A pre-conceptual endometrial test to predict pregnancy outcome in recurrent pregnancy loss

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Submitted to the University of Warwick

Division of Biomedical Sciences

Warwick Medical School

For the degree of Doctor of Medicine (MD)

March 2019

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Acknowledgements

I would like to take this opportunity to offer my most heartfelt gratitude to my supervisor Professor Siobhan Quenby for her invaluable guidance and constant support through the highs and lows of this project spanning the last 3 years.

I am also very thankful to Dr Katherine Fishwick, who provided tremendous support throughout all the laboratory work undertaken and was always willing to teach and guide me. Both my supervisor and Dr Fishwick have been integral to making this project a success.

My sincere thanks go to Professor Jan Brosens for his help and insights into the endometrium's role in recurrent reproductive failure. I would also like to thank Associate Professor Bee Tan for his help during this project.

My gratitude extends to Mr Sean James, who was the first person to teach me immunohistochemistry, laboratory techniques and provided invaluable advice. I also wish to thank Mr Peter Kimani for providing his statistical guidance and patience with my statistical queries.

I must thank all the women with recurrent reproductive failure who attended the Implantation Clinics or our Biomedical Research Unit in Reproductive Health, and agreed to participate in research. Without you, this research project and many others would not be possible.

I would like to express my sincere thanks to Mr Tarek Ghobara, Mr Stephen Keay and Dr Rina Agrawal for the huge support shown in my clinical and research work during my employ as a Clinical Research Fellow in Reproductive Medicine at University Hospitals Coventry and Warwickshire NHS Trust (UHCW).

Here, I would also like to thank all the members of the research team at the Biomedical Research Unit based at UHCW for the help and support given.

Finally, I would like to convey my special thanks to family and friends who have been immensely supportive and helped keep me focused through the challenges in completing this project.

Declaration

I, Mariam Lokman declare that:

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Medicine (MD). It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented was carried out by me except in the cases outlined below:

1. Participants were consented for endometrial research samples mainly by Professor Brosens and Professor Quenby through the Implantation Clinic. I was involved in conceiving the idea for this project, identifying women from the research clinical database, performing a proportion of the endometrial biopsies and retrieving tissue samples from the Research Tissue Bank.
2. Immunohistochemistry (IHC) and image processing: The serial sectioning of tissue blocks was performed by Mr Sean James and Dr Katherine Fishwick. In the exploratory test set, I learned and performed IHC using the autostainer then conducted image processing, interpretation and data analysis to form the study's image processing protocol in collaboration with Dr Katherine Fishwick (Chapter 2 & 4). Following this, the IHC and image processing protocol was undertaken by Dr Katherine Fishwick. The data interpretation and statistical analysis throughout this thesis was completed by me.
3. Scratch in Miscarriage (SiM) trial: This trial is a fellow MD student, Dr Valarmathy Kandavel's research project. As part of the trial, women consent for endometrial tissue samples to be used in further research and this is where I obtained data to validate the combined endometrial test designed.

List of publication

Published conference abstracts:

Lokman M, Fishwick F, Brosens J, Quenby S. Improving the pre-conceptual uNK cell test to predict pregnancy outcome in recurrent miscarriage. Human Reproduction, Volume 32, Issue suppl_1, 1 July 2017, Pages i1-i539

Lokman M, Fishwick F, Brosens J, Quenby S. A reduction in uterine Natural Killer (uNK) cell density in response to luteal-phase endometrial scratch (ES) is associated with improved pregnancy outcome. Human Reproduction, Volume 33, Issue suppl_1, 1 July 2018, Pages i1-i541.

Abstract

Recurrent pregnancy loss (RPL) and recurrent implantation failure (RIF) are major barriers to wanted pregnancies. Currently, there are few tests that reliably predict pregnancy outcome in these groups. Recent research demonstrated that an aetiological factor in early reproductive failure is impaired endometrial decidualisation due to accelerated senescence and loss of endometrial mesenchymal stem cells. Uterine Natural Killer (uNK) cells maintain homeostasis by clearing senescent decidual cells. I aimed to improve the ability of the uNK cell density test in predicting subsequent pregnancy outcomes, through a combined pre-conceptual test that includes cell senescence and proliferation markers.

Methods

This study was performed in a tertiary research 'Implantation Clinic', where the uNK cell density test is performed on mid-luteal endometrial biopsies in women with RPL and RIF. I began my investigation of a predictive test by assessing the predictive ability of the uNK test using the clinic's large retrospective database (N=281).

Next, immunohistochemistry was performed on endometrial serial sections of women with RPL using antibodies to CD56 (uNK), Ki67 (cell proliferation), HMGB2 and p16 (cell senescence) via an automated staining process. The images were analysed using Pannoramic Viewer and Image J software with colour de-convolution and thresholding technique. The findings of the exploratory test set (N=20) were used to develop a pre-conceptual combined test for outcome prediction in a larger study (N=89).

Final validation of the combined test 'uNK-glandular p16' was conducted on samples from women with RPL recruited in the setting of a prospective, randomised-controlled trial (RCT).

Results

Combining the normalised uNK and glandular p16 results improved the prediction of pregnancy outcome (p=0.0163). The predictive ability of the combined test 'uNK-glandular p16 centile' was validated in the prospective RCT setting.

Conclusion

Glandular-stromal synchrony appears to be an important determinate of pregnancy outcome and may help focus future research or therapy.

Abbreviations

ACA	anticardiolipin antibodies
AMH	anti-Mullerian hormone
ANA	antinuclear antibodies
APS	antiphospholipid syndrome
Array-CGH	array-based comparative genomic hybridisation
ART	assisted reproductive technology
AUC	area under the curve
β2GP1	β2 glycoprotein 1 antibodies
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CD56	neural cell adhesion molecule
CI	confidence interval
COX-2	cyclo-oxygenase-2
CSF-1	colony-stimulating factor
DAB	3,3-diaminobenzidine
DNA	deoxyribose nucleic acid
eMSCs	endometrial mesenchymal stem cells
ERA	endometrial receptivity test
ESHRE	European Society of Human Reproduction and Embryology
FSH	follicle stimulating hormone
GnRH	gonadotrophin releasing hormone

G-CSF	granulocyte-colony stimulating factor
HCG	human chorionic gonadotrophin
HESCs	human endometrial stromal cells
HLA	human leukocyte antigen
HMGB2	high mobility group protein 2
HPO-axis	hypothalamic-pituitary-ovarian axis
ICSI	intracytoplasmic sperm injection
IGFBP-1	insulin-like growth factor binding protein-1
IgG	immunoglobulin G
IgM	immunoglobulin M
IHC	immunohistochemistry
IL	interleukin
INF-γ	interferon gamma cytokine
IVF	in vitro fertilisation
IVIG	intravenous immunoglobulin
Ki67	nuclear protein expressed in cell proliferation
LA	lupus anticoagulant
LH	luteinising hormone
LMWH	low molecular weight heparin
MHC	major histocompatibility complex
NHS	National Health Service
NICE	National Institute of Health and Care Excellence
NK	Natural Killer cells

OR	odds ratio
P16	cyclin-dependent kinase inhibitor 2A (tumour suppressor protein)
PCOS	polycystic ovarian syndrome
PGD	pre-implantation genetic diagnosis
PGE2	prostaglandin E2
PGS	pre-implantation genetic screening
PRL	prolactin
RCT	randomised-controlled trial
RNA	ribonucleic acid
RIF	recurrent implantation failure
RM	recurrent miscarriage
ROC	receiver operating characteristic curve
RPL	recurrent pregnancy loss
T4	thyroxine
TNF-α	tumour necrosis factor-α
Th	T helper cell
TPO-Ab	thyroid peroxidase antibodies
T regs	T regulatory cell
TSH	thyroid stimulating hormone
UHCW	University Hospital Coventry and Warwickshire NHS Trust
uNK	uterine Natural Killer cells

CHAPTER 1:
INTRODUCTION

Chapter 1: Introduction

1.1 Recurrent reproductive failure

Recurrent reproductive failure in the context of this thesis, encompasses recurrent pregnancy loss (RPL) and recurrent implantation failure (RIF). Recurrent reproductive failure causes considerable distress to couples wanting to have a family. Understanding the complex processes involved in human reproduction is crucial in the effort to improve pregnancy outcomes for these couples. At present there are numerous investigations and therapies offered to couples suffering recurrent reproductive failure. Couples will invest significant time, money and take on a level of health risk in order to achieve successful childbirth, even on treatments with low success rates. Many of the treatments offered to couples do not have strong evidence of a positive effect on pregnancy outcomes. Hence further research in this area is badly needed.

In the next few chapters, I discuss human reproduction, recurrent pregnancy loss and recurrent implantation failure in further detail.

1.1.1 Human reproduction

1.1.1.1 Fertilisation

Human reproduction is dependent on a viable ovum, sperm and ability of both gametes to meet in the Fallopian tube to fertilise, unless assisted reproductive technology is used. Fertilisation of the ovum usually occurs in the ampulla of the Fallopian tube. The process of fertilisation begins with chemoattraction of the sperm to ovum and adherence to the zona pellucida surrounding the ovum (Bahat *et al.*, 2003).

Spermatozoa with active movement, particularly progressive motility comes into close contact with the ovum (secondary oocyte arrested at metaphase of Meiosis II). The acrosome reaction releases enzymes, in particular hyaluronidase, that enables penetration of the zona pellucida by spermatozoa (Holt & Van Look, 2004). The

sperm head adheres to the cell membrane of the ovum with breakdown of the area of fusion and release of the spermatozoon nucleus into the cytoplasm of the ovum. As the spermatozoon binds to the cell membrane of the ovum, the membrane becomes impenetrable to other spermatozoa. The successful spermatozoon is engulfed by the ovum's cytoplasm. The sperm head then converts into the male pronucleus and the tail detaches. Alongside this, the ovum completes meiosis II creating a female pronucleus (Ganong, 2003). Both pronuclei meet and resolve into one nucleus with a complete set of 23 chromosomes creating a zygote, which contains all essential factors to develop into a new individual. The embryonic period begins with the first division of the zygote after 24 hours of fusion.

The fertilised ovum then travels down the tube to the uterus in about 3 days. In this time, the conceptus cleaves until it becomes a morula (cluster of 8 blastomeres) and then transforms into a blastocyst (Ganong, 2003). Between day 6 to 10 after LH surge, the blastocyst will enter the implantation process with the endometrium and be supported for 9 months in a successful pregnancy.

1.1.1.2 Implantation

Implantation is the stage of pregnancy where the embryo adheres to and is embedded into the endometrium (Coughlan *et al.*, 2014). The conceptus is a blastocyst at this stage of development. The implantation window describes the endometrial receptive phase where interaction between embryo and maternal endometrium occurs (Gellersen & Brosens, 2014). Most sources state that the implantation window spans from day 7 to 9 post LH surge but there are reports that the window is wider at days 6 to 10 post LH surge (Achache & Revel, 2006; Bergh & Navot, 1992; Fanchin, 2001; Xiao *et al.*, 2010). The endometrium undergoes decidualisation in preparation for implantation. The embryonic and maternal preparation for pregnancy needs to occur in tandem to support a pregnancy successfully (Cha *et al.*, 2012; Gellersen & Brosens, 2014; Su & Fazleabas, 2015).

The process of implantation is commonly associated with a step-wise process. It involves apposition and adhesion of the blastocyst to the endometrium (Fazleabas & Kim, 2003; Genbacev *et al.*, 2003). Following this, the blastocyst breaches the luminal epithelium and decidualising stromal cells migrate and surround the invading embryo (Dey *et al.*, 2004; Quenby & Brosens, 2013). The migration of decidual stromal cells has been recorded using time-lapse imaging and in migration assays (Gellersen *et al.*, 2013; Grewal *et al.*, 2010; Grewal *et al.*, 2008; Schwenke *et al.*, 2013). This supports evidence that the endometrium plays an active role in reproductive success.

There are 5 key events that are discussed in the implantation process and adopted by the team at Warwick (Lucas *et al.*, 2013).

- **Positioning of embryo near fundus**
- **Apposition**

The two processes above occur closely together. This multi-faceted process involves the embryo finding a location to implant with a position near the fundus (Chen *et al.*, 2013; Salamonsen *et al.*, 2016). At this stage, an embryo can be flushed out as it is not yet adherent to endometrial luminal epithelium.

- **Adhesion/attachment**

The embryo initiates direct contact between trophoblast cells and endometrial luminal epithelium. This occurs via apical cell membranes of the endometrium (Lindenberg, 1991). The inner cell mass migrates to face the trophoblast on the side of apposition and the adhesion process is mediated by receptor-ligand interactions (Salamonsen *et al.*, 2016; Su & Fazleabas, 2015). A micro-environment is created by the cross-talk between the endometrium and embryo in order to support implantation.

- **Penetration through luminal epithelium**

At this stage, the decidual stromal cells migrate to encapsulate the implanting blastocyst (Quenby & Brosens, 2013). During this stage, the endometrium plays a

role as an embryo sensor. The endometrium is able to discriminate between high- and low- quality embryos (Salker *et al.*, 2010). The decidualised stromal cells have been shown not to migrate towards low quality embryos and stop implantation (Brosens *et al.*, 2014; Macklon & Brosens, 2014; Singh *et al.*, 2010; Weimar *et al.*, 2012).

- **Invasion of stroma**

Trophoblast penetrate the endometrial epithelial layer and reach the basement membrane via intercellular gaps between neighbouring epithelial cells without destroying them (Carson *et al.*, 2000). This is to ensure the embryo successfully embed in the maternal endometrium to create a haemochorial placenta.

Alongside the above process, decidualisation of the endometrium occurs. The decidualisation process starts after ovulation and involves differentiation of endometrial stromal cells, blood vessels, glands and an increase in endometrial leucocytes. The interaction between a competent embryo and endometrium initiates changes that make the endometrium more receptive and enhances decidualisation. A receptive, decidualised endometrium allows embryo invasion and encapsulation by decidualised endometrial stromal cells. The process of decidualisation underpins embryo selection at implantation or embryo loss at menstruation. I will expand on these processes next.

Decidualisation

Decidualisation occurs in response to the actions of progesterone. Progesterone nuclear receptors are highly expressed in stromal cells throughout the menstrual cycle and pregnancy (Chen *et al.*, 2009). Decidualisation of stromal cells is the differentiation of elongated fibroblast-like mesenchymal cells in the endometrial stroma to round epithelioid-like cells during the luteal phase of the menstrual cycle (Su & Fazleabas, 2015). This begins with endometrial stromal cells surrounding spiral arteries in the upper two-third of the endometrium, which is called pre-decidualization (Su & Fazleabas, 2015). This reaction persists with implantation and

spreads beyond the perivascular regions. Decidual stromal cells can migrate and are secretory; and its major secretory products are prolactin and insulin-like growth factor binding protein-1 (IGFB-1) (Gellersen *et al.*, 2007).

Endometrial response to blastocyst signals

Human chorionic gonadotrophin (HCG) is the major signalling hormone of the embryo. It is a glycoprotein hormone synthesized by trophoblast cells and acts as a LH super agonist. It extends lifespan of the corpus luteum to support pregnancy until placental progesterone is produced at around 6 to 8 weeks gestation (Hallast *et al.*, 2005; Talmadge *et al.*, 1983). HCG also exerts an endometrial epithelial response by inducing cyclooxygenase-2 (COX-2) and prostaglandin E synthase, which both control synthesis of prostaglandin E2 (PGE2) (Banerjee *et al.*, 2009). PGE2 induces cAMP and endometrial stromal cells to promote pre- decidualization during the luteal phase (Tanaka *et al.*, 1993).

Embryo invasion and encapsulation

The above cascade initiates endometrial stromal decidualisation and remodelling of stromal cell cytoskeleton. These decidualised stromal cells are intrinsically motile and have invasive capability (Weimar *et al.*, 2013). This characteristic allows the stromal cells to encapsulate the implanting blastocyst and this has been revealed on time-lapse recording (Grewal *et al.*, 2010; Grewal *et al.*, 2008). Stromal cell decidualisation and motility are essential for establishment of a successful pregnancy (Afshar *et al.*, 2012; Su & Fazleabas, 2015).

Embryo selection, rejection and menstruation

The endometrial response described serves as a biosensor of embryos. The response will either support the embryo or facilitate early rejection and menstruation (Brosens *et al.*, 2014; Teklenburg *et al.*, 2010). Decidualised stromal cells can sense poor quality embryos and will migrate towards high quality embryo and not poor-quality embryos (Teklenburg *et al.*, 2010; Weimar *et al.*, 2012). This selection process limits maternal investment in invasive but developmentally compromised human embryos (Gellersen & Brosens, 2014). In the absence of implantation or in response

to a compromised embryo, endometrial stromal cells can trigger tissue destruction which also occurs due to fall in progesterone levels causing menstruation (Leitao *et al.*, 2010; Leitao *et al.*, 2011).

Embryo nutrition

Up to 10 weeks gestation the embryonic nutrition is from glandular secretion and passive diffusion (Burton *et al.*, 2002; Salamonsen *et al.*, 2016). This is because invading trophoblast cells plug the mouths of the maternal spiral arteries in the first 10 weeks of gestation (Burton *et al.*, 1999). Blood flow is first seen in the intervillous space after this time. The initiation of blood flow into the intervillous spaces causes oxidative stress and is a second test of embryo fitness.

The importance of this separation between maternal circulation and embryo is to provide a stable, low oxygen environment during organogenesis. This critical need is to reduce risk of teratogenesis related to oxygen free radicals. Once blood flow is initiated, nutrition occurs by exchange between maternal and fetal circulations within the placenta.

1.1.2 Recurrent pregnancy loss

Recurrent pregnancy loss (RPL) causes repeated physical and psychological trauma for the couple involved (Craig *et al.*, 2002b). In the United Kingdom, recurrent pregnancy loss is defined as 3 or more consecutive miscarriages of pregnancies under 24 weeks gestation. This affects 1-2% of couples trying to conceive (Rai & Regan, 2006). However, in other countries, for example The Netherlands and North America, recurrent miscarriage is defined as 2 consecutive miscarriages. This latter definition has been adopted by the European Society of Human Reproduction and Embryology (ESHRE) in their RPL guidance. Unfortunately, management of these

couples is fraught with controversy and at times influenced by personal bias and small uncontrolled studies.

Numerous causes have been invoked to explain recurrent pregnancy loss but many are unsubstantiated (Barber *et al.*, 2010). In practice, therapies such as low molecular weight heparin (LMWH) thromboprophylaxis (Kaandorp *et al.*, 2010; Laskin *et al.*, 2009; Visser *et al.*, 2011) and immunomodulators (Ata *et al.*, 2011; Stephenson *et al.*, 2010) are frequently used despite current paucity of evidence that these approaches are effective.

Dedicated recurrent pregnancy loss clinics should aim to offer couples support in early pregnancy and where possible, within a monitored research setting. The research aim is to identify underlying causes, conduct prospective randomised controlled trials (RCT) of sufficient power and determine best treatment regimens. This set up could still be delivered in general district hospitals with input from tertiary centres. An ideal, well-orchestrated service is one that can offer early pregnancy reassurance scans, availability of cytogenetic testing and is research active in a multi-centre setting. Counselling services similar to those offered to fertility patients could be beneficial and considered in the holistic care of a select number of affected couples. Currently, care and services available to RPL couples are not uniform throughout the United Kingdom.

Underpinning the controversy of effective therapy is the fact that the pathophysiology of RPL remains unclear. One of the many difficulties in ascertaining the pathophysiology and cause of RPL is the heterogeneity of this study population.

Most miscarriages are a process of natural embryo selection and are sporadic pregnancy losses with abnormal karyotype that are not compatible with life. However, as the number of miscarriages increases so does the chance of miscarriage of a normal embryo, thereby increasing the chance of a maternal preventable pathology (Lund *et al.*, 2012; Ogasawara *et al.*, 2000) (Figure 1.2). This change in odds appears to occur after 5 miscarriages (Figure 1.3).

For patients and clinicians, it is unacceptable to delay investigations until couples experience 5 miscarriages. Therefore, it is essential to identify women who have an underlying pathology at an earlier stage to individualise management and predict outcome of subsequent pregnancies.

Although the background chance of live birth is good following 3 miscarriages, all couples suffer anxiety and need support in early pregnancy. To illustrate, a woman age 30 to 35 years with a history of 3 miscarriages has a successful pregnancy rate of 70-80% (Brigham *et al.*, 1999; Ogasawara *et al.*, 2000). New tests and treatments are needed at this stage, to identify and if possible, prevent the 20-30% who will have a repeated pregnancy loss of a normal embryo.

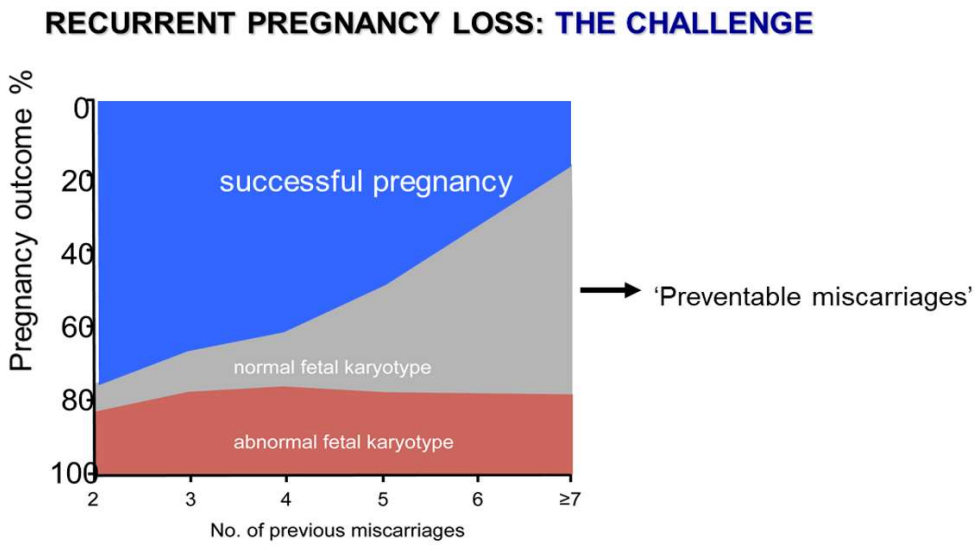

Figure 1. 1: Karyotype of miscarriages in relation to number of previous miscarriages.
Reprinted from Fertility Sterility, 73(2); Ogasawara, M., Aoki, K., Okada, S. & Suzumori, K. (2000) Embryonic karyotype of abortuses in relation to the number of previous miscarriages. Fertil Steril, 73 (2): 300-304, with permission from Elsevier

As shown in this figure, the presence of karyotypically abnormal fetuses is high in subsequent miscarriages for women with history of 2 to 4 miscarriages. Their successful pregnancy rates are reassuringly high. However, as the number of previous miscarriages reaches 5, the chances of miscarrying a normal embryo rises exponentially. It is the loss of these pregnancies that we need to implement measures to prevent.

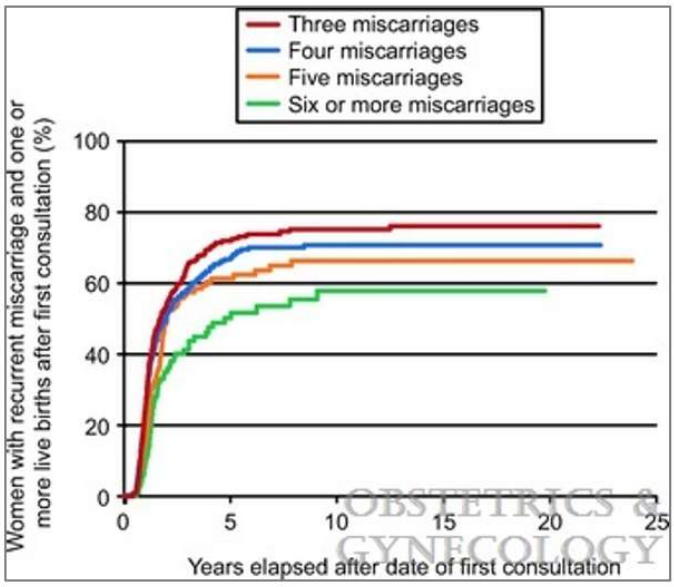

Figure 1. 2: RPL and prognosis for live birth (number of miscarriages). *Reprinted with permission from Obstet Gynecol; Lund, M., Kamper-Jorgensen, M., Nielsen, H. S., Lidegaard, O., Andersen, A. M. & Christiansen, O. B. (2012) Prognosis for live birth in women with recurrent miscarriage: what is the best measure of success? Obstet Gynecol, 119 (1): 37-43* https://journals.lww.com/greenjournal/fulltext/2012/01000/Prognosis_for_Live_Birth_in_Women_With_Recurrent.7.aspx

This figure is a Kaplan-Meier plot showing the percentage of women with RPL who have had at least one live birth after their first consultation and are grouped by the number of previous miscarriages. Successful pregnancy rates begin to decline significantly following 5 or more previous miscarriages. These findings support those shown in the previous graph (Figure 1.2).

In initial consultation with couples, the risk factors and modifiable behaviours should be discussed. The recently published ESHRE Recurrent Pregnancy Loss guidance in November 2017, is a comprehensive clinical practice guideline formed after careful consideration of the scientific evidence available and provides an aid to healthcare professionals in clinical decisions about appropriate and effective care for their patients (Bender Atik *et al.*, 2018). I will now discuss the risk factors, modifiable behaviour and investigations in RPL.

1. Risk factors

The main prognostic risk factors are age and number of previous miscarriages.

Age

Advanced female age is a risk for recurrent pregnancy loss, subfertility and obstetric complications (Nybo *et al.*, 2000; Sauer, 2015). It was found that female age ≥ 35 years doubles the risk of another pregnancy loss compared to younger women (OR 1.99; 95% CI 1.45-2.73) (Lo *et al.*, 2012). Risk of RPL increases dramatically from age 40 as demonstrated in a 5-year follow up cohort study of 987 couples (Lund *et al.*, 2012) (Figure 1.4).

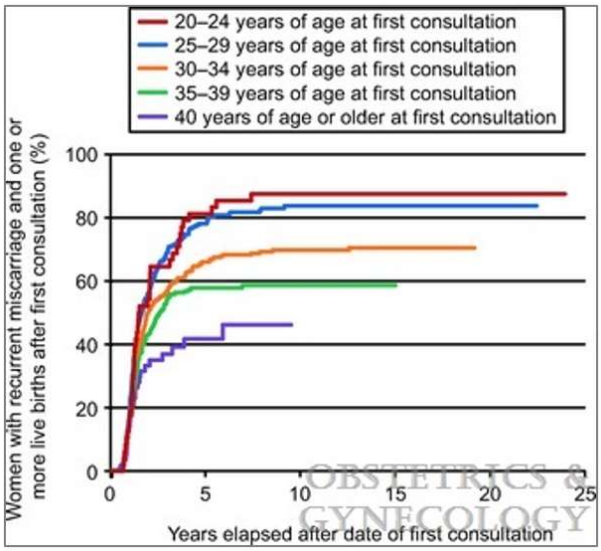

Figure 1. 3: Recurrent miscarriage and prognosis for live birth (maternal age). *Reprinted with permission from Obstet Gynecol; Lund, M., Kamper-Jorgensen, M., Nielsen, H. S., Lidegaard, O., Andersen, A. M. & Christiansen, O. B. (2012) Prognosis for live birth in women with recurrent miscarriage: what is the best measure of success? Obstet Gynecol, 119 (1): 37-43*
https://journals.lww.com/greenjournal/fulltext/2012/01000/Prognosis_for_Live_Birth_in_Women_With_Recurrent.7.aspx

This is a Kaplan-Meier plot showing the percentage of women with recurrent miscarriage who have had at least one live birth after first consultation and are grouped by age. This emphasises the impact maternal age has on the declining rate of successful pregnancy outcomes.

