2 *Experiment 4-The addition of the CD56, CD16 and CD3 in vitro diagnostic test revealed a higher PB NK and NKT yield*

This experiment compared the percentage of NK and NKT cells identified through the FC panel against the addition of CD56, CD16 and CD3 IVD test. This led to a significant difference in the number of PB NK and NKT cells (figures 4.6A-B).

A comparison with the CD45+ gated endometrial NK cells also revealed a difference. Figure 4.6C shows that the endometrial NK cell were significantly higher than the IVD determined PB NK cells.

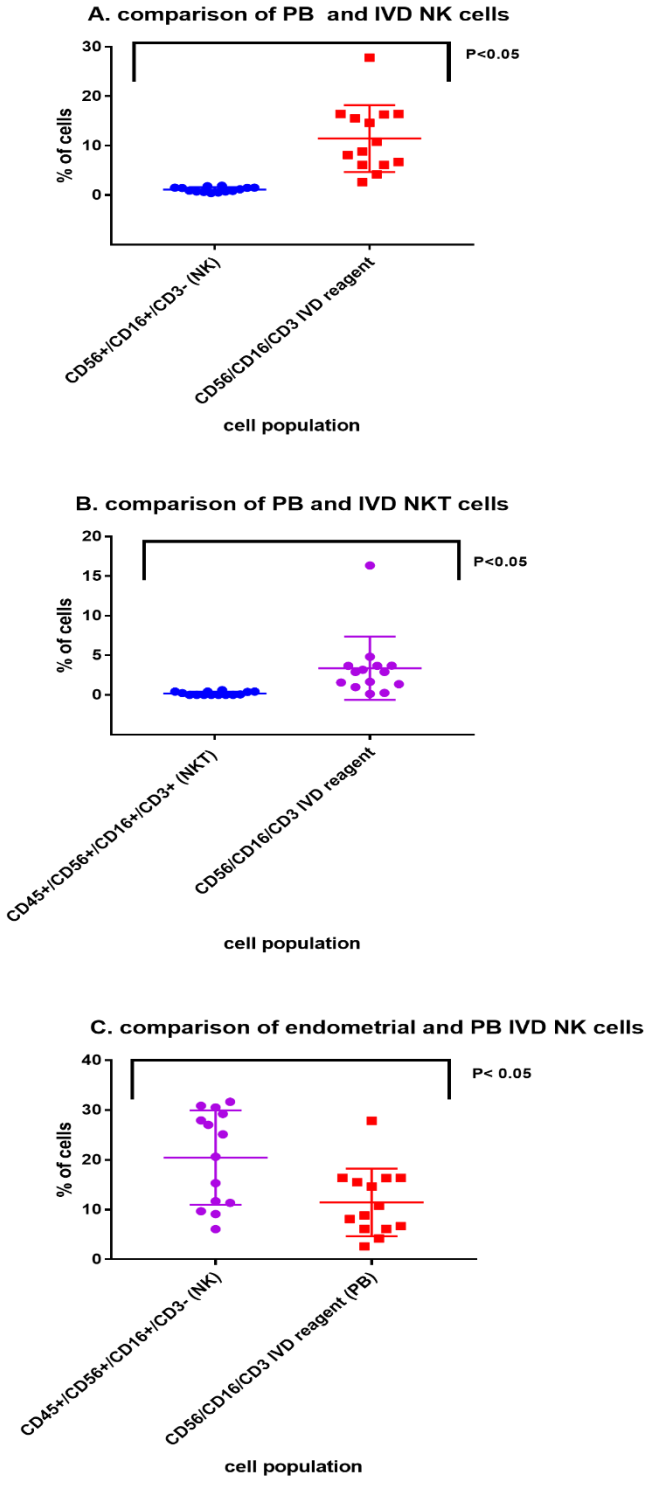


Figure 4-6- Comparison of NK and NKT cells with the addition of CD56/CD16/CD3 IVD test

The PB NK (figure 4.6A) and NKT (figure 4.6B) showed a significant difference with the in vitro diagnostic test population showing a higher mean %. The endometrial NK cells (figure 4.6C) were also compared to those identified through the IVD test. This revealed that the CD45+ gating was significantly different to the PB population.

4.3.4.3 *The endometrial immune cell populations*

These results are presented with the CD45+ gating strategy only as this was most accurate method of identifying the immune cell populations. This revealed that the highest % of cells were the CD56+ (mean %=25.31±14.78) and NK populations (mean %=19.93±12.44) (figure 4.7). The NKT number were the lowest (mean %=0.029±0.067).

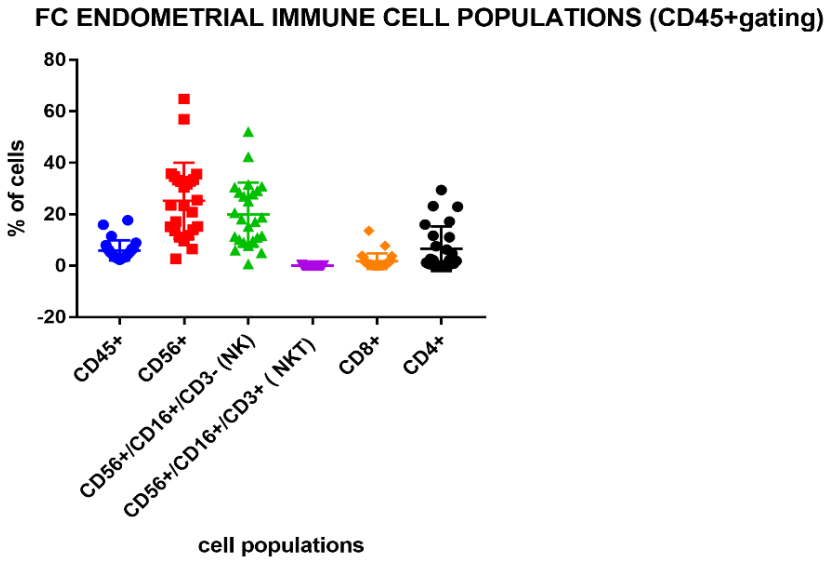


Figure 4-7- The mean % of endometrial immune cell populations derived from the FC panel (CD45+ gating strategy)

4.3.5 Comparison of paired PB and endometrial immune populations

This revealed that most of the immune cell populations except the CD8+ showed statistically significant differences in % between the PB and endometrial samples (figure 4.8).

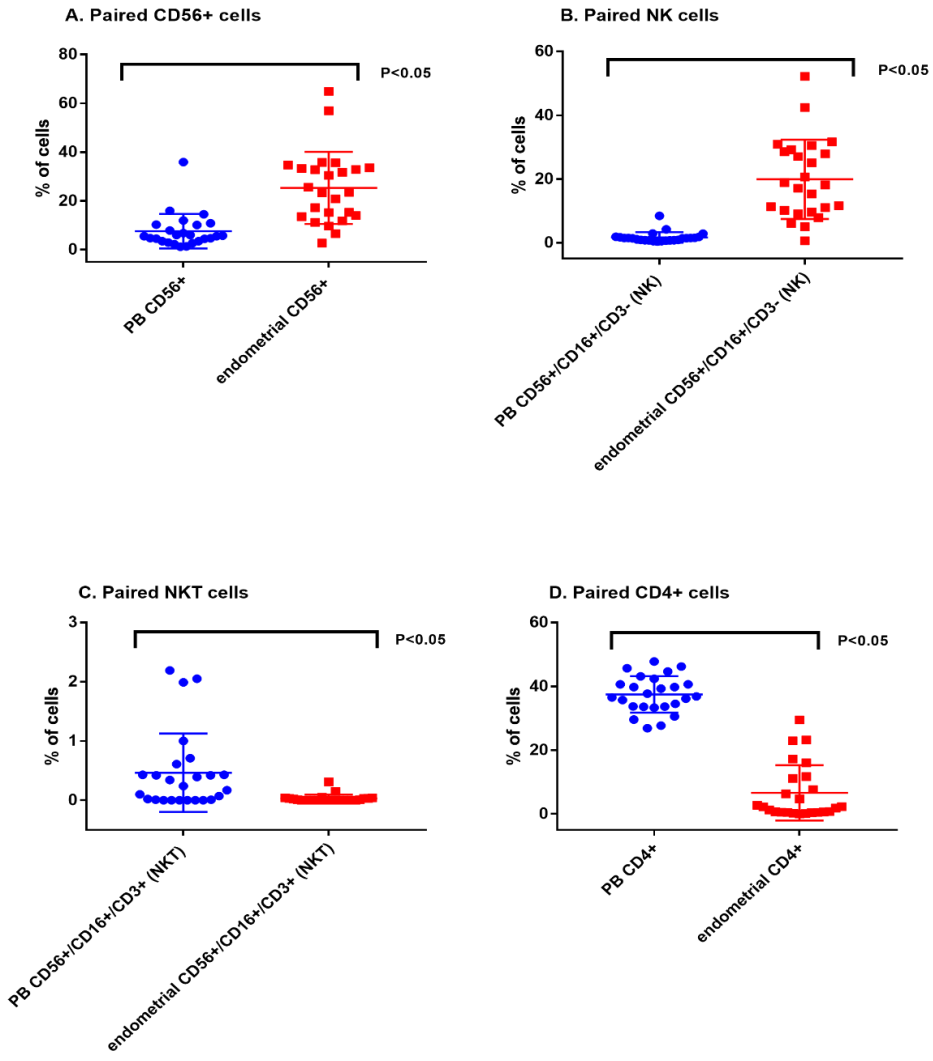


Figure 4-8- Comparison of the paired PB and CD45+ gated endometrial immune cell populations.

There were statistically significant differences in the mean % of CD56+ (figure 4.8A) and NK cells (figure 4.8B) with a higher % in the endometrial cells. The NKT (figure 4.8C) and CD4+ cells (figure 4.8D) were higher in the PB samples.

4.3.6 Comparison of cytokine expression in the PB and endometrial tissue

There were 6 cytokine populations detected with the panel. This included the individual expression of perforin and granzyme along with the co expression of both markers (perforin^{+}granzyme^{+}). This was the same for TNFα, IFNγ and a separate population of cells that co expressed TNFα and IFNγ.

4.3.7 Perforin and granzyme expression in the immune cell populations

The expression of perforin and granzyme was higher in the PB samples. Figure 4.9A shows the comparison of perforin expression only in the paired samples. A statistically significant difference was only detected in the expression of NKT cell perforin. The expression of granzyme only revealed a statistically significant difference in the NK (figure 4.9B) and CD4+ populations (figure 4.9C). The co expression of perforin^{+}granzyme^{+} only revealed a statistically significant difference in CD8+ cell population (figure 4.9D).

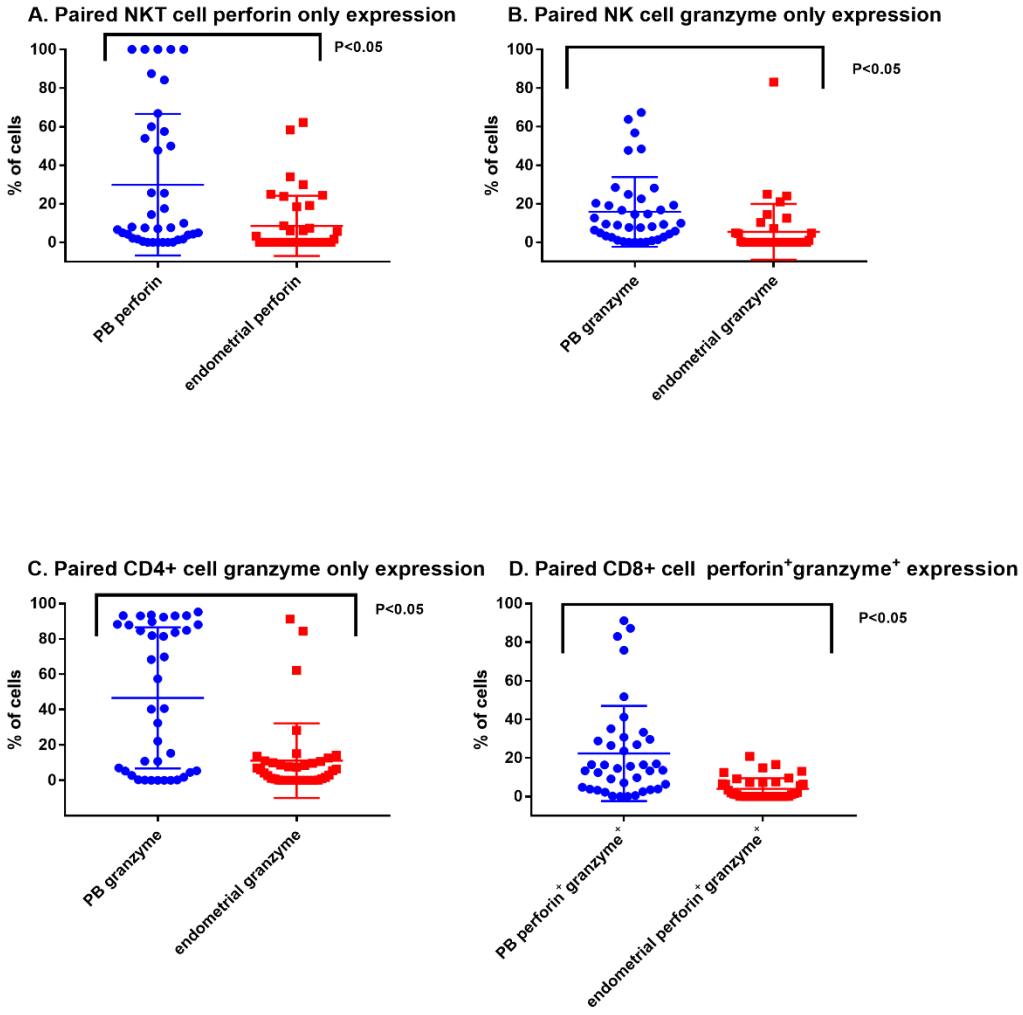


Figure 4-9- Comparison of paired PB and endometrial perforin and granzyme expression by the 4 immune cell populations.

The PB samples expressed a higher % of these cytokines compared to the endometrial tissue. The paired NKT perforin only (figure 4.9A), NK granzyme only (figure 4.9B), CD4+ granzyme only (figure 4.9C) and the co-expression of CD8+ perforin$^+$ granzyme$^+$ all showed statistically significant differences.

4.3.8 **TNFα and IFNγ expression in the immune cell populations**

This showed that only TNFα was expressed in detectable levels in both the PB and endometrial samples. The IFNγ only expression was very low and as was the co-expression of $TNF\alpha^{+}IFN\gamma^{+}$. There was only one sample in the PB group where CD4+ cell TNFα and IFNγ expression had been analysed and therefore a comparison for this variable is not presented.

The NK and CD8+ cells were the only populations to display a statistically significant difference in the expression of these cytokines between the paired samples (figures 4.10A-D).

The NK cell expression of TNFα and IFNγ only was significantly higher in the endometrial samples. This was similarly seen in the CD8+ expression of TNFα only and the co expression of both markers.

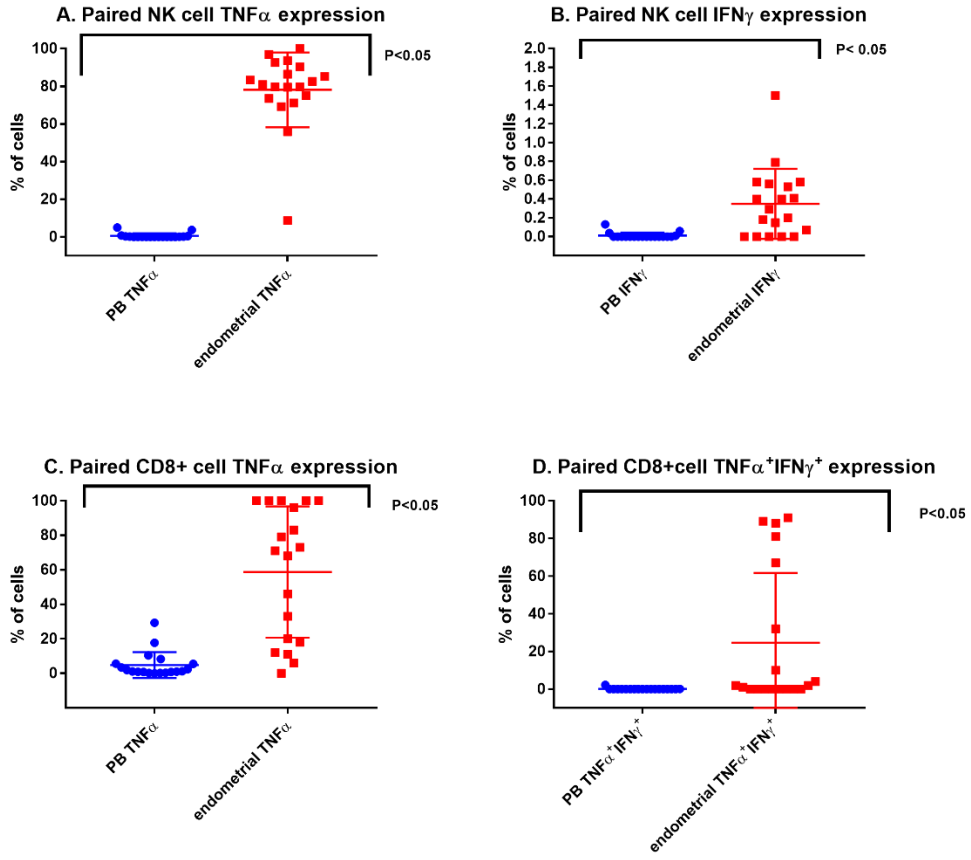


Figure 4-10- Comparison of paired PB and endometrial TNFα and IFNγ expression by the 3 immune cell populations.

The endometrial samples expressed a higher % of these cytokines compared to the PB. The paired NK TNFα only (figure 4.10A) and IFNγ only (figure 4.10B) along with, the CD8+ TNFα only (figure 4.10C) and the co-expression of CD8+ TNFα$^+$IFNγ$^+$(figure 4.10D) all showed statistically significant differences.

4.3.9 **Clinical outcomes of applying the panel**

Figure 4.11 displays the IVF clinical outcomes of the study population.

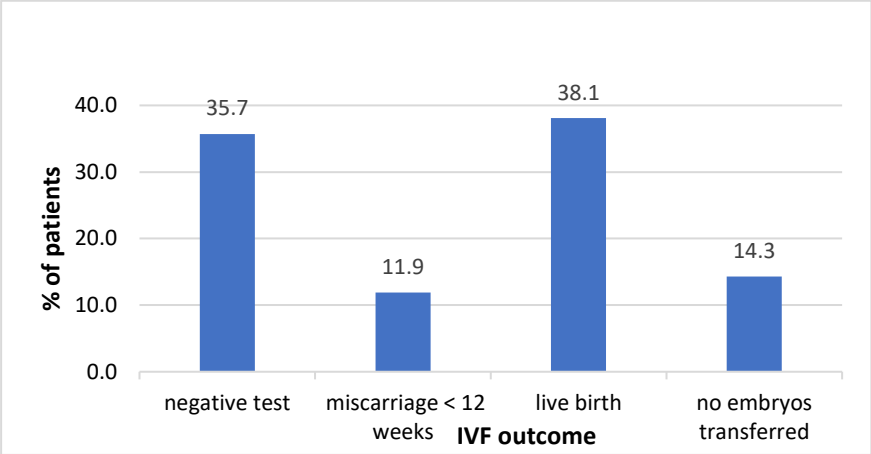


Figure 4-11- The IVF outcomes of the study patient population

This clinical outcomes will be fully discussed in Chapter 5.

However the % of patients with a negative pregnancy test and live birth was comparable (35.7% versus 38.1% respectively).

4.4 Discussion

FC has been used in reproductive immunology for the past 3 decades to determine the relationship between NK cells and pregnancy. Traditionally the mainstay of staining has involved identification of the CD56 and CD16 markers. However literature in the field has led to mixed results (Male, Gardner et al. 2012, Seshadri and Sunkara 2014, Moffett and Shreeve 2015). Studies have been mainly conducted in the recurrent miscarriage population which has now been defined as a different clinical condition to RIF, with its own distinct pathophysiology (Lachapelle, Miron et al. 1996, Shimada, Kato et al. 2004). Furthermore studies have used various populations with a mixture of fertile controls and patients with RIF who have undergone screening only, and no correlation has been made between the result and pregnancy outcome (Tang, Alfirevic et al. 2011).

Much of the research has also focused on measuring CD56 and CD16 expression without considering other immune cell populations that could play a role in implantation (Miko, Manfai et al. 2010). More importantly the functional aspects of these cell populations such as perforin or granzyme expression has not been considered.

A clearer understanding of the immunological phenotype of the endometrium in these patients complimented by the assessing the functional abilities of the different cell populations will lead to a robust screening test (Marron, Walsh et al. 2018). Furthermore it may inform targeted therapeutic strategies that could better manage RIF.

In order to address this research question, this chapter focused on the introduction of an extensive flow cytometry panel aimed at assessing both immune killer cell numbers and more importantly function in patients undergoing IVF.

The first objective of this study was to compare the IHC derived NK cell result to those generated through the FC panel. The FC panel identified 5 immune cell populations (CD56+,natural killer (NK),NKT,CD8+ and CD4+ cells) along with the expression of 6 cytokines (perforin, granzyme, TNFα, lFNγ and the co-expression for both types of cytokines).

An initial comparison was performed to determine if the two techniques correlated and could be used interchangeably. The results revealed that two techniques did not correlate and could be used in collaboration. However the clinical references of such a rationale needs further work and validation (Laird, Mariee et al. 2011).

The potential additional benefit of the FC panel was that other immune cell populations beyond NK cells could be assessed which may be related to implantation.

The mean % of the 5 immune cells population revealed that there were differences in the paired samples but this was dependent on how the endometrial cells were gated. Furthermore some cell types were more dominant in the endometrial cells i.e. NK cells and other were detected in the PB samples; NKT cells. There were some cell populations that were present equally in both cell types like the CD8+ but the expression

of certain cytokines differed according to their location, highlighting the complex nature of immune cell testing.

The expression of cytokines revealed an interesting trend with perforin and granzyme being more abundant in the PB cell populations. The expression of TNFα and IFNγ was more expressive in the endometrial cells and this may be a pattern that is more exaggerated in pregnancy success or loss.

The ability to assess both the numbers and function of immune cells is the main strength of FC. Whilst IHC is common place and easier to perform, it is only able to give a “snap shot” of the endometrial NK cells at the moment the tissue is stained. Furthermore most histopathology departments test CD56+ cells which include, NK, NKT and other CD56+ cells. The test does not differentiate between the different immune cell populations and certainly gives no assessment of functionality.

The application of this extensive FC panel has revealed that NK cells and other cell populations build a more detailed immunological profile of the PB and endometrium for an individual patient.

The large and tight gate showed the PMBC NK cells to be higher than those expressed in the endometrium. However when the CD45+ gating was applied, it was shown to be the converse with the NK cells in the endometrium being more abundant. This is likely to be the most accurate assessment and this also revealed that the CD56+ and NK cell population did not correlate with IHC result.

One of the initial hypothesis at the beginning of this thesis was that FC would be comparable to the IHC assessment of NK cells. However this results show that the two testing modalities are very different and rather than treating them as separate entities, may be used to compliment the patient profiling further. The arbitrary range of NK cells measured through IHC needs further study. It may be possible that the range varies in different patients outside the context of pregnancy and IVF.

There are only a few studies comparing NK cell analysis in paired samples of PB and endometrium by FC and IHC. These have all shown mixed results. (Santillan, Lozano et al. 2015) compared the % of CD56+ and NK cells from the blood by FC and endometrium with IHC in the context of RIF. The authors found that the results correlated between the two tissue types. This may have differed from the results of our study, as the patient group was more heterogeneous in their IVF history and not specifically defined as RIF.

Another study in the context of recurrent miscarriage (RM) (Laird, Mariee et al. 2011) authors looked at 20 patients who had PB and endometrial NK testing through FC. The results were similar to our study which revealed that CD56+ cell assessment by FC was not correlated between the two tissue types. Furthermore the subtypes of NK cells were studied with CD56 ^{dim}CD16+ and CD56^{bright} CD16- cells also not showing any correlation.

The authors also compared 25 healthy fertile controls who had IHC and FC NK testing of the endometrium. This revealed that there was a

correlation between the two techniques. Furthermore (Park, Lee et al. 2010) found that in their 22 patients with RM, the % of PB CD56+ and endometrial IHC CD56+ cells also correlated. Both these finding do not concur with the finding of this study.

The reason for this difference may be that both these studies were carried out in RM patients who did not undergo IVF and therefore the results are likely to differ due to different clinical pathologies. The study by (Laird, Mariee et al. 2011) used CD45+ to correctly identify both the PB and endometrial cells which was not performed in our study. The addition of CD45+ to the endometrial samples allowed for better cell yields and this may also have been applicable to the PB tissue.

The cytokine expression was interesting with perforin and granzyme being more abundant in the peripheral blood as opposed to TNFα and IFNγ being the predominant cytokines in the endometrium. Although the subtypes of CD56 ^{dim} and ^{bright} were not studied, this may explained the expression of these cytokines. As discussed in the background the peripheral blood is dominated with CD56+^{dim} NK cells which are cytotoxic and the main source of perforin and granzyme. This is converse in the endometrium where CD56$_{bright}$ are abundant and release TNFα and IFNγ.

The next logical step would be to assess the pregnancy outcomes in these patients to see if these trends are apparent and can be used to predict IVF success.

4.4.1 **Limitations of the study/future work**

The limitation of this work reveal that FC is a useful but a gating strategy dependent testing modality. In hindsight the addition of CD45+ from the outset would have purified the endometrial cell population and led a better distinction in the immune cell populations and markers. This may have also led to correlation with the IHC result as other studies have shown (Laird, Mariee et al. 2011, Santillan, Lozano et al. 2015).

It has been cited in the literature that two subtypes of NK cells exist. The CD56 ^{dim} are CD16+ and the most abundant in the circulation. The CD56 ^{bright} cells which lack CD16 are the most common subtype in the endometrium. This was not assessed in this study and may reveal a further distinction between the paired samples. Therefore a future modification of the panel would be to separate out the cells in the PB and endometrial samples into these subtypes.

The TNFα and IFNγ expression was extremely low. This may be due the need for an immunological stimulus to activate these cytokines or another testing modality which preserves their integrity (Chen, Piao et al. 2015).

The use of the CD56, CD16 and CD3 in vitro diagnostic test was useful but requires further validation as it may lead to an efficient assessment of paired samples. The addition of the cytokines to this in vitro diagnostic (IVD) test may streamline the application of this panel and more towards a clinically acceptable test for IVF success.

The correlation of these markers with the pregnancy outcome when these women embarked on pregnancy is the main focus in the next chapter.

Chapter 5

The IVF outcomes of applying a detailed flow cytometry panel

Chapter 5 The IVF outcomes of applying a detailed flow cytometry panel.

5.1 Introduction

The clinical IVF outcomes of the prospective study outlined in chapter 4 are presented where paired samples of peripheral blood (PB) and endometrial tissue were taken from 42 patients and the percentage (%) of natural killer (NK), NKT and T cells were determined. The functional aspects of these cell populations through the expression of cytotoxic agents; perforin, granzyme and angiogenic factors; tumour necrosis factor alpha (TNFα) and inteferon gamma (IFNγ) were also assessed. The majority of this chapter relates these to the subsequent pregnancy outcomes in order to determine if these play a part in implantation through IVF.

This work was exploratory to verify if FC could be used to develop a clinical test that would be useful in predicting pregnancy outcome through IVF.

5.2 Methods

These are fully described in Chapter 2

5.2.1.1 *Definitions used to determine clinical IVF outcomes*

Patients were asked to perform a urinary pregnancy test 12 days after embryo transfer. If the pregnancy test was positive the patients had a transvaginal scan (TVS) at 6-7 weeks gestation to confirm and date a clinical pregnancy.

Clinical pregnancy was confirmed by visualisation of an appropriately sized intrauterine gestation sac(s) with a yolk sac(s), fetal pole(s) and heartbeat(s). All these features should be present at 6-7 weeks of gestation. An early pregnancy loss was defined as the involuntary failure of a pregnancy before 24 completed weeks. In the first trimester the absence of a fetal heart or an appropriately size intrauterine gestation sac on TVS along with clinical symptoms of vaginal bleeding and or abdominal pain were used to diagnose a miscarriage. A live birth was defined as the delivery of a live fetus beyond 24 weeks gestation.

5.2.2 **Statistical analysis**

Statistical analysis of the flow cytometry data was conducted using the graph pad prism® version 7.05 package. Normality of the data was confirmed using Kolmogorov–Smirnov testing. The mean % differences of marker expression between the clinical groups; divided by pregnancy outcome were further assessed by using a one way Analysis of Variance (ANOVA) analysis. There were 4 groups; live birth, early pregnancy loss below 12 weeks, negative pregnancy test and cancelled cycles. The percentage (%) of cells are presented with mean ±SD.

Clinical data including age, BMI, cause of infertility, previous IVF and spontaneous pregnancy history was collected for all the patients. The clinical data was analysed using the SPSS version 25 (Statistical Package for Social Sciences; IBM, IL, USA). All the data was categorical and assessed using crosstabs analysis and chi squared tests. This did not yield any meaningful results as the sample size was small.

A P value<0.05 was deemed to be statistically significant.