Women should be sensitively informed that risk of pregnancy loss increases after 35 years of age. At age 40, successful pregnancy rate can be less than 50% (Lund *et al.*, 2012).

In comparison, increasing male age has an association with incidence of miscarriage but it's importance in RPL is uncertain (Sharma *et al.*, 2015).

Stress

Couples should be informed that there is no evidence that stress is a factor leading to pregnancy loss (Nelson *et al.*, 2003; Plana-Ripoll *et al.*, 2016). There are several studies that have shown the presence of higher stress levels in women with recurrent pregnancy loss compared to control groups (Kolte *et al.*, 2015; Li *et al.*, 2012). The impact of stress on risk of recurrent pregnancy loss is unclear as the increased levels of stress could be as a result rather than a cause of recurrent pregnancy loss.

Occupational and environmental exposure

There are only a few small studies that examined exposure to occupational and environmental exposure as factors in recurrent pregnancy loss. Therefore, there is insufficient information to recommend protection against a particular agent (Gold & Tomich, 1994; Pathak *et al.*, 2010). However, general advice on avoiding possible hazardous substances during any pregnancy should be given.

Chronic endometritis

Chronic endometritis is a condition where plasma cells infiltrate the endometrium. Research has shown a wide prevalence range in women with recurrent pregnancy loss depending on method of detection used (Bouet *et al.*, 2016; Cicinelli *et al.*, 2014; Kitaya, 2011; McQueen *et al.*, 2014; McQueen *et al.*, 2015; Russell *et al.*, 2013). However, screening women for endometritis is not currently recommended as there are no studies comparing the prevalence in a control group and whether treatment improves pregnancy outcomes.

Impaired endometrial decidualization

Endometrial decidualization during a menstrual cycle and early pregnancy appears to be important as a quality control point in the implantation process for normal embryos and recognition of abnormal embryos leading to rapid shed of the endometrium (menstruation) (Lucas *et al.*, 2016). An excessively receptive endometrium to implantation of abnormal embryos leads to increased rate of pregnancy loss. Further prospective studies are needed before use in clinical practice.

2. Health behaviour modifications

All couples assessed for RPL should be counselled about modifiable health behaviours that can influence pregnancy or health. Some of these behaviours are discussed below:

Smoking cessation

Couples should be advised that smoking could have a negative impact on pregnancy and should be avoided even without evidence that it reduces chance of successful pregnancy in recurrent pregnancy loss (Wilcox *et al.*, 1990; Zhang *et al.*, 2010). Smoking is known to have a strong association to poor obstetric outcomes and general health (Leung & Davies, 2015).

Body Mass index (BMI)

Studies have found obesity (BMI >30kg/m^2 according to WHO) is associated with pregnancy loss and poorer fertility treatment outcomes (Metwally *et al.*, 2008; Pandey *et al.*, 2010). Higher prevalence of RPL was found in obese women compared to those with a normal BMI (20-30 kg/m^2) (Boots & Stephenson, 2011; Lashen *et al.*, 2004). A further study found that obese women were more likely to miscarry euploid embryos (RR 1.63; 95% CI 1.08-2.47) (Boots *et al.*, 2014). It has been demonstrated that maternal obesity significantly increased the risk of miscarriage in unexplained RPL (OR 1.27; 95% CI 0.89-1.83) (Lo *et al.*, 2012). There is no evidence on the effect

of weight loss on RPL. However, studies have shown improvement of fertility with weight loss (Pandey *et al.*, 2010).

In those with low BMI <18.5 kg/m2, there is a significant association with sporadic miscarriage but not to recurrent pregnancy loss (Bellver *et al.*, 2003; Lo *et al.*, 2012; Maconochie *et al.*, 2007).

On the basis that maternal obesity and being underweight can lead to obstetric complications and impact general health, couples should be advised to aim for a healthy BMI.

Avoiding alcohol

Excessive alcohol consumption is a proven risk factor for fetal disorder (fetal alcohol syndrome). Although there is no evidence of alcohol consumption's role in recurrent pregnancy loss, a large proportion of studies have shown an increased risk of miscarriage in a dose-dependent association (Andersen *et al.*, 2012; Avalos *et al.*, 2014; Maconochie *et al.*, 2007). Therefore, couples with RPL are advised to limit alcohol consumption.

3. Investigations and management in RPL

Here, I will discuss the history, investigations and management options that need to be considered when assessing a couple with RPL.

Medical and family history

In the first visit for referral of RPL, a thorough history of medical and family history should be obtained from both the female and male partner. This would include information on risk factors and lifestyle issues as discussed previously. History on medical conditions such as thrombophilia, polycystic ovarian syndrome (PCOS), diabetes, thyroid disease and family history of hereditary thrombophilia should be taken. The information from each couple can be used to select the appropriate investigations for RPL, as not all are relevant to every couple.

Genetic factor

Chromosomal abnormalities and aneuploidy of the conceptus are recognised causes of sporadic and recurrent pregnancy loss (van den Berg *et al.*, 2012). This can be investigated by karyotyping the pregnancy tissue following a miscarriage (Mathur *et al.*, 2014). Currently, the best method of genetic analysis is array-based comparative genomic hybridisation (array-CGH) as it reduces maternal tissue contaminate (Kudesia *et al.*, 2014; Mathur *et al.*, 2014). There are limitations in the test in detection of balanced rearrangements, low-level mosaicism and minor copy number variants (Freeman *et al.*, 2006; Sahoo *et al.*, 2017). The newer technique of next generation sequencing (NGS) may change the choice of test in the near future (Shamseldin *et al.*, 2013).

Detection of chromosomal abnormalities does not translate into a prognostic test or offer of treatment. However, it can provide couples with an explanation for why the miscarriage occurred. It may also suggest an underlying condition if the couple miscarry a normal embryo.

Parental karyotyping may be beneficial in a select group of couples with RPL. The test can detect presence of reciprocal translocation, inversion, Robertsonian translocation and other abnormalities. It can be recommended based on genetic history, which include previous child born with congenital abnormalities, detection of translocation in pregnancy tissue or family history of unbalanced chromosome abnormalities. The test has very limited value in other couples, as the rate of an abnormal karyotype is very low (Franssen *et al.*, 2005; Franssen *et al.*, 2006).

This test can provide information on a contributing factor and prognosis for future pregnancies. In couples with abnormal results, the cumulative live birth rate is still good despite higher risk of RPL (Flynn *et al.*, 2014). Prenatal diagnosis or pre-implantation genetic testing can be offered to them as treatment after provision of information and genetic counselling.

Thrombophilia screen

Thrombophilia is a condition that predisposes women to venous thromboembolism and can be acquired or hereditary. Acquired thrombophilia has a strong link to RPL but the association of the hereditary form of thrombophilia with RPL is still unclear.

- ***Antiphospholipid Syndrome (APS)***

APS is the acquired form of thrombophilia. The diagnosis is made on persistent titres of antiphospholipid antibodies and vascular thrombosis and/or pregnancy complications (Miyakis *et al.*, 2006). The tests that show a strong association with early or late miscarriage are lupus anticoagulant (LA) and anticardiolipin antibodies (ACA, IgG and IgM), while β2 glycoprotein I antibodies (aβ2GPI, IgG and IgM) show possible association but not statistically significant (Opatrny *et al.*, 2006). An international consensus meeting recommended APS testing to women with a history of two or more pregnancy losses (Bender Atik *et al.*, 2018; van den Boogaard *et al.*, 2013).

It is recommended that women diagnosed with APS and a history of at least three miscarriages, be started on aspirin 75 to 100 mg daily pre-conceptually and to start prophylactic dose of heparin from date of positive pregnancy test (Bender Atik *et al.*, 2018).

- ***Hereditary Thrombophilia***

Hereditary thrombophilia includes Factor V Leiden mutation, Prothrombin gene mutation, Antithrombin, Protein C and Protein S deficiency. Due to paucity of evidence, screening and anticoagulant treatment for hereditary thrombophilia is not advocated outside of a research setting or in women with additional risk factors for thrombophilia.

Factor V Leiden mutation leads to its resistance to cleavage by activated protein C. this mutation appears to have a significant association to risk of a further pregnancy loss in the next pregnancy (Bradley *et al.*, 2012). However, there is a paucity of evidence on treatment improving pregnancy outcomes.

Prothrombin gene mutation raises plasma concentration of prothrombin. The evidence on the association between RPL and prothrombin gene mutation is not strong. The risk of a further pregnancy loss was not significant with minimal clinical utility (Bradley *et al.*, 2012; Rey *et al.*, 2003).

In antithrombin, Protein C and Protein S deficiency, there is no significant association to women with RPL or difference in live birth rate compared to controls (Matsukawa *et al.*, 2017; Rey *et al.*, 2003).

Immunological screen

- ***Human leukocyte antigen (HLA) and cytokines***

It has been suggested that increased compatibility in HLA between partners decrease immune tolerance to the fetus. Evidence for compatibility of HLA between partners does not show a difference between women with RPL and controls (Beydoun & Saftlas, 2005). Measuring cytokine levels (e.g. TNF-α) and cytokine polymorphisms should not be tested in clinical practice as the relevance and evidence is unclear (Calleja-Agius *et al.*, 2012; Choi & Kwak-Kim, 2008; Lee *et al.*, 2013; Medica *et al.*, 2009; Mueller-Eckhardt *et al.*, 1994). Therefore, HLA determination and cytokine testing in women with RPL is not recommended.

- ***Antinuclear antibodies (ANA)***

ANA are antibodies against components of cell nuclei found in many autoimmune diseases. Most studies have found an association of positive ANA with RPL and poorer pregnancy outcomes (Cavalcante *et al.*, 2015; Christiansen, 1996; Harger *et al.*, 1983; Ogasawara *et al.*, 1996; Stern *et al.*, 1998). However, the pathophysiology

is unclear and response to immunotherapy is unknown. Therefore, ANA testing has limited clinical utility.

- ***Natural Killer cells***

There are two forms of investigations, which are tests of cytotoxicity of peripheral blood NK cells and uterine NK cells in pre-conceptual endometrial biopsies from women with history of RPL. Currently, there is insufficient evidence to recommend NK cell testing for women with RPL.

There is conflicting evidence for an association of high percentage or increased cytotoxicity of NK cells in peripheral blood and RPL (Chao *et al.*, 1995; Emmer *et al.*, 2000; King *et al.*, 2010; Kwak *et al.*, 1995; Lee *et al.*, 2013; Liang *et al.*, 2012; Wang *et al.*, 2008). In addition, studies did not find that high NK cell cytotoxicity had an impact on subsequent miscarriage rates (Emmer *et al.*, 1999; Katano *et al.*, 2013; Liang *et al.*, 2012).

Most studies have shown uterine NK cells are higher in women with RPL (Clifford *et al.*, 1999; Quenby *et al.*, 2005; Shimada *et al.*, 2004; Tuckerman *et al.*, 2007). However, the percentage of uNK cell density has not been able to predict subsequent pregnancy outcome (Liu *et al.*, 2014; Tuckerman *et al.*, 2007).

There is also a significant difference between peripheral NK cells and uterine NK cells levels (Bender Atik *et al.*, 2018). This means the peripheral NK cell test is unable to identify women with RPL for immunological treatments. Furthermore, the uNK cell tests are limited by inherent test errors involving interobserver variation, timing of the biopsy in the menstrual cycle and consensus on the normal range of the test.

Metabolic and endocrine factors

- ***Thyroid Dysfunction***

Thyroid dysfunction and increased thyroid peroxidase antibodies (TPO-Ab) have been linked to aberrant fertilisation and embryogenesis which may lead to

subfertility and pregnancy loss (Vissenberg *et al.*, 2015). Hyperthyroidism is known to cause several pregnancy complications which include sporadic pregnancy loss and preterm delivery (Andersen *et al.*, 2014; Bahn *et al.*, 2011; Springer *et al.*, 2017). However, there is no evidence on its effect on RPL (Bender Atik *et al.*, 2018).

Studies report higher prevalence of hypothyroidism and increased levels of TPO-Abs in women with RPL (Rao *et al.*, 2008; Ticconi *et al.*, 2011). In hypothyroidism and subclinical hypothyroidism, no difference was found in pregnancy outcomes compared to control groups (Bernardi *et al.*, 2013; Rao *et al.*, 2008; van den Boogaard *et al.*, 2011; van Dijk *et al.*, 2016). The association between thyroid auto immunity and RPL is clearer as the odds for a further pregnancy loss in women positive for TPO-Abs appeared to be significantly higher than that of controls (Thangaratinam *et al.*, 2011; van den Boogaard *et al.*, 2011).

Hence, thyroid screening should be offered to women with RPL. Hypothyroidism should be treated with levothyroxine with monitoring of TSH and T4 levels.

- ***PCOS***

There is conflicting evidence about the prevalence of PCOS and insulin resistance in women with RPL (Craig *et al.*, 2002a; Maryam *et al.*, 2012; Okon *et al.*, 1998; Sagle *et al.*, 1988). There is also no evidence to suggest increased risk of further pregnancy loss and there was no difference in predicting live birth rate compared with controls (Liddell *et al.*, 1997; Rai *et al.*, 2000).

Therefore, assessment of PCOS, fasting insulin or glucose is not recommended.

Anatomical factors

Congenital uterine malformations show a clear association with RPL. These malformations encompass the septate uterus, bicornuate uterus, didelphic uterus and unicornuate uterus, and the prevalence of these findings are higher in women with RPL (Chan *et al.*, 2011b; Saravelos *et al.*, 2008). These women also have higher prevalence of first and second trimester miscarriage when compared to controls

(Chan *et al.*, 2011a; Venetis *et al.*, 2014). Despite this, how these malformations contribute to pregnancy loss and how treatment effects future pregnancy outcome is still unclear.

Acquired uterine malformations include submucous fibroids, endometrial polyps and intrauterine adhesions. The prevalence of these findings is again high in women with RPL but its contributory factor to further pregnancy loss is uncertain (Hooker *et al.*, 2014; Saravelos *et al.*, 2011). Further studies are required to ascertain the clinical relevance of both congenital and acquired uterine malformations. However, due to the high prevalence, all women with RPL should have an assessment for uterine anatomy.

The preferred non-invasive diagnostic test for congenital uterine malformations is a transvaginal 3D ultrasound scan (Caliskan *et al.*, 2010; Ghi *et al.*, 2009; Saravelos *et al.*, 2008). The same diagnostic technique can be used for acquired uterine abnormalities but there is no strong evidence on which method is preferred. A hysteroscopic procedure would allow for the option of treatment in the same setting.

Treatment of uterine abnormalities depends on type and severity. There is still insufficient evidence that surgical removal of submucous fibroids, endometrial polyps or intrauterine adhesions improves pregnancy outcomes in women with RPL. Hysteroscopic resection of uterine septum for RPL should be evaluated in a research setting as high-quality studies are needed (Kowalik *et al.*, 2011; Rikken *et al.*, 2017).

Male factor

There is conflicting evidence on the association of poor sperm parameters (sperm volume, count, reduced viability, abnormal morphology and reduced total progressive sperm motility) and RPL (Anifandis *et al.*, 2014; Bhattacharya, 2008; Jensen *et al.*, 2014; Pacey *et al.*, 2014; Showell *et al.*, 2014). A number of studies addressed male genetic defects and impact on RPL. The only significant association was higher miscarriage rates in men with high sperm DNA fragmentation compared to men with low sperm DNA damage (Robinson *et al.*, 2012; Zhao *et al.*, 2014).

Smoking, obesity and excessive exercise appear to cause oxidative stress that can lead to DNA fragmentation (Aitken *et al.*, 2009; Du Plessis *et al.*, 2010; Hsu *et al.*, 2009). Hence, clinicians should encourage the male partner to make healthy lifestyle modifications. More studies are needed to elucidate prognostic value of testing DNA fragmentation.

Treatment of unexplained RPL

Unexplained RPL is an area that is difficult to manage and evidence is mainly derived from poor quality or small studies. Some therapies have moderate or serious adverse effects with no significant effect on live birth rate and should not be offered or only considered in a research setting. This includes lymphocyte immunization therapy (Christiansen *et al.*, 1994; Wong *et al.*, 2014), intravenous immunoglobulin (IVIG) (Egerup *et al.*, 2015; Hutton *et al.*, 2007; Wang *et al.*, 2016), prednisolone therapy (Gomaa *et al.*, 2014; Laskin *et al.*, 1997) and heparin or low dose aspirin (de Jong *et al.*, 2014; Pasquier *et al.*, 2015; Schleussner *et al.*, 2015).

The use of progestogen in unexplained RPL remains controversial. In a couple of recent large RCTs, there was no evidence to support use of vaginal progesterone in unexplained RPL (Coomarasamy *et al.*, 2015; Kumar *et al.*, 2014). However, a Cochrane systematic review of 11 trials which included 2359 women, found moderate evidence that progestogen probably reduces the number of miscarriages compared to placebo or controls in unexplained RPL (Haas *et al.*, 2018).

Further research is required to assess the treatment potential of glucocorticoids, granulocyte-colony stimulating factor (G-CSF) and endometrial scratch (Evers, 2016; Santjohanser *et al.*, 2013; Scarpellini & Sbracia, 2009) as they have shown some beneficial effects. Current evidence does not recommend use of intravenous immunoglobulin or intralipids (Empson *et al.*, 2005; Meng *et al.*, 2016).

1.1.3 Recurrent implantation failure

Definition of Recurrent Implantation Failure

Recurrent implantation failure (RIF) has been used to describe failure of embryos to implant following repeated IVF cycles since 1983 (Potdar *et al.*, 2013). However, there is no unanimous definition of RIF in the number of failed IVF cycles or number of embryos transferred that have not implanted (Polanski *et al.*, 2014; Rinehart, 2007; Simon & Laufer, 2012). The ESHRE PGD Consortium in 2005, suggested that RIF be considered after three or more high quality embryo transfers or implantation failure with transfer of more or equal to 10 embryos in multiple transfers with exact numbers to be determined by each centre (Thornhill *et al.*, 2005). Majority of centres in UK use the definition of ≥ 3 failed IVF/ICSI cycles where good quality embryos were transferred (Tan *et al.*, 2005).

A systematic review assessing the definition of RIF was conducted by Polanski and group in 2014. It found significant heterogeneity in the definitions used. It is also unclear when implantation failure can be diagnosed. There is controversy about whether biochemical pregnancy losses should be classified as implantation failures (Coughlan *et al.*, 2014). In vitro studies have shown that HCG levels increase in developing embryos around day 7 of fertilisation and HCG is an important hormone in the implantation process. However, a clear timepoint to confirm implantation is yet unknown. The authors of the systematic review suggest RIF be defined as absence of implantation, itself defined as a negative serum HCG 14 days after oocyte collection, after 2 consecutive cycles of IVF, ICSI or FET, where the cumulative number of transferred embryos was no less than 4 cleavage stage embryos and no less than 2 blastocyst stage with all embryos being of good quality and of appropriate development stage (Polanski *et al.*, 2014).

The main limitation of the review is the fact that it spans over two decades and the definition of RIF is required to shift and evolve with time, in light of new developments (Polanski *et al.*, 2014). Most important developments are the practice

of transferring blastocyst stage embryos instead of cleavage stage and the move to single embryo transfer instead of multiple, in cycles with good quality embryos.

All of the above, highlights the need for an internationally agreed consensus on the definition of RIF. This is paramount to address the limited scientific evidence and often empirical approach to investigations and treatment of RIF, by enabling research on a focused study population.

The clinical challenge of RIF

RIF is a formidable clinical challenge for clinicians and a devastating scenario for patients. Various investigations and treatment adjuncts have been studied to improve pregnancy outcomes in women with RIF. Not all approaches are evidence-based and concerns have been raised that interventions have been introduced without sufficient supportive evidence.

A good understanding on the factors required for successful implantation is paramount to guide investigations and management in RIF. Implantation is a complex process that involves two players: the mother and the embryo. This cross-talk has been described in a previous section. A supportive maternal host environment and a good quality embryo requires synchronous interaction at the appropriate time (window of implantation) to achieve implantation and ultimately a successful pregnancy.

In the assessment of couples with RIF, there is an overlap with the clinical approach used with those who suffer with RPL. History taking is crucial from both partners and the same modifiable health behaviours are relevant as discussed in a previous section. Couples with RIF can also be affected emotionally and psychologically. There is the added financial burden in proceeding with further IVF/ICSI treatment and need for open discussions on future success rates. Fertility counselling is a crucial resource in supporting these couples in making decisions about treatment, with a pragmatic and considered approach.

In the following paragraphs, I have discussed the possible factors leading to RIF, their credence and respective treatment strategies. Currently, there is little evidence to

guide practice in this difficult clinical challenge and reflects the difference in practice across the UK (Tan *et al.*, 2005). In the midst of this, as clinicians, we meet desperate couples and it is incumbent on us to counsel based on available evidence and chances of success in future treatments.

Treatment strategies in RIF aim to review previous fertility treatment and investigate possible causal associations as a consequence of embryo or endometrial factors.

Embryo factors

The quality of the embryo is influenced by oocyte quality, paternal sperm factors, the ability to fertilise and cleave. In addition, the embryo needs to signal to the maternal endometrium to facilitate implantation. Despite the definition of RIF to only include good-quality embryos, some morphologically normal embryos of good quality still cease to develop in utero and/or fail to implant. This can be due to suboptimal local condition or intrinsic factors within the embryos.

1. **Oocyte quality**

Poor oocyte quality can be established by poor response to ovarian stimulation and functional ovarian tests. The ovarian function tests include antral follicle count, basal FSH levels and anti-Mullerian hormone levels. There is age-related association with increased chromosomal nondisjunction leading to aneuploidy and increase mitochondrial DNA damage (Wang *et al.*, 2009). Obesity also appears to affect the quality of oocyte and follicular development (Fedorcsák *et al.*, 2000). The previous ovarian stimulation protocols used should be reviewed in order to ascertain the possible oocyte contribution to RIF.

2. **Sperm contribution**

Sperm is important in the development of a normal, good quality embryo. The male partner is generally advised to stop smoking and modify lifestyle behaviour.

However, no treatment strategies aimed at improving sperm quality have been shown to improve pregnancy outcome. Investigations and treatments should be conducted in a research setting. Current evidence does not support sperm DNA fragmentation as an important association in RIF or that sperm DNA integrity testing has value (Bronet *et al.*, 2012; Coughlan *et al.*, 2015). The recent RCT comparing physiological, hyaluronan-selected-ICSI (PICSI) and ICSI, recruited 2772 couples, and showed no significant difference in outcomes of live birth rates (Miller *et al.*, 2019). There are significant differences in the way these tests are performed and interpreted.

3. Genetic cause

Structural chromosomal abnormalities in parents can lead to recurrent reproductive failure including implantation failure (Raziel *et al.*, 2002). There is a higher prevalence of balanced translocation in couples with RIF with 2.5% in unexplained RIF (Raziel *et al.*, 2002; Stern *et al.*, 1999). Karyotype testing for specific couples should be considered (Coughlan *et al.*, 2014). In the presence of structural anomaly, pre-implantation genetic diagnosis can be discussed.

However, pre-implantation genetic screening (PGS) in unexplained RIF is controversial. The frequency of aneuploidy is similar in couples with or without RIF (Baart *et al.*, 2006; Pehlivan *et al.*, 2003). In a 2006 Cochrane systematic review, nine trials met the inclusion criteria and the authors found that live birth rate was significantly reduced in the group that had IVF/ICSI with PGS (Twisk *et al.*, 2006). PGS technology has advanced in the last decade and further RCTs are urgently needed to evaluate the risk and benefit of PGS. It is also recognised that chromosomal mosaicism in blastomeres effect a large proportion of human embryos but do not necessarily lead to poor outcome (Harper *et al.*, 1995; Munne *et al.*, 1997; Voullaire *et al.*, 2000).

Maternal host environment

The only evidence-based investigations or treatment to ensure a supportive maternal host environment include identifying abnormal congenital or acquired anatomical factors and impaired endometrial function. There has been no association found between maternal thrombophilia and IVF failure (Martinelli *et al.*, 2003; Simur *et al.*, 2009). Therefore, assessing thrombophilia status should not be performed. Immunologic investigations or treatment are also controversial as evidence is not strong for an association and is conflicting (Blois *et al.*, 2011; Gellersen *et al.*, 2007; Koot *et al.*, 2012; Makrigiannakis *et al.*, 2011).

Next, I discuss the anatomical factors in RIF, the thin endometrium, treatment strategy of endometrial injury in preceding cycle to further IVF/ICSI treatment and endometrial receptivity.

1. **Anatomical factors**

Anatomical factors include the presence of uterine fibroids, uterine septum, uterine synechiae, endometrial polyps and hydrosalpinx. The evidence varies in how each of these factors and their management affect pregnancy success. Evidence is stronger for removal of hydrosalpinx, submucous fibroids and endometrial polyps (Demirol & Gurgan, 2004; Margalioth *et al.*, 2006). With these findings, ART is delayed until these factors are managed surgically to improve success rates of treatment.

Submucous fibroids distorting the uterine cavity reduce pregnancy and implantation rates (Bernard *et al.*, 2000; Farhi *et al.*, 1995; Narayan *et al.*, 1994; Varasteh *et al.*, 1999). The suggested mechanism how distorting fibroids affect pregnancy outcome is by increased uterine contractility, abnormal vascularization and chronic inflammation (Buttram & Reiter, 1981). In a systematic review and meta-analysis, hysteroscopic resection of these fibroids was found to improve pregnancy rates (Pritts *et al.*, 2009). However, there was no improvement in outcomes when myomectomy is performed for intramural fibroids that are not distorting the uterine cavity (Metwally *et al.*, 2011; Pritts *et al.*, 2009; Sunkara *et al.*, 2010).

Endometrial polyps and intrauterine adhesions may interfere with implantation rates (Dawood *et al.*, 2010; Pace *et al.*, 2003; Richlin *et al.*, 2002; Yasmin *et al.*, 2007). Hysteroscopic removal of endometrial polyps is generally performed and a systematic review showed doubling of clinical pregnancy rate in intrauterine insemination cycles following this procedure (Bosteels *et al.*, 2010). However, in a Cochrane systematic review no conclusion could be made on efficacy of endometrial polyp removal (Jayaprakasan *et al.*, 2014). Furthermore, there is insufficient evidence to support hysteroscopic adhesiolysis for intrauterine adhesions due to small numbers in studies and the possibility of recurrence of adhesions (Dawood *et al.*, 2010).

The strongest evidence available on improving pregnancy outcomes for anatomical factors is found following treatment of hydrosalpinxes. It is suggested that hydrosalpinxes can have direct embryotoxic effects, mechanical flush out and negative effect on receptivity of the endometrium (Bildirici *et al.*, 2001; Seli *et al.*, 2005). A prospective randomised controlled trial has shown that salpingectomy over no intervention improves live birth rate (LBR) (Strandell *et al.*, 1999).

In contrast, the most common congenital defect is a uterine septum. There is some evidence that a uterine septum is associated with repeated miscarriages but its role in infertility is unclear (Fedele *et al.*, 2013; Pabuçcu & Gomel, 2004; Raga *et al.*, 1997). There is insufficient evidence to recommend hysteroscopic septum resection outside research projects as reviewed by National Institute for Health and Care Excellence (NICE) guidelines in 2015 titled 'Hysteroscopic metroplasty of a uterine septum for primary infertility'. This conclusion is again confirmed by a Cochrane systematic review in 2017. The results of the nine trials that met the inclusion criteria was conflicting and there are two ongoing trials (Rikken *et al.*, 2017). A limitation of such RCTs is the low number of women with uterine septum and difficulty in recruiting.

2. Impaired endometrial function

Impaired endometrial function is an area that has been researched and multiple therapies have been suggested. However, evidence for the efficacy of these interventions is limited. The aspects of endometrial function that I will explore are 'the thin endometrium', endometrial scratch and endometrial receptivity.

The thin endometrium

One suggested optimum endometrial thickness for ART and implantation is ≥ 9mm (Noyes *et al.*, 1995). More recent studies suggest that an optimum endometrium is one with thickness of 7 or 8mm on day of HCG trigger (Liu *et al.*, 2018; Yuan *et al.*, 2016). This is a marker of endometrial function and ability to support an implanted embryo and placentation. However, a recent meta-analysis has shown that endometrial thickness is a poor predictor of pregnancy outcome casting doubt on the validity of this test (Weiss *et al.*, 2017). In particular, an endometrium thickness < 6mm, presents a difficult clinical situation to manage, 'the thin endometrium' (Abdallah *et al.*, 2012; Shufaro *et al.*, 2008). In a large Canadian cohort study of 24363 fresh and 20114 frozen cycles over 3 years, clinical pregnancy and live birth rate declined with each mm decrease in endometrial thickness <8mm in fresh cycle and <7mm in frozen cycle. However, they conclude that pregnancy outcomes were reasonable for endometrial thickness of 4 to 6mm (Liu *et al.*, 2018). These outcomes may reassure clinicians and patients who have persistently thin endometrium. This is particularly important as no therapy has been found to improve pregnancy outcomes.

The therapies that have been suggested include high dose oestrogen to prime the endometrium, use of aspirin and increasing blood flow to the endometrium (Simon & Laufer, 2012). Sildenafil is an agent that causes vasodilatation and is used to increase blood flow to the endometrium to encourage development of the endometrium in preparation for implantation. However, there have been no high quality randomised controlled trials that demonstrate that this approach is efficacious in improving live birth rates and this view is supported by a recent Cochrane systematic review (Gutarra-Vilchez *et al.*, 2018). Recent research has

explored granulocyte colony-stimulating factor as a treatment option. The evidence is conflicting but has not shown increase in live birth rates (Aleyasin *et al.*, 2016; Kunicki *et al.*, 2017; Zhang *et al.*, 2018).

Endometrial injury in ART

Mechanical stimulation of the endometrium using an endometrial sampling catheter, in the cycle preceding treatment may be beneficial in certain subgroups of women (Narvekar *et al.*, 2010; Raziel *et al.*, 2007). This is supported by a Cochrane review which found increased live birth rate following endometrial injury in women with RIF and > 2 previous embryo transfers (Nastri *et al.*, 2015). Other systematic reviews and meta-analysis found similar results (El-Toukhy *et al.*, 2012; Potdar *et al.*, 2012). It is postulated that this endometrial injury provokes an immune response that encourages the endometrium to prepare for implantation (Gnainsky *et al.*, 2010). More information is needed to understand the underlying process of mechanical stimulation.

Endometrial Receptivity Array test

Endometrial tests such as the Endometrial Receptivity Array test is a novel technique to allow estimation of when the window of implantation will take place. This test examines a small amount of tissue from the endometrial lining and analyses presence of over 200 genes associated with implantation (Garrido-Gómez *et al.*, 2013). Studies have found, this window can vary and better recognition of the 'window of implantation' may suggest options to manipulate this crucial period and facilitate the cross-talk between the embryo and its platform (Sebastian-Leon *et al.*, 2018). Moving the timing of planned embryo transfer to the appropriate time is hoped to improve the chance of successful implantation (Ruiz-Alonso *et al.*, 2013; Sebastian-Leon *et al.*, 2018). A randomised controlled trial is ongoing to assess the efficacy of this approach. Currently, there is still insufficient evidence to determine improvement of pregnancy outcomes.

Recently, the concept that RIF is more than asynchrony of endometrial maturation and therefore receptivity, has emerged. This supports the idea that endometrial receptivity is determined by a multitude of factors (Koot *et al.*, 2012). Advancements in technology have open avenues for future research allowing exploration of gene expression during decidualisation. Research has demonstrated a specific transcriptomic signature in RIF when compared to controls (Koot *et al.*, 2016; Macklon, 2017). Further work, has also shown a differential constitutive disruption of endometrial gene expression in women with RIF (Huang *et al.*, 2017). Gene expression analyses appear able to classify women by severity of RIF phenotype and has the potential to elucidate underlying mechanisms of RIF that are yet unknown.

Management of RIF

Approach to management of couples with RIF should begin with a multi-disciplinary discussion consisting of a senior fertility specialist, senior embryologist and if appropriate reproductive surgeon and fertility counsellor. A thorough review of underlying cause of infertility, previous treatment results, treatment protocol, ovarian stimulation response, oocyte quality, fertilisation rates and quality of embryos should be conducted. This will guide modification and change treatment protocols as required. Any behavioural modifications that need to be undertaken should be discussed. Following this, careful consideration should be given to further investigations or therapies based on current evidence as discussed above. Main treatment strategy is to improve quality of embryo and receptivity of the endometrium.

At present this process is problematic due to the dearth of evidence-based investigations or precision treatments available. Furthermore, there are a large number of expensive but not proven treatments.

For the rest of my thesis, I will concentrate on the endometrial contribution to reproductive failure.

1.2 Endometrial factor in RPL and RIF

In the Brosens research laboratory in Warwick Medical School, the focus has been on investigating the endometrium as a factor in recurrent reproductive failure. The Warwick paradigm is that the endometrium plays a crucial role in natural embryo selection (Brosens *et al.*, 2014; Salker *et al.*, 2010; Teklenburg *et al.*, 2010) and sustaining pregnancies.

1.2.1 Human Endometrium

The endometrium is the inner epithelial layer of the uterus. It is a highly dynamic, cycling tissue that plays an important role in embryo selection.

It consists of three layers: the stratum compactum, stratum spongiosum and stratum basalis. During the proliferative phase of the menstrual cycle, the stratum compactum and stratum spongiosum develop into the stratum functionalis.

The functionalis region is made of the epithelium, endometrial human stromal cells, endometrial glands, immune cells and spiral arteries. The functionalis layer is under the influence of hormonal changes. It thickens with proliferation and changes to a mainly secretory tissue which sloughs off during menstruation. The basalis layer remains following menstruation and contains vessels (short, straight basilar arteries) that spiral arteries originate from (Ganong, 2003).

Before puberty and following menopause, this tissue is morphologically constant. At menarche, the endometrium prepares for embryo implantation in a cyclical pattern dependent on hormonal control of the hypothalamic-pituitary-ovarian axis. The three phases are the follicular phase (proliferative), luteal phase (secretory) and menstruation if no embryo implants. This process is described next.

1.2.1.1 Hypothalamus-pituitary-ovarian axis

Hypothalamic-pituitary-ovarian axis (HPO-axis)

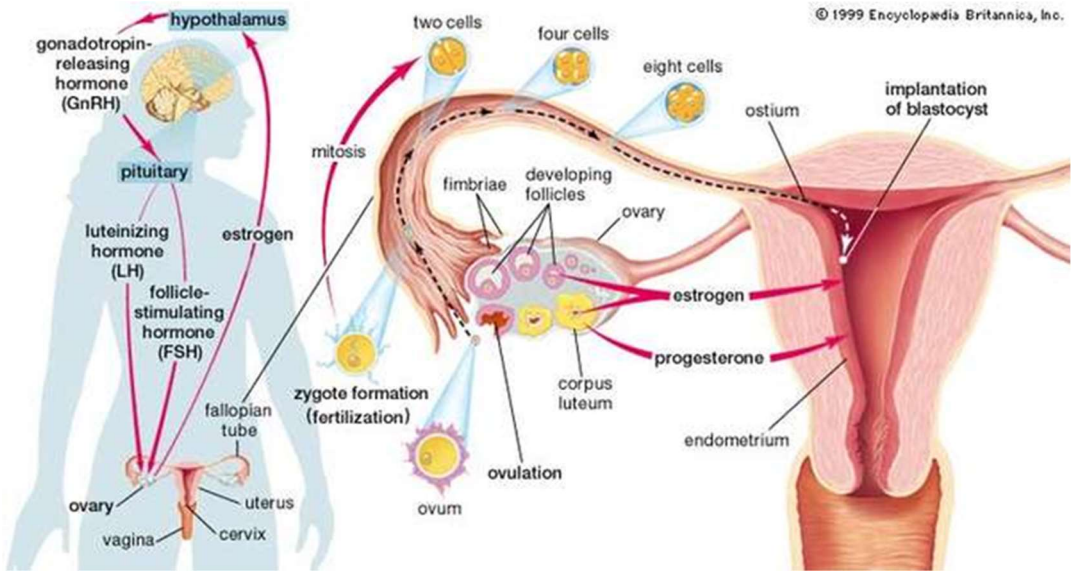

Figure 1. 4: Hypothalamic-pituitary-ovarian axis (HPO-axis) in human menstrual cycle. (By courtesy of Encyclopaedia Britannica, Inc., copyright 2013; used with permission.)

Oestrogen and progesterone are key hormones for steroid action within the endometrial cycle. These hormones are released by the ovaries. The ovaries are regulated by pituitary hormones that are under direct control of the hypothalamus. In the hypothalamus, neurons secrete pulsatile gonadotrophin-releasing hormone (GnRH) which travel along the portal vessels and bind to basophil gonadotroph cells in the anterior pituitary gland. This causes the synthesis and release of gonadotrophin hormones; follicular-stimulating hormone (FSH) and luteinising hormone (LH). FSH and LH regulate ovarian hormone production and the growth and release of oocytes.

Within each menstrual cycle, around 15 to 20 antral follicles are recruited by FSH and LH (Scheffer *et al.*, 2003). This occurs following menstruation at around day 4. At the same time, the smaller follicles release inhibin B which provides negative feedback and inhibits basal FSH secretion. Follicular development and synthesis of oestradiol are dependent on FSH, LH, granulosa cells and theca cells. As the follicles

grow, oestradiol levels increase and exerts a negative feedback on the HPO-axis. This ensures one follicle becomes dominant per cycle and ovulates. When this follicle is mature, high concentrations of oestradiol cause a change to a positive feedback at the pituitary and hypothalamus. This causes the LH surge and ovulation around 36 hours later. The corpus luteum is formed by the remaining granulosa and theca cells following ovulation. The LH surge also causes the luteinisation of these granulosa cells enabling synthesis of progesterone. The corpus luteum produces large amount of progesterone to stabilize the endometrium in preparation for pregnancy. If conception does not occur, the corpus luteum regresses and progesterone levels fall. This withdrawal causes menstruation and FSH to increase for the next cycle.

1.2.1.2 Menstrual cycle

The diagram below illustrates the interaction between hypothalamic-pituitary-ovarian axis and the endometrium.

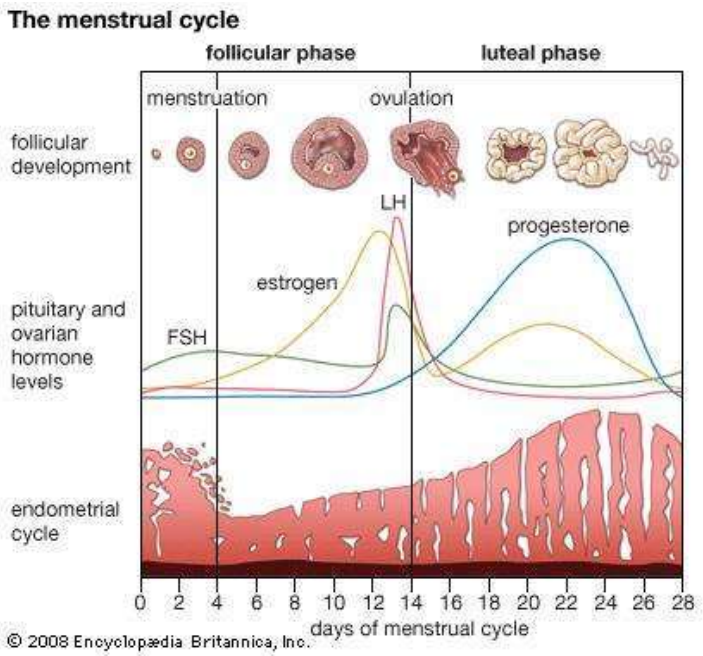

Figure 1. 5: The menstrual cycle. The HPO-axis exerts hormonal control on the ovaries which secrete oestrogen and progesterone. Oestrogen and progesterone drives proliferation and decidualisation of the endometrium. (By courtesy of Encyclopaedia Britannica, Inc., copyright 2013; used with permission.)

Endometrial changes in the menstrual cycle (28 days cycle)

1. **Proliferative Phase (follicular) – Day 4 to 14**

Following menstruation and repair, oestradiol is secreted by the developing ovarian follicles. This leads to endometrial proliferation and stimulates expression of both the oestrogen receptor and progesterone receptor across all cell types (Bouchard *et al.*, 1991). The functionalis layer of the endometrium is the site of intense mitosis creating a thickened endometrium consisting of glands supported by stroma (Brenner *et al.*, 2003a; McClellan *et al.*, 1986). Studies have demonstrated that the basalis layer may not be the source of stem cells for endometrial regeneration but changes in the microenvironment may re-programme the functionalis cells remaining to regenerate a new functionalis layer (Horne & Blithe, 2007).

The endometrial epithelium is formed again and some are ciliated. The endometrial glands grow and lengthen with the increased thickness of the endometrium but are not convoluted and do not secrete. The spiral arteries begin to wind into the stroma. The end of the proliferative phase occurs when oestradiol peak exerts a positive feedback at the pituitary and initiates the LH surge causing ovulation around 36 hours later.

2. **Luteal Phase (secretory)**

The corpus luteum is the remaining granulosa and theca cells following ovulation. It secretes progesterone which drives the transformation of the proliferative endometrium into the secretory structure required for implantation and pregnancy (Horne & Blithe, 2007). The endometrium becomes slightly oedematous and very vascular. The glands become coiled and torturous. They acquire glycogen and release glycoprotein secretions that support implantation. The spiral arteries entwine with the glands and become mature structures. The expression of oestrogen and progesterone receptors disappears about 7 to 10 days following ovulation which corresponds to the usual time of implantation (Wilcox *et al.*, 1999).

The maximal receptive period for an embryo is the implantation window, which usually lies between day 20 to 23.

The length of the luteal phase is remarkably constant at a duration of 14 days.

3. **Menstruation (Day 1 – 4)**

In the absence of conception, progesterone levels fall with the regression of the corpus luteum resulting in the loss of lysosomal integrity (Salamonsen *et al.*, 1999). The functionalis layer undergoes enzymatic degradation, the spiral arteries constrict causing tissue necrosis and the layer is sloughed off in menstrual blood, which does not coagulate due to local fibrinolytic factors (Ganong, 2003).

1.2.2 Assessing endometrial function

1.2.2.1 Decidualisation

Decidualisation is the transformation of endometrial stromal cells into specialized secretory decidual cells that provide a nutritive and immune privileged matrix essential for embryo implantation and placental development (Gellersen & Brosens, 2014). Rising progesterone levels, paracrine signals and cellular cyclic adenosine monophosphate (cAMP) production that occur in the mid-luteal phase of a menstrual cycle initiates decidualisation. Decidualisation begins in the vicinity of the spiral arterioles and then spreads to encompass the whole superficial endometrial layer as the cycle progresses. In conjunction with stromal cell decidualisation, the endometrium undergoes extensive remodelling that is affected by the influx of immune cells, predominantly uterine Natural Killer cells and macrophages.

A significant body of evidence has shown that impaired decidualisation is associated to recurrent pregnancy loss and recurrent implantation failure (Brighton *et al.*, 2017; Brosens *et al.*, 2014; Gellersen *et al.*, 2007; Lucas *et al.*, 2016; Weimar *et al.*, 2013).

Decreased endometrial mesenchymal stem cells (eMSCs) and accelerated stromal senescence has been implicated in the process of impaired decidualisation (Lucas *et al.*, 2016).

An important role of decidualisation is the ability to select normal from abnormal embryos. Decidualised human endometrial stromal cells (HESCs) from women with recurrent pregnancy loss discriminate less effectively between high and low-quality embryos and migrate more readily towards trophoblast spheroids (Salker *et al.*, 2010; Weimar *et al.*, 2012). Due to this, some women with RPL are 'superfertile' as they have increased fecundity due to the loss of endometrial ability for embryo selection and allows implantation of low-quality embryos that are lost during menstruation in normal women but end in miscarriage in 'superfertile' women (Salker *et al.*, 2010). In comparison, the endometrium in women with RIF has the opposite problem of being highly selective by inhibiting recognition and impairing the implantation of good quality embryos.

In the following paragraphs, I explore how decidualisation is important for reproductive fitness in humans.

Reproductive fitness

Historically, knowledge of early reproductive events in humans have been derived from animal models. This is due to the ethical and legal restrictions inherent in this area of study. The mouse model has been the most commonly used due to its predictability, relatively short gestation and ability to deliberately breed mice with absence of a desired gene (Melford *et al.*, 2014). However, as in most mammalian species, the murine embryo signals trigger decidualisation in an oestrous cycle. In contrast, the initiation of decidualisation in humans is uncoupled from embryonic signals (Teklenburg *et al.*, 2010). In humans, 'spontaneous' decidualisation occurs in each menstrual cycle, irrespective of the presence of an embryo (Brosens *et al.*, 2002; Gellersen *et al.*, 2007). This suggests significant difference in early pregnancy developments.

Both humans and mice develop haemochorial placentae which is the most invasive form of placentation. The difference is human embryos are more invasive and implant interstitially, deeply invading stroma cells, while the epithelium is restored over the conceptus (Melford *et al.*, 2014). Therefore, human embryos are deeply invasive and require a large investment from the mother. This investment is mainly for a single human fetus, whereas mice have on average 6 in a litter and can have up to 10 litters a year. Gross embryonic chromosomal abnormalities are also rare in most mice strain (Teklenburg *et al.*, 2010). Conversely, there is significant mosaicism in top-quality human embryos and intrinsic genomic instability. The presence of this genomic instability confers genetic diversity and adaptability; important for complex, high-functioning individuals.

Another difference from the mouse model, is the ability of mice to delay implantation. The murine uterus remains quiescent causing embryos in a blastocyst state to remain dormant (embryonic diapause) (Lopes *et al.*, 2004). A surge in ovarian oestradiol then triggers synchronized implantation of chromosomally stable murine embryos (Hamatani *et al.*, 2004). Reproductive success in mice is based on quantity, rapid breeding cycles, multiple implantation events and litter sizes (Lucas *et al.*, 2013).

In all species, reproduction has a large energy cost and it is beneficial to have ideal conditions to ensure investment in competent embryos and offspring survival. The Warwick group proposes a variable endometrial strategy for human reproductive success which safeguards the mother against prolonged investment in invasive but developmentally abnormal embryos. In humans, the endometrium plays an active role in implantation and as an embryo sensor. The process of cyclic decidualisation and menstruation is believed to be an adaptive response to the high incidence of chromosomal instability in human embryos (Teklenburg *et al.*, 2010). Decidualising stromal cells appear to have the ability to respond to individual embryos and either promote implantation and further development or facilitate early rejection (Macklon & Brosens, 2014). Therefore, failure or inadequacies of this process may disable natural embryo selection and lead to recurrent reproductive failure.

The 'permissive' and 'selective' endometrium

Cyclical decidualisation enables the endometrium to adapt and rebalance its permissive and selectivity traits. The endometrium is termed permissive during its receptive phase or window of implantation and selective out of this phase (Gellersen & Brosens, 2014). In an ordered decidual response, the endometrial stromal cells are able to discriminate low- and high-quality embryos during the window of implantation (Macklon & Brosens, 2014). This system is capable of disposing of incompetent embryos prior to implantation and safeguarding the mother from investing in abnormal embryos. There are also additional mechanisms following implantation to ensure timely rejection of failing pregnancies. An embryo that secretes insufficient HCG is deemed to lack fitness and is unable to rescue the corpus luteum to support pregnancy leading to early miscarriage in the first few weeks. Beyond this near the end of the first trimester, the transition from histiotrophic nutrition to active maternal perfusion of the placenta serves as a robust stress test of the feto-maternal interface (Hempstock *et al.*, 2003; Jauniaux *et al.*, 2000). This ensures that if a pregnancy is poised to fail, it is most likely to fail before 12 weeks gestation. The balance of endometrial and embryonic phenotypes is postulated to ensure the likelihood of a successful pregnancy outweighs that of a clinical miscarriage (Gellersen & Brosens, 2014).

Research has shown a prolonged and disordered proinflammatory decidual response in women with RPL (Salker *et al.*, 2012). This proinflammatory decidual phenotype prolongs the window of receptivity and loses its ability to discriminate between low- and high-quality embryos in cell migration assays (Salker *et al.*, 2010; Salker *et al.*, 2011; Weimar *et al.*, 2012). This highly receptive endometrium allows unhindered implantation leading to high number of subsequent pregnancy failures due to implantation of incompetent embryos. This paradigm can also explain the concept of 'superfertile women' who conceive rapidly but also have increased pregnancy losses due to aberrant embryo selection by the endometrium (Macklon *et al.*, 2002; Salker *et al.*, 2010; Wang *et al.*, 2003). Conversely, it is postulated that

excessive or lack of decidualisation could increase the barrier function of the endometrium leading to implantation failure and conception delay (Gellersen & Brosens, 2014). This may explain some of the underlying mechanisms in women with RIF.

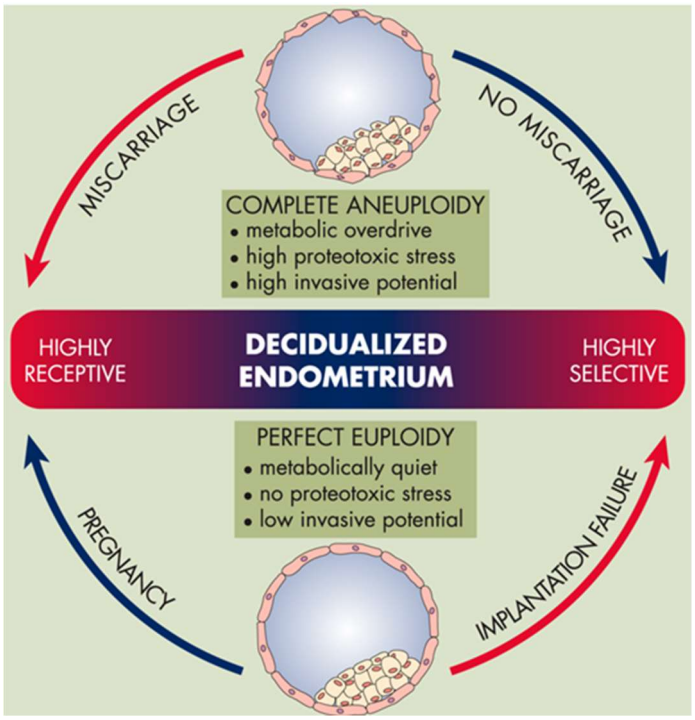

Figure 1. 6: Variable strategy of human endometrium in reproductive fitness. *Gellersen, B. & Brosens, J. J. (2014) Cyclic decidualization of the human endometrium in reproductive health and failure. Endocr Rev, 35 (6): 851-905, by permission of Endocrine Society and Oxford University Press.*

The above figure shows the interaction between the maternal endometrium and the embryo. A highly receptive endometrium encourages pregnancy but losses its ability to discriminate between high and low- quality embryos leading to higher incidence of miscarriages. A highly selective endometrium discourages implantation and may cause implantation failure of good quality embryo. However, miscarriages are less likely to happen as no implantation occurs.

Differences in RPL and RIF

Research has often combined RPL and RIF under the term recurrent reproductive failure. However, emerging evidence suggests that these are separate clinical entities that may sit on opposing ends of a spectrum of endometrial phenotypes. In reality, women's endometrial phenotype likely changes on the scale of permissive and selective traits through adaptations in menstrual cycles. Menstruation showcases the remarkable ability of the endometrium to regenerate and recruits an abundance of adult stem cells (Du *et al.*, 2012; Du & Taylor, 2010; Gargett *et al.*, 2012; Gargett & Ye, 2012).

In RPL, the endometrium appears to be highly permissive and has lost its natural embryo selection ability. However, the chances of successful pregnancy outcomes following 3 miscarriages are good with or without intervention. This may be attributed to inter-cycle variation of the endometrium and exposure to a competent embryo following a high incidence of embryo chromosomal abnormalities.

However, it is postulated that women with RIF and no history of RPL, have a highly selective endometrium. This endometrial phenotype does not only dispose of abnormal embryos but also competent ones prior to implantation. Therefore, no implantation takes place despite good quality embryos and women do not experience miscarriages. The underlying mechanism is still unclear but this paradigm supports the idea that RPL and RIF are a result of different aberrant processes (Gellersen & Brosens, 2014).

If this disparity is true, then the future enrichment of study populations in recurrent reproductive failure must recognise the difference in these two groups. Further studies examining these mechanisms and endometrial plasticity are important to understanding the underlying pathophysiology before precision interventions can be developed that ensure reproductive fitness.

1.2.2.2 Endometrial function investigations

It is clear that the human endometrium is a dynamic tissue that undergoes cyclical changes each month and requires balanced haemostasis to perform its role effectively. Research effort has focused on assessing endometrial function in the mid-luteal phase, when investigating underlying pathology and possible therapies for RPL or RIF. Assessment at this time provides information on the preparation of the endometrium for implantation. The aim of research is to identify abnormalities that lead to a highly receptive or restrictive endometrium leading to RPL or RIF, which would direct focused therapy. The following explores methods of investigation using mid-luteal endometrial biopsies.

1. **Endometrial histological dating**

As the basalis layer remains constant and is not under the influence of hormones, endometrial dating is performed on the functionalis layer. Traditionally, Noyes criteria has been used (NOYES, 1956; NOYES, 1963). It was developed on the morphological characteristics associated with the proliferative and luteal phase of a menstrual cycle.

The limitations of this method include the poor inter-observer reproducibility (Coutifaris *et al.*, 2004; Lessey *et al.*, 2000; Myers *et al.*, 2004). Histological classifications also do not address functional changes as seen with proliferative markers or steroid receptor expression. Detection and counting of proliferative cells are a better method to use in the proliferative phase. This has been measured using differing antibodies in immunohistochemistry and an example is Ki67 (Gerdes *et al.*, 1983) and phosphorylated histone H3 (Brenner *et al.*, 2003b). In addition, the technique of endometrial biopsies through Pipelle sampling disrupts the spatial arrangement of tissue and does not represent the overall complexity for accurate histological dating (Horne & Blithe, 2007).