5.3 Results

5.3.1 Patient demographics and characteristics

The patient characteristics such as age, BMI, previous IVF history are presented in table 5.1

Table 5-1- The patient population demographics in the FC study

Demographic factor		**N=42 Mean±SD**
age (yrs)		35.43±4.59
body mass index (BMI)		24.45±4.38
previous number of IVF cycles		2.05±3.11
Cause of infertility		N=42 (%)
unexplained		4 (9.5)
male		19 (45.2)
tubal		4 (9.5)
other		6 (14.3)
multifactorial		9 (21.4)
NK status with IHC testing		N=42 (%)
negative		21 (50)
positive		13 (31)
inadequate result		2 (4.8)
declined biopsy		6 (14.3)
IVF demographics		N=42 (% of patients)
type of IVF cycle	Fresh	28 (66.7)
	Frozen	13 (31)
	Ovum donation	1 (2.4)
day of embryo transfer	Day 2	8 (19)
	Day 3	3 (7.1)
	Day 5	25 (59.5)
	No embryos transferred	6 (14.3)
number of embryos transferred	0	6 (14.3)
	1	14 (33.3)
	2	22 (52.4)
endometrial thickness at embryo transfer(mm) Mean±SD		9.35± 4.43

The age of the patients ranged from 21-42 years. The BMI was normal and the common cause of infertility was male factor (45.2%).

Half (50%) of the patients tested negative for IHC NK testing and 31% were positive. There were 6 patients who declined IHC testing but all opted to participate in the study and had FC analysis. 2 patients underwent biopsy but had an inadequate result with IHC.

The patient who tested positive for NK cells by IHC (N=13) were treated with 20mg of oral prednisolone in the same regimen outlined in chapter 3.

5.3.2 **Previous IVF history**

76.2% of patients had primary infertility with no previous spontaneous pregnancies. The majority of patient had a previous cycle of IVF with around 28% having had a live birth through IVF. The number of previous IVF cycles has a wide range with 10 patients having no prior treatment to one patient having had 19 previous IVF cycles. Over half of the patients (57%) had either one or two previous unsuccessful IVF cycles. Around 20% of patient had 3 or more previous IVF cycles.

Around 66.7% of patients had a fresh IVF cycle following FC testing and over half of the patients had a double embryo transfer at day 5.

5.3.3 IVF outcomes following IHC and FC testing

The 42 patients were stratified into 4 IVF outcome groups; negative pregnancy test, early pregnancy loss, successful live birth and cancelled cycles.

15 patients (35.7%) has a negative pregnancy test and 16 (38.1%) had a successful live birth. The majority of patients who had a live birth were aged between 31-36 years. The most common cause of infertility was male factor (45.2%). 5 patients (11.9%) had a miscarriage under 12 weeks of gestation. The remaining 6 patients had cycles that were either cancelled or did not yield embryos for transfer. 3 of these patients were cancelled due to inadequate response to medication, 1 patient had a failed fertilisation and 1 patient had a freeze all cycle due a thin endometrium. The remaining patient had NK testing and participated in the sample collection but did not return for treatment. However these patients were included in the final analysis and labelled as no embryos transferred.

5.4 Application of the flow cytometry panel

As described earlier the panel identified 4 immune cell populations along with the expression for 4 cytokines. This analysis was applied to paired PB and endometrial samples.

5.4.1.1 *Flow cytometry panel-ANOVA testing and association with pregnancy outcome*

The initial analysis involved identification of the main trends through performing ANOVA testing. The patients were stratified into 4 groups according to their pregnancy outcome; negative pregnancy test, early pregnancy loss< 12 weeks, live birth and cancelled cycles.

The mean percentages of the different cell populations of CD56+, NK cells, NKT cells,CD8+ and CD4+ in the 4 clinical groups were then compared for the paired samples. Furthermore the functional aspect of these cells measured through their expression perforin, granzyme, TNFα and INFγ was also analysed.

The ANOVA revealed three markers of statistical significance when comparing patients with a negative pregnancy test to live birth (table 5.2 and figure 5.1).

Two were expressed by the PB; NKT cells; perforin and co-expression of both perforin^{+}granzyme^{+}. The NKT perforin was higher in those with a live birth. Conversely the NKT cells co-expression of perforin^{+}granzyme^{+} was higher in those with a negative pregnancy test.

The only endometrial marker that showed statistical significance was CD8+ co-expression of TNFα^{+} IFNγ^{+} which was higher in the live birth

group. All other markers were not statistically significant but their trends are discussed in the remainder of this chapter.

Table 5-2- Mean expression of statistically significant markers

Cell type and cytokine	Negative pregnancy test (Mean %)	Live birth (Mean %)	Mean difference	ANOVA P-value
PB NKT perforin	12.4	40.29	-27.89	0.000066
PB NKT perforin^{+} granzyme^{+}	59.64	25.01	34.64	0.00000074
Endometrial CD8+ TNFα^{+} IFNγ^{+}	13.33	51.95	-38.61	0.00069

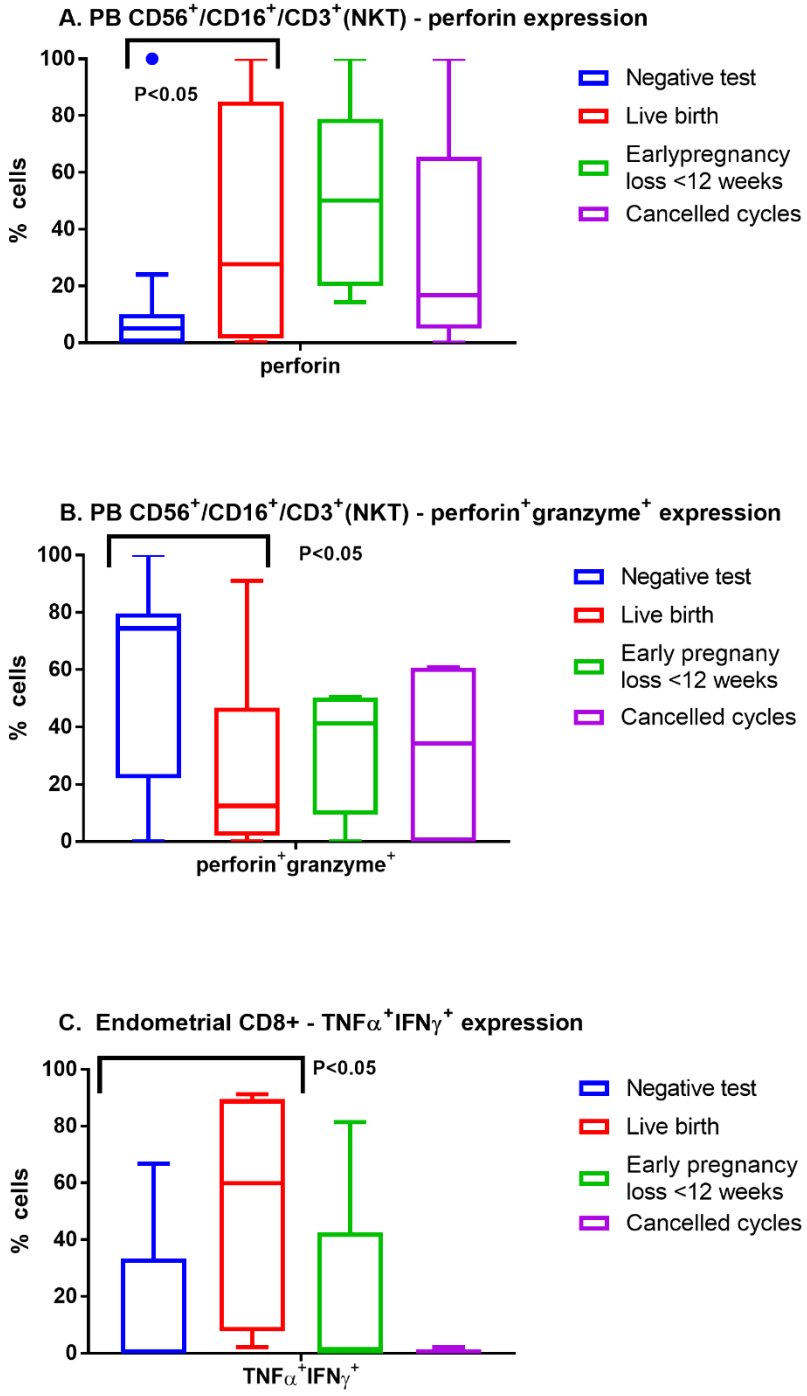


Figure 5-1- ANOVA of statistically significant cytokines

Figure 5.1A shows the expression of NKT perforin to be higher in the live birth group compared to the negative pregnancy test group. Figure 5.1B shows the expression of NKT co-expression of perforin$^+$ granzyme$^+$ to be higher in the negative pregnancy group. Figure 5.1C shows the expression of endometrial CD8+ co-expression of TNFα$^+$IFNγ$^+$ to be higher in the live birth group.

5.4.2 **PB cell populations and cytokine expression**

The mean % of cells for each of the immune populations is presented in table 5.3.

Table 5-3- The mean percentages of PB immune cell populations

Tissue type	Number of patients	Immune cell population	% of cells Mean±SD
PB	40	CD56+	7.01±6.52
		NK	2.32±2.78
		NKT	0.59±0.75
		CD8+	4.97±6.57
		CD4+	36.59±10.43

The PB samples expressed low % of NK and NKT cells. The most common immune cell population was the CD4+ cells.

5.4.3 **The expression of PB immune cell population perforin and granzyme**

The individual perforin and granzyme along with cells co- expressing both these cytokines; were plotted for the immune cell populations.

The % of NKT cells expressing perforin only was noticeably higher in the early miscarriage group compared to live birth (figure 5.2A). Furthermore there was a higher co-expression of NKT perforin^{+} and granzyme^{+} in the negative pregnancy group (~75%) as opposed to the live birth group (~10%). This was statistically significant.

Although not statistically significant the same shift in co expression of perforin^{+}granzyme^{+} was also seen in the NK cells (figure 5.2B). This shift in perforin was supported by the cells expressing granzyme only being similar in the clinical groups.

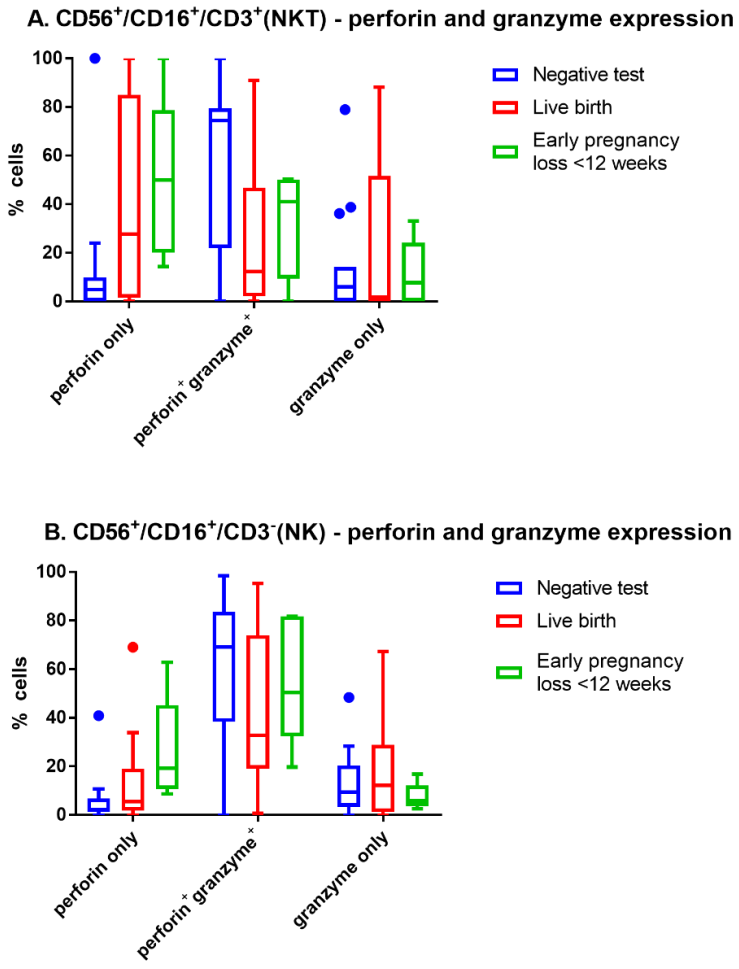


Figure 5-2- The shift of perforin and co-expression of perforin$^+$granzyme$^+$ in the PB samples

Figure 5.2A shows the statistically significant results for the NKT cells. It can be seen that the perforin only shifts to co expression of perforin$^+$granzyme$^+$; becoming more pronounced in those with a negative pregnancy test compared to the live birth. The granzyme expression is consistently low in the clinical groups. Figure 5.2B shows a similar pattern of expression in the NK cells but this is not statistically significant.

This is further illustrated by comparing the individual dot plots for 3 patients with a negative pregnancy test versus an equal number of patients who had a live birth (figure 5.3).

Figures 5.3 A-C show the dot plots of those who had a live birth and it can be seen that the majority of their NKT cells express either perforin or granzyme only with very minimal co-expression of perforin$^+$granzyme$^+$. This is contrast to the 3 plots shown below (figures

5.3 D-F) for those who had a negative pregnancy test. In these plots a shift can be seen from fewer NKT cells expressing perforin or granzyme only to a higher proportion co-expressing both cytokines.

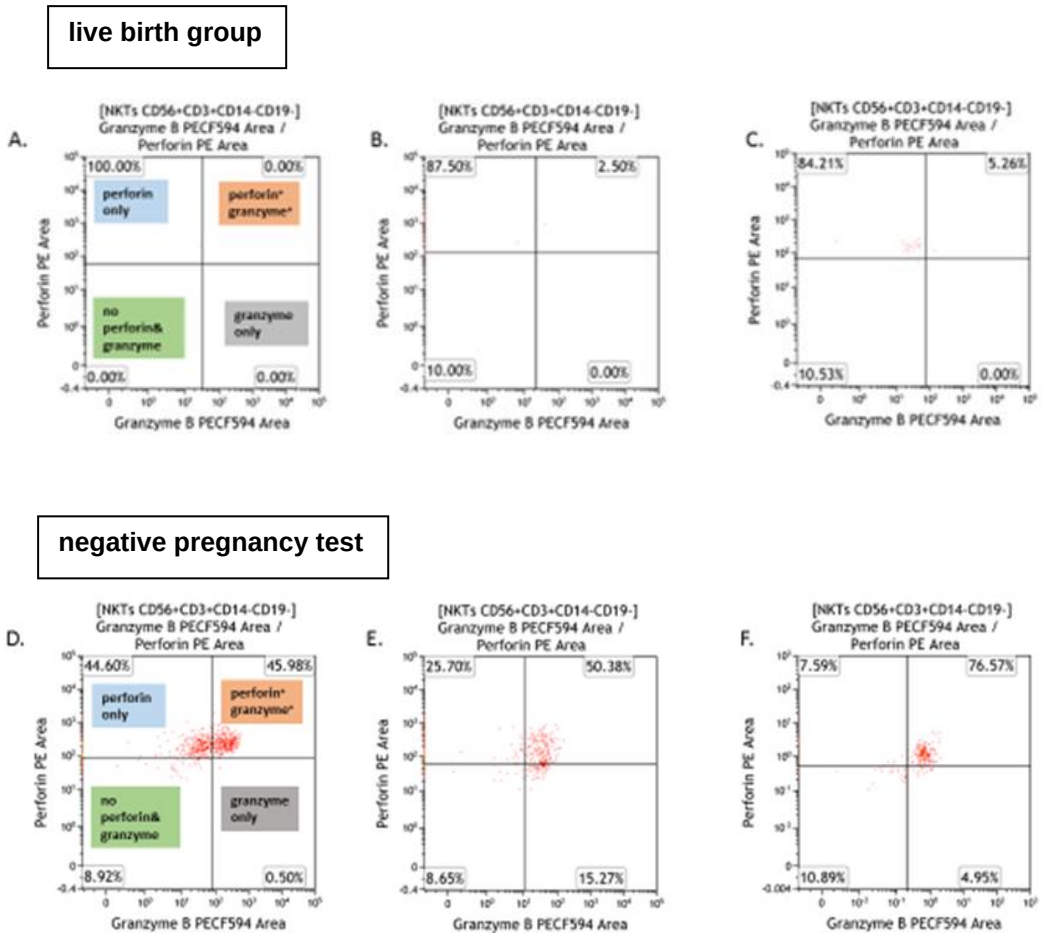


Figure 5-3- Dot plots of PB NKT perforin and granzyme expression from a subset of 3 patients

The 4 quadrants with their respective cytokine expression have been labelled in figures 6.13A and D. Figures 5.3A-C shows an example of 3 patients with a live birth where the co-expression of perforin +granzyme+ is low. A shift in expression is seen in 3 patients who had a negative pregnancy test (figures 5.3D-F).

5.4.4 The expression of PB immune cell populations TNFα and IFNγ

The expression of both of these markers was very low amongst the immune cells populations. The NK, CD8+ and CD4+ expression was negligible with the mean percentage of cells of less than 1 %. The NKT expression of TNFα was higher and showed a noticeable difference between the live birth and early pregnancy loss group, however this was not statistically significant.

5.4.5 Endometrial cell populations and cytokine expression

The endometrial cell populations expressed a higher % of NK and NKT cells compared to the PB samples. Conversely the % of CD4+ and CD8+ cells were higher in the PB than the endometrial cells (see table 5.4). However these differences were not statistically significant.

Table 5-4- The mean percentage of immune cell populations in the PB and endometrial samples

Immune cell population	% of cells (PB) N=40 Mean±SD	% of cells (endometrial) N=41 Mean±SD
CD56+	7.01±6.52	6.73±8.15
NK	2.32±2.78	5.32±6.77
NKT	0.59±0.75	0.69±1.84
CD8	4.97±6.57	3.30±6.68
CD4	36.59±10.43	9.09±13.85

As already discussed the ANOVA revealed that the co-expression of CD8+ $TNF\alpha^{+}$ $IFN\gamma^{+}$ were the only cytokines to be statistically significant. The patients with a live birth had a higher expression of these cytokines as opposed to those with a negative pregnancy test.

5.5 Discussion

This chapter revealed that there were no statistically significant differences found in the immune cell populations between the 4 clinical groups.

One of the main strengths of this study is that this panel was applied to women in a highly successful IVF centre. The clinical pregnancy rates around 10% higher than the national average (HFEA 2018). This is mainly due to stringent laboratory processes leading to the creation of optimal quality embryos and their transfer. Therefore it is reasonable to assume that if IVF did not result in a pregnancy, then endometrial receptivity or adverse maternal immunology could be contributory factors.

Looking back at the differences found in Chapter 4, the analysis revealed that the NK and NKT cells were the present in low numbers in in the PB population. This findings in this current chapter show that these cell populations may influence pregnancy through the expression of perforin and more importantly the co-expression of perforin^{+} granzyme^{+}. The % of CD8+ cells was similar in both the PB and endometrial cells but their expression of cytokines was only significant in the endometrium. The ANOVA revealed that CD8+ cells may influence pregnancy through the co-expression of TNFα^{+}IFNγ^{+} which appears to be conducive to successful pregnancy,

One of the main aims of this thesis has been to determine whether NK cell testing is useful in predicting pregnancy success through IVF. This

chapter has shown that the immune microenvironment of the endometrium and PB contains a plethora of cells, cytokines and their metabolic mediators. The prediction and influence of these markers goes beyond NK cells only.

The key finding from this chapter has corroborated the work of chapter 4 in that the immune cells not only vary according to the testing modality and tissue tested, but they also behave in an equilibrium with one another. In terms of predicting the pregnancy outcomes in an IVF cycle, it is difficult to confirm whether these cells and their expressed cytokines are the cause or symptom of pregnancy success (Lash and Bulmer 2011).

The main objective of this study was to apply the flow cytometry panel to a population of women undergoing IVF to demonstrate its clinical application and identify which of the numerous measured immunological markers correlated with IHC and successful pregnancy.

However despite extensively studying the 4 immune cell populations (CD56+, NK, NKT, CD8 and CD4 cells) in both PB and endometrial samples, no differences were seen in their mean % numbers between the IVF outcomes. This is agreement with many earlier studies (Thum, Bhaskaran et al. 2005, Baczkowski and Kurzawa 2007) and the two recent extensive systematic reviews (Tang, Alfirevic et al. 2011, Seshadri and Sunkara 2014) which have specifically looked at NK cells. However there have been a plethora of studies in the context of RIF which have shown raised NK cells in the peripheral circulation and

endometrium in those who fail their IVF treatment (Lukassen, van der Meer et al. 2003, Thum, Bhaskaran et al. 2004, Kwak-Kim and Gilman-Sachs 2008) and have recurrent miscarriage (Lachapelle, Miron et al. 1996, Michou, Kanavaros et al. 2003). Whilst RIF was not a specific inclusion criteria in this study, around 57% of the patient had previous unsuccessful treatment and hence this may have skewed the findings.

A recent large study with a similar hypothesis to this work, where over 600 patients were recruited and stratified according to their previous reproductive history (Marron, Walsh et al. 2018) found conflicting results. The participants in this observational study had endometrial FC analysis and it was noted that uterine NK cells were raised in those with a history of RIF compared to recurrent miscarriage and fertile controls. However the CD8+ and NKT cells were lowered in those with RIF.

The authors also found that CD4:CD8 ratios were higher in women with a history of infertility. This has been found in earlier work where PB CD4 and CD8 immune cells were found to be higher in those who had unsuccessful IVF treatment (Coulam and Roussev 2003).These trends may not have been present in this study due to the small sample size of patients and with a larger group and strict inclusion criteria, the associations may become more apparent. From an experimental perspective, FC is strongly dependent on the gating strategy which is arbitrary. Hence many of the preliminary studies were conducted 15-20 years before the panel optimised in this chapter was introduced. Therefore there may be variations in the results due to the these experimental nuances (Rai, Sacks et al. 2005).

The functional aspects of the immune cell populations revealed interesting results. The 3 markers identified through the ANOVA analysis were NKT perforin, co-expression of perforin^{+} granzyme^{+} by the PB population and TNFα^{+}IFNγ^{+} co-expression by the endometrial CD8+ cells. This is a remarkable observation as NKT cells have a highly cytotoxic ability which can either be detrimental to pregnancy (van den Heuvel, Peralta et al. 2007) or protective by the production of angiogenic cytokines such as IL 4 (Tsuda, Sakai et al. 2001).

The inability to detect a difference in the shift of marker expression or reciprocal changes in the endometrial cells may have been because of endometrial lymphocytes being expressed in low numbers and loss during tissue preparation. This certainly needs further validation in the protocol modification.

Although not statistically significant, studying the early pregnancy loss group revealed high expression of perforin by all the PB cell populations and lower expression of TNFα and IFNγ. This again supports the theory that a homeostatic balance exists between these cytokines that either allow pregnancy to succeed or fail.

The expression of perforin and granzyme in the peri-implantation endometrium requires further elucidation as there are limited on these markers. Evidence from animal studies suggests that although uterine NK cells contain abundant perforin and granzyme granules, they are not expressed in a successful pregnancy (Bogovic Crncic, Laskarin et al. 2005, Acar, Ustunel et al. 2011, Voskoboinik, Whisstock et al. 2015).

The release of these markers is mainly activated if there is infection or inflammation evident in the early conceptus.

There are a limited number of studies that have looked at C8+(Tc) cells in relation to implantation and their various roles have been hypothesised. Tc cells are present in abundance toward the later stages of pregnancy and the main source of IFNγ; a cytokine fundamental to successful implantation and placentation (Tilburgs, Schonkeren et al. 2010, Lissauer, Kilby et al. 2017).There is a trend for Tc cells to increase as gestation advances and therefore the role of regulating immune-tolerance by preventing viral infections such as influenza has been theorized (Liu, Diao et al. 2017). They also possess a developmental role in in facilitating the invasion of trophoblastic cells in the early placenta (Scaife, Bulmer et al. 2006).

Another theory is that of regulating NK function and impaired Tc modulation may lead to reproductive failure through this mechanism (Yoo, Kwak-Kim et al. 2012) Certainly the data from this study shows that the endometrial CD8+ co-expression of TNFα and IFNγ significantly correlated with live birth. The shift of cytokine expression was evident in this subset of cells, with the negative pregnancy group having a higher proportion of cells expressing TNFα only (~70%) versus (~40%) in the live birth group. This led to a reciprocal trend in the co-expression of $TNF\alpha^{+}IFN\gamma^{+}$ with the live birth group having around 60% of cells expressing both cytokines as opposed to minimal expression of this subset in the negative pregnancy group .The importance of highlighting this trend supports the existence of an equilibrium.

Therefore a high levels of CD8+ cells in the endometrium may lead to immune-tolerance in the early stages of the pregnancy with both TNFα and IFNγ acting as key facilitators of this process. This is supported by recent evidence that impaired Tc function is characterised by poor expression of TNFα and IFNγ (van der Zwan, Bi et al. 2018).

The expression of these angiogenic cytokines has been studied in the context of NK cell function (Moffett-King 2002, Szekeres-Bartho 2002, Hanna, Goldman-Wohl et al. 2006). These studies have shown that both are mediators of a successful pregnancy and are predominantly involved in placentation through spiral artery remodelling and trophoblastic invasion at the feto-maternal interface (Trundley and Moffett 2004, Matson and Caron 2014). TNFα is specifically responsible for trophoblastic cell growth, apoptosis and cell differentiation (Terranova, Hunter et al. 1995). However increased expression in the first trimester has been linked with early miscarriage and maternal hypertensive disease later in the pregnancy (Sharma, Singh et al. 2018).

The mechanisms whereby cytokines facilitate successful pregnancy are complex and involve bi-directional communication by the maternal immune system and the developing placenta (Szekeres-Bartho 2002). Cytokines such as TNFα and IFNγ are produced in response to the antigen expression of the placenta, which in turn is derived by the fetal tissue. Any dysregulation or imbalance in this communication forms the basis of aberrant implantation and early pregnancy loss both spontaneously and through IVF (Giudice 1994). Animal studies mostly

in mice have suggested that from an immunological perspective, a cytokine facilitated balance exists termed the Type 1 T-Helper (Th1) and Th2 response that determines a successful pregnancy (Ashkar and Croy 1999, Makhseed, Raghupathy et al. 2001). Simplistically the Th1 response is detrimental for pregnancy whereas the Th2 response is beneficial. Th2 cytokines such as interleukin (IL) 4, IL-5, IL-6, and IL-10 are produced at the maternal-fetal interface and prevent the production of destructive Th 1 cytokines namely; TNFα, IFNγ and IL-2 which are associated with implantation failure and miscarriage (Kwak-Kim, Gilman-Sachs et al. 2005).

The work presented in this chapter has shown that both cytokines were expressed in extremely low numbers in the endometrial samples and in the NK population there were no significant differences in the 4 clinical groups. This relationship needs further study as recent work by Fukui et al (Fukui, Funamizu et al. 2017) showed that NK cell derived IFNγ in the PB population was decreased in patients with implantation failure.

On the contrary earlier work a decade ago, by Thum et al showed no difference in PB TNFα and IFNγ expression between those that had a live birth and miscarriage. They hypothesised that the both these high levels cytokines facilitate NK cell activation and hence have a detrimental effect on pregnancy via this mechanism (Thum, Abdalla et al. 2007). This has been supported by other studies conducted around the same chronological time frame (Bates, Quenby et al. 2002, Fasouliotis, Spandorfer et al. 2004, Ginsburg, Xiao et al. 2005). This is partially supported by the results of this chapter as it is unclear as to the

exact relationship of TNFα and IFNγ with pregnancy outcome. However the association of NK cells with pregnancy outcome has not been confirmed.

5.5.1 **Conclusion**

FC and IHC analysis are used to test for immunological defects in the context of RIF and RM but need to be fully explored as the potential benefits are many. This has been the impetus for developing this extensive flow cytometry panel that not only measures NK cell numbers and function in the paired blood and endometrial samples, but extends this to other immune populations.

The results of this panel showed that PB NKT perforin, perforin^{+}granzyme^{+} as well as endometrial CD8+ TNFα^{+}IFNγ^{+} had an effect on pregnancy success through IVF. These associations need further validation.

The subject of NK testing is complex and throughout this chapter it has been demonstrated that immune cells do not act in isolation but form an intricate balance to bring about pregnancy success. The advantage of looking at function may highlight some of the factors that contribute or are indicative of poor IVF performance. Deciphering these trends and patterns will eventually lead to the development of a simple, evidence based clinical test which is easily accessible to patients.

5.5.2 Limitations of the study and flow cytometry panel

Immune cell populations are highly sensitive to many factors both clinical and experimental. The levels of NK cells in the peripheral blood and endometrium have been shown to fluctuate with diurnal hormonal variation (Nair, Verma et al. 2017), menstrual cycle (Sulke, Jones et al. 1985), stress (Benschop, Geenen et al. 1998) and parity (Gabrilovac, Zadjelovic et al. 1988). This is also likely to affect other immune cells which are inextricably linked to NK function and both the peripheral blood and endometrial microenvironments Hence the panel described here needs further robust validation.

The main limitation of this study was the small sample size of 42 patients with a heterogeneous reproductive history. The next logical step to eliminate clinical diversity would be to have strict inclusion criteria and apply this panel to subgroups of women such as those with 3 or more previous unsuccessful cycles. The inclusion of a control group of healthy fertile patients would strengthen the methodology further.

The gating strategies used to identify the endometrial cells evolved with experience. The addition of CD45+ revealed clearer staining and the tight gating strategy may have missed some of the cells.

Currently an extensive FC assessment as described in this thesis is not widely available and labour intensive. The addition of important antibodies through the IVD test as described in section 3.7.4 would

eliminate the need for the individual markers, and make the development of a clinical test more achievable.

Furthermore the large number of cytokines, NK receptors/cytotoxicity and profiling of specific genes that control the expression of immune cells are other scientific avenues for the work generated by this flow cytometry panel to explore.

Chapter 6

A comparison of immunohistochemistry and flow cytometry assessment of immune cell populations and their IVF outcomes

Chapter 6 A comparison of immunohistochemistry and flow cytometry assessment of immune cell populations and their IVF outcomes

6.1 Introduction

One of the main confounders in the IHC study described in chapter 3, was that women who had a positive NK result were given 20mg of oral prednisolone prior to the embryo transfer. Therefore the clinical outcome data could not be accurately compared.