2. Genomic expression

Research has studied genomic analysis as a means to classify endometrial biopsies into stages based on gene expression in each phase of the menstrual cycle. This would help overcome the inaccuracy found in histological endometrial dating. The Endometrial Receptivity Array test (ERA) used to investigate recurrent implantation failure and time the 'window of implantation' is an example. These methods are still being researched and requires specific expertise (Ruiz-Alonso *et al.*, 2013; Sebastian-Leon *et al.*, 2018). Whilst the ERA test offers promise for improving endometrial function prior to embryo transfer, the test along with the suggested management of delaying transfer or increasing progesterone administration has not been proven with a published randomised-controlled trial.

Genomic expression patterns in the endometrium can be separated based on steroid receptor patterns and the phase of the menstrual cycle with different effects in the stromal cells or the epithelial cells (Lessey, 2003; Talbi *et al.*, 2006). Genes are expressed dependent on the roles required in each phase of the menstrual cycle (Houshdaran *et al.*, 2014; Simon *et al.*, 2009; Zelenko *et al.*, 2012; Zhang & Ho, 2011).

3. Uterine immune system

Pregnancy is a unique and challenging condition for the maternal immune system. The maternal immune system must exhibit tolerance to the semi-allogenic embryo, while retaining appropriate immune responses to protect against local immune insults. This immunological paradox invokes multiple complex, overlapping and redundant mechanisms (Hyde & Schust, 2016). The mechanisms underpinning immune tolerance are crucially time and site specific. Understanding this process is complicated by limitations of human studies for logistic and ethical reasons. Hence, some insights have been garnered from animal studies only.

The immune system has two parts; the peripheral and mucosal system. The peripheral system provides defence against blood-borne pathogens through functions of spleen, lymph nodes and peripheral blood. The mucosal or innate

immune system protects against pathogens that gain entry to the body through mucosal surfaces; at the respiratory, gastrointestinal and genitourinary tract. The innate immune system compromises of T cells, B cells (including IgA-secreting plasma cells), NK cells, macrophages and dendritic cells (Hyde & Schust, 2016; Kutteh, 2014).

It is the innate immune system at the maternal-fetal interface, that confers immune tolerance needed for the establishment and maintenance of pregnancy in the pre- and peri-implantation period (Veenstra van Nieuwenhoven *et al.*, 2003). This local system is unique as it has hormonal regulation in a tissue which requires constant transformation and adaptation. There are a series of mechanisms that exert this immune tolerance; which include absence of classical major histocompatibility complex (MHC) molecules on sperm, embryonic and trophoblast cells, alteration toward anti-inflammatory Th2 driven system and a unique population of immune cells (Hyde & Schust, 2016).

Immune cell population

- Decidual granular lymphocyte (uNK cells)

The uterine Natural Killer (uNK) cells is the most abundant lymphocyte in the endometrial immune system during late luteal phase and early pregnancy (Johnson *et al.*, 1999; Loke & King, 2000a; Loke & King, 2000b; Vince & Johnson, 2000). It is the major immune cell population at the early feto-maternal interface and the key regulators in supporting trophoblast invasion and of the maternal uterine vasculature remodelling (Hanna *et al.*, 2006; Kopcow *et al.*, 2005). This is one of the reasons that it has been extensively studied.

Uterine NK cells express CD56+ CD16- a less toxic cell population compared to peripheral NK cells which mainly express CD56+Cd16+, a cytotoxic subset (Lee *et al.*, 2011). Uterine NK cells begin to increase in the mid-secretory phase and continues with a rapid rise in late luteal phase reaching 70% of uterine leucocytes (Flynn *et al.*, 2000). The levels reach a peak at the end of the first trimester (King *et al.*, 1991).

These findings suggest uNK cells play a crucial role in the establishment and maintenance of a pregnancy. Active uNK cells secrete IL-15 and increase production of cytokines, growth and angiogenic factors (Hanna *et al.*, 2006). In this manner, uNK cells contribute to embryo implantation and decidualisation of the endometrium (Manaster *et al.*, 2008). More recent research has found that uNK cells also play a role in clearing endometrial senescent cells creating space within the functionalis layer of the endometrium which facilitates invasion of the implanting embryo (Brighton *et al.*, 2017).

- NKT cell

NKT cells exhibit both NK and T cell characteristics (Boyson *et al.*, 2002; Godfrey *et al.*, 2000). NKT cell expansion is mediated by fetally expressed MHC Class I molecule from paternal genome in mice (Dang & Heyborne, 2001). NKT cells are also present in human decidua and may represent a mechanism of fetal modulation on maternal immune response to induce tolerance and be involved in formation of Th2-dominant environment (Hyde & Schust, 2016).

- T cell regulation

T cells are present in low numbers in the late secretory phases (Johnson *et al.*, 1999; Loke & King, 2000c; Vince & Johnson, 2000). The balance between effector and regulatory T cells are important for feto-maternal immune tolerance.

MHC Class I molecules present endogenous antigens to T cell receptors (TCR) on CD8+ T cells. Although gametes and most placental cells are MHC Class I and II negative, the extra villous trophoblast cell does express non-classic MHC Class I molecules HLA-G, HLA-E and HLA-C (Hyde & Schust, 2016). This pattern of MHC expression may limit allorecognition reactions and promote uNK cell-mediated cytokine modulation and maternal vascular remodelling (Hyde & Schust, 2016).

TCR on CD4+ T cells recognise antigen presented by MHC Class II molecules on antigen presenting cells and is a defence mechanism against exogenous pathogens (Hyde & Schust, 2016). These CD4+ cells are T helper and regulatory T cells.

Successful implantation requires tight control of inflammatory-type reactions. In particular, the Th 1 pro-inflammatory response which is seen in transplanted graft rejection (Zenclussen, 2013). Naive T helper cells (Th0) mature and differentiate depending on their cytokine microenvironment. T cells producing type 1 cytokines (Th1) causes a pro-inflammatory reaction in the initial process of embryo implantation and invasion of the endometrium (Mor *et al.*, 2011). This is followed by a shift to an anti-inflammatory phase with T cells releasing type 2 cytokines (Th2) to establish the embryo, support rapid fetal growth and development by maintaining uterine quiescence (Szekeres-Bartho & Polgar, 2010). Other T helper cells include regulatory T cells (T regs) which are potent suppressors of inflammatory type 1 immune responses by suppressing inflammatory cytokine INF-γ secretion (Mao *et al.*, 2010). T regs also suppress secretion of IL-4 against paternal antigens (Mjösberg *et al.*, 2007).

There are also many co-stimulatory signals which control response to antigens and assist immune tolerance by suppressing T cell activation, adaptations to promote antigen tolerance and T cell apoptosis (Freeman *et al.*, 2000; Nagamatsu *et al.*, 2011; Petroff & Perchellet, 2010).

- Dendritic cells and decidual macrophages

Dendritic cells and decidual macrophages are the major antigen presenting cells in the endometrium (Plaks *et al.*, 2008). The colony-stimulating factor (CSF-1) secreted by endometrial epithelium, is the main regulator of mononuclear phagocytic lineage and controls the function of these two cell types (Pollard, 2008). Both affect immune tolerance and regulate angiogenesis (Nagamatsu & Schust, 2010).

- B lymphocytes

There are low numbers of B lymphocytes in the decidua, which aids in immune tolerance while retain pathogen surveillance ability (Aït-Azzouzene *et al.*, 1998; Kutteh, 2014; Muzzio *et al.*, 2016). These cells produce antibodies as a central component to the humoral immunity (Hyde & Schust, 2016).

Reproductive Hormonal Control

Hormonal control of immune modulation and maintenance of semi-allogeneic fetus is provided by oestrogen, progesterone and human chorionic gonadotrophin (hCG) (Siiteri *et al.*, 1977; Wira *et al.*, 2014). High levels of oestrogen and progesterone appears to promote Th2 immune responses and enhance recruitment and proliferation of T regs (Correale *et al.*, 1998; Cutolo & Wilder, 2000; Mao *et al.*, 2010; Salem *et al.*, 2000). HCG also sustains an anti-inflammatory effect and is a chemoattractant to T reg cells at the maternal-fetal interface (Schumacher *et al.*, 2009).

In summary, the local immune system of the endometrium and of the maternal-fetal interface is a highly complex, multi-faceted process that is still not fully understood. Disruptions and aberrations in this process can lead to several complications such as RPL, RIF, preterm birth, pre-eclampsia and restricted fetal growth.

1.3 Uterine Natural Killer (uNK) cell test

One area of research, centres on the link between uNK cells and reproductive failure. As previously discussed, these lymphocytes are part of the innate immune system and are the most abundant lymphocyte found in the endometrium. uNK cells show the ability to interact with fetal trophoblasts and have a role in decidualisation, angiogenesis and clearance of acute cell senescence.

Studies in luteal phase endometrium have shown that women with recurrent reproductive failure have higher density of uNK cells than controls (Clifford *et al.*, 1999; Quenby *et al.*, 1999; Tuckerman *et al.*, 2007). The uNK cell density test is performed in mid-luteal endometrial biopsies. This test has been adopted by many centres across the world to assess women with RPL or RIF. UNK cell density correlated well with in vitro models of decidualisation suggesting that the uNK cell density is functionally important (Kuroda *et al.*, 2013). However, very little

prospective data exists to assess how good the test is at predicting pregnancy outcome (Tang *et al.*, 2011).

The normal range for uNK cell density was set by using a range determined from a group of women attending hospital in the mid-luteal phase for a laparoscopic sterilisation having completed their family with two or more live births (Quenby *et al.*, 1999; Quenby *et al.*, 2005). However, this normal range was based on a low number of samples. Recent research in China has supported these findings of a normal value of ≤ 5% (Chen *et al.*, 2017). However, we know that uNK cells increase throughout the menstrual cycle (Russell *et al.*, 2011; Russell *et al.*, 2014; Russell *et al.*, 2013) and it would be ideal to have a normal range of uNK cell density for each day of the menstrual cycle. Furthermore, to improve accuracy of testing, a UK consensus was reached and the test standardised for reliability and reproducibility across centres (Lash *et al.*, 2016).

When uNK cell density is above normal values, they appear to increase peri-implantation angiogenesis which may lead to pregnancy failure due to excessive oxidative stress (Quenby & Farquharson, 2006; Quenby *et al.*, 2009). In our research unit, recent work has shown that uNK cells are important in clearing senescent endometrial stromal cells to create spaces within the endometrium that supports implantation of an embryo (Brighton *et al.*, 2017). An imbalance of senescent cells and uNK cells disrupts this process.

It has been demonstrated that high uNK cell density can be reduced with prednisolone therapy (Quenby *et al.*, 2005). In theory, reducing high uNK cell density would lead to increased rate of live births. However, the prognostic value on pregnancy outcome of treating high uNK cell density still remains uncertain (Dan *et al.*, 2015; Tang *et al.*, 2009; Tuckerman *et al.*, 2007). This therapy also has significant adverse effects in pregnancy and it is not recommended outside a research setting. In addition, the test has the inherent error of interobserver variation and timing of the biopsy.

1.3.1 Normalisation for day of cycle

Work in our research group has investigated improving the uNK cell density test by normalising for day of cycle when the endometrial biopsy was performed. This work was instigated because uNK cell density increases throughout the menstrual cycle. The normalisation heat-map was developed based on 1997 samples and this has recently been published (Brighton *et al.*, 2017). To obtain confidence intervals, uNK cell density at each day of the cycle were fitted into a beta distribution with the 'fitdistr' function from the MASS package version 7.3-45 (Venables & Ripley, 1997) in R version 3.2.1 (Team, 2015). The resulting percentile values were smoothed with spline interpolation using the spline function of the basic statistics package of R and converted into a heatmap using the Multi Experiment Viewer (MeV) version 4.9.0 (Saeed *et al.*, 2003). In this model, values above the 75th centile is high and above 95th centile is very high. This is illustrated in Figure 1.10.

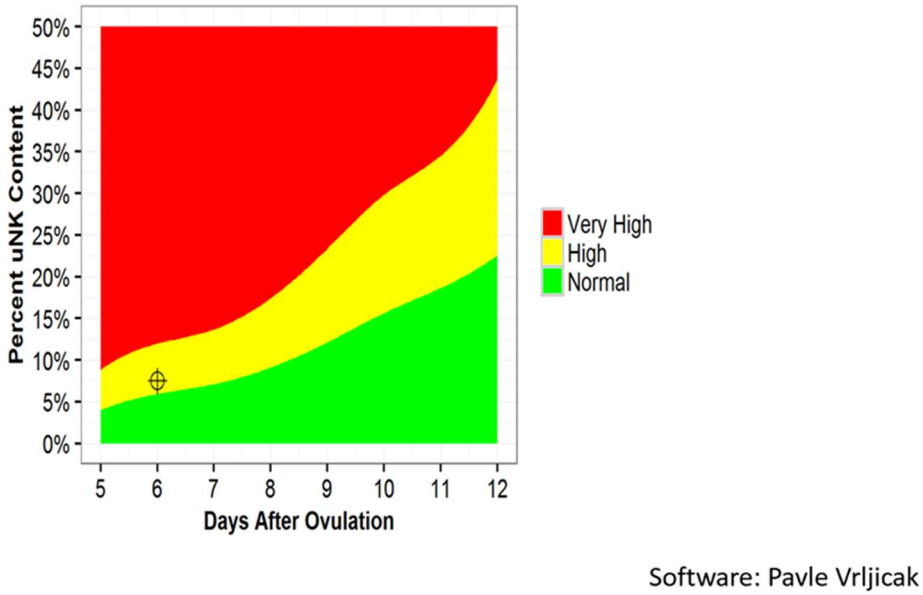

Figure 1. 7: Graph of uNK centiles based on 1997 samples of women with recurrent reproductive failure. (Images courtesy of Pavle Vrljicak and Biomedical Research Unit in Reproductive Health, joint collaboration with University of Warwick and UHCW NHS Trust). This normogram shows uNK values increase as day of cycle progresses in the menstrual cycle. uNK values between 75th and 95th centile is high and above 95th centile is very high.

1.4 Combined endometrial factor predictive test

Other groups have previously investigated combined endometrial tests. Recent work by Liu and colleagues, looked at combining the uNK density test with histological dating of endometrium to predict pregnancy outcomes (Liu *et al.*, 2014). Their results show that uNK cell density on its own was not statistically significant in outcome prediction but histological dating improved this. Another group perform uterine immune profiling with uNK cell density and levels of activation or proliferation using markers of IL-15 and IL-18 to predict pregnancy outcome and form treatment strategies (Lédée *et al.*, 2016; Lédée *et al.*, 2017).

Research from our group demonstrated that endometrial cell senescence increases during decidualisation. uNK cells have a role in clearing the senescent cells to form space within the endometrium to encourage implantation of an embryo. Therefore, it would be expected that senescence and uNK cell activity change together and can affect pregnancy outcome. On this basis, I aim to investigate a combined test of uNK cell density and cell cycle markers to evaluate its use as a predictor of subsequent pregnancy outcomes in women with RPL.

1.5 Cell cycle markers

I have reviewed the evidence of assessing decidualisation by histological findings using Noyes' et al criteria. However, this does not describe the physiological processes during decidualisation. This justified my investigation into the use of cell cycle markers. The markers of interest are that of proliferation Ki67 and senescence with loss of HMGB2 or presence of P16. I theorise that proliferation would be maximum in the proliferative phase but begin to decline in the luteal phase where senescence would become more dominant. Looking at this balance may identify endometrial mechanisms that are not synchronised or have abnormal timing.

Ki67 (Antigen KI-67) is a nuclear protein with a role in cellular proliferation and is associated with ribosomal RNA transcription (Shiozawa *et al.*, 1996). It is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). HMGB2 is the high-mobility group protein B2 which is part of the non-histone chromosomal high-mobility group protein family. The function of this is to promote DNA flexibility and repair. It maintains clonogenic populations in adult tissue. The loss of HMGB2 staining is a hallmark of replicative senescence and impaired decidualisation (Lucas *et al.*, 2016). P16 is a cyclin-dependent kinase inhibitor 2A. It is a tumour suppressor protein. It decelerates cell progression from G1 phase to S phase. Increased expression of the p16 gene as organisms age reduces the proliferation of stem cells. This reduction in the division and production of stem cells protects against cancer while increasing the risks associated with cellular senescence as is seen in cervical neoplasia (Griffin *et al.*, 2015).

1.6 Hypotheses and objectives of project

The objective of this research project is to develop and validate a pre-conceptual endometrial test that combines the endometrial cell cycle process and immune status to improve prediction of subsequent pregnancy outcome in women with recurrent pregnancy loss. I hypothesize that a pre-conceptual endometrial combined test would more accurately reflect the complexity of decidualisation, as it provides information about several processes that interplay to create a balanced microenvironment suitable for implantation. On this basis, I further hypothesize that this combined test would yield results that improves prediction of pregnancy outcome.

I have chosen to use uNK cells as they are the key immune component and most abundant immune cell in relation to decidualisation. It also has an important role in vascular remodelling and clearing senescent cells in preparation for the implantation window. I will use cell cycle markers to explore the interplay between uNK cell activity and proliferative or senescence of endometrial stroma, glandular and

luminal cells. I will use the established method of the uNK cell density test and incorporate work within our research group that has looked at normalising uNK cell density for day of cycle (uNK centile). Following this, a combined test measuring cell proliferation and senescence using immunohistochemistry (IHC) cell cycle markers would be designed.

As the first measure, I will assess the ability of the uNK cell density test in predicting pregnancy outcome in women with recurrent reproductive failure, to confirm or refute previous findings in the literature. I will then compare this to the predictive ability of the developed combined test and aim to validate the results in the setting of a prospective randomised-controlled trial.

CHAPTER 2:
MATERIALS AND METHODS

Chapter 2: Materials and methods

2.1 Ethical Approval

This research was supported by the Biomedical Research Unit in Reproductive Health; a collaboration between University Hospitals of Coventry and Warwickshire (UHCW) NHS Trust and the University of Warwick. The study was approved by the National Research Ethics – Hammersmith and Queen Charlotte's & Chelsea Research Ethics Committee (1997/5065), West Midlands – South Birmingham Research Ethics Committee (15/WM/0295) and the local Research and Development department at UHCW NHS Trust. Written informed consent was obtained from the women who attended the clinic for research endometrial samples. The study period was between December 2011 and December 2017.

2.2 Patient selection and pregnancy outcome database

The research was completed through the tertiary referral Implantation Clinic, a dedicated endometrial research clinic at UHCW NHS Trust. Women with recurrent reproductive failure (RPL and RIF) attend this clinic from across the United Kingdom. All women have their medical history recorded by medical staff and is updated to a clinical database. The information includes details of previous pregnancy outcomes, fertility treatment and time to pregnancy interval.

The clinical database is manually updated with future or ongoing pregnancy outcomes by investigators and relies on patient self-reporting of outcomes and local routine medical care. The subjects in this study were identified through this clinical database and a separate pregnancy outcome database was extracted from this information, based on pre-determined inclusion criteria.

In the initial evaluation of the uNK test, I used the pregnancy outcome database and identified 281 women with RPL and RIF who had a pregnancy within one year of their

mid-luteal phase endometrial biopsy. These biopsies were obtained between December 2011 and October 2015. The two outcome measures of interest were first trimester miscarriage (M) and live birth or pregnancy beyond 10 weeks' gestation (LB/O).

Of these women, 20 were included in the exploratory test set to explore the association of cell cycle markers and pregnancy outcome. The associations found in the exploratory test set was used to design the pre-conceptual endometrial test in a larger study of 89 women. The criterion for the larger study were age under 40, history of recurrent pregnancy loss and biopsies taken between days 7 to 9 after LH surge.

In the validation of the pre-conceptual endometrial test, I analysed 53 research endometrial samples that were collected through our local, pilot randomised-controlled trial of 'Scratch in Miscarriage' (SiM).

Finally, using the same clinical database mentioned previously, women who had two mid-luteal biopsies between one to six months apart and a pregnancy outcome within one year of the second biopsy were analysed. These biopsies were obtained between January 2016 and December 2017. 60 women who fit these criteria were identified and they included women with either recurrent pregnancy loss or implantation failure. This study sample of 60 women were analysed in this project to investigate the effect of endometrial biopsy or 'scratch' on uNK cell density and inter-cycle variation.

2.3 Endometrial sampling

In the Implantation Clinic, pre-conceptual mid-luteal phase endometrial biopsies were taken for the uNK cell density test and laboratory research. Written informed consent was obtained from women who attended the clinic after discussion of risk factors. The risk factors include vaginal spotting, vaginal bleeding and lower abdominal pain.

These biopsies were obtained using an endometrial sampler (Wallach Endocell ^{TM} sampler, Wallach Surgical Devices Inc., Trumbull, USA) and were timed at 6 to 12 days after the LH surge. The procedure was performed by first completing a transvaginal ultrasound scan to assess appropriate endometrial thickness and exclude uterine or endometrial pathology. A Cusco's speculum was then used to visualise the cervix and a disposable endometrial sampler was inserted through the cervical canal into the uterine cavity. Drawing back on the inner piston creates a vacuum suction and an endometrial sample is obtained by withdrawing the sampler in a rotational technique from the uterine fundus.

These endometrial samples are fixed in 10% neutral buffered formalin at 4^0C and processed by our research Arden Tissue Bank. Here, the samples are processed to create wax blocks which take 14 hours. In this procedure, tissue is dehydrated through a series of graded ethanol baths to displace the water, and then infiltrated with paraffin wax. The infiltrated tissues are then embedded into wax blocks. Once the tissue is embedded, it is stable for many years allowing storage of tissue for further research.

2.4 Immunohistochemistry

2.4.1 Materials

1. Adhesive microscope slides - Leica Xtra® adhesive slides, Leica

Biosystems (UK) Ltd, Milton Keynes, UK

2. Distilled water

3. Hematoxylin (<0.1%) - Leica Biosystems (UK) Ltd, Milton Keynes, UK

4. Isopropyl Alcohol

5. Phosphate buffered solution (PBS)

6. Primary antibody – 1:200 dilution (Novocastra™ Mouse Monoclonal antibody

NCL-L-CD56-504) - Leica Biosystems (UK) Ltd, Milton Keynes, UK

7. Primary antibody – 1:5 dilution (CINtec® clone E6H4 $p16^{Ink4a}$ antibody) -

Roche, Basel, Switzerland

8. Primary antibody – 1:500 dilution (HMGB2)

9. Primary antibody – 1:200 dilution (Ki67)

10. Sodium citrate buffer; 10mM sodium citrate, 0.05% Tween-20, pH 9 - Leica Biosystems (UK) Ltd, Milton Keynes, UK

11. Tris-buffered saline plus 0.05% tween20 (TBST)

12. Xylene

13. 3% (v/v) hydrogen peroxide - Leica Peroxidase Block, Leica Biosystems

(UK) Ltd, Milton Keynes, UK

14. 3, 3’ – diaminobenzedine (DAB)

2.4.2 Slide preparation and staining

The tissue wax blocks of identified subjects for cell cycle analysis were obtained from the Research Arden Tissue Bank. Each wax blocks were sliced into 3µm thick sections on a microtome. An experienced operator performed the sectioning to achieve 10 accurate serial tissue sections. The tissue sections were adhered to microscope coverslips by overnight incubation at 60°C. For each subject, one of the serial sections were stained using immunohistochemistry (IHC) with antibodies for markers of CD56 (uNK), p16 and HMGB2 (cell senescence) and Ki67 (cell proliferation).

Each antibody is made with the following dilution: CD56 1:200, Ki67 1:200, HMGB2 1:500 and p16 1:5. 30 slides are run within one cycle on the Leica Bondmax autostainer. In each cycle, 5 patients' samples were processed which included 4 IHC antibody stains per patient and 1 control slide per antibody. The IHC protocol used can be found in Figure 2.1.

The immunohistochemistry staining was performed using an automated process with the Leica Bondmax autostainer (Leica Biosystems) and a dedicated research technician performed this step. This automated process begins with de-waxing the paraffin sections using xylene and rehydration. Antigen retrieval is performed with sodium citrate antigen retrieval solution (sodium citrate buffer; 10mM sodium citrate, 0.05% Tween-20, pH 9) to make epitomes clear. Hydrogen peroxide follows to halt peroxidase activity while binding and removing blood cells. The tissue sections are then ready for staining with required antibodies (CD56, p16, HMGB2 and Ki67). The process is completed with DAB colour development and Haematoxylin counter stain. The dehydrated and cleared coverslip sections are processed through the Mirax Midi slide scanner, to obtain bright-field images which were analysed using Pannoramic Viewer v1.15.4 (3DHISTECH Ltd, Budapest, Hungary) software.

Immunohistochemistry process

E.g. CD56

Time	Process
14 hours	**A.** Preparing to wax block Formalin (fix) → Alcohol (dehydrate) → Xylene (attach wax) → Wax Block
	Slicing wax block to 3 micrometres onto slide
3 hours (1 cycle; 30 slides)	**B.** Leica machine Reverse process to wash off wax and rehydrate Buffers used to make epitome clearer Peroxide used to bind and remove blood Custom antibody to CD56 (mouse) Add human antibody to mouse labelled H-DAB Haematoxylin stain (H&E) Batch control – negative CD56 and positive control small bowel
	Covered with plastic film and then scanned into a MIRAX file

Figure 2. 1: IHC protocol. A. Process of preparing tissue wax block. B. Automated IHC staining process using the Leica Bondmax Machine and MIRAX scanner. The flowchart above demonstrates the IHC protocol used in this research project.

The tissue blocks obtained from the research tissue bank had previously undergone 14 hours of processing to create the wax block. Serial sectioning was performed on the tissue blocks to allow for the 4 different antibody stains (CD56, HMGB2, p16, Ki67). In the above example using CD56 antibody staining, the IHC process for 30 slides per cycle was completed in 3 hours. The principal of IHC is removing wax and rehydrating slide, buffering to make epitomes clearer, remove blood with peroxide, apply custom antibody then human antibody labelled H-DAB, followed by haematoxylin staining. Batch controls are used for quality control in each cycle.

2.4.3 Analysis and interpretation

IHC staining analysis

The MIRAX files created are opened using Pannoramic Viewer software in wide-screen view. Each patient file contains an image of CD56, p16, HMGB2 and Ki67 IHC stain. Within the Pannoramic viewer software, the 3 matched sites at 20x magnification for each IHC stain is captured and saved as a Tiff file. Therefore, each patient would have 12 Tiff files which are then processed in Image J software (Rasband W.S. ImageJ, National Institutes of Health).

The sites that are chosen must include an epithelial lining as it standardises the depth of tissue for analysis. This avoids inconsistencies in the densities of IHC markers measured. The 3 equal sites for each IHC stain (CD56, HMGB2, p16, Ki67) is used to obtain an average value for the whole sample. Figure 2.2 and 2.3 illustrates these processes.