The aim of this chapter is to explore the data generated through the flow cytometry (FC) panel stratified according to the immunohistochemistry (IHC) result.

The main objective is to analyse if any further differences are found in the immune cell populations and their cytokine expression

6.2 Methods

These are fully described in Chapter 2.

6.2.1 Statistical analysis

Statistical analysis of the flow cytometry data was conducted using the graph pad prism® version 7.04 package. Normality of the data was confirmed using Kolmogorov–Smirnov testing.

A paired t-test was conducted to assess any difference in the mean % of cells between the IHC and FC samples (peripheral (PB) and endometrial). This was then repeated for the comparison of cytokine expression between the different immune cell populations.

The mean % differences of marker expression between the clinical groups; stratified by pregnancy outcome were further assessed by using a one way Analysis of Variance (ANOVA) analysis.

There were 4 groups; live birth, early pregnancy loss below 12 weeks, negative pregnancy test and cancelled cycles. The percentage of cells are presented with mean ±SD.

Clinical data including age, BMI, cause of infertility, previous IVF and spontaneous pregnancy history was collected for all the patients. The clinical data was analysed using the SPSS version 25 (Statistical Package for Social Sciences; IBM, IL, USA).

A P value<0.05 was deemed to be statistically significant.

6.3 Results

6.3.1 Patient demographics and characteristics

There were 42 patients in this study. Table 6.1 displays the patient demographics stratified by IHC NK testing.

Table 6-1- The demographics of the study population stratified according to IHC testing

Demographic Factor	**Total N=42 Mean±SD**	**NK negative N=21 Mean±SD**	**NK positive N=13 Mean ±SD**	**Inadequate NK result N=2 Mean ±SD**	**Declined biopsy N=6 Mean±SD**
age (yrs)	35.43±4.59	34.71±4.86	35.85±4.14	37.5±4.95	36.33±5.16
body mass index (BMI)	24.45±4.38	25.5±5.06	23.76±3.56	21.75±1.63	23.41±4.16
previous number of IVF cycles	2.05±3.11	1.38±1.16	1.69±1.70	1.00±0.00	5.5±7.06
Cause of Infertility	N=42	NK negative (%)	NK positive (%)	Inadequate NK (%)	Declined biopsy (%)
unexplained	4	1 (25)	2 (50)	0 (0)	1 (25)
male	19	11 (57.9)	4 (21.1)	2 (10.5)	2 (10.5)
tubal	4	1 (25)	3 (75)	0 (0)	0 (0)
other	6	5 (83.3)	1 (16.7)	0 (0)	0 (0)
multifactorial	9	3 (33.3)	3 (33.3)	0 (0)	3 (33.3)

The age of the patients ranged from 21-42 years. Over half of the patients (57%) had either one or two previous unsuccessful IVF cycles. The majority of patients had male factor infertility (45.2%).

6 patients had cycles that were either cancelled or did not yield embryos for transfer. These patients were included in the final analysis and labelled as no embryos transferred.

Half (50%-21 patients) of the patients tested negative and 31% (13 patients) were positive for NK testing. There were 6 patients who declined IHC testing but all opted to participate in the study and had FC analysis. Two patients underwent biopsy but had an inadequate result

with IHC. The 13 patient with a positive NK test were treated with 20mg of oral prednisolone as outlined in chapter 3

6.3.2 **Types of samples collected**

39 patients had paired samples of both blood and endometrium analysed. One patient had PB testing only as there was an inadequate endometrial sample for FC analysis. The IHC result however was adequate and this patient tested negative. The 2 remaining patients has endometrial sample testing only due to declining the blood tests. These patients tested negative and positive respectively through IHC. The 6 patients that declined IHC testing all had paired sample testing via FC.

6.3.2.1 ***Experiment 1- comparison of the PB immune cell populations according to steroid treatment***

Figure 6.1 shows the PB immune cell populations stratified according to IHC status.

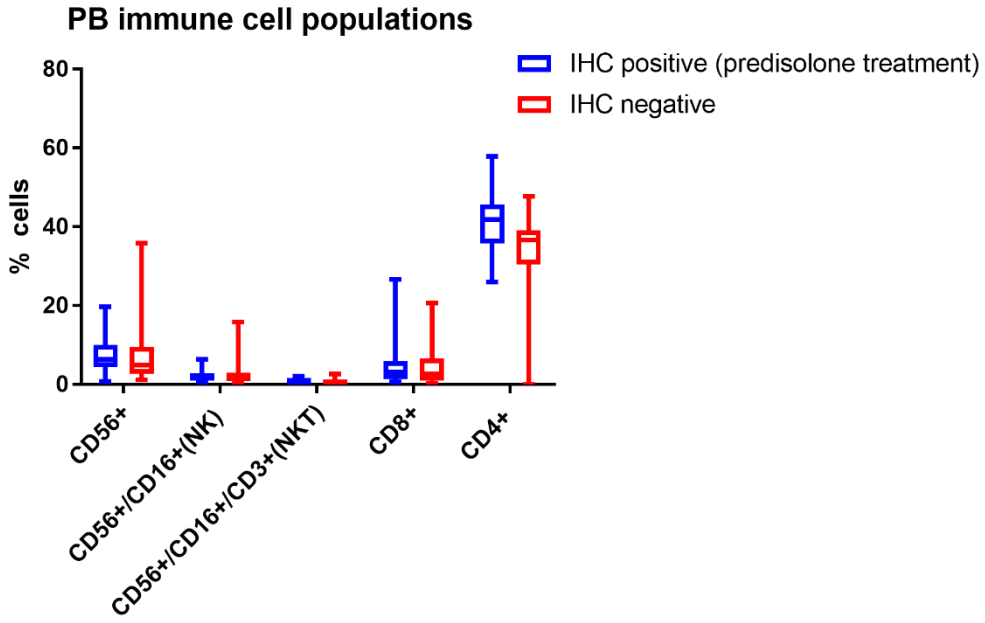


Figure 6-1- Comparison of PB immune cell population stratified by IHC status

Although there was a high % of CD4+ cells in both groups, there were no statistically significant differences in any of the immune cell populations.

6.3.2.2 *Experiment 2- comparison of the endometrial immune cell population according to steroid treatment*

A comparison of the IHC status with the three endometrial gating strategies was made. The large gating strategy showed minimal difference between the immune cell populations. The tight gating strategy showed the IHC positive group had a higher % of CD56+ cells compared to the negative group. The CD45+ gating strategy also revealed this trend along with a higher % of NK cells but overall there were no statistically significant differences between the groups.

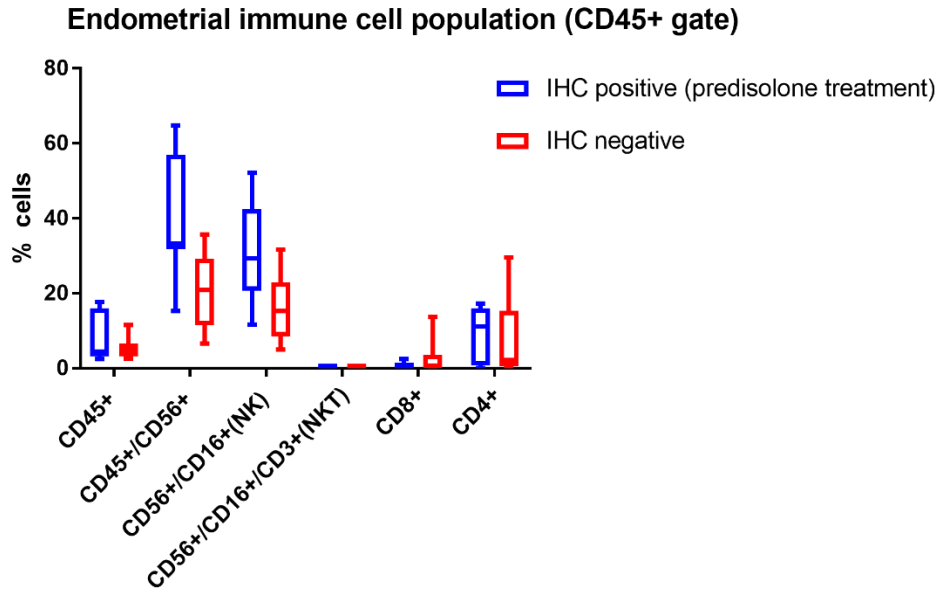


Figure 6-2- Comparison of endometrial immune cell populations stratified by IHC status

6.3.2.3 *Experiment 3- comparison of the PB perforin and granzyme expression according to IHC status*

A comparison of PB perforin and granzyme expression between the two groups revealed no statistically significant differences (figures 6.3A-C). However there were specific trends in some of the patients groups. Perforin was expressed in small amounts by all the immune cell populations. The NK and NKT cells were the main source of perforin and there were no differences in expression between the groups

The expression of ganzyme only was higher in the IHC negative patients and the highest in the CD8+ and CD4+ cell populations. The expression of both perforin^{+} and granzyme^{+} revealed that the IHC positive group expressed a high % of both cytokines across all the immune cell populations (figure 6.3C). Interestingly 50-60% of NK and NKT cells expressed perforin^{+} and granzyme^{+} in the IHC positive group.

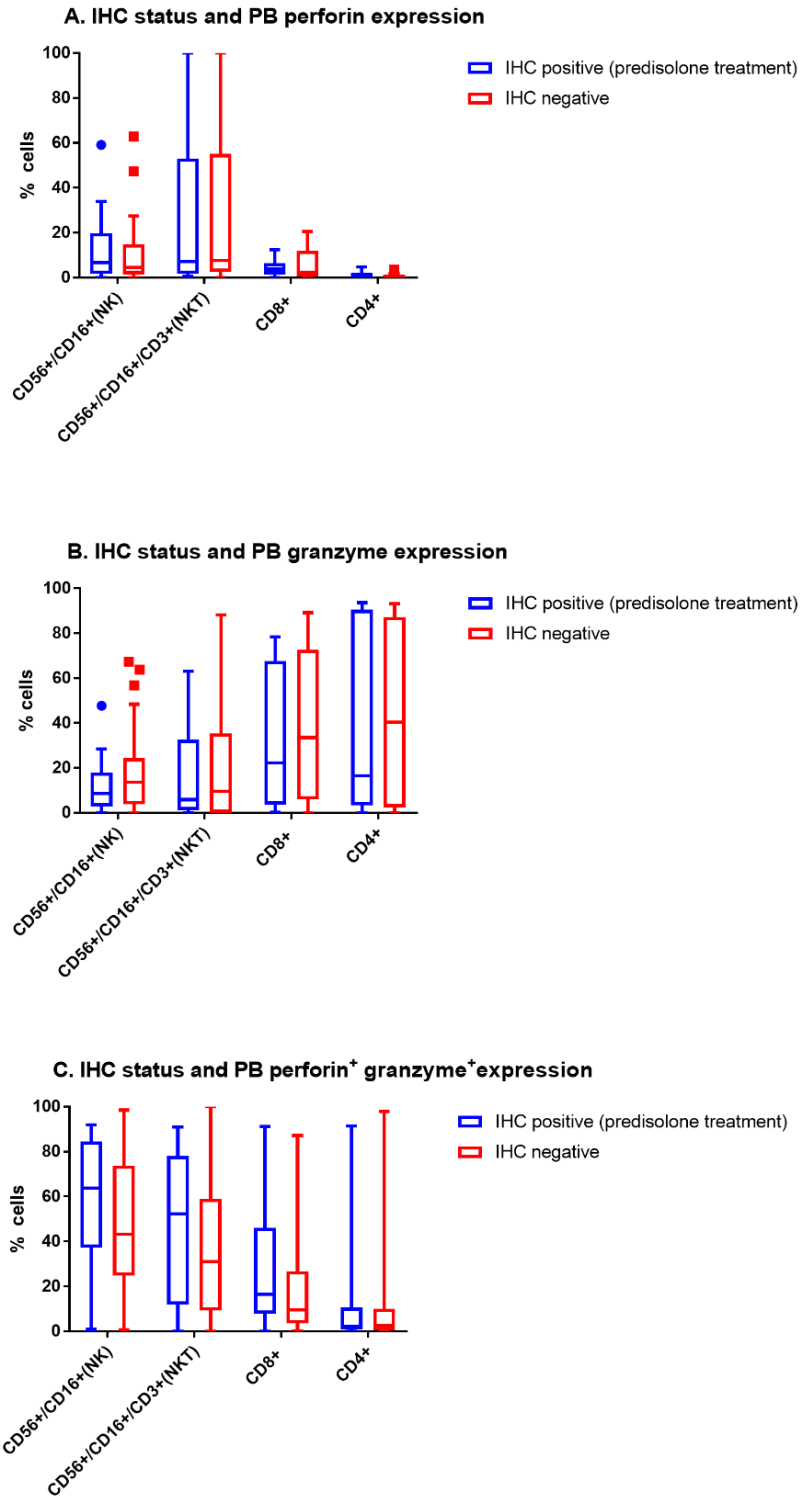


Figure 6-3- Expression of PB perforin and granzyme stratified by IHC status

6.3.2.4 *Experiment 4- comparison of endometrial perforin and granzyme expression according to IHC status*

The expression of perforin and granzyme was not as clearly defined in the endometrial cell populations (figures 6.4D-F). The main cells expressing perforin only were the NK cells and this was the same for the IHC positive and negative groups (figure 6.4D). The main cells expressing granzyme were the CD8+ and CD4+ immune populations. There were no differences in the IHC positive and negative groups (figure 6.4E).The expression of both cytokines was the highest in the NK and NKT cells and similar to the PB samples. Although not statistically significant, the % of NK cells expressing perforin^{+} and granzyme^{+} was around 80% in the IHC positive group compared to 50% in IHC negative group (figure 6.4F).

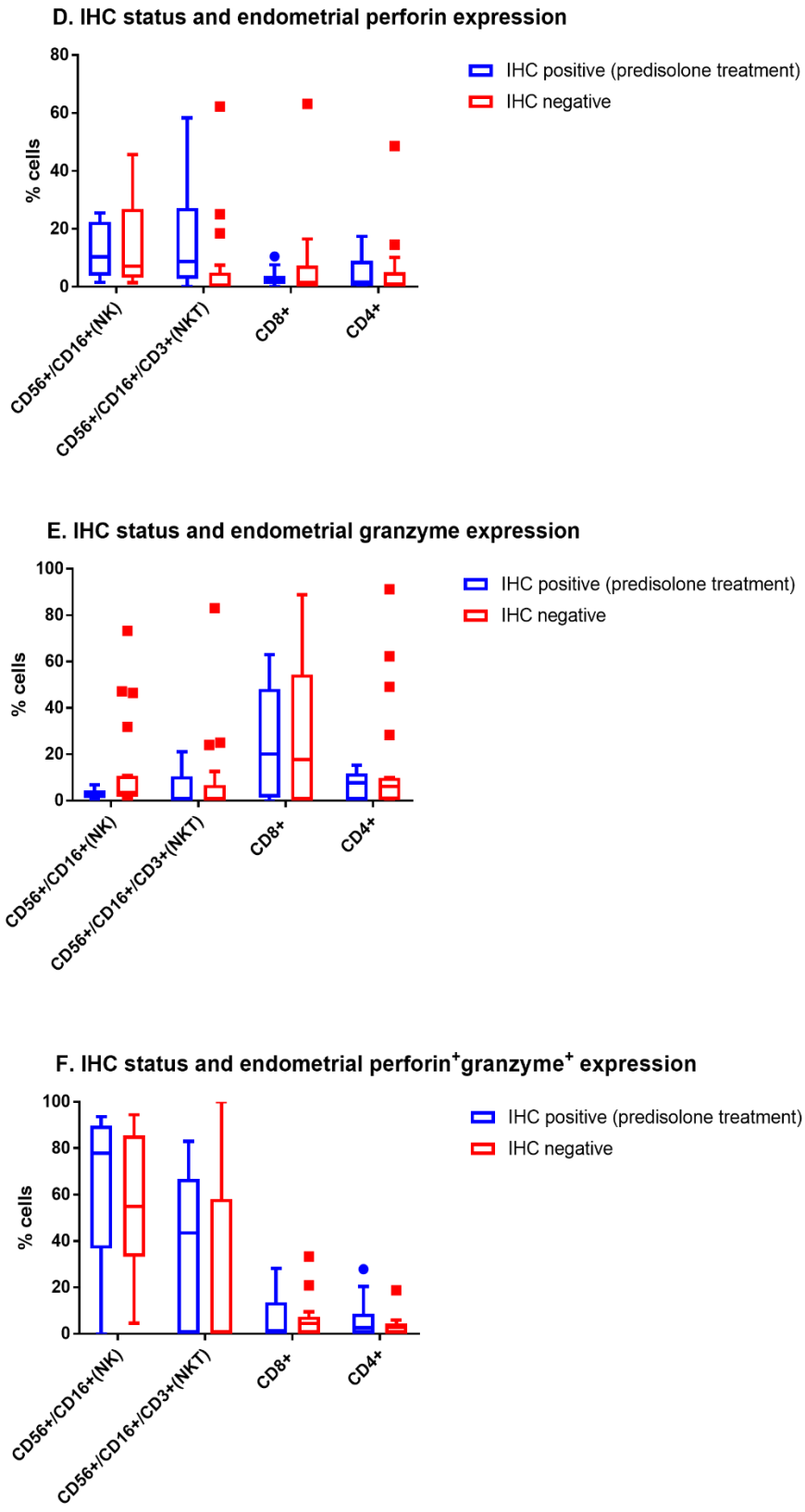


Figure 6-4- The expression of endometrial perforin and granzyme stratified by IHC status

6.3.2.5 *Experiment 5- comparison of PB and endometrial TNFα and IFNγ expression according to IHC status*

Both these cytokines were expressed in small amounts (0-20%) in the PB and endometrial samples .There were no statistical significant differences in the % of cells expressing TNFα and IFNγ.

6.3.3 Pregnancy outcomes between the IHC positive and negative groups

Table 6.2 shows the pregnancy outcomes of the patients according to the NK status.

Table 6-2 The IVF outcomes of the patient study group according to IHC status

Outcome		**IHC NK result (n=34)**				
		NK negative N=21 (%)	**NK positive N=13 (%)**	**Declined biopsy N=6 (%)**	**Inadequate NK result N=2 (%)**	**P value**
pregnancy test	negative	7 (33.3)	4 (30.8)	4 (66.7)	0 (0)	0.41
	positive	10 (47.6)	7 (53.8)	2(33.3)	2 (100)	
clinical pregnancy	yes	9 (42.9)	6 (46.2)	0 (0)	2 (100)	0.12
	no	1 (4.76)	1 (7.69)	2(33.3)	0	
early miscarriage <12 weeks	yes	1 (4.76)	0 (0)	0 (0)	0	0.36
	no	8 (38.1)	6 (46.2)	0 (0)	2 (100)	
live birth	yes	8 (38.1)	6 (46.2)	0 (0)	2 (100)	0.26
	no	0 (0)	0 (0)	6 (100)	0	
cycle cancelled/ no embryo transferred		4(19)	2 (15.4)	0 (0)	0 (0)	0.63

10 patients (47.6%) with negative NK tests had a positive pregnancy test versus 7 in the IHC positive group (53.8%).

Two patients (9.52%) in the negative and 1 patient (7.69%) in the positive group has a pregnancy loss under 12 weeks.

There were 8 live births (38.1%) in the NK negative group and 6 (46.2%) in the positive group.

The remainder of this chapter will analyse the FC immune cell populations and their cytokine expression stratified by pregnancy outcomes and IHC NK testing.

The IHC positive group were treated with prednisolone. This analysis was performed to see if there were any differences detected in the treatment group as opposed to those with an IHC negative status.

6.3.3.1 *Comparison of PB immune cell populations by IHC status and IVF outcomes*

Figure 6.5 shows the distribution of the PB immune cell populations in the IHC positive (prednisolone treated) and negative patients.

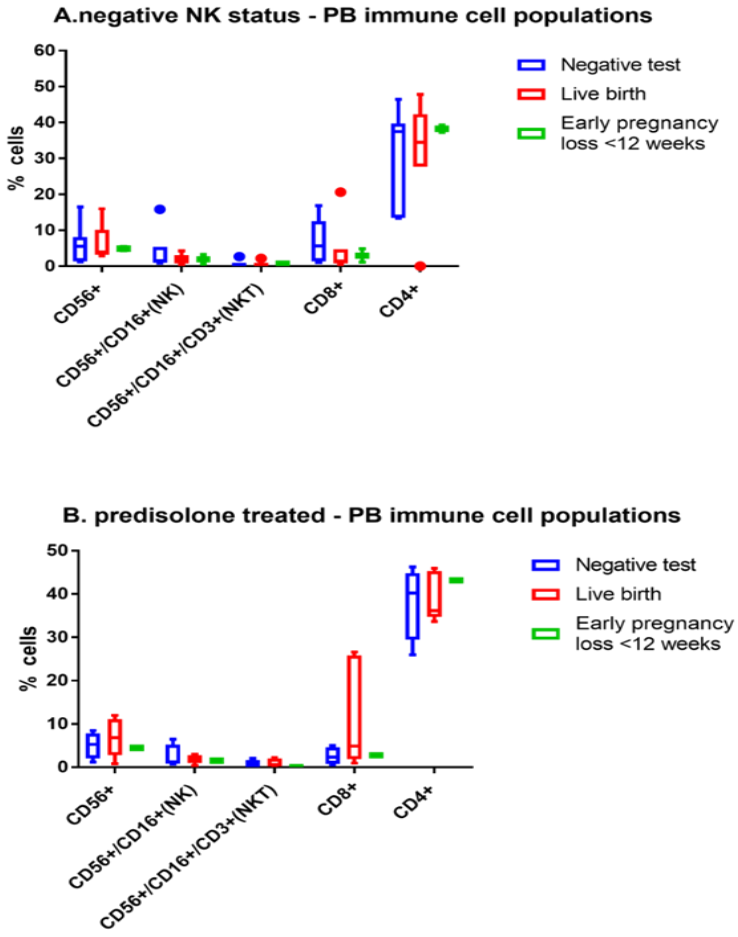


Figure 6-5- The FC immune cell populations according to IHC status in the PB samples.

Figure 6.5A shows the immune cell populations in the NK negative group alongside the prednisolone treated group (figure 6.5B).

There were no statistically significant differences between the IHC groups. However both groups expressed PB CD4+cells in equal amounts.

6.3.3.2 *Comparison of endometrial immune cell populations by IHC status and IVF outcomes*

In the endometrial samples, the NK and NKT cells were expressed in the highest % by the prednisolone treated patients (figure 6.6B). This live birth patients in this group also expressed a higher % of NK and NKT cells.

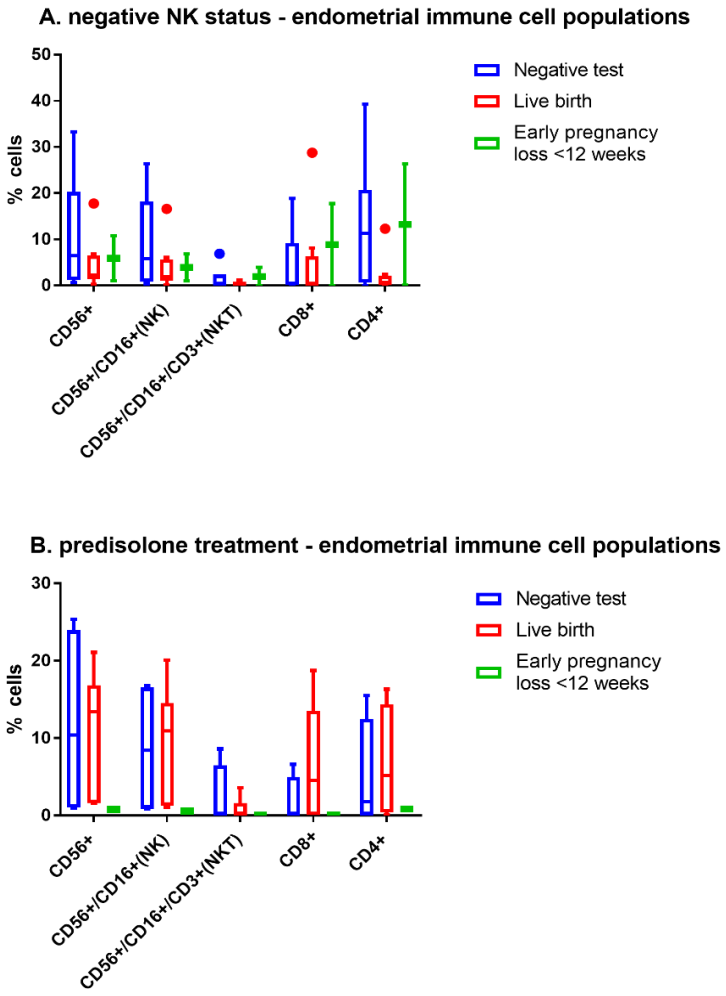


Figure 6-6- The FC immune cell populations according to IHC status in the endometrial samples.

Figure 6.6A shows the immune cell populations in the NK negative group alongside the prednisolone treated group (figure 6.6B).

6.3.3.3 *Comparison of PB perforin and granzyme expression by IHC status and IVF outcomes*

There were no statistically significant differences between the groups for the expression of these cytokines. However the co-expression of perforin$^+$ and granzyme$^+$ was the highest in the NK and NKT cells as discussed already. All the immune cell populations expressed both these cytokines in a higher % in those patients who had a negative tests as opposed to those that had a live birth (figure 6.7).

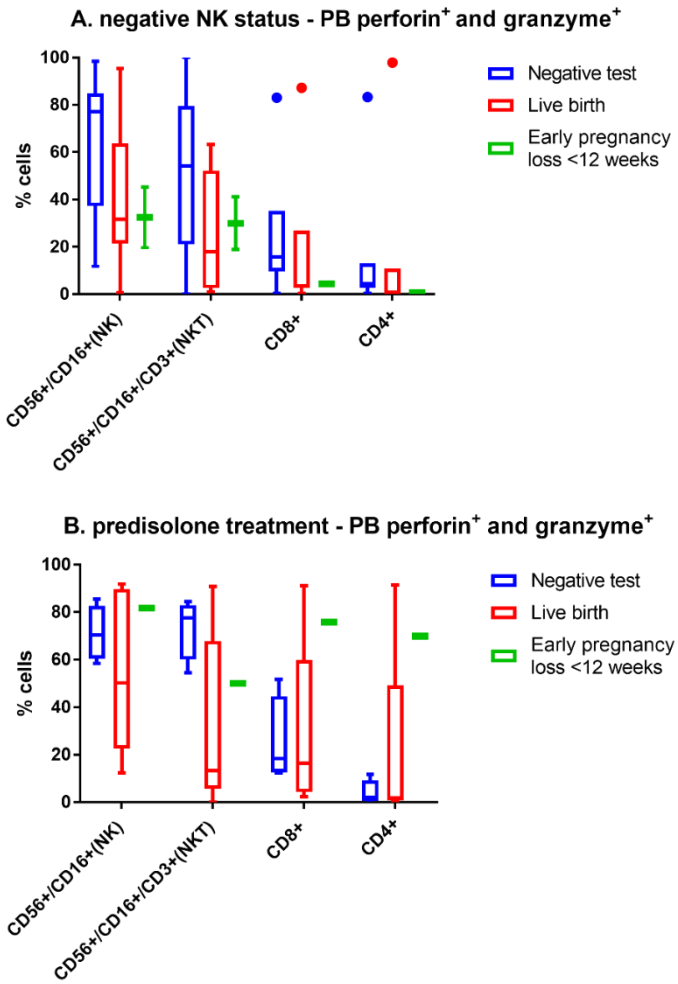


Figure 6-7- Comparison of PB perforin$^+$ and granzyme$^+$ expression by IHC status and IVF outcomes.

Figure 6.7A shows the co-expression of perforin$^+$ and granzyme$^+$ in the NK negative group alongside those treated with prednisolone (figure 6.7B).

6.3.3.4 *Comparison of endometrial perforin and granzyme expression by IHC status and IVF outcomes*

There were no statistically significant differences between the groups. The co-expression of perforin$^+$ and granzyme$^+$ was the highest in the NK and NKT cells. However converse to the PB, all immune cell populations expressed these cytokines in a higher % in those patients who had a live birth as opposed to those that had a negative test. (figure 6.8A-B).

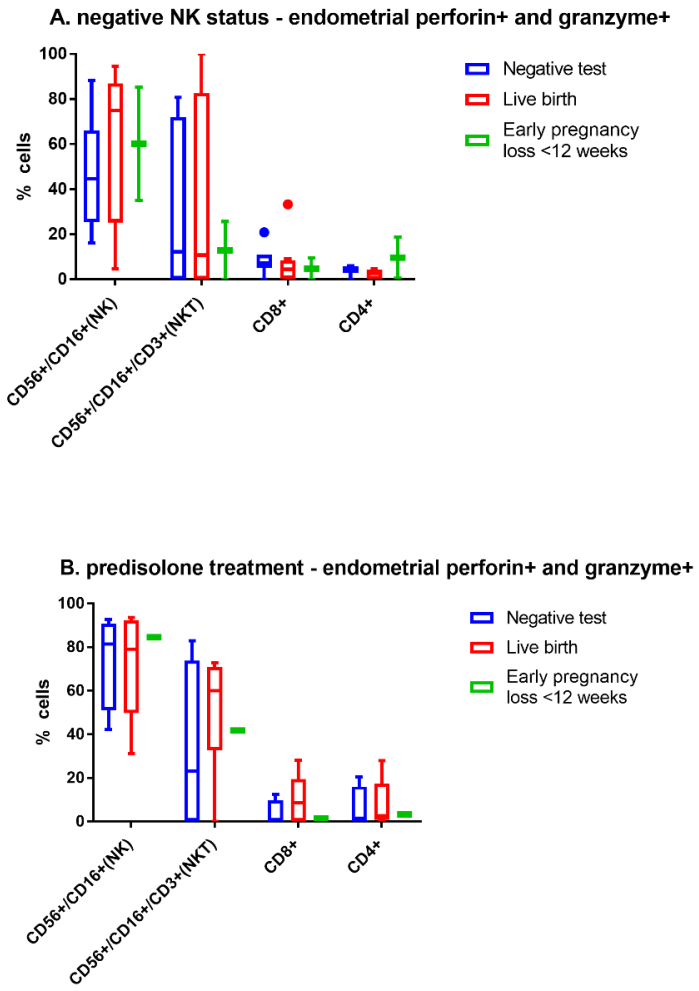


Figure 6-8- Comparison of endometrial perforin$^+$ and granzyme$^+$ co- expression by IHC status and IVF outcomes.

Figure 6.8A shows the co-expression of perforin$^+$ and granzyme$^+$ in the NK negative group alongside those treated with prednisolone (figure 6.8B).

6.3.3.5 *Comparison of PB and endometrial TNFα and IFNγ expression*

There was very little expression of these cytokines due to the sample size. The PB NK and NKT cells were the main source of TNFα and there are no discernible differences between the prednisolone treated and NK negative groups. This was also similar in the endometrial samples.

6.4 Discussion

Stratification of the patients into IHC positive (prednisolone treated) and NK negative and correlating this to the FC findings and IVF outcomes revealed a more detailed analysis of the patient data. Although the IHC results were different to the FC endometrial CD56+ and NK cells in Chapter 5, splitting the 42 patients into NK positive and negative showed some interesting findings.

The first observation was that those treated with prednisolone had a higher % of both NK and NKT cells that the NK negative group .This confers that IHC and FC results may in part may be correlated although this was not statistically significant. The reason for this may have been due to patient selection or the effects of prednisolone treatment altering the immune profile in those with a positive NK result. The microenvironment of the endometrium may also have been affected by the biopsy taking process which induces a local inflammatory response by altering the cytokine expression of TNFα and IL15 (Potdar, Gelbaya et al. 2012, Gnainsky, Granot et al. 2015).

The influence of prednisolone on immune cell populations and their cytokines is an unclear area in reproductive medicine (Robertson, Jin et al. 2016). The purpose of corticosteroid drugs is to mimic the effects of the endogenous hormone; cortisol. This is a stress hormone that has a variety of effects including resolving inflammation and in particular alterations in the function of immune cells (Franchimont 2004, Boomsma and Macklon 2008). The endometrium in particular has a specialised capacity to convert inert forms of cortisol to more potent types. This phenomenon is driven by progesterone and the decidual stromal cells (Kuroda, Venkatakrishnan et al. 2013) thereby supporting the role of endogenous steroids being beneficial for implantation (Dekel, Gnainsky et al. 2014). Studying uterine cortisol signalling process in animal models, has shown a lack of endogenous steroids and their receptors leads to aberrant implantation (Whirledge, Oakley et al. 2015). Conversely excessive cortisol can be harmful with an association with adverse placental and fetal growth patterns (Fowden and Forhead 2015).

Studies have shown that NK cells in the uterine environment express receptors for cortisol (Henderson, Saunders et al. 2003, Milne, Henderson et al. 2005) and hence are likely to fluctuate in number and activity in the presence of synthetic steroids. NKT cells in the context of respiratory disease have also been shown to respond in a similar manner and the data within the context of early pregnancy is limited (Hodge and Hodge 2016). Although there is evidence to suggest that immunosuppression has a role in the context of reproductive failure

(Quenby, Kalumbi et al. 2005, Boomsma, Keay et al. 2012), administration of steroids may actually diminish a nurturing process and in some patients may even be harmful (Reynolds, Labad et al. 2013).

The expression of cytokines and in particular perforin and granzyme was noticeable. It revealed that the expression of NK and NKT co-expression of perforin^{+} granzyme^{+} was generally high in both groups. However the expression in the IHC positive patients was higher than the IHC negative group. This pattern was similarly seen in the PB and endometrial samples. It is known that the endometrium comprises of CD56 ^{bright} NK cells which lack CD16 and are weakly cytotoxic (Fukui, Fujii et al. 1999). It is hypothesised that it is the abundance of these cells that facilitate immune-tolerance of the developing fetus. Therefore the cytotoxic role of perforin^{+}granzyme^{+} may be needed in the initial stages of implantation to provide the initial inflammatory response and trigger immune-tolerance.

However when the immune cell populations were correlated with the pregnancy outcomes there were some distinct observations. In both patient groups (IHC positive and negative); those with a negative pregnancy test had a higher NK co-expression of perforin^{+} granzyme^{+} than those attaining a live birth (figure 6.7).

This pattern was similar for the NKT cell co-expression of perforin^{+}granzyme^{+}. However the expression of these cytokines was much higher in the IHC positive patients (figure 6.7B) who had a negative pregnancy test (~80%) compared to the (~55%) in the IHC

negative groups (figure 6.7A). Although these results were not statistically significant, it may suggest that an over expression of NKT perforin^{+}granzyme^{+} is detrimental for pregnancy. It can also be argued that steroid treatment may have altered this pattern in the IHC positive group. It is difficult to infer if the NKT co-expression of perforin^{+}granzyme^{+} leads to a poor outcome in IVF or is an indicator of this pathological process.

In the endometrial samples, another pattern of expression was evident. In the IHC negative group, the NKT co-expression of perforin^{+}granzyme^{+} was low (~10%) regardless of IVF cycle outcome. However in the NK cells, the expression of these cytokines was highest in the live birth group (~80%) compared to the negative pregnancy group. The converse was true in the IHC positive group. In this analysis, the co-expression of NK perforin^{+}granzyme^{+} was equally high in the negative pregnancy and live birth groups (80%). However the NKT cell expression of these cytokines was higher in the live birth group.

This shift in expression was also seen in Chapter 5 with the NKT co-expression of PB perforin^{+} granzyme^{+} being detrimental to pregnancy. The difference in the NKT co-expression of perforin^{+}granzyme^{+} was statistically significant between the negative pregnancy test and live birth groups. This difference was not statistically different in the endometrial cells.

Looking at the literature, there is evidence that supports that NK cells in the peripheral blood are cytotoxic and release perforin and granzyme in

order to treat infection and carcinogenic factors (Koopman, Kopcow et al. 2003).The role of the peripheral NK cells in pregnancy development is poorly understood and much of the literature is focused around the uterine NK cells (de Andres, Fernandez-Paredes et al. 2017).However studies looking at the IVF population have found that the % of NK cells are raised in the PB of women who have unsuccessful treatment or RIF (Thum, Bhaskaran et al. 2004, Sacks, Yang et al. 2012).The data from this study also shows that a higher percentage of perforin^{+}granzyme^{+} was evident in those that did not achieve a live birth through IVF. This was in turn was related to an IHC positive result. This may be a simplistic perspective on the relationship of NK cells and pregnancy success in IVF and certainly there are multiple immune cell populations and mechanisms at play.

Although very little is known about NKT cells, they comprise a small subset of lymphocytes that have immunological properties similar to NK cells and conventional T cells This confers numerous capabilities such as to being able to release Th1 and Th2 cytokines which either promote or hinder pregnancy (Bendelac, Savage et al. 2007). Furthermore they are able to induce cytotoxicity through the release of perforin, the fas ligand and TNFα (Di Pietro and Falcone 2014). These multiple functions allow NKT cells to either have a protective or deleterious effect on their target organ either by promoting inflammation or immune tolerance.

The intricate details of this complex interaction needs further elucidation, the work in this chapter alludes to the idea of immune cell population being both self-regulating and responsive to their

environment. They may function in similar manner to hormones with a paracrine and/or autocrine function according to the triggers and tissue they interact with This phenomenon is certainly true of NKT cells which can either stimulate or prevent immune response depending on their environment (Kronenberg 2005). This adaptive “plasticity” and versatility is likely to be highly influential in pregnancy and is paradoxically the main challenge of being able to develop a suitable clinical test to capture this response.

The data on TNFα and IFNγ was not clear to interpret. Findings from chapter 6 highlighted that those with a live birth had a higher endometrial CD8+ co- expression of TNFα and IFNγ. However when stratifying the patients by IHC result, this revealed that both groups of patients had a high expression of endometrial NK TNFα and IFNγ. There was also some expression by the CD8+ cells. However there were no differences seen between the IHC negative and positive groups Furthermore there was also no pattern or differences seen in the IVF pregnancy outcomes. The expression of TNFα and IFNγ was negligible in the PB samples and this was also evident when correlating with the IVF cycle outcome.

6.4.1 Limitations of the study and further work

The main limitation in this study was the confounding factor of prednisolone treatment given to those who were IHC positive. This was a common theme running through both studies in Chapter 3 and Chapter 5.

Therefore an attempt was made to stratify this group of patients to check if there were any differences from those that were IHC negative. This did revealed some differences in the expression of NK and NKT cell perforin^{+} granzyme^{+} which in part explains how these cells function. The IVF outcome data showed some difference but it is difficult to clarify whether these were corrected with the steroid treatment.

In order to explore this, a comparison with matched controls who had NK testing with IHC and did not have steroid treatment would dispel some uncertainties. An attempt was made to find a group of patients from the IVF centre but this was not possible.

The next progression in this research would be to design a study where patients are initially tested with both IHC and FC and blinded to the results. They are then randomised to placebo versus prednisolone treatment. The IVF outcomes are then correlated for the 4 groups generated in the study which would include IHC negative given placebo and steroid treatment versus those who were IHC positive with the same stratification. This would allow a comparison of 4 different clinical groups and clarify whether there is a difference in the pregnancy rates between those that are IHC positive and negative. It would allow a

comparison of whether steroids are an effective treatment for those undergoing IVF.

Another perspective would be to study a cohort of IHC positive patients with the FC panel before and after the administration of steroids. This could also be complimented with in vitro studies with both PB and endometrial cells treated with differing doses of prednisolone and comparing this to a baseline profile of the FC panel.

Chapter 7

A Systematic review on immune therapies for women with history of unsuccessful implantation undergoing subsequent IVF/ICSI treatment

Chapter 7 A Systematic review on immune therapies for women with history of unsuccessful implantation undergoing subsequent IVF/ICSI treatment

7.1 Introduction

In this chapter, the evidence for ten most commonly used immunotherapies in the management of unexplained recurrent implantation failure (RIF) will be systemically reviewed. The main objective is to determine whether their administration results in improved live birth and miscarriage rates in women undergoing subsequent IVF treatment. The data collection and extraction were conducted using validated methodology from the Cochrane collaboration (Cochrane Handbook for Systematic Reviews of Interventions (version 5.1.0) (Higgins 2011).

This systematic review was deemed necessary for both clinical and scientific reasons. The significance of natural killer (NK) cells in RIF has been systematically reviewed in two large meta-analysis (Tang, Alfirevic et al. 2011, Seshadri and Sunkara 2014) as have the treatments used to treat raised levels (Polanski et al 2014). The results have been inconclusive and NK cell testing should only be reserved for the research setting. However simplifying the research question and evaluating whether immunotherapies should be given to women with unexplained RIF as is commonplace in many clinics (Kwak-Kim, Han et al. 2013) has not been fully evaluated to date. The majority of systematic reviews have included one immunotherapy and both

randomised and non-randomised studies. Therefore a logical starting point would be to collate the immunotherapies used to treat unexplained RIF.

The following immunotherapies: glucocorticoids, tumour necrosis factor alpha (TNFα) inhibitors, peripheral blood mononuclear cells (PBMC), intravenous immune globulin (IVIG), lymphocyte immune therapy (LIT), intralipid, granulocyte colony stimulating factor (G-CSF), leukocyte inhibitory factor (LIF), heparin and aspirin were included. Only randomised controlled trials (RCTs) where immunotherapy was given to women with a history of unexplained RIF before further assisted reproduction were reviewed. Patients with known auto-immune or thrombophillic conditions or gynaecological cancer were excluded.

7.1.1 **Summary of review findings**

Two authors; SA and HA searched the Cochrane Gynaecology and Fertility Group Specialised Register, the Cochrane Central Register of Studies Online (CRSO), MEDLINE, Embase, CINAHL and PsycINFO from inception to September 2017. The reference lists of key relevant articles, trial registers and conference proceedings were hand searched and researchers in the field were contacted.

The review included 19 studies with a total of 1748 patients. G-CSF was used in 8 studies, PBMC were used in 3 studies, heparin in 4 studies (one compared with aspirin), intralipid in 2 studies and LIF in 2 studies. No studies using glucocorticoids, LIT, IVIG and TNFα inhibitors met the criteria for inclusion.

The evidence showed that intralipid probably improves the live birth rate compared to no treatment (RR 2.13, 95% CI 1.35 to 3.36; participants = 303; studies = 2; I^2 = 6%, moderate certainty evidence).

Similarly heparin may slightly improve the live birth rate compared to aspirin (RR 2.56, 95% CI 1.17 to 5.58; participants = 126; studies = 1; I^2 = 0, low certainty evidence).

Overall there was insufficient evidence to determine a difference in miscarriage rate between intralipid (RR 0.43, 95% CI 0.12 to 1.63; participants = 203 patients = 1; I^2 = 0, low certainty evidence), PMBC (RR 0.20, 95% CI 0.08 to 0.51; participants = 54; studies = 1; very low certainty evidence) and LIF (RR 0.61, 95% CI 0.14 to 2.64; participants = 149; studies = 1; low certainty evidence) compared to no treatment.

Virtually all the included studies did not report on drug side effects.

In conclusion this review highlighted that there is some evidence that intralipid and heparin probably have some beneficial effects in RIF patients. However these findings have to be interpreted with extreme caution.

7.2 Review Objectives

The aim of this review was to determine whether immunotherapy results in improved live birth rates in women with a history of unexplained RIF who are undergoing assisted reproduction.

7.3 Materials and Methods

7.3.1 Types of studies

Only randomised trials comparing immune therapies for women with a history of unexplained failed implantation who were subsequently undergoing IVF treatment were included. Quasi- and pseudo-randomised trials were not included.

7.3.2 Types of participants

7.3.2.1 *Inclusion criteria*

Women with a history of unexplained RIF who were undergoing IVF/ICSI treatment and were receiving a form of immune therapy.

RIF was defined as two unsuccessful attempts at implantation in which fresh or frozen embryos were transferred.

7.3.2.2 *Exclusion criteria*

Women with gynaecological cancer

Women with known autoimmune conditions

Women with known thrombophilic conditions

Women undergoing IVF/ICSI treatment with donor eggs

7.3.3 **Types of interventions**

The following immune therapies were considered in women with a history of failed implantation who are undergoing IVF/ICSI treatment.

- Intralipid
- PBMC
- Heparin
- Aspirin
- G-CSF
- LIF
- Glucocorticoids
- TNF-α inhibitors
- IVIG
- LIT

These immune therapies were compared with no treatment each other or with placebo. Combination of therapies were also included.

7.3.4 **Types of outcome measures**

All outcomes were reported per woman randomised

7.3.4.1 ***Primary and secondary outcomes***

There were two primary outcomes:-

- Live birth rate per woman, defined as delivery of a live fetus after 24 completed weeks of gestation
- Miscarriage rate per woman, defined as the involuntary loss of a pregnancy before 24 weeks gestation

The secondary outcome of drug side effects was also analysed

7.3.5 **Search methods and strategies for identification of studies**

All published and unpublished RCTs of immune treatment versus no treatment, another immune treatment or placebo were obtained using the strategies described below.

7.3.6 **Electronic searches**

The following electronic databases, trial registers and web sites were searched, from inception to September 2017:-

Cochrane Gynaecology and Fertility Group (CGF) Specialised Register of controlled trials

CRSO

MEDLINE

Embase

PsycINFO

CINAHL

PubMed

LILACS

DARE database

There were no language restrictions in these searches. The search terms commonly used included: 'IVF', 'glucocorticoids', and 'immunotherapy'.

Trial registers were also searched for ongoing and registered trials and included the following platforms. The reference list of trials and systematic reviews retrieved by the search were hand searched and personal contact was made with experts in the field to obtain any additional data.

7.3.7 Data collection and analysis

Data collection and analysis was conducted in accordance with the Cochrane Handbook for Systematic Reviews of Interventions (version 5.1.0) (Higgins 2011).

7.3.8 Selection of studies

The titles and abstracts retrieved were read by one author independently. The citations that were irrelevant were removed and the full text of the remaining trials was retrieved. Two review authors (SA and HA) independently examined the full text articles for compliance with the inclusion criteria and selected trials eligible for inclusion in the review. Authors corresponded with trial investigators to clarify trial eligibility. Disagreements were resolved by consensus or by discussion with a third author (PC). The selection process was documented in a PRISMA flow chart (figure 7.1)

7.3.9 Data extraction and management

A data extraction form was designed and used to extract data from eligible trials. Two review authors independently extracted data (SA and HA) and any disagreement between these authors was resolved by a third author (PC).

7.3.10 Assessment of risk of bias in included studies

The Cochrane risk of bias assessment tool was used to assess the risk of bias in the included trials. The following were assessed by two authors (SA and PC): sequence generation; allocation concealment; blinding of participants, providers and outcome assessors; completeness of outcome data; selective outcome reporting; and other potential sources of bias

7.3.11 Measures of treatment effect

For dichotomous data such as live birth, the number of events in the control and intervention groups of each trial was used to calculate Risk Ratio (RR). Relative risk was used wherever possible and results reported as percentages were be converted to event numbers to facilitate this. A Peto odds ratio (OR) was used in analyses with no events in either the intervention or control group. There was no continuous data as all review outcomes were binary. Every outcome was presented with 95% confidence intervals after entering the data into the RevMan 5.3 software (Copenhagen: The Nordic Cochrane Centre, The Cochrane collaboration, 2012).

7.3.12 Assessment of heterogeneity

Statistical heterogeneity was examined by inspecting the scatter in the data points on the graphs and the overlap in their confidence intervals. In addition, it was assessed by the use of the I^2 statistic. An I^2 statistic greater than 50% was considered an indicator of substantial heterogeneity (Higgins 2011).

7.3.13 Data synthesis

The data from primary trials were combined using a fixed-effect model for the interventions. The Rev Man 5.3 software (Copenhagen: The Nordic Cochrane Centre, The Cochrane collaboration, 2012) was used to perform meta-analyses using the Mantel-Haesnzel method.

7.4 Results

7.4.1 Results of the search

The search retrieved 3351 articles from database searches and hand-searching of the reference lists of relevant articles. After removal of duplicate references and abstract screening, 27 studies were potentially eligible for inclusion and were retrieved in full-text.5 studies were excluded and there were no studies awaiting further classification.19 studies were included in the review with three studies having an abstract which was superseded with the full study. 13 studies were included in the meta-analysis (figure 7.1).

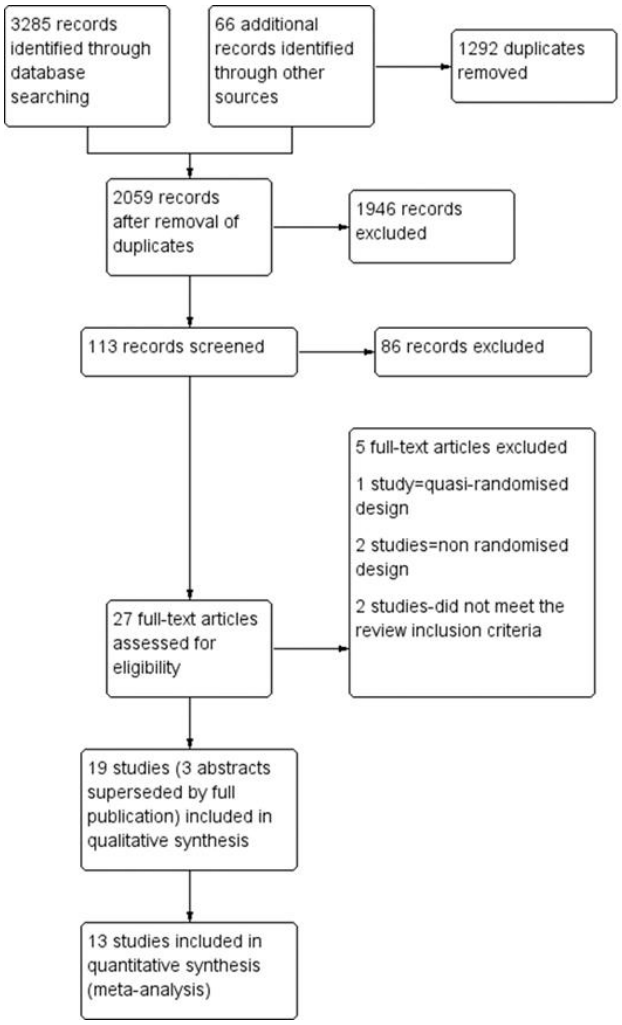


Figure- 7-1- PRISMA study flow diagram

7.4.2 **Included studies**

19 studies were included in the review;

(Aleyasin, Abediasl et al. 2016),(Brinsden, Ndukwe et al. 2003),(Brinsden, Alam et al. 2009),(Cambiaghi and Leao 2012),(Colicchia A, Pergolini I et al. 2011),(Davari Tanha, Kaveh et al. 2016),(El-Khayata and El Sadek 2015),(Gamaleldin I, Lenguerrand E et al. 2016),(Hamdi, Danaii et al. 2015),(Kim, You et al. 2011),(Madkour, Bouamoud et al. 2016),(Makrigiannakis 2011),(Miraj, Eftekhar et al. 2015),(Obidniak, Gzgzyan et al. 2016),(Pergolini, Gravotta et al. 2013) ,(Scarpellini and Sbracia 2011),(Scarpellini and Sbracia 2013),(Urman, Ata et al. 2009),(Yu, Zhang et al. 2016)

Three studies were included in abstract form and the superseded full text (Davari Tanha, Kaveh et al. 2016),(Hamdi, Danaii et al. 2015),(Madkour, Bouamoud et al. 2016).

7.4.3 **Study design and setting**

7.4.3.1 ***Design***

Seventeen of the included trials had a parallel design with a total of 1748 women.

Two studies had 3 groups of patients (Davari Tanha, Kaveh et al. 2016);(Obidniak, Gzgzyan et al. 2016) with both testing G-CSF. The first of these studies had an intervention arm with intrauterine (IU) G-CSF and 2 control arms with IU saline or the passage of a cook catheter alone.(Obidniak, Gzgzyan et al. 2016) used both subcutaneous (SC) and IU G-CSF as well as control group that had no intervention.

7.4.3.2 *Setting*

A variety of different settings were used to recruit patients into the various studies.Table 7 1 shows the setting for the included for the included studies

Table 7-1- clinical setting of the included studies

Setting	Studies
IVF clinic	Brinsden 2009 Cambiaghi 2012 Collichia 2011 Madkour 2016 Pergolini 2013 Urman 2009 Yu 2016
Fertility clinic or hospital	Aleyasin 2016 Davari-Tanha 2016 Hamdi 2015 Miraj 2015
Location not specified:	Brinsden 2003 El-Khayat 2015 Gamaleldin 2016 Kim 2011 Makrigiannakis 2011 Obidniak 2016 Scarpellini 2011 Scarpellini 2013

Studies were conducted in the following countries:

Brazil; (Cambiaghi and Leao 2012), China; (Yu, Zhang et al. 2016), Egypt; (El-Khayata and El Sadek 2015), Greece ; (Makrigiannakis 2011), Iran; (Aleyasin, Abediasl et al. 2016), (Davari Tanha, Kaveh et al. 2016),(Hamdi, Danaii et al. 2015) and (Miraj, Eftekhar et al. 2015), Italy; (Colicchia A, Pergolini I et al. 2011), (Pergolini, Gravotta et al. 2013), (Scarpellini and Sbracia 2011) and (Scarpellini and Sbracia 2013), Morocco; (Madkour, Bouamoud et al. 2016), Russia; (Obidniak, Gzgzyan et al. 2016), South Korea; (Kim, You et al. 2011), Turkey; (Urman, Ata et al. 2009), UK; (Brinsden, Ndukwe et al. 2003) and (Gamaleldin I, Lenguerrand E et al. 2016).

The locations of the 28 recruitment centres were not specified in one study (Brinsden, Alam et al. 2009). However the authors conducted a pilot study in 2003 within the UK using a similar protocol.

7.4.3.3 ***Participants***

The age of the patients ranged between 20 and 42 years.

11 studies had patients undergoing fresh IVF and 2 studies had patient with embarking on frozen embryo replacement (FER). Only one study included both fresh and frozen cycles.

5 studies did not specify the IVF treatment administered.Table 7.2 outlines the type of IVF treatment given across studies.

Table 7-2 Type of IVF treatment in the included studies

Type of IVF treatment			
fresh IVF cycle	**frozen cycle**	**fresh and frozen cycles**	**Not reported**
Aleyasin 2016	Obidniak 2016	Davari-Tanha 2016 91 Fresh cycles 9 frozen	Brinsden 2003
Brinsden 2009	Yu 2016		Gameleldin 2016
Cambiaghi 2012			Pergolini 2013
Collichia 2011			Scarpellini 2011
El-Khayat 2015			Scarpellini 2013
Hamdi 2015			
Kim 2011			
Makrigiannakis 2011			
Madkour 2016			
Miraj 2015			
Urman 2009			

7.4.3.4 ***Definitions of RIF***

There were two main definitions of RIF across studies.

Ten studies included defined RIF as women who previously underwent at least two consecutive failed cycles of IVF (Brinsden, Alam et al. 2009), (Cambiaghi and Leao 2012),(Colicchia A, Pergolini I et al. 2011),(El-Khayata and El Sadek 2015),(Hamdi, Danaii et al. 2015),(Madkour, Bouamoud et al. 2016),(Miraj, Eftekhar et al. 2015),(Obidniak, Gzgzyan et al. 2016),(Pergolini, Gravotta et al. 2013) and (Urman, Ata et al. 2009).

Eight studies defined RIF as women who underwent at least 3 previously failed consecutive cycles; (Aleyasin, Abediasl et al. 2016), (Brinsden, Ndukwe et al. 2003), (Davari Tanha, Kaveh et al. 2016), (Kim, You et al. 2011), (Makrigiannakis 2011), (Scarpellini and Sbracia 2011),(Scarpellini and Sbracia 2013) and (Yu, Zhang et al. 2016).

One study did not state a definition for RIF nor report any information on the previous number of failed cycles (Gamaleldin I, Lenguerrand E et al. 2016).

7.4.4 **Interventions**

7.4.4.1 ***G-CSF studies***

G-CSF was used in 8 studies (Aleyasin, Abediasl et al. 2016),(Cambiaghi and Leao 2012),(Davari Tanha, Kaveh et al. 2016),(Kim, You et al. 2011),(Miraj, Eftekhar et al. 2015),(Obidniak, Gzgzyan et al. 2016),(Scarpellini and Sbracia 2011) and (Scarpellini and Sbracia 2013).

Four studies used SC G-CSF(Aleyasin, Abediasl et al. 2016),(Cambiaghi and Leao 2012) (Scarpellini and Sbracia 2011) and (Scarpellini and Sbracia 2013).

All the studies has different control groups. Five studies gave G-CSF versus no treatment (Aleyasin, Abediasl et al. 2016),(Cambiaghi and Leao 2012) (Kim, You et al. 2011),(Miraj, Eftekhar et al. 2015) and (Obidniak, Gzgzyan et al. 2016).

Two studies gave the G-CSF intervention versus SC saline (Scarpellini and Sbracia 2011) and (Scarpellini and Sbracia 2013) and 1 study (Davari Tanha, Kaveh et al. 2016) gave GCSF vs IU saline. This study also included a control group of patients who had the passage of a cook catheter only.

(Aleyasin, Abediasl et al. 2016) used a single dose of 300µg 1 hour before the embryo transfer whereas (Cambiaghi and Leao 2012) used 0.25ml of G-CSF on alternate days from the day of embryo transfer to the confirmation of the presence of the fetal heart on USS post embryo transfer.

The two studies by Scarpellini et al;(Scarpellini and Sbracia 2011) and (Scarpellini and Sbracia 2013)used differing dosages of SC G-CSF. (Scarpellini and Sbracia 2011) used a daily dose of 1.5mg per kilogram of body weight from the day of embryo transfer to the pregnancy test 2 weeks later.(Scarpellini and Sbracia 2013) used a dose of 60mg daily from the day of embryo transfer to a pregnancy test conducted 2 weeks later.

Two studies used IU G-CSF (Davari Tanha, Kaveh et al. 2016) and (Miraj, Eftekhar et al. 2015) used 300µg/ml of IU G-CSF administered with a cook catheter on the day of egg ovum collection. This study also had two control groups with one group having 1ml of IU saline given in the same way or just the passage of the cook catheter (Miraj, Eftekhar et al. 2015) used IU 300mg/0.5mls administered on the day of eggcollection.

(Obidniak, Gzgzyan et al. 2016) used both intrauterine and subcutaneous G-CSF at a dosage of 30 million international units/ml. The SC G-CSF was given on the day of embryo transfer whilst the IU arm patients were given it 5 days before the transfer. This study also had a no intervention control group.

One study did not define the route of administration (Kim, You et al. 2011).

7.4.4.2 ***Heparin***

Heparin was used in four studies; (Colicchia A, Pergolini I et al. 2011); (Hamdi, Danaii et al. 2015); (Pergolini, Gravotta et al. 2013); (Urman, Ata et al. 2009).

Three studies used subcutaneous heparin (Hamdi, Danaii et al. 2015);(Urman, Ata et al. 2009), (Pergolini, Gravotta et al. 2013) whilst the mode of administration was not stated in(Colicchia A, Pergolini I et al. 2011).