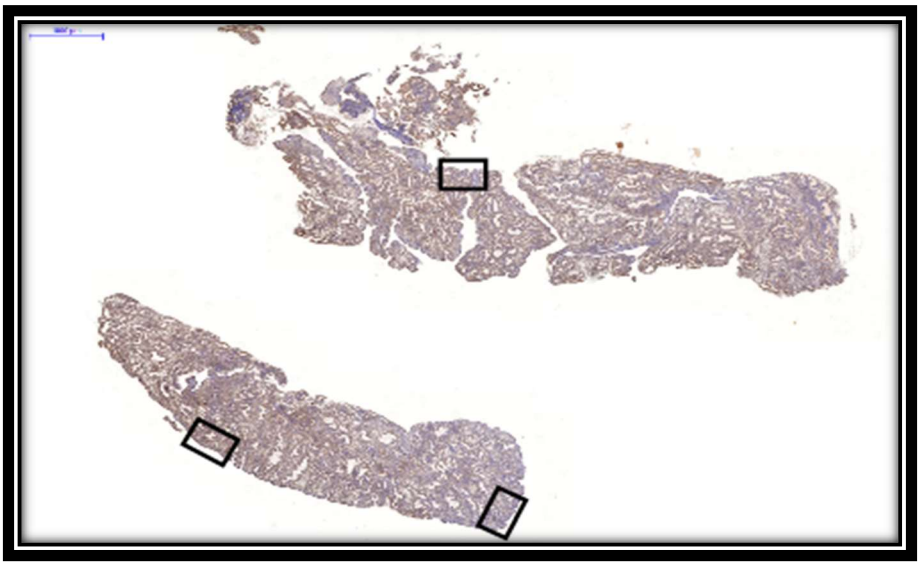

Figure 2. 2: MIRAX file in Pannoramic Viewer software. Low power magnification of a tissue section stained with CD56 showing how three representative areas would be chosen for analysis at higher magnification.

The above image is an example of a stained section for CD56 marker. Within each MIRAX file 3 areas are selected to calculate the average density of IHC marker for the corresponding sectioned slide.

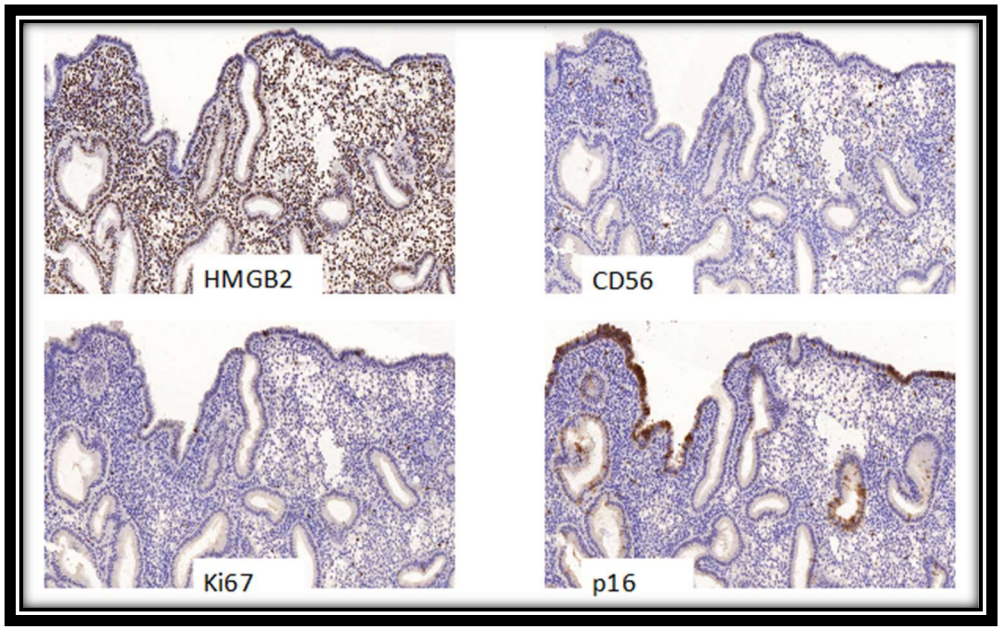

Figure 2. 3: Serial sections of (a) HMGB2 (b) CD56 (c) Ki67 and (d) p16 antibody staining from the same patient biopsy. This diagram illustrates the similar areas within the tissue sample that can be identified using serial sectioning.

Serial sections of 3 micrometres thickness were performed by experienced technicians to obtain slides with similar image to allow good comparisons across the four IHC markers used (CD56, p16, HMGB2 and Ki67).

Cell cycle marker density

In order to calculate the density of IHC stain of custom antibody for each image created by the IHC protocol in Figure 2.2 and 2.3, I initially used cell counter in Image J and overlaid each image to identify which cells showed cell senescence or proliferation. However, this process was laborious and time-consuming. The accuracy of this method was more reliant on the quality of serial sectioned images. Due to the difficulty of consistently producing top quality serial sectioned images and feasibility of running a predictive test in large numbers with processes that are time-consuming, I decided to use the recognised, standardised method now used in uNK cell density tests (Drury *et al.*, 2011; Drury *et al.*, 2013; Lash *et al.*, 2016).

This standardised method used colour de-convolution and thresholding technique in Image J software. Colour de-convolution using Image J Plugin splits the image into single colour channels of brown (e.g. CD56+ staining) and blue (haematoxylin cell nucleus staining) (Ruifrok & Johnston, 2001) as seen in Figure 2.4. Next, we use the thresholding function in Image J to set the level of staining against background threshold (Figure 2.4). The separated images in the 2 colour channels allow the colour intensity of the antibody over background endometrial stromal cells to be calculated and provide percentage of density of antibody marker within image. For example, the uNK percentage from these data were calculated as CD56 / stromal cells × 100 (Drury *et al.*, 2013). The average of 3 sites per patient and antibody is used to represent density in each corresponding endometrial section slide.

Localisation

Looking at areas of interest within a sample requires manually generating separate images of the endometrial stroma, lumen and glands for each IHC stained marker per patient (Figure 2.5). The protocol established in Figure 2.1 to 2.5 are repeated and the average of 3 sites is calculated.

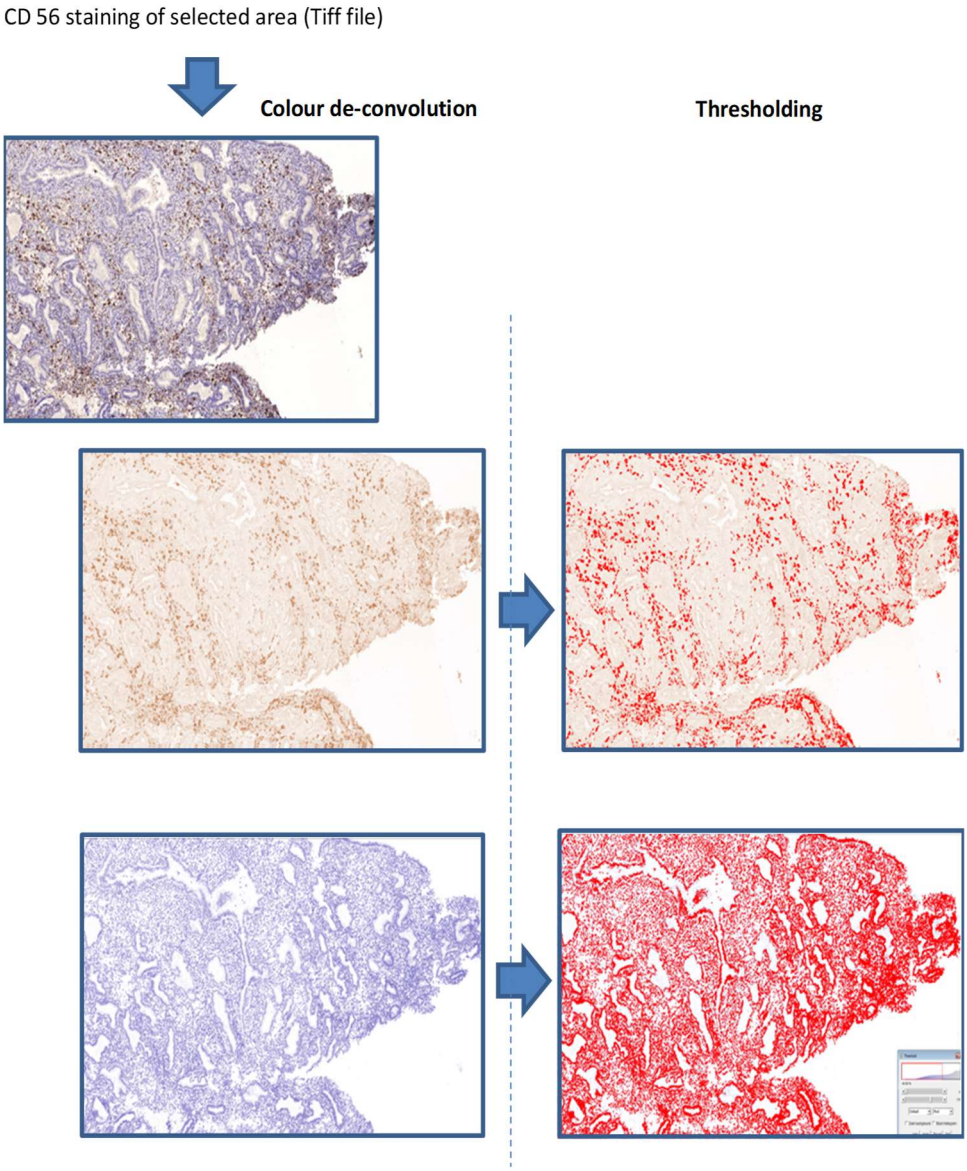

Figure 2. 4: Colour de-convolution and thresholding technique. Each Tiff file (20x magnification) of an area within a sectioned slide underwent colour de-convolution then thresholding to calculate the percentage of density within the selected area. An average of 3 selected areas is calculated for each IHC marker.

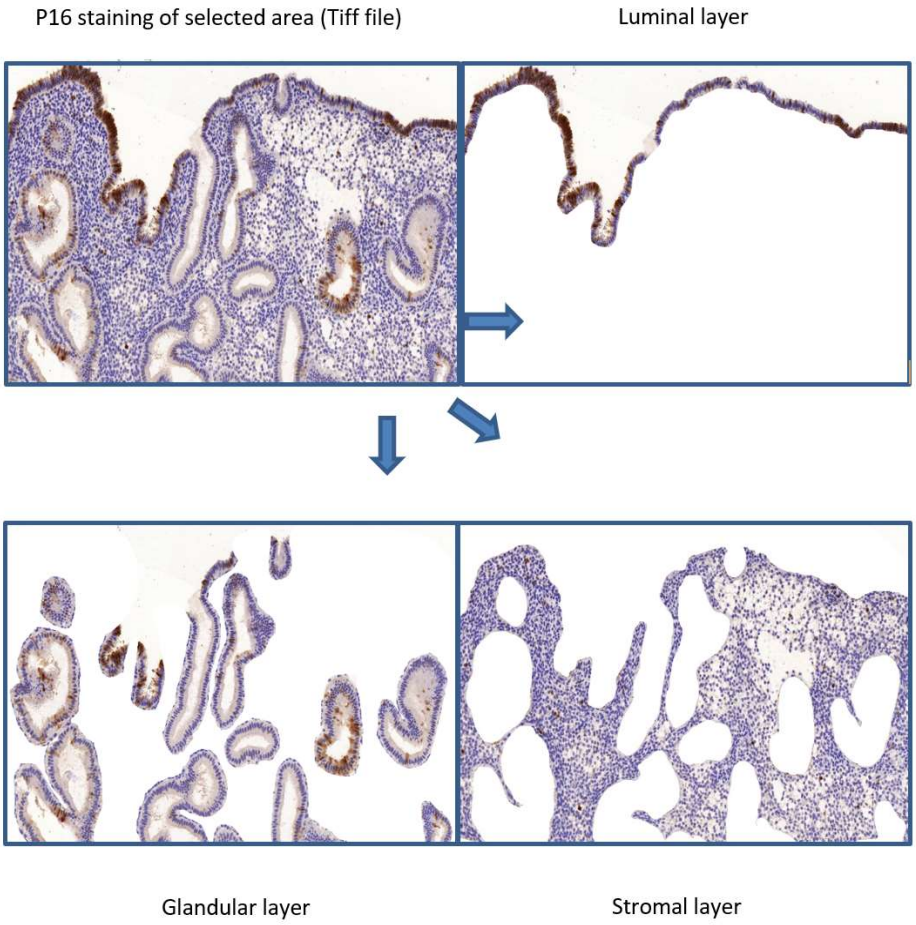

Figure 2. 5: Generating separate images to determine localisation of IHC markers. Average values for each IHC marker localised for the endometrial stroma, gland and lumen can be calculated.

The above figure illustrates how each image of a selected area can be edited in Image J to generate separate images of localised areas (luminal, glandular and stromal layer). This allows comparison of IHC markers within prescribed areas of interest. Each separate image underwent colour de-convolution and thresholding technique to measure IHC marker density.

2.5 Statistical Analysis

Data from the pregnancy outcome database were analysed with IBM® SPSS® Statistics V22.0 statistical software. Binary logistic regression and chi-squared test was used. I also formulated a receiver operating characteristic (ROC) curve to validate the predictive ability of the developed combined pre-conceptual endometrial test.

Data in the combined test were analysed with GraphPad Prism 6 (GraphPad Software Inc.). Mann-Whitney U test and Kruskal-Wallis test was performed where appropriate. Data in the inter-cycle variation tests were analysed using this software. Wilcoxon test was performed to compare the paired biopsy values.

Statistical significance was reached when $P < 0.05$.

CHAPTER 3:
EVALUATION OF uNK TEST AS PREDICTOR OF OUTCOME

Chapter 3: Evaluation of uNK cell test as predictor of outcome

3.1 Background

As discussed in my introduction chapter, the uNK cell density has been shown to be high in recurrent reproductive failure. Research on the prognostic ability of uNK cell density test has been inconclusive. Here, I tested the hypothesis that uNK cell density test has the ability to predict subsequent pregnancy outcome in women with recurrent pregnancy loss or recurrent implantation failure. This test was repeated for the locally developed uNK centile test to investigate whether normalising the uNK cell density for day of cycle, increases accuracy and predictive ability of the test.

3.1.1 Hypothesis

In the uNK cell density test, a result of 5% or more was classified as high based on previous research (Chen *et al.*, 2017; Quenby *et al.*, 1999; Quenby *et al.*, 2005; Tang *et al.*, 2011; Tang *et al.*, 2013). In the uNK centile test, a result on the 75th centile or above was classified as high for the purpose of this study (Brighton *et al.*, 2017). Therefore, my hypothesis is women with a high uNK cell density are less likely to have a live birth or pregnancy beyond 10 weeks. My second hypothesis is the uNK centile would be more accurate than uNK cell density in predicting women who are less likely to have a live birth or ongoing pregnancy.

3.1.2 Methods

This was a retrospective cohort study. I collated data on 281 women who fulfilled the criteria of history of recurrent reproductive failure and a recorded pregnancy event within one year of their mid-luteal endometrial biopsy. The primary outcome of interest was pregnancy outcome, which was divided into two groups; live birth or ongoing pregnancy beyond 10 weeks gestation and first trimester miscarriage.

The demographic details analysed and investigated as confounding factors included age, parity, presence of subfertility and previous pregnancy losses. I was unable to assess body mass index (BMI) as a confounder as this was not recorded in a large proportion of this study sample.

I processed the 281 women's data using binary logistic regression to address the effect of confounding factors on the calculated probability in the above statistical tests. This will assess the adjusted odds ratio of live birth or pregnancy beyond 10 weeks using the uNK cell density and uNK centile test.

3.2 Results

3.2.1 Demographics

Demographic details; n=281

	All subjects (n=281)	Live birth / ongoing (n=185)	Miscarriage (n=96)	p value
Age; Mean (±SD)	34.8 (4.28)	34 (4.17)	35 (4.45)	P= 0.089[1]
uNK cell density (%); Median (IQR)	5.33 (2.59 - 9.54)	4.78 (2.5 - 8.33)	6.75 (2.87 - 11.99)	P= 0.017[2]
uNK centile; Median (IQR)	48.5 (19.8 - 72.0)	46.5 (18 - 70.5)	53.8 (27.8 - 75.9)	P= 0.050[2]
No of previous miscarriages; Median (IQR)	3 (2 - 5)	3 (2 - 4)	4 (2 - 5)	P= 0.005[2]
Parity				
Nulliparous; Frequency (%)	202 (71.9)	134 (66.3)	68 (33.7)	P= 0.886[3]
Multiparous; Frequency (%)	79 (28.1)	51 (64.6)	28 (35.4)	
Fertility				
Recurrent miscarriage; Frequency (%)	163 (58)	105 (64.4)	58 (35.6)	P= 0.644[3]
Infertility; Frequency (%)	118 (42)	80 (67.8)	38 (32.2)	

[1] t test, [2] Mann-Whitney U test, [3] X^2 test

Table 3. 1: Demographic details in uNK evaluation test. Distribution of maternal age, uNK cell test, previous history of miscarriages, parity and fertility in the study sample is demonstrated above and compared in each pregnancy outcome group; (i) live birth or ongoing pregnancy beyond 10 weeks gestation and (ii) miscarriage.

In evaluating the uNK cell density test, a total of 281 women were identified retrospectively from the Implantation Clinic's clinical database. The demographic information for this cohort study is found in Table 3.1. The women were divided into two groups based on the primary outcome of interest (Group 1 – live birth or ongoing pregnancy beyond 10 weeks gestation and Group 2 – first trimester

miscarriage). A normality test was performed and data for uNK cell density, uNK centile, previous history of miscarriages, parity and fertility did not follow a normal distribution (Shapiro-Wilk test).

In this study, 185 (65.8%) women were successful in achieving a live birth or a pregnancy beyond 10 weeks gestation. There was no significant difference in age (p=0.089), parity (p=0.886) and fertility (p=0.644) in Group 1 and Group 2. The mean age of all subjects was 34.8 (±4.28), 202 (71.9%) women were nulliparous, 163 (58%) women had history of RPL and 118 (42%) women had subfertility.

The most significant difference between the two groups was number of previous miscarriages with a median of 4 (IQR 2-5) in the miscarriage group compared the median of 3 (IQR 2-4) in the live birth or ongoing pregnancy beyond 10 weeks group (p=0.005).

In this demographic data, the uNK test was assessed without use of cut off value for a high result to investigate the distribution between the two groups. There was a significant difference in uNK cell density (p=0.017) in the two groups with median of 4.78% (IQR 2.5-8.33%) in Group 1 and median of 6.75% (IQR 2.87-11.99%) in Group 2. In contrast, the uNK centile test was not significant (p=0.05) with median 46.5 centile (IQR 18-70.5) in Group 1 and median 53.8 centile (IQR 27.8-75.9).

3.2.2 The uNK cell density test and its predictive ability

uNK density: Binary Logistic Regression (n=281)

Characteristics	ongoing/LB ratio (%)	odds ratio (95% CI); p value unadjusted	adjusted
Age		0.95 (0.90 to 1.01); 0.09	0.95 (0.89 to 1.01); 0.09
uNK density test			
normal* (< 5%)	102/144 (70.8)	0.63 (0.39 to 1.04); 0.07	0.65 (0.39 to 1.09); 0.10
high (≥ 5%)	83/137 (60.6)		
Previous miscarriages			
<2*	70/97 (72.2)	0.003	0.003†
3 or 4	79/111 (71.2)	0.95 (0.52 to 1.74); 0.87	0.74 (0.33 to 1.67); 0.47
5 or more	36/73 (49.3)	0.38 (0.20 to 0.71); 0.003	0.27 (0.11 to 0.67); 0.005
Parity			
Multiparous*	51/79 (64.6)	0.86 (0.52 to 1.42); 0.56	0.82 (0.44 to 1.51); 0.52
Nulliparous	134/202 (66.3)		
Fertility			
Subfertility*	80/118 (67.8	1.08 (0.63 to 1.87); 0.78	1.32 (0.62 to 2.81); 0.48
RPL	105/163 (64.4)		

LB - Live Birth

*Reference category; †Global P-value

Table 3. 2: Binary logistic regression to investigate ability of uNK cell density test to predict pregnancy outcome. Confounding factors included in the test were maternal age, number of previous miscarriages, parity and fertility. The only significant factor in predicting pregnancy outcome was 5 or more previous miscarriages (p=0.005).

To test the hypothesis that women with a high uNK cell density test are less likely to have a live birth or ongoing pregnancy beyond 10 weeks gestation, I conducted a binary logistic regression on all demographic data collected to account for confounding factors. I divided the data describing the number of previous miscarriages into three binned groups (<2 miscarriages, 3-4 miscarriages and ≥ 5 miscarriages) as this provides information on severity of phenotypic presentation.

Results were expressed as [odds ratio (OR); 95% confidence interval (CI); P value]. The uNK density cut off value of 5% was used, where a high value was ≥ 5%.

This regression analysis showed that uNK cell density test was not a good predictor of outcome in subsequent pregnancy [adjusted OR: 0.65; 95% CI: (0.39 – 1.09); p=0.1] (Tab. 3.2). Number of previous miscarriages was the only significant predictor of outcome when there were 5 or more miscarriages [OR: 0.27; 95% CI: (0.11 – 0.67); p=0.005]. Factors such as age, parity and fertility did not appear to effect outcome prediction.

3.2.3 Normalising the uNK cell density test to improve its predictive ability

It has been shown that uNK cell density gradually rises in the secretory phase of the menstrual cycle (Johnson *et al.*, 1999; Loke & King, 2000c; Vince & Johnson, 2000). This suggests that a single cut off value of 5% may not effectively identify women with poorer outcomes and supports the findings in this cohort study. The uNK centile test normalises for day of the cycle the biopsy was taken. Therefore, it is hypothesised that the uNK centile test is more accurate and would predict subsequent pregnancy outcome better. For the purpose of this cohort study, a high uNK centile was a value of ≥ 75th centile.

The binary logistic regression was repeated substituting uNK cell density with the corresponding uNK centile value. Results were expressed as [odds ratio (OR); 95% confidence interval (CI); P value].

uNK centiles: Binary Logistic Regression (n=281)

		odds ratio (95% CI); p value	
Characteristics	ongoing/LB ratio (%)	unadjusted	adjusted
Age		0.95 (0.90 to 1.01); 0.09	0.95 (0.89 to 1.01); 0.07
uNK centile			
normal* (< 75th)	156/227 (68.7)	0.53 (0.29 to 0.97); 0.04	0.53 (0.28 to 1.00); 0.05
high (≥ 75th)	29/54 (53.7)		
Previous miscarriages			
<2*	70/97 (72.2)	0.003	0.004†
3 or 4	79/111 (71.2)	0.95 (0.52 to 1.74); 0.87	0.76 (0.34 to 1.70); 0.50
5 or more	36/73 (49.3)	0.38 (0.20 to 0.71); 0.003	0.28 (0.12 to 0.69); 0.006
Parity			
Multiparous*	51/79 (64.6)	0.86 (0.52 to 1.42); 0.56	0.78 (0.42 to 1.43); 0.42
Nulliparous	134/202 (66.3)		
Fertility			
Subfertility*	80/118 (67.8	1.08 (0.63 to 1.87); 0.78	1.23 (0.58 to 2.64); 0.59
RPL	105/163 (64.4)		

LB - Live Birth

*Reference category; †Global P-value

Table 3. 3: Binary logistic regression to investigate ability of uNK centile test to predict pregnancy outcome. The same confounding factors as used to evaluate the uNK density test were included in this test. The only significant factor in predicting pregnancy outcome after adjustment was 5 or more previous miscarriages (p=0.006). However, the unadjusted value for uNK centile was significant (p=0.04) suggesting that it is a marginally better prognostic test than uNK density.

The analysis showed that the uNK centile was significant as a predictor of live birth or ongoing pregnancy prior to adjustment for confounding factors [OR: 0.53; 95% CI: (0.29 – 0.97); p=0.04]. The significance level was lost when adjusted for confounding factors [adjusted OR: 0.53; 95% CI: (0.28 – 1.00); p=0.05] (Tab. 3.3). However, it did perform better than the uNK cell density test [adjusted OR: 0.65; 95% CI: (0.39 – 1.09); p=0.1]. As seen in the first logistic regression analysis, the only significant predictor was number of previous miscarriages when there were 5 or more miscarriages [OR: 0.28; 95% CI: (0.12 – 0.69); p=0.005].

3.2.4 uNK test in Recurrent Pregnancy Loss

My research is aimed to design an endometrial test for women with recurrent pregnancy loss. Therefore, I have extracted the data on women without subfertility issues from this database. There are 163 women with 'RPL only' in this study sample. I have repeated the binary logistic regression for these women to ascertain whether there is any difference in the results. The following tables illustrate the predictive ability of uNK density and uNK centile test in women with 'RPL only'.

uNK density: Binary Logistic Regression (n=163)

		odds ratio (95% CI); p value	
Characteristics	ongoing/LB ratio (%)	unadjusted	adjusted
Age		0.99 (0.92 to 1.06); 0.70	0.99 (0.92 to 1.07); 0.85
uNK density test			
normal* (< 5%)	25/41 (61)	0.97 (0.51 to 1.84); 0.92	1.05 (0.53 to 2.07); 0.90
high (≥ 5%)	80/122 (66)		
Previous miscarriages			
< 2*	8./9 (89)	0.003	0.003
3 or 4	66/90 (73)	0.34 (0.04 to 2.90); 0.33	0.35 (0.04 to 2.94); 0.33
5 or more	31/64 (48)	0.12 (0.01 to 0.99); 0.049	0.12 (0.01 to 0.99); 0.05
Parity			
Multiparous*	38/61 (62)	1.16 (0.60 to 2.24); 0.66	0.91 (0.44 to 1.90); 0.81
Nulliparous	67/102 (66)		

Table 3. 4: Binary logistic regression to investigate predictive ability of the uNK test in women with RPL only. The data shows that both the uNK density is unable to predict pregnancy outcome in this group of women.

uNK centile: Binary Logistic Regression (n=163)			
		odds ratio (95% CI); p value	
Characteristics	ongoing/LB ratio (%)	unadjusted	adjusted
Age		0.99 (0.92 to 1.06); 0.70	0.99 (0.92 to 1.07); 0.86
uNK centile test			
normal* (<75th)	17/27 (63)	0.86 (0.36 to 2.05); 0.74	0.92 (0.37 to 2.29); 0.86
high (≥ 75th)	88/136 (65)		
Previous miscarriages			
< 2*	8./9 (89)	0.003	0.003
3 or 4	66/90 (73)	0.34 (0.04 to 2.90); 0.33	0.35 (0.04 to 2.93); 0.33
5 or more	31/64 (48)	0.12 (0.01 to 0.99); 0.049	0.12 (0.01 to 0.99); 0.05
Parity			
Multiparous*	38/61 (62)	1.16 (0.60 to 2.24); 0.66	0.92 (0.45 to 1.90); 0.83
Nulliparous	67/102 (66)		

Table 3. 5: Binary logistic regression to investigate predictive ability of the uNK test in women with RPL only. The data shows that both the uNK centile is unable to predict pregnancy outcome in this group of women.

The statistical analyses show that the uNK density and uNK centile test are unable to predict pregnancy outcome in women with RPL. The odds ratios are [OR: 1.05; 95% CI: (0.53 – 2.07); p=0.90] and [OR: 0.92; 95% CI: (0.37 – 2.29); p=0.86] respectively. There was no difference between the uNK density or uNK centile test. It also confirms that number of previous miscarriages is the only significant factor in pregnancy outcome prediction in this study.

3.3 Discussion

3.3.1 Limitations

The limitations of this cohort study can be divided into data collection and inherent error of the uNK test. Bias is introduced during data collection as it is a retrospective study and the recording of pregnancy outcomes are reliant on patient reporting. Some of these women would have also received medical intervention in the form of progesterone and glucocorticoid therapy.

Over the five-year duration of this study, the uNK cell density test has been calculated by multiple investigators and the method of calculation changed from a counting method using point picker to image analysis using Image J software. The counting method is more accurate but time consuming. Therefore, it is not an efficient method to process large number of samples. The image analysis is calculated on the area of CD56 which is a uNK cell surface marker over the area of the stromal nuclear marker (haematoxylin staining). The known test intra-observer error in image analysis is about 20% as analysed in our laboratory. In the uNK centile test, there is an added error of patient reported ovulation tests which can be reported imprecisely by women.

3.3.2 Conclusions

The strength of this cohort study is the large sample size. The results show that the uNK cell density test is not a good prognostic test of subsequent pregnancy outcome.

The uNK centile calculation more than halved the women with a high uNK test value in comparison to the uNK cell density test in the whole recurrent reproductive failure dataset (n=54 and n=137 respectively). However, the uNK density and uNK centile tests are unable to predict pregnancy outcome in women with recurrent reproductive failure.

It is clear that the number of previous miscarriages is the only significant factor that predicts pregnancy outcome in this study. These findings support previous evidence that history of miscarriages is a good predictor of subsequent pregnancy outcome. However, this does not assist in managing most women with recurrent reproductive failure because it is not modifiable.

Recent scientific advances in the physiology of decidualisation would suggest a complex system with interplay of several components that when balanced, supports a karyotypically normal embryo. These components include cell senescence and mesenchymal stem cells (Lucas *et al.*, 2016). In my research, I proceeded to explore biomarkers for cell cycle proliferation and senescence in the mid-luteal phase endometrium and focused on women with recurrent pregnancy loss. I investigated whether knowledge gained could improve the predictive ability of the uNK test by combining multiple factors that constitute decidualisation.

CHAPTER 4:
EXPLORATORY TEST SET FOR PREDICTOR MARKERS

Chapter 4: Exploratory test set for predictor markers

4.1 Background

In this research, I have concentrated on exploring a predictive model for subsequent pregnancy outcome in women with recurrent pregnancy loss. The premise for this model is a combined pre-conceptual endometrial test incorporating multiple factors within the endometrium that are important for decidualisation and enabling support of normal karyotypic embryos.

The uNK cell test has been adopted widely (Quenby and colleagues in UK, Sacks and colleagues in Australia, Li and colleagues in Hong Kong) because;

1. uNK cells have consistently been found to be higher in reproductive failure (Quenby *et al.*, 1999; Tuckerman *et al.*, 2007)
2. uNK cell density correlates negatively with markers of decidualisation (Kuroda *et al.*, 2013)
3. uNK cell density has been used as a marker of local cortisol deficiency (Kuroda *et al.*, 2013)
4. uNK cell density test has been used to identify women who may benefit from steroid to prevent repeated pregnancy loss (Tang *et al.*, 2011)

However, the test has limitations as described in previous chapters.

Decidualisation involves a number of processes as well as uNK cell infiltration. Recent findings within our biomedical research unit has shown that impaired decidualisation is associated with recurrent pregnancy loss due to accelerated senescence and loss of endometrial mesenchymal stem cells (eMSCs) as it limits differentiation capacity of the endometrium (Lucas *et al.*, 2016). It was found that knockdown of the chromatin protein HMGB2 (high mobility group protein 2) in human endometrial stromal cells (HESCs) promotes senescence and impairs decidualisation.

Other researchers have investigated the possibility of tests to predict subsequent pregnancy outcomes. LéDée and colleagues have shown that immune profiling of uNK cell density by investigating the activity and proliferation of uNK cell improves prognostic ability of the uNK test (Lédée *et al.*, 2016; Lédée *et al.*, 2017). Research by Li and colleagues, also found that histological dating of endometrium improves prognostic ability of the uNK cell density test (Liu *et al.*, 2014). Histological dating includes assessment of the development of glands and spiral arteries in the decidualisation process.

We aimed to improve the uNK test by combining it with biomarkers of proliferation and senescence to the stroma, glands and lumen of the endometrium. We also aimed to explore the importance of these components as separate entities.

Immunohistochemistry was used to detect biomarkers of uNK cells (CD56), cell proliferation (Ki67) and cell senescence (HMGB2 and p16).

Considering all these factors, the following hypotheses were developed.

4.1.1 Hypotheses

1. Reduced proliferation indicated by low Ki67 activity in human endometrial stromal, luminal or glandular cells is associated with recurrent pregnancy loss

2. Increased ratio of proliferation indicated by Ki67 in uNK cells indicates active uNK cells and is associated with recurrent pregnancy loss

3. Increased senescence indicated by raised p16 and loss of HMGB2 in human endometrial stromal, luminal or glandular cells is associated with recurrent pregnancy loss

4. A balance of endometrial cell proliferation and senescence support decidualisation and improves chance of supporting an embryo leading to a live birth

4.1.2 Methods

This study is an exploratory test using cell cycle biomarkers to design a predictive model of subsequent pregnancy outcome in women with recurrent pregnancy loss. In order to improve the uNK cell test, I selected an exploratory test set which consisted of women attending our 'Implantation Clinic'. I included women with recurrent pregnancy loss, a biopsy between LH + 6 to 10 days and known pregnancy outcome within one year of their biopsy (n=20). This exploratory study took place prior to the development of uNK centile. Therefore, a normal uNK cell density was defined as below 5% in this test set. I pre-defined 4 groups and identified 5 women into each group. The groups were defined as:

A: High uNK cell density and subsequent live birth (uNK false positive test)

B: High uNK cell density and subsequent miscarriage (uNK true positive test)

C: Normal uNK cell density and subsequent live birth (uNK true negative test)

D: Normal uNK cell density and subsequent miscarriage (uNK false negative test)

This exploratory set was devised to include women who had a further miscarriage and a more severe phenotype to make any difference between the groups more pronounced to enable detection. Each patient's history and pregnancy outcome are reported in Table 4.1.

uNK test	Age	No of miscarriages	Parity	Outcome
High	41	4	0	Live Birth
	36	3	0	(Group A)
	38	3	0	
	39	3	1	
	42	5	1	
	41	6	0	Miscarriage
	32	3	0	(Group B)
	38	9	2	
	40	4	0	
	43	19	0	
Normal	28	3	0	Live Birth
	36	4	0	(Group C)
	35	3	1	
	30	2	0	
	40	7	1	
	27	11	0	Miscarriage
	29	5	0	(Group D)
	43	8	0	
	30	7	0	
	30	8	0	

Table 4. 1: Demographic details in exploratory test set. Women with more severe phenotype were chosen for this test set to enable detection of differences between groups. Group A denotes the false positive uNK test, Group B the true positive uNK test, Group C the true negative uNK test and Group D the false negative uNK test.

All endometrial samples underwent the same immunohistochemistry (IHC) process as outlined in chapter 2. The MIRAX files created were analysed using Pannoramic Viewer and Image J software. The identified cell cycle markers used for this exploratory analysis were CD56 (uNK), HMGB2 and p16 (cell senescence) and Ki67 (cell proliferation). We compared the trend of each cell cycle marker in the 4 defined groups to determine whether an association exists between density of cell cycle markers, it's localisation within the endometrium and pregnancy outcome.

4.2 Results

4.2.1 Proliferative and senescent cell ratio

In the early stages of this exploratory study, I used a cell counting and image stacking method in Image J software, to identify single cells and corresponding IHC markers of interest. I describe the cell counting method below. This method is reliant on good serial sections to identify the same cell for each IHC marker and is time-consuming. Hence, after the initial results in this chapter, I adopted the method of thresholding and localisation as described in Chapter 2.

Methods of cell counting

1. Each patient had four images in TIFF file with IHC staining for CD56, Ki67, HMGB2 and p16.

2. The images of CD 56, Ki67 and p16 staining were overlain using image stacker and grid to allow identification of single cells on the corresponding IHC serial section. This helps identify whether a uNK cell (CD56) or stromal cell (haematoxylin) is senescent (p16) or proliferative (Ki67).

3. The cell counter is then used to count the total number of uNK cells (CD56), the stromal cells (haematoxylin) and the corresponding uNK and stromal cells that are proliferative ($CD56^{Ki67+}$ and $stromal^{Ki67+}$) or senescent ($CD56^{p16+}$ and $stromal^{p16+}$).

4. The following formula was used to calculate ratio of proliferative uNK cells, ratio of senescent uNK cells, ratio of proliferative stromal cells and ratio of senescent stromal cells.

    - $[(uNK^{Ki67+})/\text{all uNK cells}]*100$
    - $[(uNK^{p16+})/\text{all uNK cells}]*100$
    - $[(stromal^{Ki67+})/\text{all stromal cells}]*100$
    - $[(stromal^{p16+})/\text{all stromal cells}]*100$

5. HMGB2 staining was assessed separately to calculate the loss of the marker as sign of senescence [(HMGB2 loss/all stromal cells] *100.

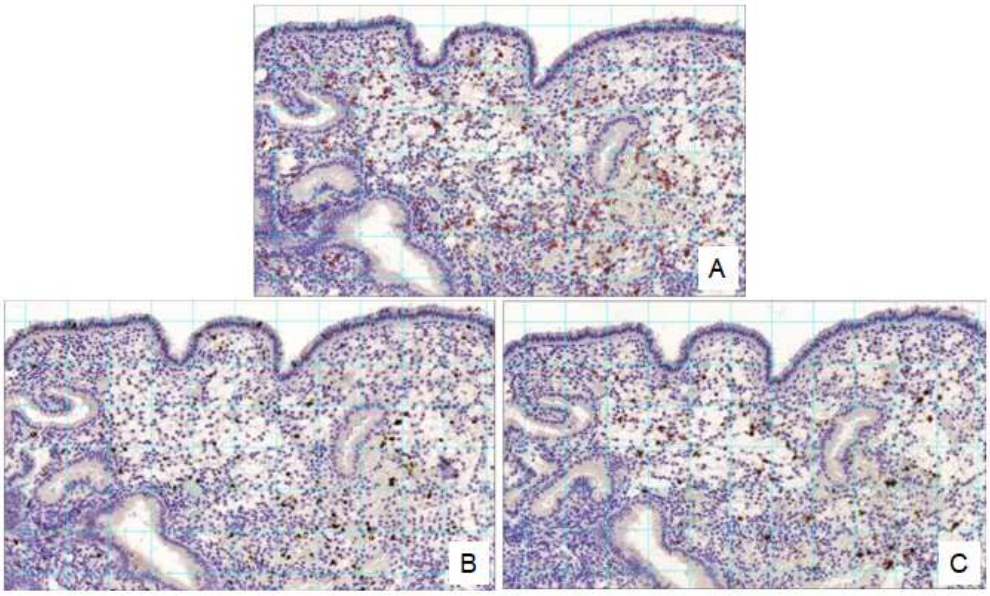

Figure 4. 1: An example of serial section from a patient used in image stack and cell counting to calculate proliferative and senescent uNK cells. A) CD56 stained slide B) Ki67 stained slide C) p16 stained slide. The blue grid was used to align the image on top of each other so that I could identify which CD 56 cells were also Ki67 or p16 positive.

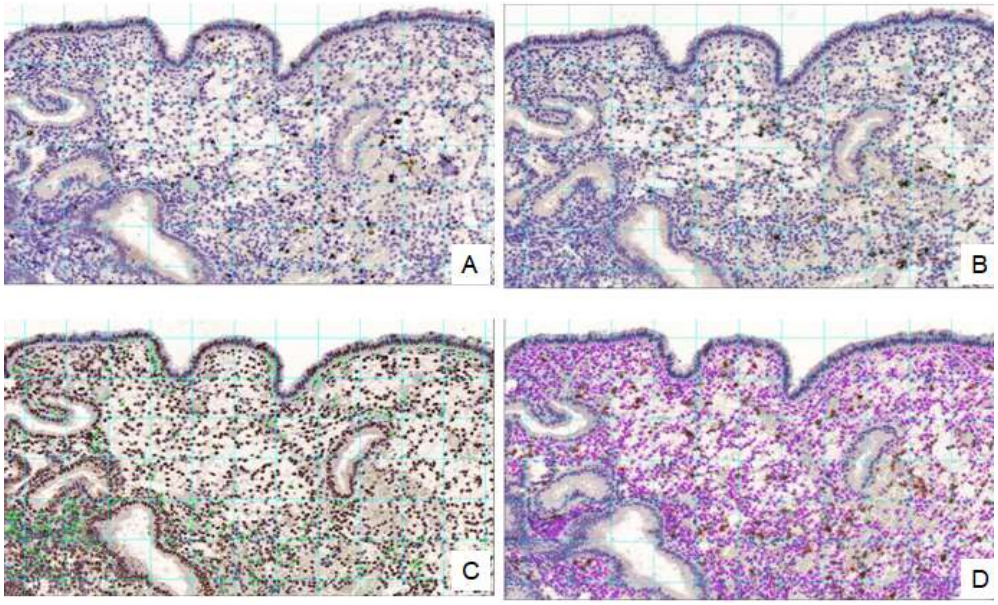

Figure 4. 2: An example of serial section from a patient used in image stack and cell counting to calculate proliferative and senescent stromal cells. A) Ki67 stained slide B) p16 stained slide C) HMGB2 stained slide D) Counted stromal cells

The results showed no significant difference in proliferative or senescent uNK cell ratios in the four groups; as shown in Figure 4.3.

There was significance in the difference of proliferative and senescent stromal cells across the groups (Figure 4.4). However, the difference did not discriminate between pregnancy outcomes. Higher levels of stromal proliferation and senescence are present when uNK cell density is high. Therefore, it does not add value to the uNK test.

In addition, HMGB2 loss in stromal cells did not significantly differentiate between the groups (Figure 4.5).

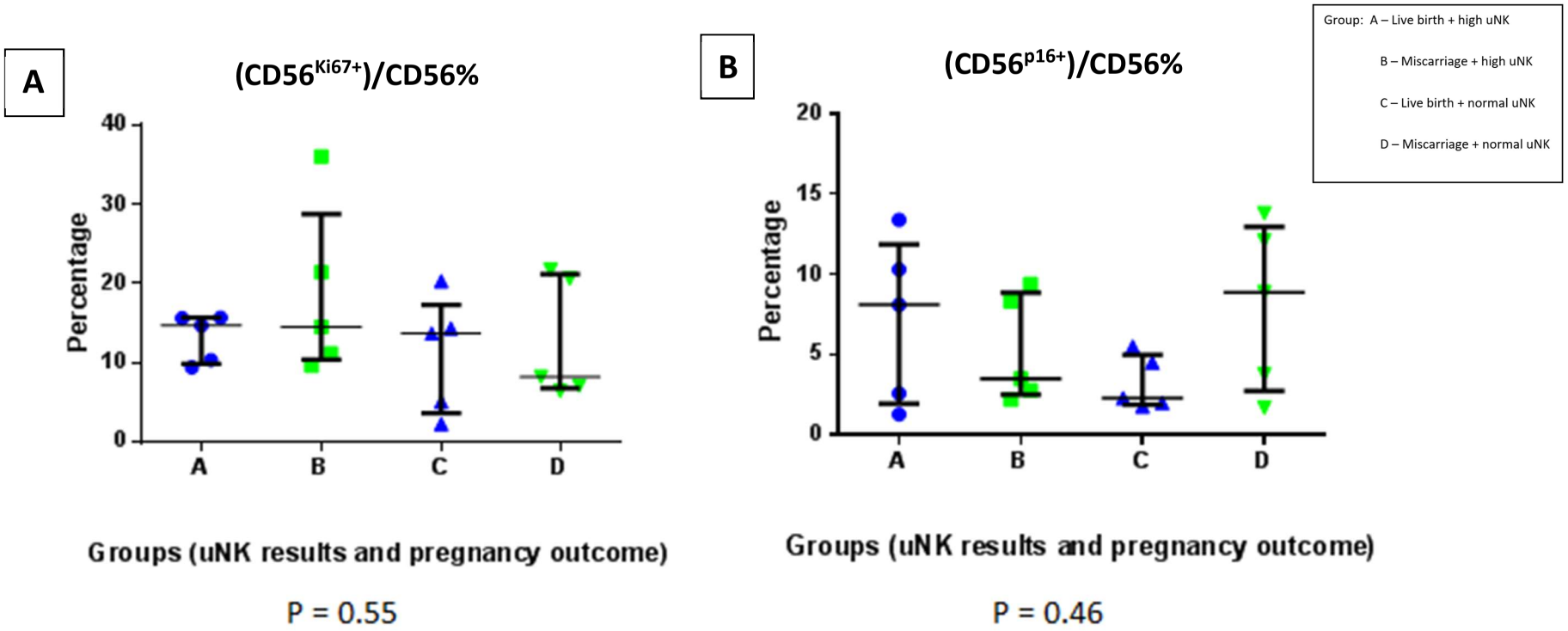

Figure 4. 3: Ratio of uNK proliferation and senescence in the 4 pre-determined groups. A) Graph A illustrates the percentage of proliferative uNK cells ($CD56^{Ki67+}$) over whole sample proliferation for each group. B) Graph B shows the percentage of senescent uNK cells ($CD56^{p16+}$) over whole sample senescence for each group. Both show no significant difference using Kruskal-Wallis test. This data is unable to separate women based on pregnancy outcome.

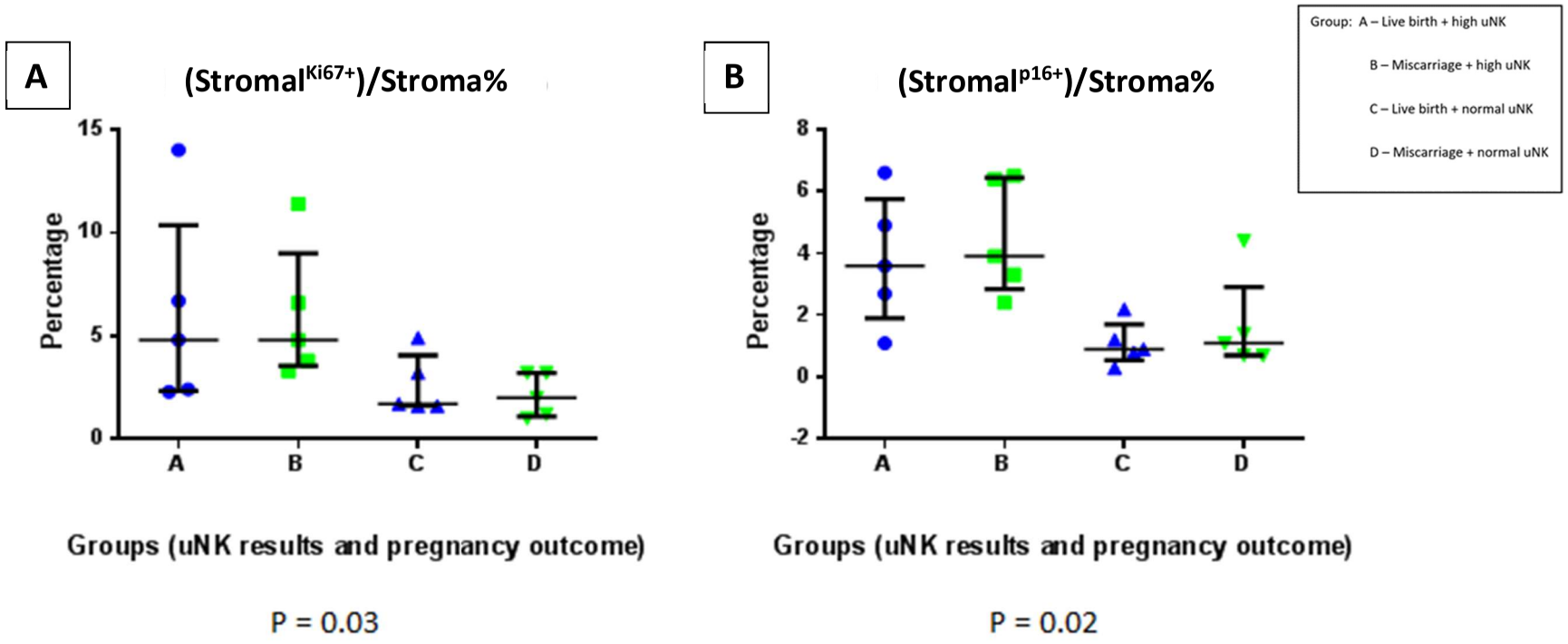

Figure 4. 4: Ratio of stromal proliferation and senescence in the 4 pre-determined groups. groups. A) Graph A illustrates the percentage of proliferative stromal cells (stroma^{Ki67+}) over whole sample proliferation for each group. B) Graph B shows the percentage of senescent stromal cells (stroma^{p16+}) over whole sample senescence for each group. Both show a significant difference using Kruskal-Wallis test. This data is able to separate women based on uNK result and not pregnancy outcome. Therefore, not adding information to the uNK test.

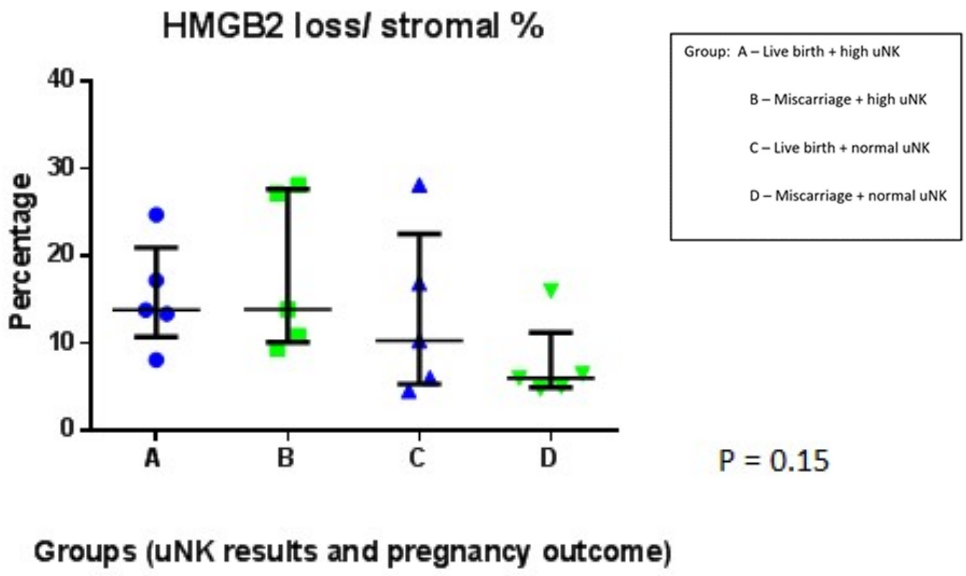

Figure 4. 5: Comparison of loss of HMGB2 in stromal cells between the 4 pre-determined groups. This graph measures stromal senescence using HMGB2 loss in stromal cells as a proportion of total stromal cell within sample image used. There was no significant difference between the groups (Kruskal-Wallis test).

4.2.2 Localisation of proliferation and senescence in stromal, glandular and luminal cells.

The colour de-convolution and thresholding technique described in Chapter 2 was used for the localisation of cell proliferation and senescence test. The results are shown in Figures 4.6 to 4.9.

Proliferation in the stroma, glandular and luminal cells were not significantly different between the four groups. Interestingly, stromal proliferation appeared to be high when uNK cell density were high.

Stromal senescence measured by HMGB2 loss and p16 levels were also not significant.

However, HMGB2 levels reached statistically significant levels when comparing the four groups in the glandular and luminal cells (p=0.04 and p=0.03 respectively) (Figure 4.7). P16 levels were also significantly different across the groups in the glandular and luminal cells (p=0.02 and p=0.03 respectively) (Figure 4.8).

Further analysis, showed that raised glandular and luminal p16 levels in the presence of high uNK cell density were associated with further pregnancy loss (p=0.02 and p=0.03 respectively) (Figure 4.8). However, only HMGB2 loss in the lumen differentiated the groups by pregnancy outcome data irrespective of uNK cell density (p=0.003) (Figure 4.9). It was found that HMGB2 loss in the lumen was associated with further pregnancy loss.