Three studies gave the heparin intervention against no treatment (Colicchia A, Pergolini I et al. 2011);(Hamdi, Danaii et al. 2015);(Urman, Ata et al. 2009), whilst (Pergolini, Gravotta et al. 2013) gave the intervention against 100mg of aspirin. The route of aspirin administration was not defined.

Both (Colicchia A, Pergolini I et al. 2011) and (Pergolini, Gravotta et al. 2013) used Dalteparin at a dose of 2500IU twice daily on the day of embryo transfer up to the time of the pregnancy test.(Colicchia A, Pergolini I et al. 2011) did not define the timing of the pregnancy test whereas (Pergolini, Gravotta et al. 2013) continued the heparin until 12 days after embryo transfer.

(Hamdi, Danaii et al. 2015) used 5000IU of subcutaneous heparin daily from the day of egg ovum collection to the pregnancy test 15 days later. The results section did not report all the outcomes outlined in protocol.

(Urman, Ata et al. 2009) was the only study to use enoxaparin which was given at a daily dosage of 1mg per kilogram of body weight. This

was started from the day of egg collection until the pregnancy test date 12 days later. If the pregnancy test was positive the heparin was continued until 12 weeks of pregnancy.

7.4.4.3 ***PBMC***

PBMC were used in three studies (Madkour, Bouamoud et al. 2016); (Makrigiannakis 2011). All three studies gave the PB intervention against no treatment (Madkour, Bouamoud et al. 2016); (Makrigiannakis 2011); (Yu, Zhang et al. 2016)

One study was available in abstract form (Makrigiannakis 2011) whilst the remaining two studies were available in full text (Madkour, Bouamoud et al. 2016); (Yu, Zhang et al. 2016).

Two studies were set in single centre (Madkour, Bouamoud et al. 2016); (Yu, Zhang et al. 2016),whereas (Makrigiannakis 2011) did not state their setting.

PBMC were given intrauterine in 2 studies (Madkour, Bouamoud et al. 2016); (Yu, Zhang et al. 2016)

One study has 3 treatment groups, PBMC versus (vs) untreated PBMC vs no treatment (Makrigiannakis 2011)

7.4.4.4 ***Intralipid***

Intralipid was used in two studies (El-Khayata and El Sadek 2015); (Gamaleldin I, Lenguerrand E et al. 2016). (El-Khayata and El Sadek 2015) gave intralipid versus no intervention whereas (Gamaleldin I, Lenguerrand E et al. 2016) used a placebo which was not defined.

Both studies were available as abstracts and did not state their setting.

The route of intervention was intravenous in (El-Khayata and El Sadek 2015), where 20% intralipid was given between 4-7 days of ovarian stimulation. Patients who had a positive pregnancy test 2 weeks after embryo transfer were given another dose. (Gamaleldin I, Lenguerrand E et al. 2016) also administered 20% intralipid but in this study it was given 6-7 days before embryo transfer. A repeat dose was also given if the patient had a positive pregnancy test. The mode of administration was undefined.

7.4.4.5 ***LIF***

LIF was used in 2 studies (Brinsden, Ndukwe et al. 2003); (Brinsden, Alam et al. 2009). An undefined placebo was used a control group in both studies.

One study was available as an abstract (Brinsden, Ndukwe et al. 2003) and 1 study was available in full text paper (Brinsden, Alam et al. 2009).

Both studies were multi-centred and used 150µg of subcutaneous LIF which was administered just before embryo transfer and repeated for the following 7 days. A 1g dose of oral paracetamol was also given alongside the course of LIF.

There were no studies using glucocorticoids, LIT, IVIG, aspirin and TNF-α inhibitors that met the criteria for inclusion.

7.4.5 **Outcome measures**

Eight studies reported live birth (Brinsden, Ndukwe et al. 2003);(Brinsden, Alam et al. 2009); (El-Khayata and El Sadek 2015); (Gamaleldin I, Lenguerrand E et al. 2016); (Makrigiannakis 2011); (Pergolini, Gravotta et al. 2013); (Urman, Ata et al. 2009); (Yu, Zhang et al. 2016).

Five studies reported miscarriage rate (Brinsden, Alam et al. 2009);(Davari Tanha, Kaveh et al. 2016);(El-Khayata and El Sadek 2015);(Madkour, Bouamoud et al. 2016); (Obidniak, Gzgzyan et al. 2016).

(Obidniak, Gzgzyan et al. 2016) was the only study to state that no patients has been affected by side effects.

(El-Khayata and El Sadek 2015) reported 2 cases of congenital middle ear anomaly in the intralipid group.

7.4.5.1 ***Excluded studies***

Five studies were excluded. One study was Quasi randomised design (Fawzy and El-Refaeey 2014); 2 studies were cohort design and non randomised (Makrigiannakis, Benkhalifa et al. 2015); (Yoshioka, Fujiwara et al. 2006). 2 studies included patients with additional clinical conditions such as at least 1 thrombophilia (Qublan, Amarin et al. 2008); or evidence of autoimmune disease as well as RIF (Stephenson and Fluker 2000).

7.4.5.2 *Risk of bias in included studies*

In order to evaluate the quality of the data, a risk of bias assessment focusing on seven main domains: random sequence generation, allocation concealment, blinding, incomplete data outcome, selective reporting and other potential bias was performed. The findings are summarised in figures 7.2 and 7.3.

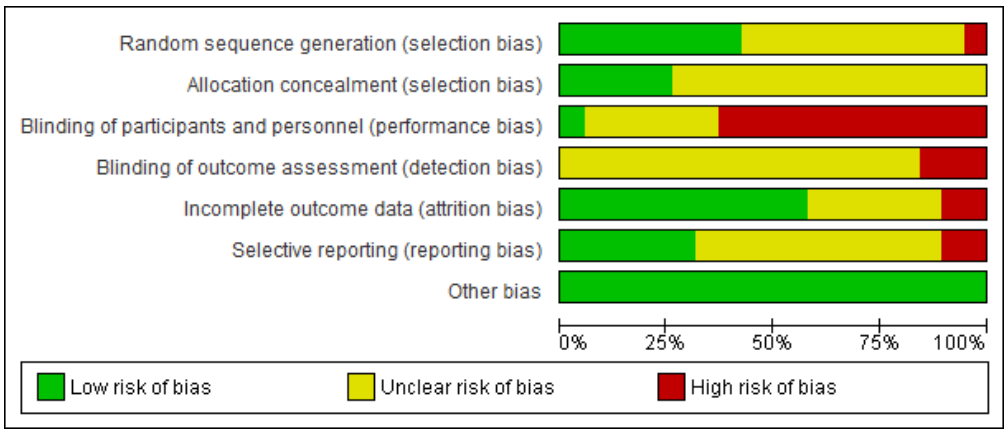


Figure- 7-2- Risk of bias graph

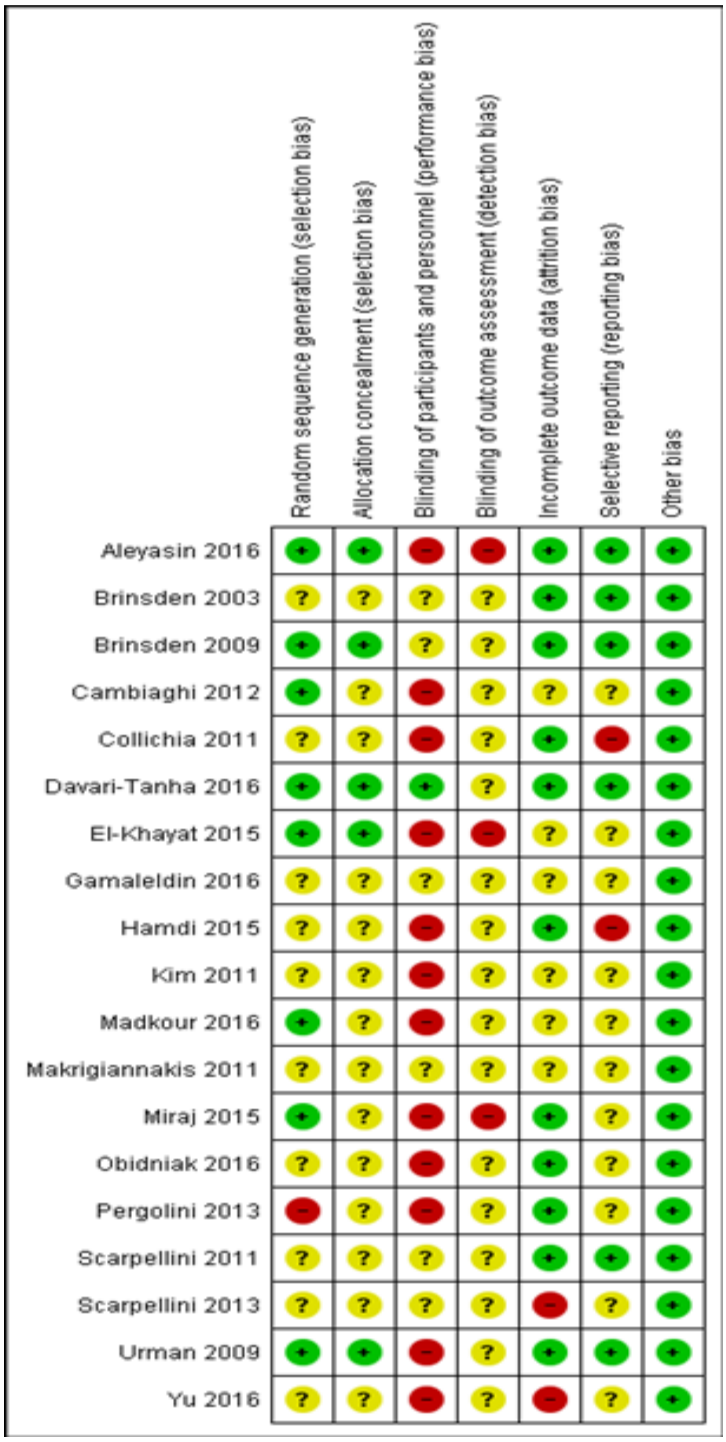


Figure- 7-3- Risk of bias summary

The review authors' judgements about each risk of bias item for each included study

7.4.5.2.1 Allocation-selection bias

7.4.5.2.1.1 Sequence Generation

Eight trials were at low risk of selection bias due to sequence generation (Aleyasin, Abediasl et al. 2016); (Brinsden, Alam et al. 2009); (Cambiaghi and Leao 2012); (Davari-Tanha 2016); (El-Khayat 2015); (Madkour 2016); (Miraj 2015); (Urman 2009).

10 studies had unclear risk due to lack of detail of their methods of sequence generation (Brinsden, Alam et al. 2009); (Collichia 2011); (Gamaleldin 2016);(Hamdi 2015); (Kim 2011); (Makrigiannakis 2011); (Obidniak, Gzgzyan et al. 2016); (Scarpellini 2011); (Scarpellini 2013); (Yu 2016) and 1 study was at high risk of bias in this domain (Pergolini 2013).

7.4.5.2.1.2 Allocation concealment

Six trials were at low risk of bias for allocation concealment (Aleyasin, Abediasl et al. 2016); (Brinsden, Alam et al. 2009); (Davari-Tanha 2016) ; (Urman 2009). The other 14 trails were at unclear risk of bias for allocation concealment (Brinsden, Ndukwe et al. 2003); (Cambiaghi and Leao 2012); (Collichia 2011); (Gamaleldin 2016); (Hamdi 2015); (Kim 2011); (Madkour 2016); (Makrigiannakis 2011); (Miraj 2015); (Obidniak, Gzgzyan et al. 2016); (Pergolini 2013); (Scarpellini 2011); (Yu 2016).

7.4.5.2.2 Blinding-performance bias and detection bias

7.4.5.2.2.1 Performance bias

Due to nature of studies, blinding was often not possible. Twelve trials had no means of blinding and were at high risk of performance and detection bias (Aleyasin, Abediasl et al. 2016); (Cambiaghi and Leao

2012); (Hamdi 2015); (Kim 2011); (Madkour 2016); (Miraj 2015);(Obidniak, Gzgzyan et al. 2016) (Obidniak 2016); (Urman 2009; Yu 2016 6 studies risk was unclear due to lack of data Brinsden 2003); (Brinsden, Alam et al. 2009); (Gamaleldin 2016); (Makrigiannakis 2011); (Scarpellini 2011); (Scarpellini 2013) and one trial was at low risk (Davari-Tanha 2016).

7.4.5.2.2.2 Detection bias

The majority of studies had an unclear risk of assessment blinding due to a lack of reporting of relevant data for most outcomes (Brinsden, Ndukwe et al. 2003); (Brinsden, Alam et al. 2009); Cambiaghi 2012; Collichia 2011; Davari-Tanha 2016.Gamaleldin 2016; Hamdi 2015; Kim 2011; Madkour 2016; Makrigiannakis 2011;(Obidniak, Gzgzyan et al. 2016); (Pergolini 2013); (Scarpellini 2011);(Scarpellini 2013); (Urman 2009); (Yu 2016). The remaining 3 studies were at high risk of bias in this domain as outcome assessors were not blinded (Aleyasin 2016; El-Khayat 2015); (Miraj 2015).

7.4.5.2.3 **Incomplete outcome data- attrition bias**

Six studies were at unclear risk of bias in this domain (Cambiaghi 2012); (El-Khayat 2015); Gamaleldin 2016; (Madkour 2016); (Makrigiannakis 2011); (Kim 2011), two at high risk (Scarpellini 2013); (Yu 2016); and 11 had low risk of attrition bias (Aleyasin 2016); (Brinsden 2003); (Brinsden, Alam et al. 2009); (Collichia 2011); (Davari-Tanha 2016); (Hamdi 2015); (Miraj 2015); (Obidniak, Gzgzyan et al. 2016); (Pergolini 2013); (Yu 2016).

7.4.5.2.4 **Selective reporting- reporting bias**

Seven trials were at low risk of reporting bias, (Aleyasin, Abediasl et al. 2016); (Brinsden, Ndukwe et al. 2003); (Brinsden, Alam et al. 2009);(Cambiaghi and Leao 2012); (Davari Tanha, Kaveh et al. 2016); (Scarpellini and Sbracia 2011); (Urman, Ata et al. 2009).

11 were assessed as having unclear risk (El-Khayata and El Sadek 2015); (Gamaleldin I, Lenguerrand E et al. 2016); (Kim, You et al. 2011); (Madkour, Bouamoud et al. 2016); (Makrigiannakis 2011); (Miraj, Eftekhar et al. 2015); (Obidniak, Gzgzyan et al. 2016); (Yu, Zhang et al. 2016) and 1 trial was at high risk of this bias (Hamdi, Danaii et al. 2015).

7.4.6 **Effects of interventions**

7.4.6.1 ***Intralipid vs no treatment***

7.4.6.1.1 **Live birth rate**

Both included studies (El-Khayata and El Sadek 2015);(Gamaleldin I, Lenguerrand E et al. 2016) reported this outcome with a total of 303 participants. (Gamaleldin I, Lenguerrand E et al. 2016) quoted a RR of 1.56 (CI 0.74-3.27) whilst (El-Khayata and El Sadek 2015) reported a RR of 1.6 (CI 1.28-2.13). However the authors of the later study did not specify what the abbreviation RR represented. Therefore a meta-analysis was performed with the relative risk calculated from the percentages quoted for each group in both studies. This showed that intralipid probably improves the live birth rate in those given this intervention (RR 2.13, 95% CI 1.35 to 3.36; participants = 303; studies = 2; I2 = 6% moderate certainty evidence) (figure 8.4).

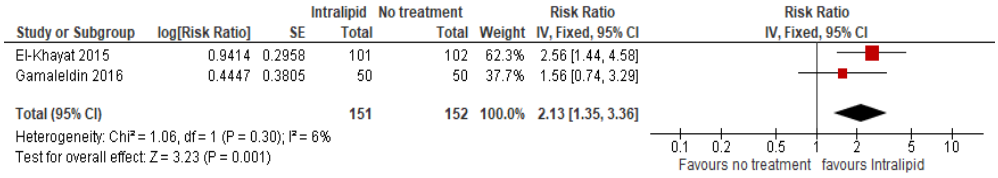


Figure- 7-4- Live birth rate for intralipid vs no treatment

7.4.6.1.2 Miscarriage rate

This was reported by one study (El-Khayata and El Sadek 2015) with a total of 203 participants. The results showed insufficient evidence to show a difference between the study groups as the confidence interval crossed the line of no effect (RR 0.43, CI 0.12 to 1.63; participants=203 ; studies=1; I^2 = 0 ,low certainty evidence) (figure 8.5).

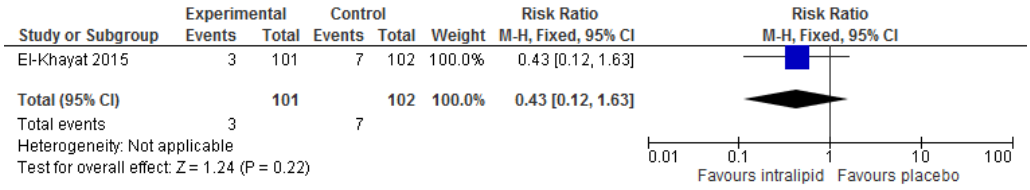


Figure- 7-5- Miscarriage rate for intralipid vs no treatment

(El-Khayata and El Sadek 2015) reported 2 cases of congenital middle ear anomaly in the intralipid group.

7.4.6.2 *PBMC vs no treatment*

7.4.6.2.1 Live birth rate

2 studies (Makrigiannakis 2011); (Yu, Zhang et al. 2016) reported this outcome with a total of 318 patients.

A meta-analysis could not be performed as (Yu, Zhang et al. 2016) reported results per embryo transferred with 32/93 embryos achieving pregnancy in the PBMC vs 12/105 in the control arm.

(Makrigiannakis 2011) reported their results as percentages with 39.5%of participants achieving a live birth in the cortisol releasing

hormone (CRH) treated PBMC cells vs 25% in the untreated PBMC group vs 7% who had no intervention. This was a three group comparison and therefore unsuitable for further analysis. Furthermore the authors did not report the total number of patients in each group and therefore event numbers could not be calculated.

7.4.6.2.2 **Miscarriage rate**

One study (Madkour, Bouamoud et al. 2016) reported this outcome. A meta-analysis was performed which showed a probable improvement in the miscarriage rate of those given PBMC treatment (RR 0.20, 95% CI 0.08 to 0.51; participants = 54; studies = 1, I^2 = 0%; very low certainty evidence) (figure 7.6).



Figure- 7-6- Miscarriage rate for PBMC vs no treatment

7.4.6.3 *Heparin vs no treatment*

7.4.6.3.1 **Live birth rate**

Only one study (Urman, Ata et al. 2009) reported this outcome with a total of 150 participants. The results showed insufficient evidence to show a difference between the study groups as the confidence interval crossed the line of no effect seen in the heparin group (RR 1.30, 95% CI 0.80 to 2.12; participants = 150; studies = 1; I^2 = 0%, low certainty evidence)(figure 7.7).

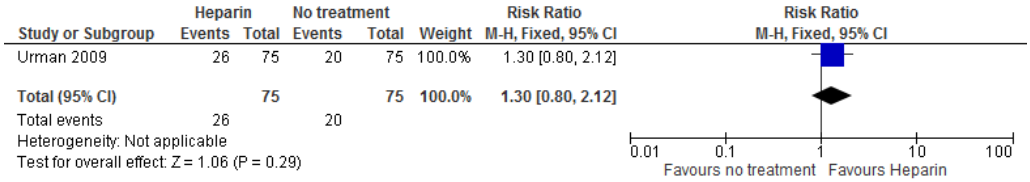


Figure 7-7- Live birth rate for heparin vs no treatment

The miscarriage rate or drug side effects were not reported in any of the include studies for heparin.

7.4.6.4 *Heparin vs Aspirin*

7.4.6.4.1 Live birth rate

This was reported in one study (Pergolini, Gravotta et al. 2013) outcome with a total of 126 participants. Although there was a possible improvement seen in the heparin group, the evidence was graded as low certainty (RR 2.56, 95% CI 1.17 to 5.58; participants = 126; studies = 1; I^2 = 0, low certainty evidence) (figure 7.8).

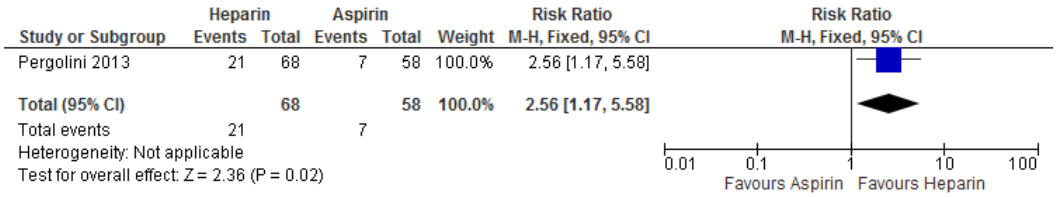


Figure 7-8- Live birth rate for heparin vs aspirin

7.4.6.5 *G-CSF vs no treatment*

7.4.6.5.1 Live birth rate

This was reported by any study in this comparison

7.4.6.5.2 Miscarriage rate

This was not reported by any study using SC administration. However 2 studies reported this outcome (Davari Tanha, Kaveh et al. 2016); (Obidniak, Gzgzyan et al. 2016) using the IU route with a total of 43 participants. The meta-analysis for this sub group showed there was insufficient evidence to determine if IU G-CSF made a difference to miscarriage rate as the CI was very wide and a Peto OR had to be applied as (Davari Tanha, Kaveh et al. 2016) had no miscarriages in their control group (Peto OR 0.94, 95% CI 0.17 to 5.11: participants=43;studies=2,I^2=0%) low certainty evidence (figure 7.9).

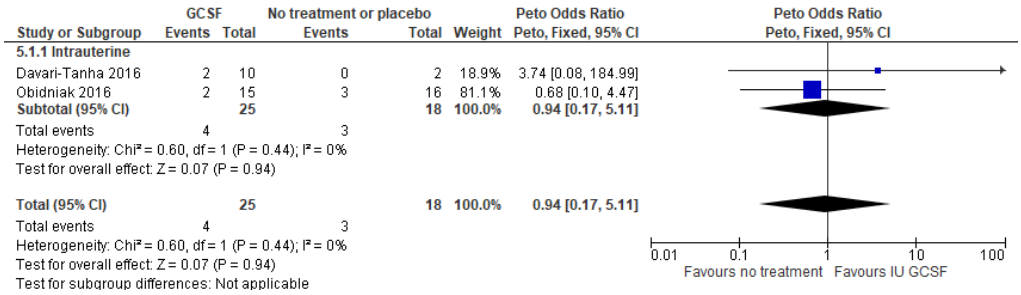


Figure 7-9- Miscarriage rate for IU G-CSF vs no treatment

(Obidniak, Gzgzyan et al. 2016) was the only study to report on drug side effects with no participants being affected.

7.4.6.6 *LIF vs placebo*

7.4.6.6.1 Live birth rate

Both included studies reported on this outcome with a total of 208 participants (Brinsden, Ndukwe et al. 2003) and (Brinsden, Alam et al. 2009) There was insufficient evidence to determine if there was a difference in live birth in those given LIF treatment as the confidence interval was extremely wide and crossed the line of no effect.(RR 1.68, 95% CI 0.06 to 48.49; participants = 208; studies = 2; I^2 = 82%, very low certainty evidence) (figure 7.10).

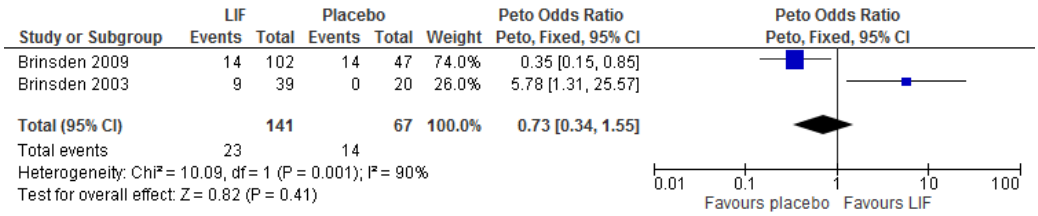


Figure 7-10- Live birth rate for LIF vs placebo treatment

Furthermore a Peto OR had to be applied as (Brinsden, Ndukwe et al. 2003) reported no live births in the control group (Peto OR 0.73, 95% CI 0.34 to 1.55; participants = 208; studies = 2; I^2 = 90%, very low certainty evidence) This study also had unbalanced groups with a significantly

higher number of participants with male factor infertility making the risk of bias high. Therefore the findings have to be interpreted with caution and cannot be generalised to the RIF population.

7.4.6.6.2 **Miscarriage rate**

(Brinsden, Alam et al. 2009) was the only study to report on this outcome with a total of 149 participants. The results showed insufficient evidence to show a difference between the LIF and placebo treatment as the confidence interval crossed the line of no effect (RR 0.61, 95% CI 0.14 to 2.64; participants = 149; studies = 1,I^2 =0%, low certainty evidence) (figure 7.11).

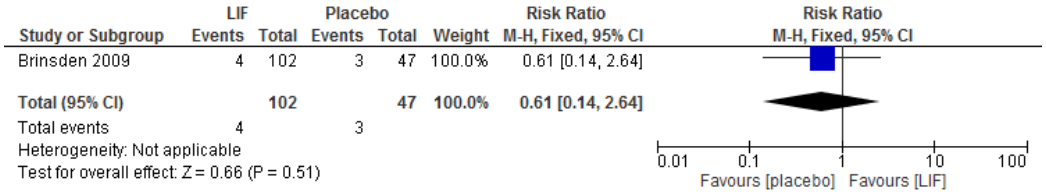


Figure 7-11- Miscarriage rate for LIF vs placebo

7.5 Discussion

The main aim of this review was to investigate whether the administration of commonly used immunotherapies, improve pregnancy outcomes in unexplained RIF women undergoing IVF. Overall there is no clear evidence that administration of most of the immunotherapies improve live birth or miscarriage rate. However limited benefit for the use of intralipid, and heparin was seen.

However there were variations in the dosage, regimen and use of placebos with many studies not stating their full methodology. The lack of reporting adverse outcomes such as drug side effects was highly evident. This along with varying criteria for selecting patients with RIF made most of the data inadequate for inclusion in the meta-analysis and caution has to be exercised when applying the results to clinical practice.

It is widely known in the literature that there is no consensus or globally accepted definition for RIF (Polanski, Baumgarten et al. 2014). We wanted to include as many studies as possible and hence chose the definition of at least 2 unsuccessful cycles. Despite finding a good numbers of studies, 11 out of the 19 studies were in conference abstract format. Studies reported different outcomes ranging from chemical pregnancy rate to live birth. The dosage, administration and duration of treatment also varied across studies.

We found 2 studies that used intralipid treatment and both showed an improvement in live birth rate in those given the intervention (El-

Khayata and El Sadek 2015),(Gamaleldin I, Lenguerrand E et al. 2016) Although the quality of evidence was moderate, these findings have to be interpreted with extreme caution as both were conference abstracts with small participant numbers.

The studies using PB were heterogeneous and currently this immune therapy is not widely used. In (Yu, Zhang et al. 2016) the PBMC were co cultured with HCG for 24 hours before IU administration. (Makrigiannakis 2011) cultured the PBMC with CRH for 48 hours whilst (Madkour, Bouamoud et al. 2016) incubated the PBMC only for 72 hours. A meta-analysis could not be performed for live birth but showed an overall beneficial effect on implantation and clinical pregnancy rates. The miscarriage rate was also lower in the PBMC group. The quality of evidence was moderate and analysed using one study.

G-CSF was the most frequently used intervention, none of the studies reported live birth.

The evidence for the use of the LIF was the least robust. Both included studies were by the same study group and showed high heterogeneity (I^2 statistic=82%) and as a result the quality of evidence had to be downgraded and classified as low. In the earlier study (Brinsden, Ndukwe et al. 2003), the study groups were not matched equally as there was significantly higher number of patients with male factor infertility in control group. This led to a lower pregnancy rate in this group. Therefore the findings of this study cannot be generalised to all women with RIF.

There were 8 studies (Brinsden, Ndukwe et al. 2003),(Brinsden, Alam et al. 2009),(El-Khayata and El Sadek 2015),(Gamaleldin I, Lenguerrand E et al. 2016),(Makrigiannakis 2011),(Pergolini, Gravotta et al. 2013),(Urman, Ata et al. 2009) and (Yu, Zhang et al. 2016) that reported on live birth which is ultimately the outcome that both clinicians and patients want to know. However there were questionable results with intralipid, PBMC treatment and heparin showing a higher live birth in the intervention group. The data on heparin for only available from one study. The use of LIF was not significant with very wide confidence intervals. Interestingly the most commonly used intervention included in this review was G-CSF and none of the 8 studies reported on live birth. A possible explanation for this is the high number of studies being in abstract form with limited reporting. The difficulty in collecting data at birth in these patients along with reporting bias may explain the trend further. A robust strategy to contact authors in order to obtain outcome data and clarify methodology was carried out but very little correspondence was achieved.

The miscarriage rate was only reported in 5 studies (Brinsden, Alam et al. 2009); (Davari-Tanha 2016); (El-Khayat 2015); (Madkour 2016); (Obidniak 2016). A meta-analysis was only possible for IU G-CSF which showed a non-significant result in a total of 43 patients. This again highlights the reporting bias and limited information gained from conference abstracts.

One of the most significant findings in this review is that we found NO studies using steroids as a treatment for RIF. There have been two

large cochrane systematic reviews on the use of steroids in women undergoing routine IVF with the inclusion of 14 studies (Boomsma, Keay et al. 2007, Boomsma, Keay et al. 2012).However none of these were relevant or met our criteria. Furthermore the use of novel interventions such as PBMC's and LIF need to be clarified along with their safety profiles. Only 2 studies reported on adverse effects which is strikingly low given that most of these treatments are not fully licensed in the context of RIF ((El-Khayata and El Sadek 2015); (Obidniak, Gzgzyan et al. 2016) This is where further studies are needed and future research should be directed.

The majority of studies (13 trials) had no means of blinding and were at high risk of performance and detection bias. There was evidence of poor reporting of study methods and lack of studies investigating the desired outcomes. The evidence for live birth was of moderate quality in the intralipid studies and low in heparin studies. The evidence for the miscarriage rate was deemed to be low to very low and this was influenced by a lack of reporting for this outcome across all trials.

The potential biases in this review were minimised by adherence to the Cochrane methodology. All abstracts and studies were screened by two independent reviewers. The data extraction was also conducted independently by two reviewers. Discrepancies in the data extraction and other areas of uncertainty were resolved through team discussion.

7.5.1 Agreements and disagreements with other studies or reviews

This is the first systematic review that has comprehensively pooled outcome data for commonly used immune therapies in the context of RIF. There is already a respectable pool of systematic reviews looking at the effectiveness of aspirin;(Dentali, Ageno et al. 2012) (Gelbaya, Kyrgiou et al. 2007);(Khairy, Banerjee et al. 2007);(Siristatidis, Basios et al. 2016), heparin (Akhtar, Sur et al. 2013) (Seshadri, Sunkara et al. 2012), steroids (Boomsma, Keay et al. 2012), and IVIG (Clark, Coulam et al. 2006); (Matteo, Greco et al. 2013) in women undergoing IVF. The major reviews on aspirin, steroids and heparin have concluded that the evidence for their benefit in IVF is currently lacking and definitive well designed trials for each intervention are desperately needed. The use of IVIG however has been shown to be beneficial but the studies included by (Matteo, Greco et al. 2013), had patients with an autoimmune or thrombophillic condition requiring immunotherapy. Therefore these findings cannot be extrapolated to our review question.

We have identified 4 systematic reviews looking specifically at RIF. Both (Potdar, Gelbaya et al. 2013) and (Seshadri, Sunkara et al. 2012) have looked at heparin in the context of RIF whilst (Zhao, Xu et al. 2016) and (Kamath, Chittawar et al. 2017) have collated evidence on GCSF. The latter 2 reviews have also included women with a thin endometrium but sub group analyses on RIF patients were been performed.

(Potdar, Gelbaya et al. 2013) included 3 studies in their systematic review. 2 studies were randomised in their design (Urman, Ata et al. 2009); (Qublan, Amarin et al. 2008) whilst (Berker, Taşkın et al. 2011)was quasi-randomised.(Seshadri, Sunkara et al. 2012) performed a sub group analysis of women with RIF using (Berker, Taşkın et al. 2011)and (Urman, Ata et al. 2009) with a pooled OR for live birth showing no statistically significant difference between the two groups. We excluded (Qublan, Amarin et al. 2008) in our review, as 83 included patients also had at least one thrombophilic defect. Furthermore (Potdar, Gelbaya et al. 2013) showed an improvement in the live birth rate and a reduction in the miscarriage rate with heparin compared with controls.

(Zhao, Xu et al. 2016)included 6 studies looking at the role of GCSF in women undergoing ART. The authors included 2 studies of women with RIF (Aleyasin, Abediasl et al. 2016) and,(Scarpellini and Sbracia 2011), both of which have been included in our review. Despite the authors not reporting on live birth, higher clinical pregnancy rates for women with RIF were found when compared to control group. The authors (Kamath, Chittawar et al. 2017) also concluded that in the RIF population, the administration of SC GCSF was associated with a higher clinical pregnancy rate compared to no intervention. The authors of both these review are in agreement with our conclusion that well designed RCTs are required before offering both heparin and G-CSF for clinical use.

There have been no systematic reviews on intralipid, PB and LIF use in unexplained RIF and most of the evidence of its use has been through in-vitro and small case control studies.

(Ndukwe 2011) is the only non-randomised study using intralipid that may be comparable to this review. The authors assessed the clinical pregnancy rate in 50 women with RIF defined as three or more failed IVF cycles following the administration of IV 20 % intralipid solution between day 4 and 9 of ovarian stimulation. If the pregnancy test post embryo transfer was positive a second infusion was given within 7 days. The intervention group were compared to a group of 46 women who had no intralipid. The results reported a higher clinical pregnancy rate in the intralipid group, but this study was not conducted in the context of unexplained RIF.

There have been 3 non randomised case control studies using PB treatment in the context of RIF that have all shown a beneficial effect on clinical pregnancy rates ((Makrigiannakis, Benkhalifa et al. 2015);(Okitsu, Kiyokawa et al. 2011); (Yoshioka, Fujiwara et al. 2006)).

More recently (Li, Wang et al. 2017) has developed the work on PB treatment further using a similar protocol to (Yoshioka, Fujiwara et al. 2006). In this study 633 patients with a history of at least one unsuccessful embryo transfer cycle were either given HCG treated PBs cultured 2 days before embryo transfer (ET) or no additional treatment. The PB treated group comprised of 294 patients with the remaining 339 patients having no additional treatment. The HCG treated PBMC were

administered into the uterine cavity 24 hours before the embryo transfer. All patients then underwent a FER with either a day 3 or day 5 embryo. The results were reported per embryo transfer cycle and there was a significant increase in the clinical pregnancy rate in those treated with PB group. However the live birth rate was not significant.

A subgroup analysis of patients with 4 or more previous implantation failures showed a significant increase in the both the clinical pregnancy and live birth rates. These findings are consistent with those of (Okitsu, Kiyokawa et al. 2011) who also stratified their results into patients with at least 3 previous implantation failures.

The women in (Okitsu, Kiyokawa et al. 2011) and (Li, Wang et al. 2017) were tested and excluded if there was presence of auto-immune or thrombophilic disease. The remaining studies do not specify this as an exclusion criteria. A significant limitation of these studies is that women were not randomised and chose to undergo treatment and therefore both operators and patients were unblinded. However the results of our meta-analysis does support these findings and therefore further work is required in this field.

7.6 Conclusions

This review highlights that there is weak evidence to suggest that intralipid, and heparin may be beneficial in some RIF patients. However these findings have to be interpreted with extreme caution.

Despite the overwhelming lack of good evidence for most of these interventions, there are some associations that these therapies have potential beneficial impact on the early conceptus. Therefore an effort to return to the basic science in order to understand the therapeutic mechanisms of these treatments should be made. This will hopefully allow the clinical community to understand their exact mode of action on the maternal immune system in the IVF setting. The principle of correctly identifying women who need immune therapy also requires further thought and exploration. Currently there are no robust immune screening tests that allow clinicians to screen and identify patients who are at risk of RIF. This is where the basic science and clinical research needs to be directed.

This systematic review has highlighted that before immune therapies are given, the testing for immune cells in the context of RIF needs clarification. The findings also need to be accepted with extreme caution as many of the immunotherapies were used in a heterogeneous group of patients and varied regimen. This supports the hypotheses and work presented in this thesis.

Chapter 8

Discussion and suggestions for future work

Chapter 8 Discussion and suggestions for further work

There were 7 hypothesis posed as the beginning of this thesis:-

1. The prevalence of raised NK status will be greater than 20% in women undergoing IVF treatment (Chapter 3)

The prevalence of raised NK status in the total population was 61.6% (CI 54.9-68.3) which supports the hypothesis.

2. An increase in the number of previous unsuccessful IVF cycles will lead to a higher prevalence of raised NK status (Chapter 3)

The work in Chapter 3 revealed that this was not a linear relationship. The prevalence of raised NK cells was highest in those who has 3 or more unsuccessful cycle (71.4% CI 47.8-95.1) and the lowest in those with 2 previous unsuccessful cycles (43.6% 28.0-59.1).Surprisingly the prevalence in women with no previous IVF treatment was also high 66.7% (CI 54.4-78.9).

3. A raised NK status will be associated with a reduced chance of live birth (Chapter 3)

There were no differences in livebirth with when the NK result was stratified into low to high severity. However those with a NK cell level greater than 50% had a lower live birth rate, even though these patients were given steroid treatment; crude OR 0.72 (0.24-2.19) but this was not statistically significant (P value 0.87).

4. The application of a flow cytometry panel will lead to the identification of other immune cell populations and provide a functional aspect to testing (Chapter 4 and 5)

The application of the FC panel revealed the expression of perforin and granzyme was noticeable in the peripheral blood (PB) samples as opposed to the presence of TNFα and IFNγ in the endometrium. This pattern may be more exaggerated in pregnancy success or loss.

5. The FC panel will reveal immune cell populations or cytokines that are associated with IVF success (Chapter 5)

The FC panel showed that PB NKT perforin, perforin $^{+}$granzyme^{+} as well as endometrial CD8+ TNFα^{+}IFNγ^{+} had an effect on pregnancy success through IVF. These associations need further validation.

6. Stratification according to IHC NK status will reveal further trends in the FC data (Chapter 6)

The work in chapter revealed that patients who had IHC and FC had a high expression of both NK and NKT cells in those that IHC positive. Furthermore the co-expression of perforin^{+} granzyme^{+} may in part explain how they are functionally related to the pregnancy outcome.

7. A systematic review of the literature will support the use of oral steroid treatment as an immune therapy for RIF (Chapter 7)

The systematic review did not support the use of steroid treatment in those with unexplained RIF as no relevant studies were identified.

The work in this thesis has confirmed that other immune populations apart from NK cells play a part in implantation. Furthermore a novel finding was made to support that a balance and shift in cytokines namely perforin and granzyme occurs to bring about or hinder early pregnancy. It must also be noted that the studies in this thesis were conducted in an IVF clinic with good pregnancy results and if a cycle was successful then the contributory role of the endometrium was paramount.

The tissue of interest; the human endometrium is a unique biological fabric that undergoes a monthly cycle of proliferation, differentiation and shedding. During a woman's lifetime this intricate process occurs hundreds of times and is significantly altered with pregnancy. This coupled with the immune system and NK cell life cycle also exhibiting change makes this area both challenging and interesting to research.

The measurement of immune cells in the circulation and endometrium is a complex and controversial area of reproductive medicine (Moffett and Shreeve 2015, Sacks 2015). As discussed in the background to this topic, the clinical criteria, reference range and most appropriate test to apply is still largely variable and poorly understood.

One of the main challenges when assessing the human immune system is that it is under the influence of many internal homeostatic and external factors. This coupled with the similar dynamic and intricate events of pregnancy makes this area both interesting and scientifically demanding. Therefore developing a clinical test that can adequately

assess two multifaceted processes must also possess multi-functionality.

The main aim of this thesis was to go “back to basics” and address some of disparities with NK cell testing before considering the administration of Immunotherapies. A logical starting point in this research was to gather evidence and unravel some of the discrepancies that exist regarding the significance of NK cells in implantation and whilst some of the trends from existing research have been found, their association still need further work. There is certainly a pathological basis to RIF, of which an immune imbalance is either the symptom or contributory cause.

One of the main conclusions is that looking at NK cells by their numbers or percentage in relation to pregnancy success is far too simplistic. Although a significantly higher prevalence of raised NK status was found, the clinical implications of this were not corroborated. The logistic regression in chapter 3 failed to establish if a positive IHC status was associated with the prediction of live birth through IVF. There was a declining trend in being IHC positive with age and increasing embryo quality but these are already known factors for success in IVF.

The work with the FC panel supported the notion that NK cells must be thought of as a dynamic entity which are part of a wider immunological profile that either causes an imbalance or homeostasis within the pregnancy conducive endometrium. The notion that a group of women who suffer from dysregulation of the endometrium leading to aberrant

decidualisation and implantation is a plausible one and is supported by some of the work presented in this thesis. One of the key features of this work has been to identify some of the markers that contribute to this; namely; perforin, co-expression of perforin^{+}granzyme^{+} and TNFα^{+}IFNγ^{+}.

Both IHC and FC are featured heavily in the context of NK testing and RIF. However this work is agreeable with the conclusions in the literature that before testing is offered the definition of RIF needs standardisation so that test techniques are used appropriately and validated (Polanski, Baumgarten et al. 2014, Lash, Bulmer et al. 2016).

One of the main exploratory themes was to study these techniques in the IVF population and correlate this with pregnancy outcome in the next cycle. This has been sparsely studied in the literature previously (Laird, Mariee et al. 2011) and much of the data was generated 2-3 decades ago (Fukui, Fujii et al. 1999, Thum, Bhaskaran et al. 2004).

The comparison of IHC and paired PB and endometrial samples in Chapters 4 and 5 led to some interesting observations. The main theme across the two analyses was that of reciprocal shifts being observed in the expression of immune cells and their cytokines depending on the tissue type and certainly in the clinical outcomes.

The introduction of the FC panel led to the discovery that NKT and CD8+cells may play a significant part. The relationship between the cytokine expression was novel as was the shift from cytotoxic makers to the more angiogenesis inducing properties; TNFα in successful

pregnancies. This link between the co expression of perforin^{+}granzyme^{+} and TNFα needs further exploration. Simplistically it would seem that the co-expression of perforin^{+}granzyme^{+} is associated with a negative outcome whereas TNFα is a facilitator of successful pregnancy. This is logical as perforin and granzyme are mediators of inflammation and protect against infection, malignancy and inflammation. It would seem in the context of early pregnancy, this may lead to detrimental effects on implantation. Conversely an abundance of angiogenic factors such as TNFα is likely to beneficial for pregnancy. This shift may in part explain the inflammatory and anti-inflammatory balance that occurs to facilitate implantation. Furthermore it is known that other immune cells such as dendritic cells(DCs) and macrophages also play a significant role in implantation and immune-tolerance (Mor, Cardenas et al. 2011, Moffett and Colucci 2014). Hence the inclusion of these cells are further avenues this panel can explore.

The use of prednisolone is patients who were positive for NK testing was a common theme in this thesis. Chapter 6 was an attempt to stratify the study population into those treated with prednisolone and NK negative patients. This led to the observation that steroid treatment was not entirely beneficial for a mixed population of women undergoing IVF. The immune cell populations present in the endometrium at the time of implantation are all sensitive to the effects of endogenous steroids. This is a pivotal step needed to bring about a state of immune-tolerance. There is likely to be a group of women who have a resistance or abnormal response to steroid fluctuations and hence would benefit from

exogenous steroid treatment. The development of a screening test to identify this cohort of women is another research area needing clarification. Assessing paired samples before and after steroid is one of the major modification that could be made to the FC panel in order to investigate this further.

One of the trends in the literature is that of moving away from testing a single cellular component and moving towards a panel of cells and/or markers i.e. an immune profile that collectively act synergistically to bring about pregnancy (Ledee, Prat-Ellenberg et al. 2017).The FC panel described here and its combination with IHC is a progressive step in achieving this goal. The functionality of cytokines such as perforin and granzyme could be further explored with techniques such enzyme-linked immunosorbent assay (ELISA) to quantify the total expression of key predictors of pregnancy success.

The direction of work in this field includes testing modalities such as genetic profiling and epigenetic assessment of the endometrium (Egea, Puchalt et al. 2014, Valdes, Schutt et al. 2017) in order to provide detailed information about the correct time for implantation. The introduction of “omics” focusing on the genes and proteins encoding for endometrial receptivity in order to build a profile of an individual patients endometrium is also proving useful in some IVF centres (Diaz-Gimeno, Ruiz-Alonso et al. 2013, Edgell, Rombauts et al. 2013, Ruiz-Alonso, Blesa et al. 2013).Although there is a wide variation in the study populations and techniques employed, ultimately identifying correct window of implantation and transfer of embryo, will not only reduce

cycle failure but target focused treatment. This coupled with immune testing with a robust FC panel will provide combined testing for patients and provide a multi-functional dimension to testing.

The systematic review highlighted the stark lack of good quality evidence for any therapeutic intervention in RIF. This is concerning as patients are being given many of the interventions studied. Therefore a reliable screening test(s) and or randomised controlled trail (RCT) s need to be developed before any of the interventions are integrated into clinical practice.

8.1 Limitations and suggestions for further work

The main criticism of the two laboratory studies presented here and in the literature is the lack of fertile controls included in the research. This would certainly work towards setting a reference for many of the immune cells and their patterns of expression in and outside of pregnancy.

One of the main confounders in this thesis was the both studies outlined in chapter 3 and 5 included patients who were given oral prednisolone treatment if they tested IHC NK positive. Whilst this did not allow direct comparison of outcome between negative and positive results, a subgroup analysis showed **NO** linear relationship with severity of NK results and pregnancy outcome. This certainly needs further analysis with a larger groups of patients. A cohort study of stratifying patients according to number of IVF cycles and comparing their demographic and pregnancy outcomes may reveal a more significant trend.

Another potential confounder was the effects of taking the endometrial biopsy which may have led to a change in the immune profile of the endometrium. There is evidence to suggest the passing a device into the endometrium can improve implantation, clinical pregnancy and live birth (Potdar, Gelbaya et al. 2012). There is also emerging evidence that sampling uterine fluid at the time of implantation, is a safe procedure and less invasive than an endometrial biopsy (Hannan, Nie et al. 2012). The fluid is rich in proteins and immune cells that could be used as a surrogate markers for receptivity (Edgell, Evans et al. 2018, Fitzgerald, Evans et al. 2018). Therefore applying the FC panel to PB, endometrium prior to the IVF cycle and correlating this with uterine fluid at the time of the embryo transfer would allow trends to be analysed further.

The work in Chapter 5 highlights a potential correlation of IHC and FC as do other studies (Park, Lee et al. 2010, Santillan, Lozano et al. 2015). Therefore optimisation of the panel and patient selection may lead to peripheral blood being used for testing rather than endometrial sampling which can be invasive.

The next logical progression from this work would be to apply this flow cytometry panel to a much larger groups of patients with the inclusion of fertile controls after introducing a power calculation. The ability to combine some of the markers such as those demonstrated by the addition of the CD56, CD16 and CD3 in vitro diagnostic test may help to streamline the workload involved and may be the first step towards developing a clinical test. The addition of further immune cell

populations such as DCs, macrophages and regulatory T cells would expand testing. Furthermore cytokines such as IL2, 6 and 15 would delineate some of the mechanisms and pathways in which these cell functions.

The immunotherapies described in Chapter 7 such as prednisolone and intralipid could be introduced into the panel to explore the effects of these on the various immune cell populations and their markers. The may subsequently guide treatment dosage and efficacy.

Ultimately designing and implementing a (RCT of prednisolone versus placebo in women with normal/raised uterine NK levels will answer many important questions in this area. This would lead to 4 arms in the study as shown in figure 8.1.

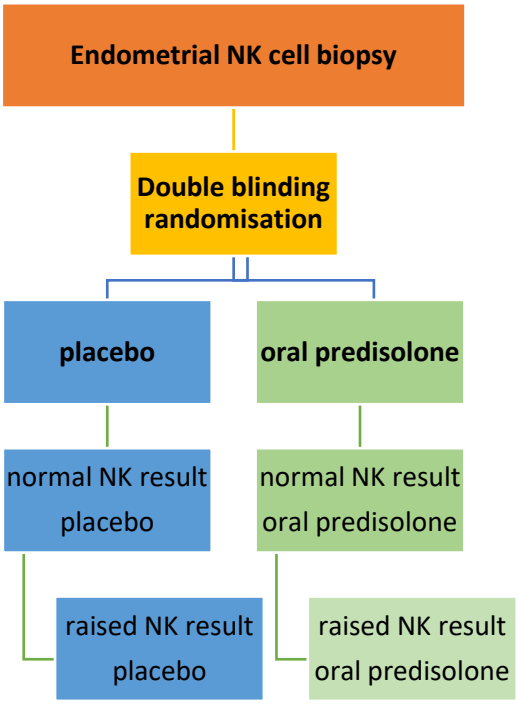


Figure 8-1- RCT design of NK testing and prednisolone

The study would involve offering all women NK testing through IHC and flow cytometry panel followed by randomisation to either placebo or prednisolone. This would lead to a control arm of normal NK results who have placebo compared to those given prednisolone. The latter group would provide information about the use of steroids in women undergoing IVF. The remaining groups would be comprised of those with raised NK levels who were randomised to placebo and prednisolone. This would answer the therapeutic benefits of this therapy in the context of NK testing and IVF.

8.2 Conclusion

The maternal immune system plays an important role in implantation and early pregnancy. The functions and properties of the many immune cells involved are still yet to be fully defined. There is growing evidence that NK cells are heavily involved in successful and more importantly unsuccessful implantation in the context of IVF. However there is no consensus as how to measure these cells and what constitutes a detrimental levels for pregnancy success.

The work in this thesis had attempted to answer some of the discrepancies that exist and had established that IHC and FC are complimentary techniques when looking at the significance of NK cells in implantation during IVF. It has also shown that FC can not only accurately define immune cell populations but is able to look at their function in different clinical groups such as RIF patients.

The advantages of FC have been explored and future possibilities of being able to define pathways in which NK cells and their immune counterparts in the endometrium and blood can predict pregnancy outcome is an exciting prospect.

It envisaged that in the future this panel can be adapted into a clinically accessible, simple and effective test that is available to patients, and the clinics managing their IVF journey.

References

Abuzakouk, M., et al. (1996). "Collagenase and Dispase enzymes disrupt lymphocyte surface molecules." Journal of Immunological Methods **194**(2): 211-216.

Acar, N., et al. (2011). "Uterine natural killer (uNK) cells and their missions during pregnancy: a review." Acta Histochem **113**(2): 82-91.

Achache, H. and A. Revel (2006). "Endometrial receptivity markers, the journey to successful embryo implantation." Hum Reprod Update **12**(6): 731-746.

Akhtar, M. A., et al. (2013). "Heparin for assisted reproduction." Cochrane Library(8).

Alecsandru, D. and J. A. Garcia-Velasco (2015). "Immune testing and treatment: still an open debate." Hum Reprod **30**(8): 1994.

Aleyasin, A., et al. (2016). "Granulocyte colony-stimulating factor in repeated IVF failure, a randomized trial." Reproduction **151**(6): 637-642.

Alhalabi, M., et al. (2011). Prednisolone improves implantation in ICSI patients with high peripheral CD69+ NK Cells. Human Reproduction,

Ansell, J. D., et al. (1974). "Binucleate and polyploid cells in the decidua of the mouse." J Embryol Exp Morphol **31**(1): 223-227.

Aplin, J. D. (2000). "The cell biological basis of human implantation." Baillieres Best Pract Res Clin Obstet Gynaecol **14**(5): 757-764.

Arias-Stella, J. (2002). "The Arias-Stella reaction: facts and fancies four decades after." Adv Anat Pathol **9**(1): 12-23.

Arruvito, L., et al. (2007). "Expansion of CD4+CD25+and FOXP3+ regulatory T cells during the follicular phase of the menstrual cycle: implications for human reproduction." J Immunol **178**(4): 2572-2578.

Artis, D. and H. Spits (2015). "The biology of innate lymphoid cells." Nature **517**(7534): 293-301.

Ashkar, A. A. and B. A. Croy (1999). "Interferon-gamma contributes to the normalcy of murine pregnancy." Biol Reprod **61**(2): 493-502.

Ashkar, A. A. and B. A. Croy (2001). "Functions of uterine natural killer cells are mediated by interferon gamma production during murine pregnancy." Semin Immunol **13**(4): 235-241.

Ashkar, A. A., et al. (2000). "Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy." J Exp Med **192**(2): 259-270.

Athukorala, C., et al. (2010). "The risk of adverse pregnancy outcomes in women who are overweight or obese." BMC Pregnancy Childbirth **10**: 56.

Baczkowski, T. and R. Kurzawa (2007). "Immunophenotypic profiles of peripheral blood lymphocytes on the day of embryo transfer in women undergoing in vitro fertilization." Folia Histochem Cytobiol **45 Suppl 1**: S73-77.

Balen, A. H. and A. J. Rutherford (2007). "Management of infertility." BMJ: British Medical Journal **335**(7620): 608.

Barber, E. M. and J. W. Pollard (2003). "The uterine NK cell population requires IL-15 but these cells are not required for pregnancy nor the resolution of a Listeria monocytogenes infection." J Immunol **171**(1): 37-46.

Bates, M. D., et al. (2002). "Aberrant cytokine production by peripheral blood mononuclear cells in recurrent pregnancy loss?" Hum Reprod **17**(9): 2439-2444.

Beer, A. E. and R. E. Billingham (1976). Immunobiology of mammalian reproduction, Prentice-Hall.

Beer, A. E., et al. (1996). "Immunophenotypic profiles of peripheral blood lymphocytes in women with recurrent pregnancy losses and in infertile women with multiple failed in vitro fertilization cycles." Am J Reprod Immunol **35**(4): 376-382.

Bell, S. (1990). "Decidualization and relevance to menstruation." Contraception and Mechanisms of Endometrial Bleeding. C. D'Arcangues, IS Fraser, JR Newton & V. Odlind, Eds **188**.

Belva, F., et al. (2007). "Medical outcome of 8-year-old singleton ICSI children (born >or=32 weeks' gestation) and a spontaneously conceived comparison group." Hum Reprod **22**(2): 506-515.

Bendelac, A., et al. (2007). "The biology of NKT cells." Annu Rev Immunol **25**: 297-336.

Benschop, R. J., et al. (1998). "Cardiovascular and immune responses to acute psychological stress in young and old women: a meta-analysis." Psychosom Med **60**(3): 290-296.

Bergh, P. and D. Navot (1992). "The impact of embryonic development and endometrial maturity on the timing of implantation." Fertil Steril **58**(3): 537-542.

Berker, B., et al. (2011). "The role of low-molecular-weight heparin in recurrent implantation failure: a prospective, quasi-randomized, controlled study." Fertility and sterility **95**(8): 2499-2502.

Billingham, R. E., et al. (1953). "Actively acquired tolerance of foreign cells." Nature **172**(4379): 603-606.

Biron, C. A., et al. (1999). "Natural killer cells in antiviral defense: function and regulation by innate cytokines." Annu Rev Immunol **17**(1): 189-220.

Bogovic Crncic, T., et al. (2005). "Perforin and Fas/FasL cytolytic pathways at the maternal-fetal interface." Am J Reprod Immunol **54**(5): 241-248.

Bonatz, G., et al. (1992). "Macrophage- and lymphocyte-subtypes in the endometrium during different phases of the ovarian cycle." Int J Gynaecol Obstet **37**(1): 29-36.

Bonney, E. A. (2017). "Alternative theories: Pregnancy and immune tolerance." J Reprod Immunol **123**: 65-71.

Boomsma, C. and N. Macklon (2006). "What can the clinician do to improve implantation?" Reproductive BioMedicine Online **13**(6): 845-855.

Boomsma, C. M., et al. (2007). "Peri-implantation glucocorticoid administration for assisted reproductive technology cycles." Cochrane Database Syst Rev(1): Cd005996.

Boomsma, C. M., et al. (2012). "Peri-implantation glucocorticoid administration for assisted reproductive technology cycles." Cochrane Database Syst Rev **6**: CD005996.

Boomsma, C. M. and N. S. Macklon (2008). "Does glucocorticoid therapy in the peri-implantation period have an impact on IVF outcomes?" Curr Opin Obstet Gynecol **20**(3): 249-256.

Borzychowski, A. M., et al. (2005). "Changes in systemic type 1 and type 2 immunity in normal pregnancy and pre-eclampsia may be mediated by natural killer cells." Eur J Immunol **35**(10): 3054-3063.

Bosteels, J., et al. (2010). "The effectiveness of hysteroscopy in improving pregnancy rates in subfertile women without other gynaecological symptoms: a systematic review." Human reproduction update **16**(1): 1-11.

Braud, V. M., et al. (1998). "HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C." Nature **391**(6669): 795-799.

Brinsden, P. R., et al. (2009). "Recombinant human leukemia inhibitory factor does not improve implantation and pregnancy outcomes after assisted reproductive techniques in women with recurrent unexplained implantation failure." Fertility and sterility **91**: 1445-1447.

Brinsden, P. R., et al. (2003). "Does recombinant human leukaemia inhibitory factor improve implantation in women with recurrent failure of assisted reproduction treatment?" **18**(Suppl 1): 18, Abstract no: O-050.

Brosens, I., et al. (2017). "The impact of uterine immaturity on obstetrical syndromes during adolescence." Am J Obstet Gynecol **217**(5): 546-555.

Brosens, I., et al. (2011). "The "Great Obstetrical Syndromes" are associated with disorders of deep placentation." Am J Obstet Gynecol **204**(3): 193-201.

Brosens, J. J., et al. (2009). "A role for menstruation in preconditioning the uterus for successful pregnancy." Am J Obstet Gynecol **200**(6): 615 e611-616.

Brosens, J. J., et al. (2002). "The myometrial junctional zone spiral arteries in normal and abnormal pregnancies: a review of the literature." Am J Obstet Gynecol **187**(5): 1416-1423.

Bulmer, J. N. and P. M. Johnson (1985). "Antigen expression by trophoblast populations in the human placenta and their possible immunobiological relevance." Placenta **6**(2): 127-140.

Bulmer, J. N. and G. E. Lash (2005). "Human uterine natural killer cells: a reappraisal." Molecular immunology **42**(4): 511-521.

Bulmer, J. N. and G. E. Lash (2015). "The Role of Uterine NK Cells in Normal Reproduction and Reproductive Disorders." Adv Exp Med Biol **868**: 95-126.

Bulmer, J. N., et al. (1991). "Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies." Human Reproduction **6**(6): 791-798.

Buster, J. E., et al. (1985). "Biologic and morphologic development of donated human ova recovered by nonsurgical uterine lavage." American journal of obstetrics and gynecology **153**(2): 211-217.

Cakmak, H. and H. S. Taylor (2011). "Implantation failure: molecular mechanisms and clinical treatment." Hum Reprod Update **17**(2): 242-253.

Callewaert, D. M., et al. (1991). "Hormone specific regulation of natural killer cells by cortisol Direct inactivation of the cytotoxic function of cloned human NK cells without an effect on cellular proliferation." FEBS letters **285**(1): 108-110.