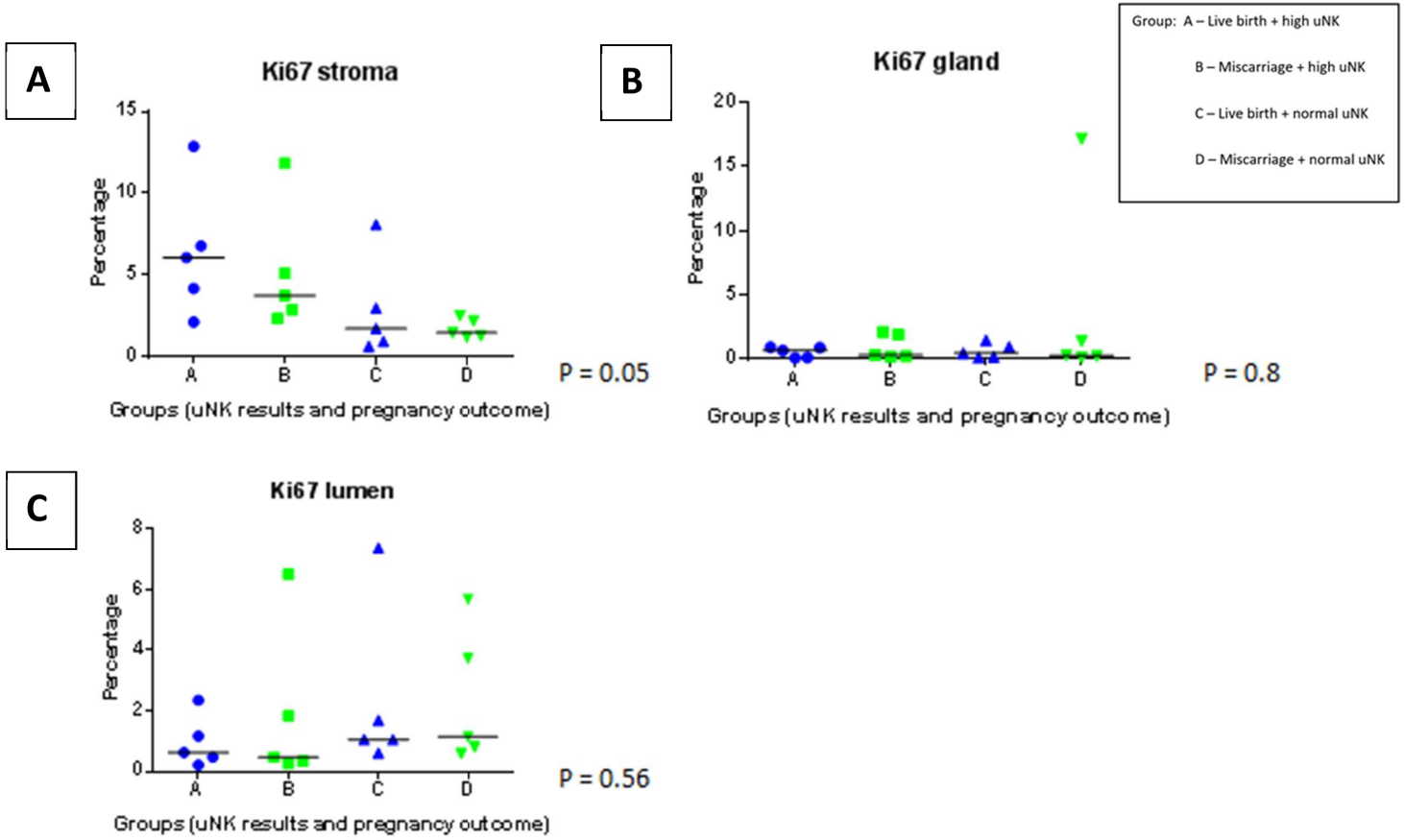

Figure 4. 6: Cell proliferation within endometrial stroma, glands and lumen. The graphs depict the proportion of endometrial A) stroma, B) glandular and C) luminal cells that stain with Ki67. The results are not significant and is unable to separate the groups based on pregnancy outcome. However, the trend in stroma proliferation appears to be high when uNK cell density is high. (Kruskal-Wallis test)

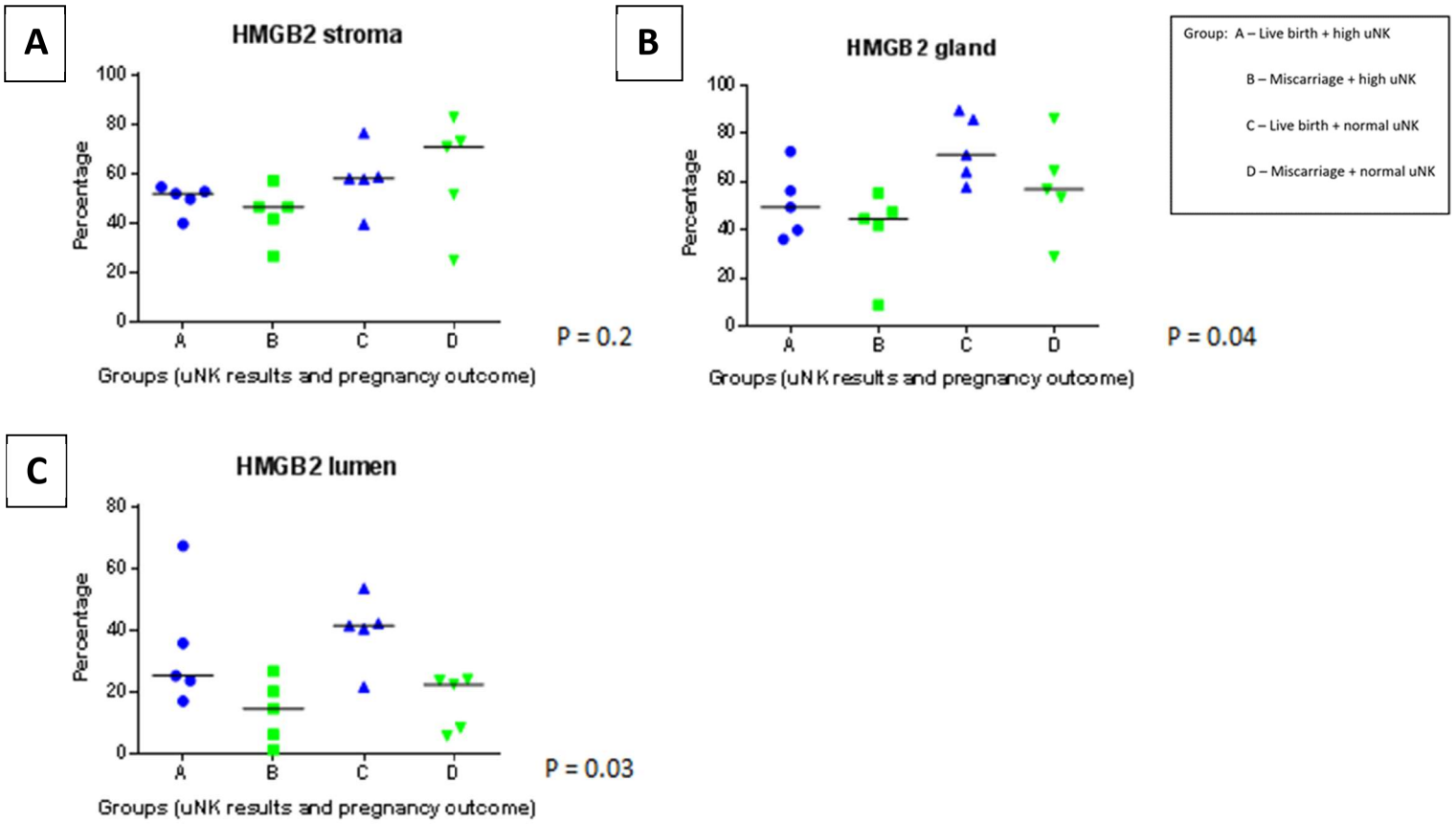

Figure 4. 7: Cell senescence within endometrial stroma, glands and lumen using HMGB2. The graphs depict the proportion of endometrial A) stroma, B) glandular and C) luminal cells that stain with HMBG2. The results were significant in graph B and C. However, only HMGB2 loss in the lumen was able to discriminate based on pregnancy outcome with HMGB2 loss indicating further pregnancy loss. (Kruskal-Wallis test).

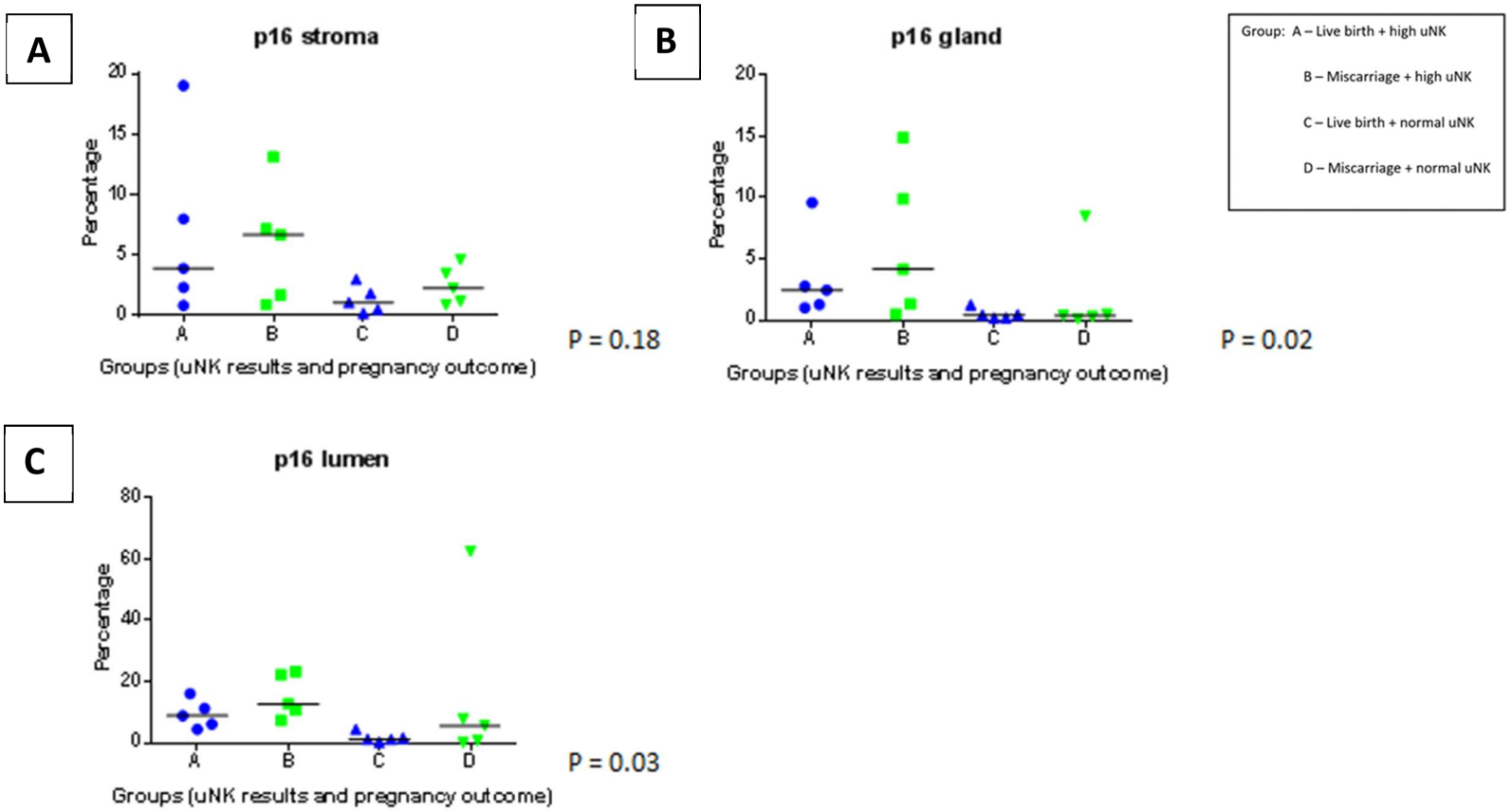

Figure 4. 8: Cell senescence within endometrial stroma, glands and lumen using p16. The graphs depict the proportion of endometrial A) stroma, B) glandular and C) luminal cells that stain with p16. The results were significant in graph B and C. Raised levels of p16 in glands and lumen in the presence of high uNK density were able to identify women who had a further miscarriage. (Kruskal-Wallis test)

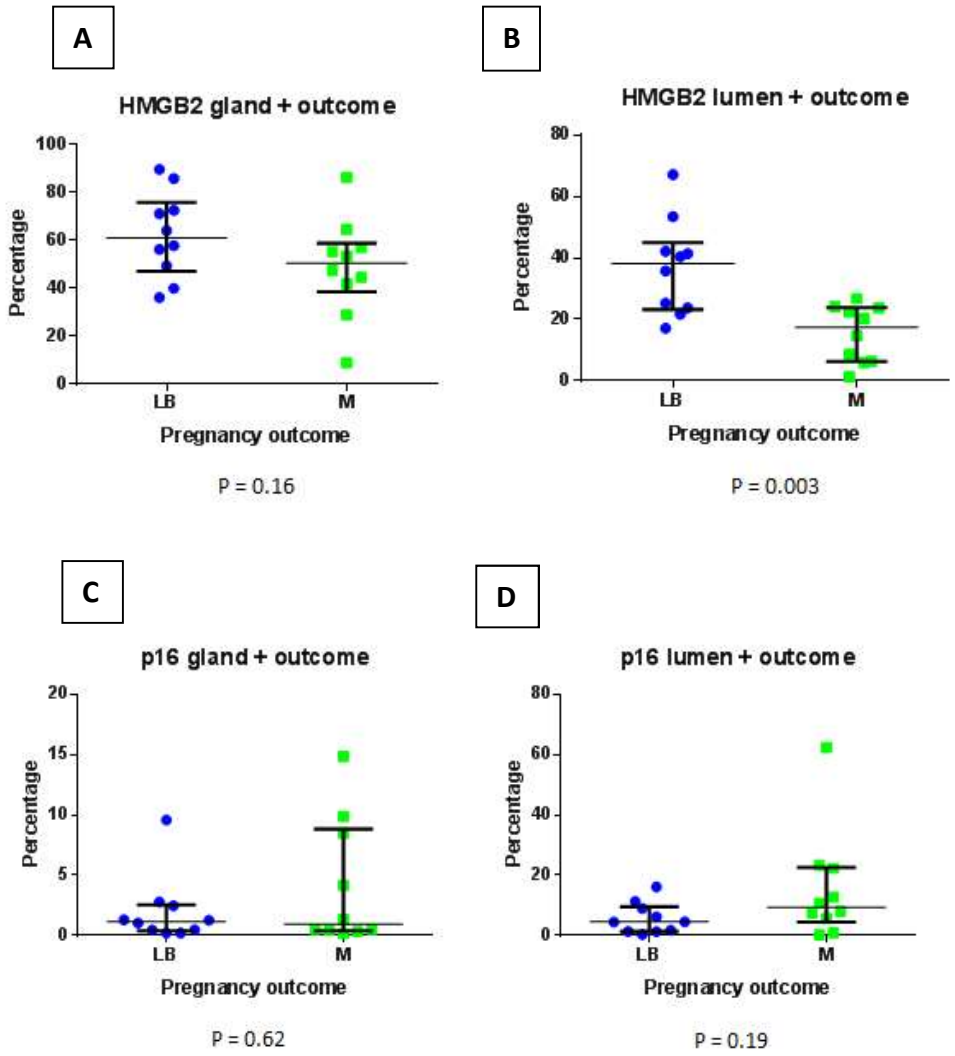

Figure 4. 9: Endometrial cell senescence and pregnancy outcome. I performed Mann-Whitney test on the significant results in Figure 4.7 and 4.8 to investigate whether the tests can discriminate based on pregnancy outcome irrespective of uNK result. The graphs illustrate A) HMGB2 loss in glandular cells, B) HMGB2 loss in luminal cells, C) p16 presence in glandular cells and D) p16 presence in luminal cells. HMGB2 loss in luminal cells was the only significant factor in association with further pregnancy loss irrespective of uNK result.

4.3 Discussion

This exploratory data set was useful. It looked to test the hypotheses generated prior to the study.

- Proliferative markers

> ***Hypothesis 1:*** Reduced proliferation indicated by low Ki67 activity in human endometrial stromal, luminal or glandular cells is associated with recurrent pregnancy loss
>
> ***Hypothesis 2:*** Increased ratio of proliferation indicated by Ki67 in uNK cells indicates active uNK cells and is associated with recurrent pregnancy loss

The study tested whether the ratio of CD56 or stromal cells stained with Ki67, a proliferative marker, would predict pregnancy outcome. The results showed that cell proliferation using Ki67 did not add significant information to uNK cell density test as suggested by LéDée and colleagues (Lédée *et al.*, 2016; Lédée *et al.*, 2017). It must be noted that I have used a different method to measure uNK proliferation and it is likely to be a less accurate way of measuring this. Although we used a small number of patients, this method was time-consuming and thousands of cells were counted. We should have been able to detect big differences in the Ki67 positive cells but did not find this. Therefore, the study did not support Hypothesis 1 or 2.

- Senescent Markers

Hypothesis 3: Increased senescence indicated by raised p16 and loss of HMGB2 in human endometrial stromal, luminal or glandular cells is associated with recurrent pregnancy loss

Hypothesis 4: A balance of endometrial cell proliferation and senescence support decidualisation and improves chance of supporting an embryo leading to a live birth

In the effort to test Hypothesis 3 and 4, I found a combination of positive and negative results dependent on localisation of the marker to the lumen, gland or stroma. The findings are summarised below:

- Glandular p16 marker appeared to identify women with high uNK density and subsequent miscarriage
- Stromal p16 and Ki67 ratios varied in the same manner as the uNK density
- HMGB2 loss in lumen appeared to identify women with a subsequent miscarriage irrespective of uNK density

The cell senescence marker in the glands (glandular p16), was found to help differentiate false positive test from true positive test. Senescence (p16) was higher in the glands of women with true positive test (high uNK and subsequent miscarriage) compared to the false positive test (high NK and subsequent live birth). We hypothesize that the natural progression of endometrial glandular development is due to senescence, denoting cell maturation, after the mid-luteal phase peak differentiation. This finding supports that of Liu and colleagues, who suggest that glandular development identified using histological Noyes criteria improved the uNK test. We hoped that using IHC would be more objective than pathologist subjective histological dating (Coutifaris *et al.*, 2004; Lessey *et al.*, 2000; Myers *et al.*, 2004).

Interestingly, stromal p16 senescent marker and Ki67 proliferative marker ratios varied in the same manner as the uNK density. The reason of this is elucidated in a further publication describing a role for uNK cells in clearing senescent cells (Brighton *et al.*, 2017). These new findings mean that uNK cell density will reflect stromal senescence. Further work suggested that clearing senescent cells leads to stimulation of stem cells and proliferation that could have been detected with Ki67 (Brighton *et al.*, 2017). Other work in our laboratory, had suggested that impaired stromal senescence would be useful in predicting outcome but I found no evidence to support this in the exploratory test set (Lucas *et al.*, 2016).

HMGB2 was also found to be lower in the luminal layer in those with a miscarriage irrespective of the uNK cell test result. This addition of a luminal compartment layer has never been explored before. However, in terms of technicality of the test, a glandular test is easier to calculate than a luminal one as it can be difficult to find a good length of intact luminal tissue due to the endometrial sampling process

The limitation of this study is that it is a retrospective study and this introduces bias in data collection. Another confounding factor is that the proportion of miscarriages due to chromosomal abnormality is unknown. Therefore, the results need to be tested on a larger scale to confirm the hypothetical association of senescent markers and subsequent pregnancy outcomes. Finally, the findings of the larger study would need to be evaluated in a prospective study setting to reduce bias.

Based on the results of this exploratory study, I have selected p16 and HMBG2 in the glands and lumen for further development in a larger study sample of 89 women. The aim would be to validate the resulting combined pre-conceptual endometrial test in a prospective RCT setting.

CHAPTER 5:

‘COMBINED PRE-CONCEPTUAL ENDOMETRIAL TEST’ DEVELOPMENT STUDY

Chapter 5: 'Combined pre-conceptual endometrial test' development study

5.1: Background

It was found in the exploratory test set, that loss of HMGB2 in endometrial luminal layer and increased P16 with high CD56 in endometrial glandular layer appear associated with a subsequent pregnancy loss. I designed a study protocol to test the validity of the hypotheses made. I aim to develop a pre-conceptual endometrial test using knowledge gained and assess its ability to improve prediction of subsequent pregnancy outcome of the uNK test.

5.1.1 Testing hypotheses

The hypotheses tested are;

1. Endometrial luminal senescence (HMGB2) is associated with further pregnancy loss despite uNK cell density
2. Mature glandular development indicated by increase endometrial glandular senescence (p16), when associated with high uNK cell density is associated with further pregnancy loss
3. Endometrial stromal senescence does not predict subsequent pregnancy outcome

5.1.2 Methods

In this study protocol, a systematic approach of investigating cell senescence in each localised area of the endometrial stroma, glands and lumen was used. IHC staining was performed for HMGB2 and p16 on serial sections of each area mentioned and I explored associations with respective uNK cell density and subsequent pregnancy outcomes.

In order to establish this study, I identified a larger retrospective study sample of 89 patients with age under 40, recurrent pregnancy loss, an endometrial biopsy taken at LH +7 to +9 and a pregnancy event within 1 year of their biopsy. Each IHC stained serial section was processed using localisation, colour de-convolution and thresholding technique in Image J software as described in Chapter 2.

The study sample was derived from two groups:

1. Subsequent live birth or ongoing pregnancy beyond 10 weeks (LB/O)
2. Subsequent first trimester miscarriage (M)

Recent work in our research laboratory has formulated the uNK centile heat-map which represents the uNK density normalised for day of cycle the endometrial biopsy was taken and is based on 1997 samples. I have discussed the uNK centile values in more depth in Chapter 1. In this study, I have adopted the uNK centile due to the increased accuracy of uNK activity based on timing of biopsies.

5.2 Results

5.2.1 Demographic data

In this study, I developed a pre-conceptual endometrial test based on 89 women with recurrent pregnancy loss fulfilling the criteria stated above. 44 women had a live birth or ongoing pregnancy beyond 10 weeks gestation following their endometrial biopsy and 45 women had a further miscarriage. These two groups did not differ in maternal age or parity but there was a significant difference in the number of previous miscarriages. Women with a further miscarriage had a median of four miscarriages and those who had a positive pregnancy outcome had a median of 3 (p= 0.02; Mann-Whitney U test).

	PREGNANCY BEYOND 10 WEEKS N= 44	MISCARRIAGE N= 45	P VALUE
AGE, MEAN (SD)	34 (4.1)	33 (3.3)	0.44
NO OF PREVIOUS MISCARRIAGES MEDIAN (IQR)	3 (3-4)	4 (3-6)	0.02
PARITY NULLIPAROUS (%)	28 (64)	34 (76)	0.22

Table 5. 1: Demographic data of large retrospective study of 89 women. There was no significant difference in maternal age or parity in women who had an ongoing pregnancy beyond 10 weeks gestation or a further miscarriage. However, women with a miscarriage had more previous miscarriages (p=0.02, Mann-Whitney U test).

5.2.2 Assessing uNK and senescent factors in predicting pregnancy outcome

As the calculated uNK centile and senescent markers' density is not from a normal distribution, I applied the Mann-Whitney statistical test to investigate an association between uNK centile or cell senescence and pregnancy outcome. This statistical test investigates whether the distribution of uNK or senescent markers differ in both groups of women.

Statistical significance was reached when $p < 0.05$.

In this study, I found that the uNK centile test was not a predictor of subsequent pregnancy outcome (p=0.55) (Figure 5.1). The distribution between both groups were similar. This supports previous findings in the literature.

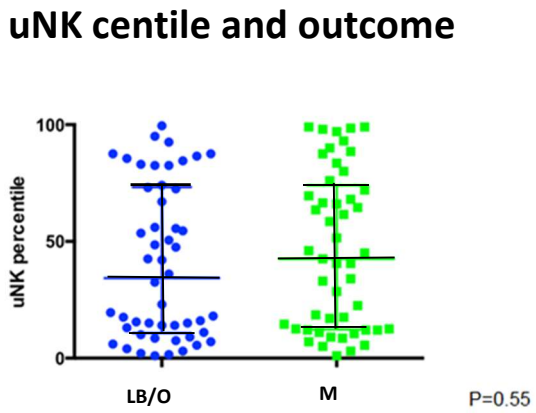

Figure 5. 1: uNK centile and prediction of pregnancy outcome. There is no difference between the uNK centile value in the groups who had a subsequent live birth or ongoing pregnancy beyond 10 weeks and the group of women who had a further miscarriage (p= 0.55; Mann-Whitney U test).

Next, the HMGB2 loss was measured in endometrial stroma, glands and lumen. There was no statistically significant difference between women with positive pregnancy outcome to those with further miscarriage when assessing cell senescence using HMGB2 loss (stromal p value = 0.4, glandular p value = 0.6, luminal p value = 0.92). The graphs are presented in Figure 5.2.

Cell senescence measurement using p16 antibody was more discerning between the two groups of women. The density of p16 in endometrial glands and lumen were significantly different between women with positive pregnancy outcome and those with further miscarriage (p = 0.01; p = 0.04 respectively). In comparison, p16 density in the stroma did not discriminate between the two groups of women (p = 0.37). This is illustrated in Figure 5.3.

Work in our research unit analysed p16 IHC staining using a heat-map similar to the uNK centile to increase accuracy due to timing of biopsies. The formulated p16 centile value is the normalised P16 density for day of cycle based on 308 samples (Brighton *et al.*, 2017). I converted the p16 density values to centile and repeated the statistical analysis discussed in the last paragraph.

The levels of p16 senescence in the endometrial glands and lumen between the two groups of women became more statistically significant, when p16 centile values were used instead of p16 density (p = 0.005; p = 0.015 respectively) (Figure 5.3, 5.4). The p16 centile in the endometrial stroma still showed no significant difference between women who had a positive pregnancy outcome or women with a further miscarriage (p = 0.47).

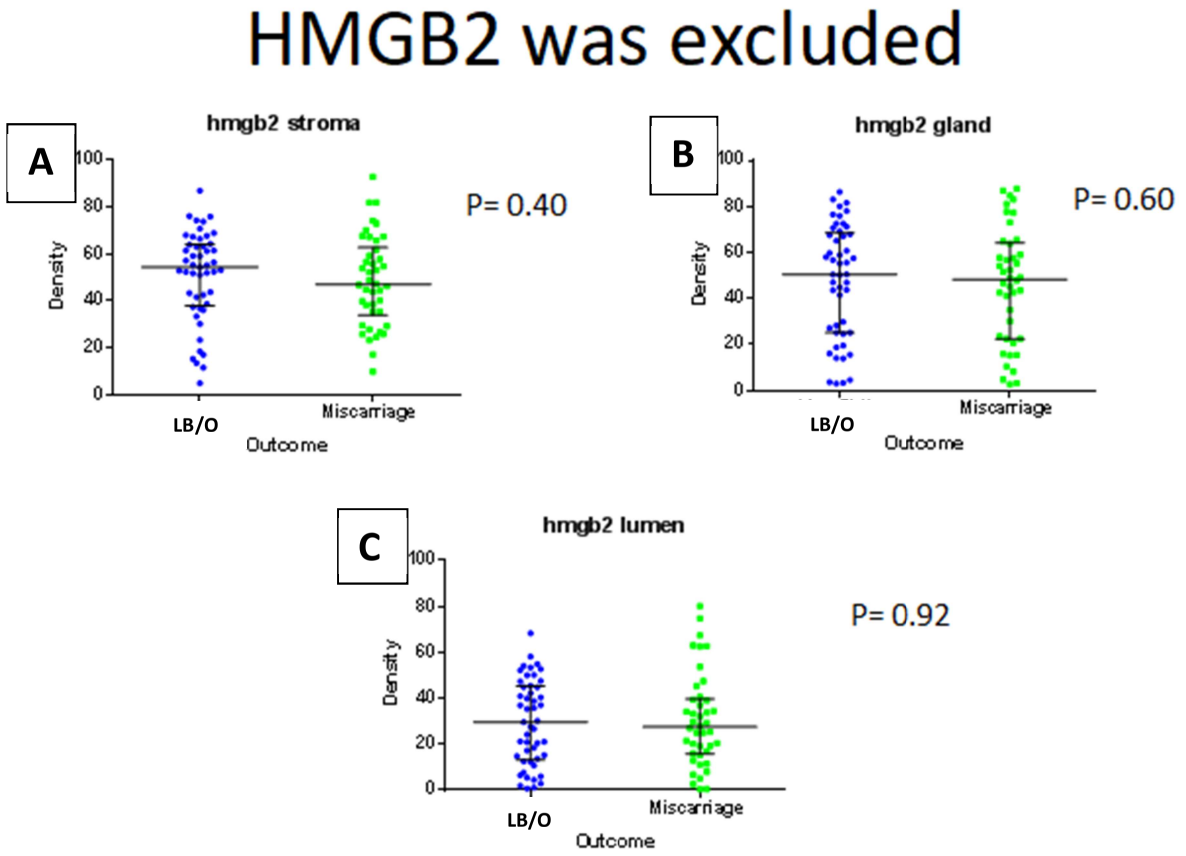

Figure 5. 2: HMGB2 and prediction of pregnancy outcome. These graphs illustrate HMGB2 loss in A) endometrial stroma, B) glands and C) lumen. There was no significant difference of HMGB2 levels between women with positive pregnancy outcome or a further miscarriage. (Mann-Whitney test)