Cambiaghi, A. S. and R. B. F. Leao (2012). "Granulocyte-colony stimulation factor (G-CSF) may improve pregnancy rate in patients with repeated implantation failures." **98 Suppl 1**(3): abstract number S186-S187.

Carp, H. J., et al. (1990). "Immunization by paternal leukocytes for prevention of primary habitual abortion: results of a matched controlled trial." Gynecol Obstet Invest **29**(1): 16-21.

Carson, D. D., et al. (2000). "Embryo implantation." Developmental biology **223**(2): 217-237.

Cartwright, J. E., et al. (2010). "Remodelling at the maternal–fetal interface: relevance to human pregnancy disorders." Reproduction **140**(6): 803-813.

Cartwright, P. S., et al. (1986). "Performance of a new enzyme-linked immunoassay urine pregnancy test for the detection of ectopic gestation." Annals of emergency medicine **15**(10): 1198-1199.

Cerwenka, A. and L. L. Lanier (2001). "Natural killer cells, viruses and cancer." Nature Reviews Immunology **1**(1): 41-49.

Cha, J., et al. (2012). "Mechanisms of implantation: strategies for successful pregnancy." Nat Med **18**(12): 1754-1767.

Chakraborty, D., et al. (2011). "Natural killer cells direct hemochorial placentation by regulating hypoxia-inducible factor dependent trophoblast lineage decisions." Proc Natl Acad Sci U S A **108**(39): 16295-16300.

Chan, R. W., et al. (2004). "Clonogenicity of human endometrial epithelial and stromal cells." Biol Reprod **70**(6): 1738-1750.

Chen, C. P., et al. (2015). "Expression of Interferon gamma by Decidual Cells and Natural Killer Cells at the Human Implantation Site: Implications for Preeclampsia, Spontaneous Abortion, and Intrauterine Growth Restriction." Reprod Sci **22**(11): 1461-1467.

Chen, L., et al. (2010). "Inhibition of HLA-G expression via RNAi abolishes resistance of extravillous trophoblast cell line TEV-1 to NK lysis." Placenta **31**(6): 519-527.

Chen, S.-J., et al. (2011). "Immunologic regulation in pregnancy: from mechanism to therapeutic strategy for immunomodulation." Clinical and Developmental Immunology **2012**.

Chen, X., et al. (2017). "Measurement of uterine natural killer cell percentage in the periimplantation endometrium from fertile women and women with recurrent reproductive failure: establishment of a reference range." Am J Obstet Gynecol **217**(6): 680.e681-680.e686.

Chen, Z., et al. (2014). "Collagenase digestion down-regulates the density of CD27 on lymphocytes." Journal of Immunological Methods **413**: 57-61.

Christian, M., et al. (2002). "Mechanisms of decidualization." Reprod Biomed Online **4 Suppl 3**: 24-30.

Clancy, K. B., et al. (2013). "Systemic inflammation is associated with ovarian follicular dynamics during the human menstrual cycle." PLoS One **8**(5): e64807.

Clark, D. A. (2014). "Popular myths in reproductive immunology." J Reprod Immunol **104-105**: 54-62.

Clark, D. A., et al. (2006). "Is intravenous immunoglobulins (IVIG) efficacious in early pregnancy failure? A critical review and meta-analysis for patients who fail in vitro fertilization and embryo transfer (IVF)." Journal of assisted reproduction and genetics **23**(1): 1-13.

Colicchia A, et al. (2011). Role of heparin in embryo implantation in women without thrombophilia: a pilot study.Vol 26 ,Abstract number: i151-i160 P090. Human Reproduction

Cooper, T. G. (2012). The epididymis, sperm maturation and fertilisation, Springer Science & Business Media.

Costanzo, M. C., et al. (2015). "OMIP-027: Functional analysis of human natural killer cells." Cytometry A **87**(9): 803-805.

Coughlan, C., et al. (2014). "Recurrent implantation failure: definition and management." Reproductive BioMedicine Online **28**(1): 14-38.

Coulam, C. B. and C. Goodman (2000). "Increased pregnancy rates after IVF/ET with intravenous immunoglobulin treatment in women with elevated circulating C56+ cells." Early Pregnancy **4**(2): 90-98.

Coulam, C. B. and R. G. Roussev (2003). "Correlation of NK cell activation and inhibition markers with NK cytoxicity among women experiencing immunologic implantation failure after in vitro fertilization and embryo transfer." J Assist Reprod Genet **20**(2): 58-62.

Coulam, C. B. and R. G. Roussev (2003). "Increasing circulating T-cell activation markers are linked to subsequent implantation failure after transfer of in vitro fertilized embryos." Am J Reprod Immunol **50**(4): 340-345.

Croxatto, H., et al. (1978). "Studies on the duration of egg ovum transport by the human oviduct." Am J Obstet Gynecol **132**: 629-634.

Cutting, R. (2018). "Single embryo transfer for all." Best Pract Res Clin Obstet Gynaecol.

Dan, S., et al. (2015). "Effect of Prednisolone Administration on Patients with Unexplained Recurrent Miscarriage and in Routine Intracytoplasmic Sperm Injection: A Meta-Analysis." Am J Reprod Immunol **74**(1): 89-97.

Das, M. and H. E. Holzer (2012). "Recurrent implantation failure: gamete and embryo factors." Fertil Steril **97**(5): 1021-1027.

Davari Tanha, F., et al. (2016). "The role of G-CSF in recurrent implantation failure – a randomized double blind placebo control trial." Placenta **45**: 116-116.

de Andres, C., et al. (2017). "Activation of Blood CD3(+)CD56(+)CD8(+) T Cells during Pregnancy and Multiple Sclerosis." Front Immunol **8**: 196.

De Geyter, C., et al. (2018). The European IVF-monitoring Consortium (EIM) ‡ for the European Society of Human Reproduction and Embryology (ESHRE).

De los Santos, M., et al. (2003). "Implantation rates after two, three, or five days of embryo culture." Placenta **24**: S13-S19.

Dekel, N., et al. (2014). "The role of inflammation for a successful implantation." Am J Reprod Immunol **72**(2): 141-147.

Dentali, F., et al. (2012). "Low-dose aspirin for in vitro fertilization or intracytoplasmic sperm injection: a systematic review and a meta-analysis of the literature." Journal of Thrombosis and Haemostasis **10**(10): 2075-2085.

Dey, S., et al. (2004). "Molecular cues to implantation." Endocr Rev **25**(3): 341-373.

Di Pietro, C. and M. Falcone (2014). "The role of invariant NKT cells in organ-specific autoimmunity." Front Biosci (Landmark Ed) **19**: 1240-1250.

Diaz-Gimeno, P., et al. (2013). "The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity." Fertil Steril **99**(2): 508-517.

Diedrich, K., et al. (2007). "The role of the endometrium and embryo in human implantation." Hum Reprod Update **13**(4): 365-377.

Dokras, A., et al. (1993). "Human blastocyst grading: an indicator of developmental potential?" Hum Reprod **8**(12): 2119-2127.

Dons'koi, B. V., et al. (2014). "Peripheral blood natural killer cells activation status determined by CD69 upregulation predicts implantation outcome in IVF." Immunobiology **219**(3): 167-171
.
Dosiou, C. and L. C. Giudice (2005). "Natural killer cells in pregnancy and recurrent pregnancy loss: endocrine and immunologic perspectives." Endocr Rev **26**(1): 44-62.

Duc-Goiran, P., et al. (1999). "Embryo–maternal interactions at the implantation site: a delicate equilibrium." European Journal of Obstetrics & Gynecology and Reproductive Biology **83**(1): 85-100.

Dunn, C. L., et al. (2003). "Decidualization of the human endometrial stromal cell: an enigmatic transformation." Reprod Biomed Online **7**(2): 151-161.

Duvan, C. I., et al. (2006). "Does addition of low-dose aspirin and/or steroid as a standard treatment in nonselected intracytoplasmic sperm injection cycles improve in vitro fertilization success? A randomized, prospective, placebo-controlled study." J Assist Reprod Genet **23**(1): 15-21.

Edgell, T. A., et al. (2018). "Assessment of potential biomarkers of pre-receptive and receptive endometrium in uterine fluid and a functional evaluation of the potential role of CSF3 in fertility." Cytokine **111**: 222-229.

Edgell, T. A., et al. (2013). "Assessing receptivity in the endometrium: the need for a rapid, non-invasive test." Reproductive BioMedicine Online **27**(5): 486-496
.
Egea, R. R., et al. (2014). "OMICS: Current and future perspectives in reproductive medicine and technology." Journal of Human Reproductive Sciences **7**(2): 73-92.

El-Khayata, W. and M. El Sadek (2015). "Intralipid for repeated implantation failure (RIF): A randomized controlled trial." Fertil Steril **1)**: e26.

El-Toukhy, T., et al. (2012). "Local endometrial injury and IVF outcome: a systematic review and meta-analysis." Reproductive BioMedicine Online **25**(4): 345-354.

El Costa, H., et al. (2009). "Effector functions of human decidual NK cells in healthy early pregnancy are dependent on the specific engagement of natural cytotoxicity receptors." J Reprod Immunol **82**(2): 142-147.

Enders, A. C. and S. Schlafke (1967). "A morphological analysis of the early implantation stages in the rat." American Journal of Anatomy **120**(2): 185-225.

Evers, J. L. (2002). "Female subfertility." The Lancet **360**(9327): 151-159.

Faas, M. M. and P. De Vos (2017). "Uterine NK cells and macrophages in pregnancy." Placenta **56**: 44-52.

Fasouliotis, S. J., et al. (2004). "Maternal serum levels of interferon-gamma and interleukin-2 soluble receptor-alpha predict the outcome of early IVF pregnancies." Hum Reprod **19**(6): 1357-1363.

Fatemi, H. M. and B. Popovic-Todorovic (2013). "Implantation in assisted reproduction: a look at endometrial receptivity." Reprod Biomed Online **27**(5): 530-538.

Fawzy, M. and A. A. El-Refaeey (2014). "Does combined prednisolone and low molecular weight heparin have a role in unexplained implantation failure?" Arch Gynecol Obstet **289**: 677-680.

Ferraretti, A., et al. (2011). "ESHRE consensus on the definition of 'poor response'to ovarian stimulation for in vitro fertilization: the Bologna criteria." Human Reproduction **26**(7): 1616-1624.

Fitzgerald, H. C., et al. (2018). "Idiopathic infertility in women is associated with distinct changes in proliferative phase uterine fluid proteins." Biol Reprod **98**(6): 752-764.

Fowden, A. L. and A. J. Forhead (2015). "Glucocorticoids as regulatory signals during intrauterine development." Exp Physiol **100**(12): 1477-1487.

Franchimont, D. (2004). "Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies." Ann N Y Acad Sci **1024**: 124-137.

Fraser, R., et al. (2012). "Impaired decidual natural killer cell regulation of vascular remodelling in early human pregnancies with high uterine artery resistance." The Journal of pathology **228**(3): 322-332.

Fukui, A., et al. (1999). "Natural killer cell subpopulations and cytotoxicity for infertile patients undergoing in vitro fertilization." Am J Reprod Immunol **41**(6): 413-422.

Fukui, A., et al. (1999). "Natural killer cell subpopulations and cytotoxicity for infertile patients undergoing in vitro fertilization." American journal of reproductive immunology **41**(6): 413-422.

Fukui, A., et al. (2017). "Expression of natural cytotoxicity receptors and cytokine production on endometrial natural killer cells in women with recurrent pregnancy loss or implantation failure, and the expression of natural cytotoxicity receptors on peripheral blood natural killer cells in pregnant women with a history of recurrent pregnancy loss." J Obstet Gynaecol Res **43**(11): 1678-1686.

Fukui, A., et al. (2008). "Intracellular cytokine expression of peripheral blood natural killer cell subsets in women with recurrent spontaneous abortions and implantation failures." Fertil Steril **89**(1): 157-165.
Gabrilovac, J., et al. (1988). "NK cell activity and estrogen hormone levels during normal human pregnancy." Gynecol Obstet Invest **25**(3): 165-172.

Gamaleldin I, et al. (2016). Intralipid in women with recurrent implantation failure in IVF/ICSI cycles. A double blinded randomised controlled trial. Vol 31, Abstract number i2284-i2285 P-36. 32nd Annual Meeting of ESHRE.

Gardner, D. K., et al. (2004). "Single blastocyst transfer: a prospective randomized trial." Fertil Steril **81**(3): 551-555.

Gelbaya, T., et al. (2007). "Low-dose aspirin for in vitro fertilization: a systematic review and meta-analysis." Human reproduction update **13**(4): 357-364.

Ginsburg, E. S., et al. (2005). "T-helper 2 and 3 type immunity to trophoblast in successful in vitro fertilization-embryo transfer." Fertil Steril **83**(6): 1659-1664.

Giudice, L. (1999). "Implantation and endometrial function." Molecular biology in reproductive medicine. Parthenon Publishing Group, New York, NY, USA, London, UK.

Giudice, L. C. (1994). "Growth factors and growth modulators in human uterine endometrium: their potential relevance to reproductive medicine." Fertil Steril **61**(1): 1-17.

Glasser, S. R., et al. (2004). The endometrium, CRC Press.

Gnainsky, Y., et al. (2015). "Biopsy-induced inflammatory conditions improve endometrial receptivity: the mechanism of action." Reproduction **149**(1): 75-85.

Gnainsky, Y., et al. (2010). "Local injury of the endometrium induces an inflammatory response that promotes successful implantation." Fertil Steril **94**(6): 2030-2036.

Gnoth, C., et al. (2005). "Definition and prevalence of subfertility and infertility." Human Reproduction **20**(5): 1144-1147.

Gomaa, M. F., et al. (2014). "Combined oral prednisolone and heparin versus heparin: the effect on peripheral NK cells and clinical outcome in patients with unexplained recurrent miscarriage. A double-blind placebo randomized controlled trial." Arch Gynecol Obstet **290**(4): 757-762.

Goswamy, R. K., et al. (1988). "Decreased uterine perfusion—a cause of infertility." Human Reproduction **3**(8): 955-959.

Guidice, L. C. and M. Conti (2009). "Growth factors and the development and function of the reproductive organs." Semin Reprod Med **27**(1): 3-4.

Günther, V., et al. (2018). "Active Immunisation with Partner Lymphocytes in Female Patients Who Want to Become Pregnant - Current Status." Geburtshilfe und Frauenheilkunde **78**(3): 260-273.

Gurka, G. and R. E. Rocklin (1987). "Reproductive immunology." Jama **258**(20): 2983-2987.

Guzeloglu-Kayisli, O., et al. (2007). "Basic aspects of implantation." Reprod Biomed Online **15**(6): 728-739.

Hamdi, K., et al. (2015). "The role of heparin in embryo implantation in women with recurrent implantation failure in the cycles of assisted reproductive techniques (without history of thrombophilia)." Journal of Family and Reproductive Health **9**(2): 59-64.

Hanna, J., et al. (2006). "Decidual NK cells regulate key developmental processes at the human fetal-maternal interface." Nat Med **12**(9): 1065-1074.

Hannan, N. J., et al. (2012). "Uterine lavage or aspirate: which view of the intrauterine environment?" Reprod Sci **19**(10): 1125-1132.

Harris, L. K., et al. (2010). "Trophoblast-and vascular smooth muscle cell-derived MMP-12 mediates elastolysis during uterine spiral artery remodeling." The American journal of pathology **177**(4): 2103-2115.

Hawkins, S. M. and M. M. Matzuk (2008). "The Menstrual Cycle." Annals of the New York Academy of Sciences **1135**(1): 10-18.

Hazeldine, J. and J. M. Lord (2013). "The impact of ageing on natural killer cell function and potential consequences for health in older adults." Ageing research reviews **12**(4): 1069-1078.

Heffner, L. J. and D. J. Schust (2010). The reproductive system at a glance, John Wiley & Sons.

Hempstock, J., et al. (2003). "The contribution of placental oxidative stress to early pregnancy failure." Human pathology **34**(12): 1265-1275.

Henderson, T. A., et al. (2003). "Steroid receptor expression in uterine natural killer cells." J Clin Endocrinol Metab **88**(1): 440-449.

HFEA (2009). "Latest UK IVF figures-2008."

HFEA (2012). "Latest UK fertility treatment data and figures: 2009–2010." HFEA, Finsbury Tower, London.

HFEA (2018). Human Fertilisation and Embryology Authority. Fertility treatment 2014-2016; trends and figures. www.hfea.gov.uk/media/2544/hfea-fertility-treatment-2014-2016-trends-and-figures.pdf. London.

Hiby, S. E., et al. (2010). "Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2." J Clin Invest **120**(11): 4102-4110.

Higgins (2011). Cochrane Handbook for Systematic Reviews of Interventions Version 5.1.0, Available from www.handbook.cochrane.org.

Hill, J. A., et al. (1995). "T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion." Jama **273**(24): 1933-1936.

Hillier, S. G., et al. (1996). Scientific essentials of reproductive medicine, Saunders.

Hodge, G. and S. Hodge (2016). "Steroid Resistant CD8(+)CD28(null) NKT-Like Pro-inflammatory Cytotoxic Cells in Chronic Obstructive Pulmonary Disease." Front Immunol **7**: 617.

Homan, G. F., et al. (2007). "The impact of lifestyle factors on reproductive performance in the general population and those undergoing infertility treatment: a review." Hum Reprod Update **13**(3): 209-223.

Hu, Y., et al. (2006). "Decidual NK cells alter in vitro first trimester extravillous cytotrophoblast migration: a role for IFN-γ." The Journal of Immunology **177**(12): 8522-8530.

Hung, T.-H., et al. (2001). "In vitro ischemia-reperfusion injury in term human placenta as a model for oxidative stress in pathological pregnancies." The American journal of pathology **159**(3): 1031-1043.

Hustin, J., et al. (1990). "Histological study of the materno-embryonic interface in spontaneous abortion." Placenta **11**(6): 477-486.

Hyde, K. J. and D. J. Schust (2016). "Immunologic challenges of human reproduction: an evolving story." Fertil Steril **106**(3): 499-510.

Jiang, R., et al. (2017). "Abnormal ratio of CD57(+) cells to CD56(+) cells in women with recurrent implantation failure." Am J Reprod Immunol **78**(5).

Johnson, M. H. (2012). Essential reproduction, John Wiley & Sons.

Jokhi, P. P., et al. (1994). "Screening for cytokine messenger ribonucleic acids in purified human decidual lymphocyte populations by the reverse-transcriptase polymerase chain reaction." The Journal of Immunology **153**(10): 4427-4435.

Jones, R. E. and K. H. Lopez (2013). Human reproductive biology, Academic Press.

Jung, Y. S., et al. (2018). "Physical Inactivity and Unhealthy Metabolic Status Are Associated with Decreased Natural Killer Cell Activity." Yonsei Med J **59**(4): 554-562.

Kalkunte, S. S., et al. (2009). "Vascular endothelial growth factor C facilitates immune tolerance and endovascular activity of human uterine NK cells at the maternal-fetal interface." The Journal of Immunology **182**(7): 4085-4092.

Kamath, M. S., et al. (2017). "Use of Granulocyte-colony stimulating factor in assisted reproductive technology: A systematic review and meta-analysis." European Journal of Obstetrics & Gynecology and Reproductive Biology **214**: 16-24.

Khairy, M., et al. (2007). "Aspirin in women undergoing in vitro fertilization treatment: a systematic review and meta-analysis." Fertility and sterility **88**(4): 822-831.

Kim, C. H., et al. (2011). "Effect of granulocyte colony-stimulating factor on pregnancy outcome following IVF/ICSI in patients with repeated implantation failure." Hum Reprod **26 Suppl 1**: i244 Abstract no: P-318.

King, A. (2000). "Uterine leukocytes and decidualization." Hum Reprod Update **6**(1): 28-36.

King, A., et al. (2000). "HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells." European journal of immunology **30**(6): 1623-1631.

King, A., et al. (2000). "Recognition of trophoblast HLA class I molecules by decidual NK cell receptors--a review." Placenta **21 Suppl A**: S81-85.

Kofod, L., et al. (2017). "Endometrial immune markers are potential predictors of normal fertility and pregnancy after in vitro fertilization." Am J Reprod Immunol **78**(3).

Koopman, L. A., et al. (2003). "Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential." J Exp Med **198**(8): 1201-1212.

Kosova, G., et al. (2015). "Evolutionary forward genomics reveals novel insights into the genes and pathways dysregulated in recurrent early pregnancy loss." Hum Reprod **30**(3): 519-529.

Koyama, A., et al. (2013). Emerging Options for Emergency Contraception.

Kronenberg, M. (2005). "Toward an understanding of NKT cell biology: progress and paradoxes." Annu. Rev. Immunol. **26**: 877-900.

Kronenberg, M. and L. Gapin (2002). "The unconventional lifestyle of NKT cells." Nat Rev Immunol **2**(8): 557-568.

Kuon, R. J., et al. (2017). "Uterine natural killer cells in patients with idiopathic recurrent miscarriage." Am J Reprod Immunol **78**(4).

Kuroda, K., et al. (2013). "Elevated periimplantation uterine natural killer cell density in human endometrium is associated with impaired corticosteroid signaling in decidualizing stromal cells." J Clin Endocrinol Metab **98**(11): 4429-4437.

Kwak-Kim, J. and A. Gilman-Sachs (2008). "Clinical implication of natural killer cells and reproduction." Am J Reprod Immunol **59**(5): 388-400.

Kwak-Kim, J., et al. (2013). "Current trends of reproductive immunology practices in in vitro fertilization (IVF) - a first world survey using IVF-Worldwide.com." Am J Reprod Immunol **69**(1): 12-20.

Kwak-Kim, J. Y., et al. (2005). "T helper 1 and 2 immune responses in relationship to pregnancy, nonpregnancy, recurrent spontaneous abortions and infertility of repeated implantation failures." Chem Immunol Allergy **88**: 64-79.

Kwak-Kim, J., et al. (2003). "Increased T helper 1 cytokine responses by circulating T cells are present in women with recurrent pregnancy losses and in infertile women with multiple implantation failures after IVF." Human Reproduction **18**(4): 767-773.

Lachapelle, M.-H., et al. (1996). "Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome." The Journal of Immunology **156**(10): 4027-4034.

Lachapelle, M. H., et al. (1996). "Flow-cytometric characterization of hematopoietic cells in non-pregnant human endometrium." Am J Reprod Immunol **35**(1): 5-13.

Lachapelle, M. H., et al. (1996). "Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome." J Immunol **156**(10): 4027-4034.

Laird, S. M., et al. (2011). "Measurements of CD56+ cells in peripheral blood and endometrium by flow cytometry and immunohistochemical staining in situ." Human Reproduction **26**(6): 1331-1337.

Lash, G., et al. (2010). "Review: functional role of uterine natural killer (uNK) cells in human early pregnancy decidua." Placenta **31**: S87-S92.
Lash, G. E. (2015). "Molecular Cross-Talk at the Feto-Maternal Interface." Cold Spring Harb Perspect Med **5**(12).

Lash, G. E. and J. N. Bulmer (2011). "Do uterine natural killer (uNK) cells contribute to female reproductive disorders?" J Reprod Immunol **88**(2): 156-164.

Lash, G. E. and J. N. Bulmer (2011). "Do uterine natural killer (uNK) cells contribute to female reproductive disorders?" Journal of reproductive immunology **88**(2): 156-164.

Lash, G. E., et al. (2016). "Standardisation of uterine natural killer (uNK) cell measurements in the endometrium of women with recurrent reproductive failure." J Reprod Immunol **116**: 50-59.

Lash, G. E., et al. (2006). "Interferon-γ inhibits extravillous trophoblast cell invasion by a mechanism that involves both changes in apoptosis and protease levels." The FASEB Journal **20**(14): 2512-2518.

Lash, G. E., et al. (2010). "Review: Functional role of uterine natural killer (uNK) cells in human early pregnancy decidua." Placenta **31 Suppl**: S87-92.

Lash, G. E., et al. (2006). "Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy." Journal of leukocyte biology **80**(3): 572-580.

Le Bouteiller, P. and M. P. Piccinni (2008). "Human NK cells in pregnant uterus: why there?" Am J Reprod Immunol **59**(5): 401-406.

Le Bouteiller, P. and J. Tabiasco (2006). "Killers become builders during pregnancy." Nat Med **12**(9): 991-992.

Ledee-Bataille, N., et al. (2004). "A new role for natural killer cells, interleukin (IL)-12, and IL-18 in repeated implantation failure after in vitro fertilization." Fertil Steril **81**(1): 59-65.

Ledee-Bataille, N., et al. (2002). "Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation." Hum Reprod **17**(1): 213-218.

Ledee, N., et al. (2017). "Uterine immune profiling for increasing live birth rate: A one-to-one matched cohort study." J Reprod Immunol **119**: 23-30.

Ledee, N., et al. (2018). "Impact of prednisone in patients with repeated embryo implantation failures: Beneficial or deleterious?" J Reprod Immunol **127**: 11-15.

Lee, B.-C., et al. (2016). "Adipose Natural Killer Cells Regulate Adipose Tissue Macrophages to Promote Insulin Resistance in Obesity." Cell metabolism **23**(4): 685-698.

Lee, J. Y., et al. (2011). "Role of endometrial immune cells in implantation." Clin Exp Reprod Med **38**(3): 119-125.

Lee, K. Y., et al. (2007). "Mouse models of implantation." Trends in Endocrinology & Metabolism **18**(6): 234-239.

Lee, N., et al. (1998). "HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A." Proceedings of the National Academy of Sciences **95**(9): 5199-5204.

Lee, S. K., et al. (2015). "Immune Cells in the Female Reproductive Tract." Immune network **15**(1): 16-26.

Lessey, B. A. (2011). "Assessment of endometrial receptivity." Fertil Steril **96**(3): 522-529.

Li, S., et al. (2017). "Intrauterine administration of hCG-activated autologous human peripheral blood mononuclear cells (PBMC) promotes live birth rates in frozen/thawed embryo transfer cycles of patients with repeated implantation failure." J Reprod Immunol **119**: 15-22.

Li, X. F., et al. (2001). "Angiogenic Growth Factor Messenger Ribonucleic Acids in Uterine Natural Killer Cells 1." The Journal of Clinical Endocrinology & Metabolism **86**(4): 1823-1834.

Lima, P. D., et al. (2014). "Leukocyte driven-decidual angiogenesis in early pregnancy." Cell Mol Immunol **11**(6): 522-537.

Lissauer, D., et al. (2017). "Maternal effector T cells within decidua: The adaptive immune response to pregnancy?" Placenta **60**: 140-144.

Liu, B., et al. (2014). "The prognostic value of uNK cell count and histological dating in the mid-luteal phase of women with reproductive failure." Eur J Obstet Gynecol Reprod Biol **181**: 171-175.

Liu, S., et al. (2017). "The role of decidual immune cells on human pregnancy." J Reprod Immunol **124**: 44-53.

Loke, Y. and A. King (1995). Human implantation: cell biology and immunology, Cambridge University Press.

Loke, Y. and A. King (2000). "Immunology of implantation." Best Practice & Research Clinical Obstetrics & Gynaecology **14**(5): 827-837.
Lonergan, P., et al. (1996). "Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development in vitro." Biol Reprod **54**(6): 1420-1429.

López-Botet, M., et al. (2000). NK cell recognition of non-classical HLA class I molecules. Semin Immunol, Elsevier.

Lukassen, H. G., et al. (2004). "Hormonal stimulation for IVF treatment positively affects the CD56bright/CD56dim NK cell ratio of the endometrium during the window of implantation." Mol Hum Reprod **10**(7): 513-520.

Lukassen, H. G., et al. (2003). "The proportion of follicular fluid CD16+CD56DIM NK cells is increased in IVF patients with idiopathic infertility." J Reprod Immunol **60**(1): 71-84.

Ma, W.-g., et al. (2003). "Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation." Proceedings of the National Academy of Sciences **100**(5): 2963-2968.

Maddur, M. S., et al. (2011). "Inhibition of differentiation, amplification, and function of human TH17 cells by intravenous immunoglobulin." J Allergy Clin Immunol **127**(3): 823-830 e821-827.

Madkour, A., et al. (2016). "Intrauterine insemination of cultured peripheral blood mononuclear cells prior to embryo transfer improves clinical outcome for patients with repeated implantation failures." Zygote **24**(1): 58-69.

Makhseed, M., et al. (2001). "Th1 and Th2 cytokine profiles in recurrent aborters with successful pregnancy and with subsequent abortions." Hum Reprod **16**(10): 2219-2226.

Makrigiannakis, A. (2011). "New approaches in treating recurrent implantation failures." **22 Suppl 2**: S105.

Makrigiannakis, A., et al. (2015). "Repeated implantation failure: A new potential treatment option." Eur J Clin Invest **45**(4): 380-384.

Makrigiannakis, A., et al. (2008). Fetomaternal Immunotolerance." American journal of reproductive immunology **60**(6): 482-496.

Makrigiannakis, A., et al. (2011). "Recent advances in understanding immunology of reproductive failure." J Reprod Immunol **90**(1): 96-104.

Male, V., et al. (2012). "Isolation of cells from the feto-maternal interface." Curr Protoc Immunol **Chapter 7**: Unit 7 40 41-11.

Manaseki, S. and R. F. Searle (1989). "Natural killer (NK) cell activity of first trimester human decidua." Cell Immunol **121**(1): 166-173.

Manaster, I., et al. (2008). "Endometrial NK cells are special immature cells that await pregnancy." The Journal of Immunology **181**(3): 1869-1876.

Manyonda, I. T. (2006). The immunology of human reproduction, CRC Press.

Margalioth, E. J., et al. (2006). "Investigation and treatment of repeated implantation failure following IVF-ET." Hum Reprod **21**(12): 3036-3043.

Mariee, N., et al. (2012). "The observer and cycle-to-cycle variability in the measurement of uterine natural killer cells by immunohistochemistry." J Reprod Immunol **95**(1-2): 93-100.

Marron, K., et al. (2018). "Detailed endometrial immune assessment of both normal and adverse reproductive outcome populations." J Assist Reprod Genet.

Masuda, A., et al. (2016). "An improved method for isolation of epithelial and stromal cells from the human endometrium." J Reprod Dev **62**(2): 213-218.

Matson, B. C. and K. M. Caron (2014). "Uterine natural killer cells as modulators of the maternal-fetal vasculature." Int J Dev Biol **58**(2-4): 199-204.

Matteo, M., et al. (2013). "Preliminary evidence for high anti-PLAC1 antibody levels in infertile patients with repeated unexplained implantation failure." Placenta **34**(4): 335-339.

Matteo, M. G., et al. (2007). "Normal percentage of CD56bright natural killer cells in young patients with a history of repeated unexplained implantation failure after in vitro fertilization cycles." Fertil Steril **88**(4): 990-993.

Matzinger, P. (2002). "The danger model: a renewed sense of self." Science **296**(5566): 301-305.

Maybin, J. A. and H. O. D. Critchley (2015). "Menstrual physiology: implications for endometrial pathology and beyond." Hum Reprod Update **21**(6): 748-761.

Medawar, P. B. (1953). Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. Symp Soc Exp Biol.

Michael, A. E. and A. T. Papageorghiou (2008). "Potential significance of physiological and pharmacological glucocorticoids in early pregnancy." Hum Reprod Update **14**(5): 497-517.

Michou, V. I., et al. (2003). "Fraction of the peripheral blood concentration of CD56+/CD16-/CD3- cells in total natural killer cells as an indication of fertility and infertility." Fertil Steril **80 Suppl 2**: 691-697.

Miko, E., et al. (2010). "Possible role of natural killer and natural killer T-like cells in implantation failure after IVF." Reprod Biomed Online **21**(6): 750-756.

Milne, S. A., et al. (2005). "Leukocyte populations and steroid receptor expression in human first-trimester decidua; regulation by antiprogestin and prostaglandin E analog." J Clin Endocrinol Metab **90**(7): 4315-4321.

Miraj, S., et al. (2015). "Efficacy of transvaginal perfusion of granulocyte colony stimulating factor on recurrent implantation failure: Randomized control trial." **13 Suppl 1**(4): 16.

Mitri, F., et al. (2016). "Current tools for the optimization of embryo transfer technique for recurrent implantation failure." Minerva Ginecol **68**(4): 431-449.

Moffett-King, A. (2002). "Natural killer cells and pregnancy." Nature Reviews Immunology **2**(9): 656-663.

Moffett, A. and F. Colucci (2014). "Uterine NK cells: active regulators at the maternal-fetal interface." The Journal of clinical investigation **124**(5): 1872-1879.

Moffett, A., et al. (2004). "Natural killer cells, miscarriage, and infertility." BMJ **329**(7477): 1283-1285.

Moffett, A. and N. Shreeve (2015). "First do no harm: uterine natural killer (NK) cells in assisted reproduction." Human Reproduction **30**(7): 1519-1525.

Moore, K. and A. Dalley (1999). Clinically Orientated Anatomy, Pennsylvania, Lippincott Williams and Wilkins.

Mor, G., et al. (2017). "The unique immunological and microbial aspects of pregnancy." Nat Rev Immunol.

Mor, G. and I. Cardenas (2010). "The immune system in pregnancy: a unique complexity." Am J Reprod Immunol **63**(6): 425-433.

Mor, G., et al. (2011). "Inflammation and pregnancy: the role of the immune system at the implantation site." Annals of the New York Academy of Sciences **1221**(1): 80-87.

Moraru, M., et al. (2012). "Intravenous immunoglobulin treatment increased live birth rate in a Spanish cohort of women with recurrent reproductive failure and expanded CD56(+) cells." Am J Reprod Immunol **68**(1): 75-84.

Mote, P. A., et al. (1999). "Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle." J Clin Endocrinol Metab **84**(8): 2963-2971.

Muktabhant, B., et al. (2012). "Interventions for preventing excessive weight gain during pregnancy." Cochrane Database Syst Rev **4**(4): CD007145-CD007145.

Nair, R. R., et al. (2017). "Immune-endocrine crosstalk during pregnancy." Gen Comp Endocrinol **242**: 18-23.

Nakayama, T., et al. (2002). "Human peripheral blood mononuclear cells (PBMC) in early pregnancy promote embryo invasion in vitro: HCG enhances the effects of PBMC." Hum Reprod **17**(1): 207-212.

Nancy, P. and A. Erlebacher (2014). "T cell behavior at the maternal-fetal interface." Int J Dev Biol **58**(2-4): 189-198.

Nardo, L. G., et al. (2015). "British Fertility Society Policy and Practice Committee: adjuvants in IVF: evidence for good clinical practice." Hum Fertil (Camb) **18**(1): 2-15.

Naruse, K., et al. (2009). "Localization of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitors for MMPs (TIMPs) in uterine natural killer cells in early human pregnancy." Human Reproduction **24**(3): 553-561.

Nastri, C. O., et al. (2013). "Endometrial scratching performed in the non-transfer cycle and outcome of assisted reproduction: A randomized controlled trial." Ultrasound in Obstetrics and Gynecology **42**(4): 375-382.

Naumova, E., et al. (2016). "Natural killer cells, ageing and cancer." Cancer Immunol Immunother **65**(4): 367-370.

Navot, D., et al. (1991). "The window of embryo transfer and the efficiency of human conception in vitro." Fertil Steril **55**(1): 114-118.

Ndukwe, G. (2011). "Recurrent embryo implantation failure after in vitro fertilisation: improved outcome following intralipid infusion in women with elevated T Helper 1 response." Hum Fertil (Camb) **14**(2): 21-22.

Nederlof, I., et al. (2017). "The seed to success: The role of seminal plasma in pregnancy." J Reprod Immunol **123**: 24-28.

Nelson, S. M. and I. A. Greer (2008). "The potential role of heparin in assisted conception." Hum Reprod Update **14**(6): 623-645.

NICE (2013). Fertility: assessment and treatment for people with fertility problems. N. I. f. H. a. C. Excellence. London UK.

Norwitz, E. R. (2006). "Defective implantation and placentation: laying the blueprint for pregnancy complications." Reproductive BioMedicine Online **13**(4): 591-599.

Norwitz, E. R., et al. (2001). "Implantation and the survival of early pregnancy." N Engl J Med **345**(19): 1400-1408.

Obidniak, D., et al. (2016). "Effect of colony-stimulating growth factor on outcome of frozen-thawed embryo transfer in patients with repeated implantation failure." Fertility and sterility **106**: e134-e135.

Okada, H., et al. (2004). "Interleukin-1 inhibits interleukin-15 production by progesterone during in vitro decidualization in human." J Reprod Immunol **61**(1): 3-12.

Okitsu, O., et al. (2011). "Intrauterine administration of autologous peripheral blood mononuclear cells increases clinical pregnancy rates in frozen/thawed embryo transfer cycles of patients with repeated implantation failure." J Reprod Immunol **92**(1-2): 82-87.

Oktem, O. and K. Oktay (2008). "The ovary." Annals of the New York Academy of Sciences **1127**(1): 1-9.

Oliver, C., et al. (1999). "Antigen phenotype of cultured decidual stromal cells of human term decidua." J Reprod Immunol **45**(1): 19-30.

Padubidri, V. and S. N. Daftary (2014). Shaw's Textbook of gynecology, Elsevier Health Sciences.

Park, D. W., et al. (2010). "Peripheral blood NK cells reflect changes in decidual NK cells in women with recurrent miscarriages." Am J Reprod Immunol **63**(2): 173-180.

Parr, M. B. and E. L. Parr (1989). The implantation reaction. Biology of the Uterus, Springer**:** 233-277.

Parrillo, J. E. and A. S. Fauci (1979). "Mechanisms of glucocorticoid action on immune processes." Annual review of pharmacology and toxicology **19**(1): 179-201.

Patel, A. S., et al. (2017). "Prediction of male infertility by the World Health Organization laboratory manual for assessment of semen analysis: A systematic review." Arab journal of urology.

Perchellet, A. L., et al. (2013). "Maternal CD4(+) and CD8(+) T cell tolerance towards a fetal minor histocompatibility antigen in T cell receptor transgenic mice." Biol Reprod **89**(4): 102.

Perdu, S., et al. (2016). "Maternal obesity drives functional alterations in uterine NK cells." JCI Insight **1**(11): e85560.

Perez-Villar, J. J., et al. (1997). "The CD94/NKG2-A inhibitory receptor complex is involved in natural killer cell-mediated recognition of cells expressing HLA-G1." The Journal of Immunology **158**(12): 5736-5743.

Pergolini, I., et al. (2013). "Low molecular weight heparin versus cardioaspirin in embryo implantation in women undergoing ICSI without inherited trombophilia: A randomized pilot study." Human Reproduction **28**: i13.

Polanski, L. T., et al. (2014). "Interventions to improve reproductive outcomes in women with elevated natural killer cells undergoing assisted reproduction techniques: a systematic review of literature." Hum Reprod **29**(1): 65-75.

Polanski, L. T., et al. (2014). "What exactly do we mean by 'recurrent implantation failure'? A systematic review and opinion." Reprod Biomed Online **28**(4): 409-423.

Popat, V. B., et al. (2008). "The Menstrual Cycle." Annals of the New York Academy of Sciences **1135**(1): 43-51.

Potdar, N., et al. (2012). "Endometrial injury to overcome recurrent embryo implantation failure: a systematic review and meta-analysis." Reproductive BioMedicine Online **25**(6): 561-571.

Potdar, N., et al. (2013). "Adjunct low-molecular-weight heparin to improve live birth rate after recurrent implantation failure: a systematic review and meta-analysis." Human reproduction update **19**(6): 674-684.

Pradeu, T. and E. L. Cooper (2012). "The danger theory: 20 years later." Front Immunol **3**: 287.

Psychoyos, A. (1986). "Uterine receptivity for nidationa." Annals of the New York Academy of Sciences **476**(1): 36-42.

Qublan, H., et al. (2008). "Low-molecular-weight heparin in the treatment of recurrent IVF–ET failure and thrombophilia: A prospective randomized placebo-controlled trial." Human Fertility **11**(4): 246-253.

Qublan, H. S., et al. (2006). "Acquired and inherited thrombophilia: implication in recurrent IVF and embryo transfer failure." Human Reproduction **21**(10): 2694-2698.

Quenby, S. and R. Farquharson (2006). "Uterine natural killer cells, implantation failure and recurrent miscarriage." Reproductive BioMedicine Online **13**(1): 24-28.

Quenby, S., et al. (2005). "Prednisolone reduces preconceptual endometrial natural killer cells in women with recurrent miscarriage." Fertil Steril **84**(4): 980-984.

Quenby, S., et al. (2009). "Uterine natural killer cells and angiogenesis in recurrent reproductive failure." Human Reproduction **24**(1): 45-54.

Quenby, S., et al. (2009). "Uterine natural killer cells and angiogenesis in recurrent reproductive failure." Hum Reprod **24**(1): 45-54.

Raga, F., et al. (1997). "Reproductive impact of congenital Müllerian anomalies." Human Reproduction **12**(10): 2277-2281.

Rai, R., et al. (2005). "Natural killer cells and reproductive failure--theory, practice and prejudice." Hum Reprod **20**(5): 1123-1126.

Ramathal, C. Y., et al. (2010). Endometrial decidualization: of mice and men. Semin Reprod Med, NIH Public Access.

Redman, C., et al. (1984). "Class 1 major histocompatibility complex antigens on human extra-villous trophoblast." Immunology **52**(3): 457.

Reynolds, R. M., et al. (2013). "Transmitting biological effects of stress in utero: implications for mother and offspring." Psychoneuroendocrinology **38**(9): 1843-1849.

Richardson, A., et al. (2015). "A clinically useful simplified blastocyst grading system." Reprod Biomed Online **31**(4): 523-530.

Rinehart, J. (2007). "Recurrent implantation failure: definition." J Assist Reprod Genet **24**(7): 284-287.

Roberts, S. A. and C. Stylianou (2012). "The non-independence of treatment outcomes from repeat IVF cycles: estimates and consequences." Hum Reprod **27**(2): 436-443.

Robertson, M. J. and J. Ritz (1990). "Biology and clinical relevance of human natural killer cells." Blood **76**(12): 2421-2438.

Robertson, S. A., et al. (2016). "Corticosteroid therapy in assisted reproduction–immune suppression is a faulty premise." Human Reproduction **31**(10): 2164-2173.

Robertson, S. A., et al. (2016). "Corticosteroid therapy in assisted reproduction - immune suppression is a faulty premise." Hum Reprod **31**(10): 2164-2173.

Robson, A., et al. (2012). "Uterine natural killer cells initiate spiral artery remodeling in human pregnancy." The FASEB Journal **26**(12): 4876-4885.

Ruan, Y. C., et al. (2014). "Ion channels in the endometrium: regulation of endometrial receptivity and embryo implantation." Hum Reprod Update **20**(4): 517-529.

Ruiz-Alonso, M., et al. (2013). "The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure." Fertil Steril **100**(3): 818-824.

Russell, P., et al. (2013). "The distribution of immune cells and macrophages in the endometrium of women with recurrent reproductive failure. III: Further observations and reference ranges." Pathology **45**(4): 393-401.

Sacks, G. (2015). "Enough! Stop the arguments and get on with the science of natural killer cell testing." Hum Reprod **30**(7): 1526-1531.

Sacks, G., et al. (2012). "Detailed analysis of peripheral blood natural killer cells in women with repeated IVF failure." Am J Reprod Immunol **67**(5): 434-442.

Salamonsen, L. A. and L. J. Lathbury (2000). "Endometrial leukocytes and menstruation." Hum Reprod Update **6**(1): 16-27.

Salamonsen, L. A. and D. E. Woolley (1999). "Menstruation: induction by matrix metalloproteinases and inflammatory cells." J Reprod Immunol **44**(1-2): 1-27.

Salamonsen, L. A., et al. (2002). "Leukocyte networks and human endometrial remodelling." J Reprod Immunol **57**(1-2): 95-108.

Salker, M. S., et al. (2011). "Deregulation of the serum- and glucocorticoid-inducible kinase SGK1 in the endometrium causes reproductive failure." Nat Med **17**(11): 1509-1513.

Santillan, I., et al. (2015). "Where and when should natural killer cells be tested in women with repeated implantation failure?" J Reprod Immunol **108**: 142-148.

Sargent, I. L., et al. (2007). "Immunoregulation in normal pregnancy and pre-eclampsia: an overview." Reprod Biomed Online **14 Spec No 1**: 111-117.

Scaife, P. J., et al. (2006). "Effector activity of decidual CD8+ T lymphocytes in early human pregnancy." Biol Reprod **75**(4): 562-567.

Scarpellini, F. and M. Sbracia (2011). "The use of G-CSF for implantation failure in IVF: A clinical trial." Fertil Steril **96**: S93.

Scarpellini, F. and M. Sbracia (2013). "G-CSF treatment in the implantation failure with a fixed dose of 60mcg/day: Preliminary data of a controlled trial." Hum Reprod **28**: i145-i146.

Schatz, F., et al. (1997). "Physiological and clinical implications of decidualization-associated protease activity." Ann N Y Acad Sci **828**: 175-179.

Schoenwolf, G. C., et al. (2012). Larsen's Human Embryology: with STUDENT CONSULT Online Access, Elsevier Health Sciences.

Schulke, L., et al. (2008). "Endometrial dendritic cell populations during the normal menstrual cycle." Hum Reprod **23**(7): 1574-1580.

Seibel, M. M. and M. L. Taymor (1982). "Emotional aspects of infertility." Fertil Steril **37**(2): 137.

Seshadri, S., et al. (2012). "Effect of heparin on the outcome of IVF treatment: a systematic review and meta-analysis." Reproductive BioMedicine Online **25**(6): 572-584.

Seshadri, S. and S. K. Sunkara (2014). "Natural killer cells in female infertility and recurrent miscarriage: a systematic review and meta-analysis." Hum Reprod Update **20**(3): 429-438.

Sharkey, A. M., et al. (2008). "Killer Ig-like receptor expression in uterine NK cells is biased toward recognition of HLA-C and alters with gestational age." J Immunol **181**(1): 39-46.

Sharkey, A. M. and S. K. Smith (2003). "The endometrium as a cause of implantation failure." Best Pract Res Clin Obstet Gynaecol **17**(2): 289-307.

Sharma, K., et al. (2018). "First-Trimester Inflammatory Markers for Risk Evaluation of Pregnancy Hypertension." J Obstet Gynaecol India **68**(1): 27-32.

Shimada, S., et al. (2004). "No difference in natural killer or natural killer T-cell population, but aberrant T-helper cell population in the endometrium of women with repeated miscarriage." Hum Reprod **19**(4): 1018-1024.

Silberstein, S. and G. Merriam (2000). "Physiology of the menstrual cycle." Cephalalgia **20**(3): 148-154.

Simon, A. and N. Laufer (2012). "Assessment and treatment of repeated implantation failure (RIF)." J Assist Reprod Genet **29**(11): 1227-1239.

Simon, C., et al. (2000). "Paracrine regulators of implantation." Baillieres Best Pract Res Clin Obstet Gynaecol **14**(5): 815-826.

Siristatidis, C. S., et al. (2016). "Aspirin for in vitro fertilisation." Cochrane Database of Systematic Reviews(11).

Smith, S. D., et al. (2009). "Evidence for immune cell involvement in decidual spiral arteriole remodeling in early human pregnancy." The American journal of pathology **174**(5): 1959-1971.

Snell, R. S. (2007). Clinical anatomy by systems, Lippincott Williams & Wilkins.

Sojka, D. K., et al. (2014). "Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells." Elife **3**: e01659.

Solana, R., et al. (1999). "Natural killer cells in healthy aging." Exp Gerontol **34**(3): 435-443.

Song, J. Y. and I. Fraser (1995). "Effects of progestogens on human endometrium." Obstetrical & gynecological survey **50**(5): 385-394.

Spandorfer, S. D., et al. (2000). "An Analysis of the Effect of Age on Implantation Rates." Journal of assisted reproduction and genetics **17**(6): 303-306.

Stephenson, M. D. and M. R. Fluker (2000). "Treatment of repeated unexplained in vitro fertilization failure with intravenous immunoglobulin: a randomized, placebo-controlled Canadian trial." Fertil Steril **74**(6): 1108-1113.

Stevens, A. and J. S. Lowe (1997). Human histology, Mosby London.

Strowitzki, T., et al. (2006). "The human endometrium as a fertility-determining factor." Hum Reprod Update **12**(5): 617-630.

Sulke, A. N., et al. (1985). "Variation in natural killer activity in peripheral blood during the menstrual cycle." Br Med J (Clin Res Ed) **290**(6472): 884-886.

Sun, J. C., et al. (2009). "Adaptive immune features of natural killer cells." Nature **457**(7229): 557-561.

Szekeres-Bartho, J. (2002). "Immunological relationship between the mother and the fetus." Int Rev Immunol **21**(6): 471-495.

Tabibzadeh, S. and A. Babaknia (1995). "The signals and molecular pathways involved in implantation, a symbiotic interaction between blastocyst and endometrium involving adhesion and tissue invasion." Molecular Human Reproduction **1**(4): 179-202.

Tang, A. W., et al. (2011). "Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: a systematic review." Hum Reprod **26**(8): 1971-1980.

Tang, A. W., et al. (2013). "A feasibility trial of screening women with idiopathic recurrent miscarriage for high uterine natural killer cell density and randomizing to prednisolone or placebo when pregnant." Hum Reprod **28**(7): 1743-1752.

Taylor, E. and V. Gomel (2008). "The uterus and fertility." Fertil Steril **89**(1): 1-16.

Taylor, E. B. and J. M. Sasser (2017). "Natural killer cells and T lymphocytes in pregnancy and pre-eclampsia." Clin Sci (Lond) **131**(24): 2911-2917.

Teklenburg, G., et al. (2010). "Natural selection of human embryos: decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation." PLoS One **5**(4): e10258.

Templer, S. and G. Sacks (2016). "A blessing and a curse: is high NK cell activity good for health and bad for reproduction?" Hum Fertil (Camb) **19**(3): 166-172.

Terranova, P. F., et al. (1995). "Tumor necrosis factor-alpha in the female reproductive tract." Proc Soc Exp Biol Med **209**(4): 325-342.

Thiruchelvam, U., et al. (2013). "The importance of the macrophage within the human endometrium." J Leukoc Biol **93**(2): 217-225.

Thum, M. Y., et al. (2007). "The relationship of systemic TNF-alpha and IFN-gamma with IVF treatment outcome and peripheral blood NK cells." Am J Reprod Immunol **57**(3): 210-217.

Thum, M. Y., et al. (2008). "Prednisolone suppresses NK cell cytotoxicity in vitro in women with a history of infertility and elevated NK cell cytotoxicity." Am J Reprod Immunol **59**(3): 259-265.

Thum, M. Y., et al. (2004). "An increase in the absolute count of CD56dimCD16+CD69+ NK cells in the peripheral blood is associated with a poorer IVF treatment and pregnancy outcome." Hum Reprod **19**(10): 2395-2400.

Thum, M. Y., et al. (2005). "Simple enumerations of peripheral blood natural killer (CD56+ NK) cells, B cells and T cells have no predictive value in IVF treatment outcome." Hum Reprod **20**(5): 1272-1276.

Thurin, A., et al. (2004). "Elective single-embryo transfer versus double-embryo transfer in in vitro fertilization." New England Journal of Medicine **351**(23): 2392-2402.

Tilburgs, T., et al. (2010). "Human decidual tissue contains differentiated CD8+ effector-memory T cells with unique properties." J Immunol **185**(7): 4470-4477.

Tilburgs, T. and J. L. Strominger (2013). "CD8+ effector T cells at the fetal-maternal interface, balancing fetal tolerance and antiviral immunity." Am J Reprod Immunol **69**(4): 395-407

Toth, B., et al. (2011). "Disorders of implantation – a
re there diagnostic and therapeutic options?" J Reprod Immunol **90**(1): 117-123.

Trundley, A. and A. Moffett (2004). "Human uterine leukocytes and pregnancy." Tissue Antigens **63**(1): 1-12.

Tsuda, H., et al. (2001). "Characterization of NKT cells in human peripheral blood and decidual lymphocytes." Am J Reprod Immunol **45**(5): 295-302.

Tuckerman, E., et al. (2010). "Uterine natural killer cells in peri-implantation endometrium from women with repeated implantation failure after IVF." J Reprod Immunol **87**(1-2): 60-66.

Ubaldi, F., et al. (2002). "Low dose prednisolone administration in routine ICSI patients does not improve pregnancy and implantation rates." Hum Reprod **17**(6): 1544-1547.

Urbanowicz, R. A., et al. (2009). "Altered effector function of peripheral cytotoxic cells in COPD." Respir Res **10**: 53.

Urbanowicz, R. A., et al. (2010). "Enhanced effector function of cytotoxic cells in the induced sputum of COPD patients." Respir Res **11**: 76.

Urman, B., et al. (2009). "Luteal phase empirical low molecular weight heparin administration in patients with failed ICSI embryo transfer cycles: a randomized open-labeled pilot trial." Human Reproduction **24**(7): 1640-1647.

Usadi, R. S., et al. (2003). "Temporal and morphologic characteristics of pinopod expression across the secretory phase of the endometrial cycle in normally cycling women with proven fertility." Fertil Steril **79**(4): 970-974.

Valdes, C. T., et al. (2017). "Implantation failure of endometrial origin: it is not pathology, but our failure to synchronize the developing embryo with a receptive endometrium." Fertil Steril **108**(1): 15-18.

van den Heuvel, M. J., et al. (2007). "Decline in number of elevated blood CD3(+) CD56(+) NKT cells in response to intravenous immunoglobulin treatment correlates with successful pregnancy." Am J Reprod Immunol **58**(5): 447-459.

van der Zwan, A., et al. (2018). "Mixed signature of activation and dysfunction allows human decidual CD8(+) T cells to provide both tolerance and immunity." Proceedings of the National Academy of Sciences of the United States of America **115**(2): 385-390.

van Loendersloot, L., et al. (2014). "Prediction models in in vitro fertilization; where are we? A mini review." J Adv Res **5**(3): 295-301.

van Montfoort, A. P., et al. (2006). "In unselected patients, elective single embryo transfer prevents all multiples, but results in significantly lower pregnancy rates compared with double embryo transfer: a randomized controlled trial." Human Reproduction **21**(2): 338-343.

Vitagliano, A., et al. (2018). "Endometrial scratch injury for women with one or more previous failed embryo transfers: a systematic review and meta-analysis of randomized controlled trials." Fertility and sterility **110**(4): 687-702.e682.

Vitale, C., et al. (2004). "The corticosteroid-induced inhibitory effect on NK cell function reflects down-regulation and/or dysfunction of triggering receptors involved in natural cytotoxicity." Eur J Immunol **34**(11): 3028-3038.

von Rango, U. (2008). "Fetal tolerance in human pregnancy—a crucial balance between acceptance and limitation of trophoblast invasion." Immunology letters **115**(1): 21-32.

Voskoboinik, I., et al. (2015). "Perforin and granzymes: function, dysfunction and human pathology." Nat Rev Immunol **15**(6): 388-400.

Voullaire, L., et al. (2002). "Chromosome abnormalities identified by comparative genomic hybridization in embryos from women with repeated implantation failure." Mol Hum Reprod **8**(11): 1035-1041.
Waller, D. G. and T. Sampson (2013). Medical pharmacology and therapeutics, Elsevier Health Sciences.

Wang, H. and S. K. Dey (2006). "Roadmap to embryo implantation: clues from mouse models." Nat Rev Genet **7**(3): 185-199.

Wang, J., et al. (2013). "Differential activation of killer cells in the circulation and the lung: a study of current smoking status and chronic obstructive pulmonary disease (COPD)." PLoS One **8**(3): e58556.

Whirledge, S. D., et al. (2015). "Uterine glucocorticoid receptors are critical for fertility in mice through control of embryo implantation and decidualization." Proc Natl Acad Sci U S A **112**(49): 15166-15171.

Wilcox, A. J., et al. (1988). "Incidence of early loss of pregnancy." New England Journal of Medicine **319**(4): 189-194.

Wilkens, J., et al. (2013). "Uterine NK cells regulate endometrial bleeding in women and are suppressed by the progesterone receptor modulator asoprisnil." J Immunol **191**(5): 2226-2235.

Winger, E. E., et al. (2011). "Elevated preconception CD56+ 16+ and/or Th1:Th2 levels predict benefit from IVIG therapy in subfertile women undergoing IVF." Am J Reprod Immunol **66**(5): 394-403.

Wold, A. S. and A. Arici (2005). "Natural killer cells and reproductive failure." Curr Opin Obstet Gynecol **17**(3): 237-241.
Wong, A. W. Y., et al. (2014). "Do uterine natural killer cell numbers in peri-implantation endometrium predict hypertensive disorder in pregnancy in women with a history of reproductive failure?" J Reprod Immunol **106**: 34-40.

Xu, B., et al. (2005). "Glucocorticoids inhibit placental cytokines from cultured normal and preeclamptic placental explants." Placenta **26**(8-9): 654-660.

Yagel, S. (2009). "The developmental role of natural killer cells at the fetal-maternal interface." American journal of obstetrics and gynecology **201**(4): 344-350.

Yang, H. L., et al. (2019). "Decidual stromal cells promote the differentiation of CD56(bright) CD16(-) NK cells by secreting IL-24 in early pregnancy." Am J Reprod Immunol: e13110.

Yang, X., et al. (2019). "Ovarian and endometrial immunity during the ovarian cycle." J Reprod Immunol **133**: 7-14.

Yedwab, G., et al. (1976). "The temperature, pH, and partial pressure of oxygen in the cervix and uterus of women and uterus of rats during the cycle." Fertil Steril **27**(3): 304-309.

Yoo, J. H., et al. (2012). "Peripheral blood NK cell cytotoxicities are negatively correlated with CD8(+) T cells in fertile women but not in women with a history of recurrent pregnancy loss." Am J Reprod Immunol **68**(1): 38-46.

Yoshioka, S., et al. (2006). "Intrauterine administration of autologous peripheral blood mononuclear cells promotes implantation rates in patients with repeated failure of IVF-embryo transfer." Hum Reprod **21**: 3290-3294.

Yu, N., et al. (2016). "Intrauterine administration of autologous peripheral blood mononuclear cells (PBMCs) activated by HCG improves the implantation and pregnancy rates in patients with repeated implantation failure: a prospective randomized study." American journal of reproductive immunology **76**(3): 212-216.

Zamai, L., et al. (1998). "Natural killer (NK) cell–mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells." J Exp Med **188**(12): 2375-2380.

Zhang, J., et al. (2011). "Natural killer cell-triggered vascular transformation: maternal care before birth?" Cellular & molecular immunology **8**(1): 1-11.

Zhang, J., et al. (2016). "To serve and to protect: the role of decidual innate immune cells on human pregnancy." Cell Tissue Res **363**(1): 249-265.

Zhang, J. H., et al. (2009). "DBA-lectin reactivity defines natural killer cells that have homed to mouse decidua." Placenta **30**(11): 968-973.

Zhang, T., et al. (2017). "Successful treatment with intrauterine delivery of dexamethasone for repeated implantation failure." Am J Reprod Immunol **78**(6).

Zhao, J., et al. (2016). "Whether G-CSF administration has beneficial effect on the outcome after assisted reproductive technology? A systematic review and meta-analysis." Reproductive Biology and Endocrinology **14**(1): 62.

Zhou, J., et al. (1997). "Glucocorticoid regulation of natural cytotoxicity: effects of cortisol on the phenotype and function of a cloned human natural killer cell line." Cell Immunol **178**(2): 108-116.