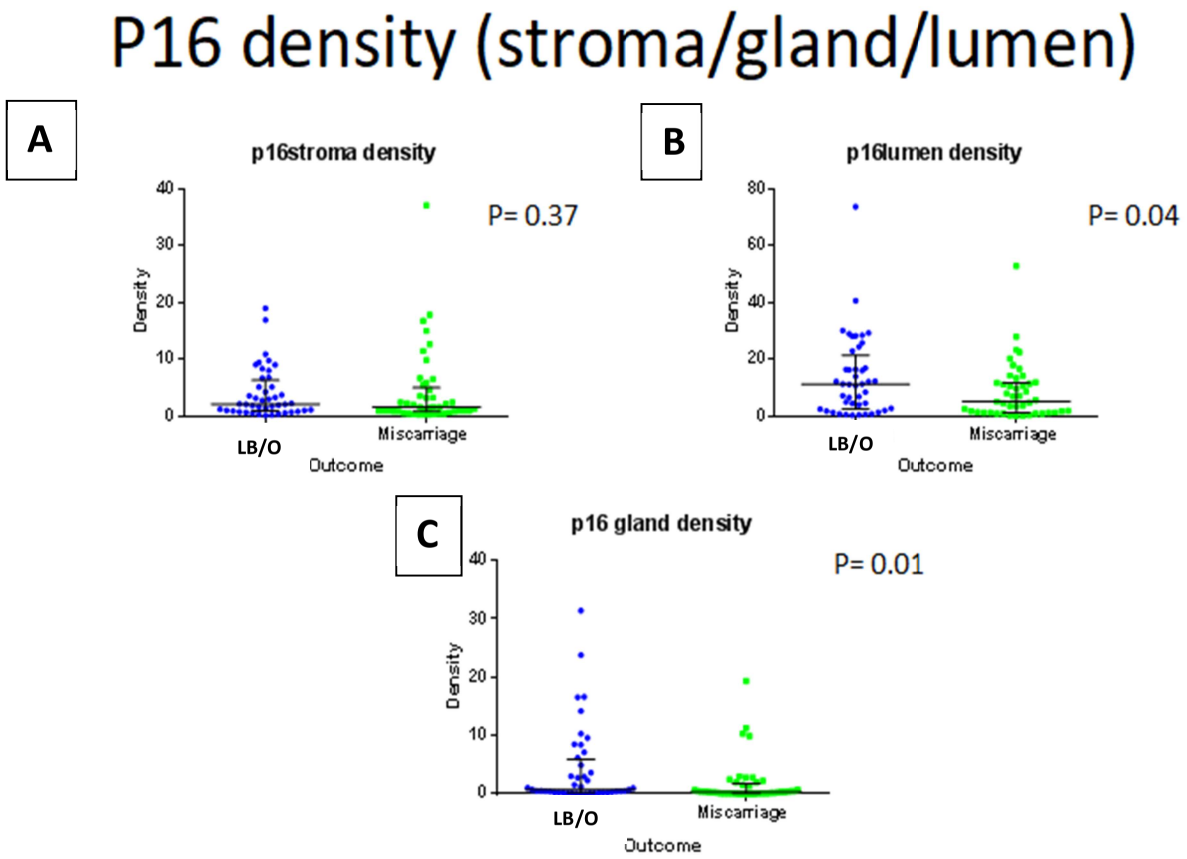

Figure 5. 3: p16 and prediction of pregnancy outcome. These graphs illustrate p16 levels in A) endometrial stroma, B) lumen and C) glands. There was significant difference of p16 levels between women with positive pregnancy outcome or a further miscarriage in the endometrial glands and lumen. p16 levels were higher in women with a positive pregnancy outcome. (Mann-Whitney test)

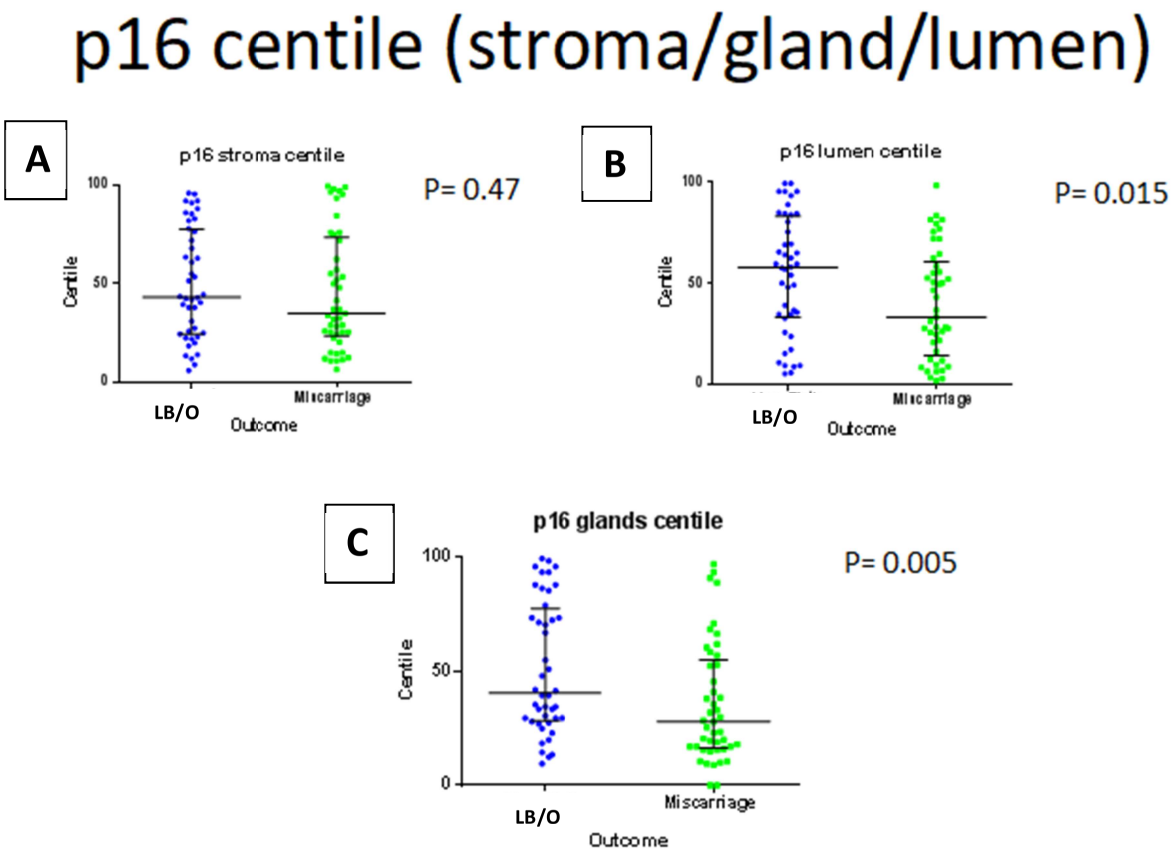

Figure 5. 4: p16 centile and pregnancy outcome. These graphs illustrate p16 centiles in A) endometrial stroma, B) lumen and C) glands. There was significant difference of p16 centile levels between women with positive pregnancy outcome or a further miscarriage in the endometrial glands and lumen. p16 levels were higher in women with a positive pregnancy outcome. The significance was more pronounce in endometrial glands. (Mann-Whitney test)

Based on these findings, I have excluded the use of HMGB2 in a combined pre-conceptual endometrial test as the levels were nearly identical in both groups. Focussing on p16 centiles, the most statistically significant discriminating factor between women with a positive pregnancy outcome and those with further miscarriage is glandular p16 centiles. Therefore, I have used the glandular p16 centiles to investigate logistic regression modelling with the aim to clarify its relationship with uNK centiles and pregnancy outcomes.

5.2.3 Regression Modelling to improve uNK test

Logistic regression modelling was used to formulate an equation that describes the relationship of uNK and glandular p16 values with pregnancy outcomes. The CD56 and p16 values were logged to make them a linear co-variable. Whilst the model described the outcomes in relation to uNK and glandular p16 values, the number of previous miscarriages is by far the largest factor in the model as shown in Table 5.2. The regression model also did not indicate that the endometrial test results were significant factors after removing the factor of number of previous miscarriages from the test equation (Table 5.3).

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	log56	.735	.418	3.088	1	.079	2.085
	log16g	-.504	.268	3.549	1	.060	.604
	logpmisc	3.461	1.220	8.051	1	.005	31.846
	plb	-.463	.564	.673	1	.412	.630
	Constant	-2.390	.768	9.691	1	.002	.092

a. Variable(s) entered on step 1: log56, log16g, logpmisc, plb.

Table 5. 2: Logistic regression modelling using factors of uNK (CD56) centile, glandular p16 centile and number of previous miscarriages.

Variables in the Equation							
		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	log56	.629	.378	2.759	1	.097	1.875
	log16g	-.425	.243	3.048	1	.081	.654
	Constant	-.632	.314	4.062	1	.044	.532
a. Variable(s) entered on step 1: log56, log16g.							

Table 5. 3: Logistic regression modelling using factors of uNK (CD56) centile and glandular p16 centile only.

The logistic regression model was complicated and clinically, was no improvement on asking women how many miscarriages they had. In addition, the model did not make any biological sense. Hence, I planned a new approach. Instead of using the Log values I went back to using the centile values of uNK and glandular p16, as this has a similar effect on making the data more linear. In addition, I used the findings of the regression modelling of subtracting the glandular p16 from the uNK cell value.

This enabled me to come up with a simple, clinically usable formula of 'uNK – glandular P16' centile. This has biological meaning because it assesses; (i) the synchronicity of the stromal and glandular decidualisation process, (ii) CD56 cells and their target senescent cells and (iii) stromal/glandular interactions.

5.2.4 Endometrial factor synchronicity test

I returned to the hypothesis that synchronicity of endometrial factors in decidualisation is paramount to successful pregnancy outcome. uNK cells play a role in clearing senescent cells and suggests a balance in the activity of uNK cells and presence of cell senescence is required to support pregnancy (Brighton *et al.*, 2017). Furthermore, uNK cell activity in the stroma and glandular maturation or differentiation appears to improve prediction of subsequent pregnancy as seen in previous work using Noyes criteria in histological dating (Liu *et al.*, 2014).

Hence, a test that combines stromal and glandular development is likely to assess the synchronicity of the various compartments in the endometrial preparation for pregnancy. We hypothesise that disruption of this process would lead to impaired decidualisation. Here, I used uNK (CD56) centiles as a surrogate marker for stromal development and glandular p16 centiles for glandular development. Therefore, I designed the formula of 'uNK minus glandular p16' centile test to assess this synchronicity.

Results are expressed as [median 'difference value' (Q1-Q3)]. The results show that a larger positive value of difference between uNK and glandular p16 centiles is associated with further miscarriage (13 (-14 to 39.3)) as compared to in women who had a live birth (-11 (-35.3 to 15.1)) (Figure 5.5). This difference in medians is significant using Mann-Whitney test (p=0.016). The significance is further supported by narrow inter-quartile ranges. This would suggest high uNK cell density that exceeds the predicted level of senescence is more prevalent in subsequent poor pregnancy outcome. Based on these findings, we would be able to set a cut off 'difference value' of 13. In this study, a value of ≤ 13 reflects 75% of live births or ongoing pregnancies beyond 10 weeks gestation and 50% of women with a further miscarriage. This cut off point is demonstrated by the red line in Figure 5.5.

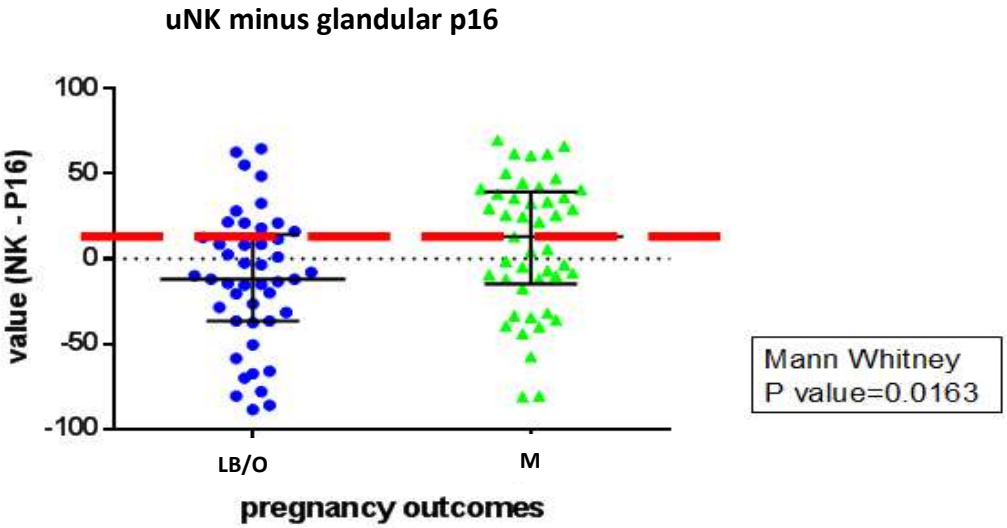

Figure 5. 5: Comparison of 'uNK minus glandular p16' centile value and pregnancy outcome. There is a significant difference between the 2 groups of women and the confidence intervals are narrow (p=0.0163). The cut-off value of 13 denoted by the red line in this graph, could potentially be used to predict live birth rate in a subsequent pregnancy.

The histogram below is a pictorial depiction of the results demonstrated in Figure 5.5. It illustrates a shift of 'difference values' to the right of 0, in women who have a further miscarriage. This means that uNK centile values are generally higher than glandular p16 centiles in women with a further miscarriage.

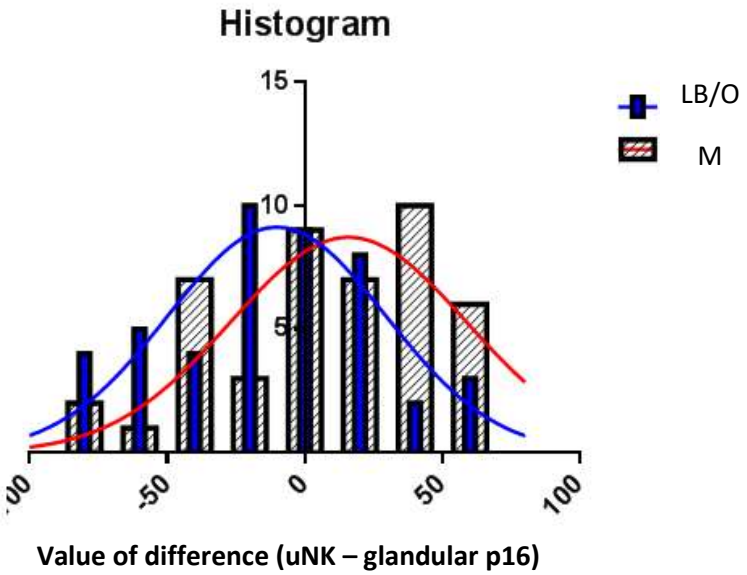

Figure 5. 6: Histogram showing distribution of 'uNK – glandular p16 centile'. The blue bars and line represent women with a live birth or pregnancy beyond 10 weeks gestation (LB/O) and, the striped bars and red line represent women with a further miscarriage (M). The histogram shows a shift to the right in women with poor pregnancy outcome. These women appear to have proportionately higher uNK centiles to glandular p16 centiles.

5.2.5 Validity of uNK – glandular p16 centile test

To test the validity in outcome prediction of 'uNK – glandular p16' centile test, I performed a receiver operating characteristic (ROC) analysis.

The accuracy of a test depends on how well the test separates the group being tested into those with and without the disease in question. Accuracy is measured by the area under the ROC curve. An area of 1 represents a perfect test. An area of 0.5 represents a test that is unable to differentiate between the two groups and is no better than chance. The traditional academic point system for the accuracy of a diagnostic test is as below (Tape, Thomas G. The Area Under an AUC Curve. University of Nebraska Medical Center. http://gim.unmc.edu/dxtests/Default.htm):

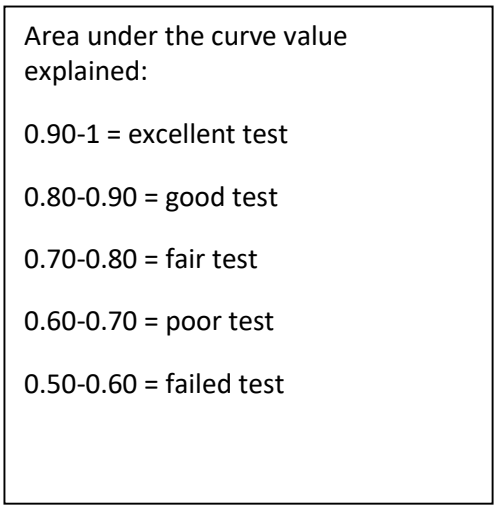

Figure 5. 7: Concept of area under curve in receiver operating characteristics. The ROC curve assesses the sensitivity and specificity of a test and determines the robustness of a test. An excellent test is a test that has a high true positive rate and a low false positive rate.

In this study, I have shown the ability to predict a further miscarriage is improved in area under the curve from 54% using uNK centile alone to 65% with 'uNK – glandular p16' centile test (Figure 5.8). The AUC value suggests that although better, the 'uNK – glandular p16' test has a poor ability as a predictive test. Clinically, the 'uNK – glandular p16' centile test is not yet robust to reliably predict pregnancy outcome. However, it may identify women who would benefit from further intervention or therapy. In the setting of future research in endometrial factor of recurrent reproductive failure, the test may help focus on a target population with interruption to synchronicity between uNK cells and glandular cell senescence.

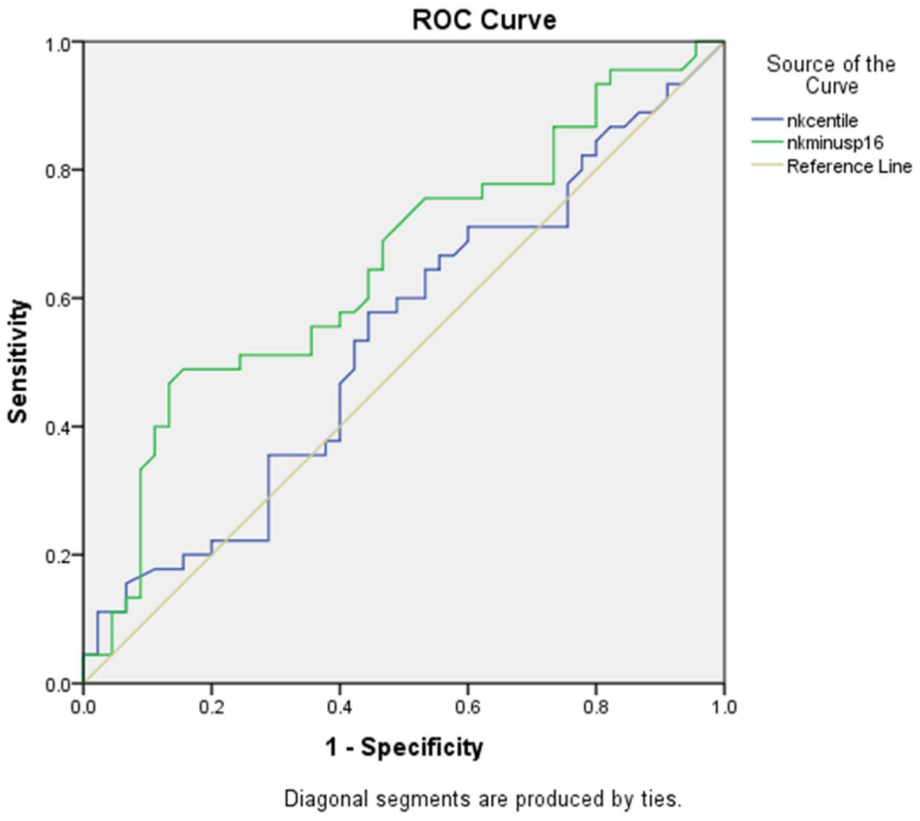

Figure 5. 8: ROC curve showing ability of two tests; i) uNK centile test and ii) 'uNK – glandular p16' centile test, in predicting a further miscarriage. The AUC for the uNK centile test and 'uNK-glandular p16' centile test was 0.54 and 0.65 respectively.

In order to determine the significance of the difference between the two ROC curve analyses, I compared the two AUC values of the uNK centile and 'uNK – glandular p16' centile test using Hanley & McNeil statistical formula. The table below shows that the improvement seen in predictive ability of 'uNK – glandular p16' centile test is significant with p value 0.011.

Variable	AUC	SE [a]	95% CI [b]
uNK_cent	0.537	0.0616	0.428 to 0.643
uNKminusp16	0.647	0.0588	0.539 to 0.746

[a] Hanley & McNeil, 1982
[b] Binomial exact

Pairwise comparison of ROC curves

uNK_cent ~ uNKminusp16	
Difference between areas	0.110
Standard Error [a]	0.0431
95% Confidence Interval	0.0256 to 0.195
z statistic	2.555
Significance level	P = 0.0106

[a] Hanley & McNeil, 1983

Table 5. 4: Hanley & McNeil statistical test; this statistical test compares two AUC values to determine whether there is a significant difference between two tests in its ability to predict outcome. The 'uNK – glandular p16' centile test is an improved predictive test compared to uNK centile test with a significant p value of 0.011.

5.3 Discussion

5.3.1 Endometrial stromal and glandular cell synchronicity

To elucidate whether uNK cell density and glandular cell senescence change together in the endometrium to encourage implantation, I designed a test formula of 'uNK – glandular p16' centile test. This was extracted from conclusions of the large test development study which showed an association between glandular p16 centile and improved pregnancy outcomes. This formula was analysed for all 89 women from the test development study. The value was plotted for each patient and divided into two defined groups. The groups were women with subsequent first trimester miscarriage and women with subsequent ongoing pregnancy beyond 10 weeks'

gestation or live birth. The difference between uNK and glandular cell senescent activity was compared in the two groups.

The loss of synchronicity denoted by higher values of uNK cells in comparison to glandular senescence was found to be more prevalent in women with a further miscarriage (Figure 5.5 and Figure 5.6). This could describe an overactivity of uNK cells or delayed glandular development leading to impaired decidualisation. A threshold value of 13 depicts the difference value point at which below it, identifies 75% of women with ongoing pregnancy or live birth and 50% of women who have a further miscarriage. I aim to use this threshold in a prospective RCT setting to evaluate this combined pre-conceptual endometrial test of endometrial stroma and gland development (uNK – glandular p16 centile test).

The limitations of this combined test are apparent in the ROC curve presented in the results. It shows that the 'uNK – glandular p16' centile test improves prediction over uNK test alone. However, with an area under the curve of 0.65, the combined test is still poor in differentiating women with a good pregnancy outcome to those with a further first trimester miscarriage. The limitations are caused by miscarriage of karyotypically abnormal embryos, the unknown effect of an endometrial biopsy and variation between menstrual cycles (inter-cycle variation). These factors may also prevent any pre-conceptual endometrial test from achieving excellent predictive ability. Therefore, it is possible that the best we can expect to achieve is marginal improvement in prediction of subsequent pregnancy outcomes. However, the improvement may still be relevant clinically.

I will attempt to address these limitations in the next chapter evaluating the combined test.

CHAPTER 6:
EVALUATION OF COMBINED TEST

Chapter 6: Evaluation of combined test

6.1 Background

The combined test 'uNK – glandular p16' centile test has been shown to improve predictive ability of pregnancy outcome of the uNK test. However, it is not yet a robust test for clinical use. In this chapter, I aim to evaluate this test and its limitations in a randomised-controlled trial setting to investigate reproducibility and address limitations.

6.1.1 Evaluating the combined test

The hypotheses tested:

- Synchronicity of endometrial stromal and glandular development are important for appropriate decidualisation and endometrial preparation for pregnancy.
- Endometrial stromal development is assessed by uNK cell density based on day of menstrual cycle. This hypothesis was made base on the fact that stromal cell proliferation followed changes in uNK cell density (Figure 4.6) and the role of uNK cells clearing stromal senescent cells, which also stimulates stem cells and proliferation (Brighton *et al.*, 2017).
- Endometrial glandular development is assessed by senescence of glandular cells measured by p16. This is due to senescence reflecting the mature state of a cell.
- Higher levels of uNK cell density based on day of cycle compared to glandular senescence is more prevalent in women with further miscarriage. This may reflect loss of synchronicity of the endometrial stromal and glandular compartments leading to pregnancy loss. This could describe an overactivity of uNK cells or delayed glandular development leading to impaired decidualisation.

Limitations to address:

- Miscarriage of karyotypically abnormal embryo
- Inter-cycle variation or effect of endometrial biopsy

6.1.2 Methods

Randomised-controlled trial (RCT) setting

There is a registered, pilot randomised controlled trial in our unit that is assessing the effect of endometrial scratch (biopsy) against 'sham-procedure' in recurrent miscarriage (Scratch in Miscarriage (SiM) trial). This pilot trial is a fellow research student's research project. I played a role as an investigator on the trial. I helped recruit and provide care for women entered into this trial. The trial received ethical approval from NHS National Research Ethics 2015 as mentioned in Chapter 2. The inclusion criteria for this trial was age 18 to 42 with history of at least two miscarriages and currently trying to conceive. It has reached its target of 109 recruited patients. The recruited patients provided written consent to the trial and for research samples to be stored.

The endometrial samples were taken using the same method as described in Chapter 2. Endometrial samples that were obtained in the intervention arm have been stored in our research Tissue Bank for future research.

In order to investigate the reproducibility of the combined test of 'uNK – glandular p16 centiles' as a predictive test, I retrieved these samples from tissue bank to process using IHC as per study protocol. Each patient had a calculated uNK and glandular p16 centile. The value of 'uNK – glandular p16 centiles' was derived from these values. The outcome measure of interest to test the predictive ability of the combined test, was the first pregnancy event within 3 menstrual cycles from the endometrial biopsy.

The patients were divided into groups based on their pregnancy outcome:

1. Women with a live birth
2. Women with first trimester miscarriage of karyotypically abnormal embryo
3. Women with further first trimester miscarriage
4. Women not achieved pregnancy in the 3 months

I have separated the women who miscarried a karyotypically abnormal embryo into their own group because this showed the correct response of the endometrium to the conceptus. Therefore, they represent an appropriate endometrial response and are distinct from women who miscarry a normal embryo, or where we do not have this information.

The values of uNK and glandular p16 centiles underwent statistical analysis as per study protocol in Chapter 5. Using the cut-off value of ≤13, I then divided patients into two groups to compare their subsequent pregnancy outcome. Group 1 has a 'difference value' of ≤13 and Group 2 a 'difference value' of >13.

To support the validity and reproducibility of this combined test, a cut-off value of 13 would be able to discriminate between women more likely to have a live birth to those with a poor pregnancy outcome. This will assess the importance of endometrial synchronicity between uNK cell density and glandular cell senescence.

Cytogenetic testing

During the course of this RCT study, cytogenetic testing was offered to all women who had a further miscarriage following current guidelines in recurrent pregnancy loss. Appointments were set up by the research team to discuss the results. The test may find normal or abnormal karyotype of embryo, and whether parental karyotype is advised. This effort was also made to address the study limitation of pregnancy loss attributable to karyotypically abnormal embryos, which hinders many research studies involving recurrent pregnancy loss.

6.2 Results

6.2.1 SiM trial and limitations

In the SiM trial, 55 patients were randomly allocated to the intervention arm. In the intervention arm, women underwent an endometrial scratch with a Wallach endometrial sampler in the mid-luteal phase of the menstrual cycle and advised to try to conceive from the next menstrual cycle. The primary outcome for my project, was assessed as the first pregnancy achieved or not within the first three months after the intervention as any positive effect of the biopsy on the endometrium was thought to last 3 months. We also believe that after 3 months the endometrial sample is not likely to reflect the endometrium at the time of implantation.

In this study sample (n=55), 31 women conceived within 3 months and 24 did not. This meant a large proportion could not be analysed for pregnancy outcome (44%). The trial was designed to obtain biopsy from women with recurrent miscarriage and collect pregnancy outcome prospectively. It was hypothesised that most women would conceive within the 3 months of the biopsy because women with recurrent miscarriage would have a highly receptive endometrium. However, this was not reflected in the results of the trial. The problem with the trial was loss of 34 women because they conceived before recruitment or randomisation to the endometrial scratch procedure. This group of women are likely to represent the superfertile women with highly receptive endometrium. The inability to recruit these women may explain to a degree, the proportion of women who did not conceive within the 3-month period. Please refer to the consort diagram in Figure 6.1 for illustration.

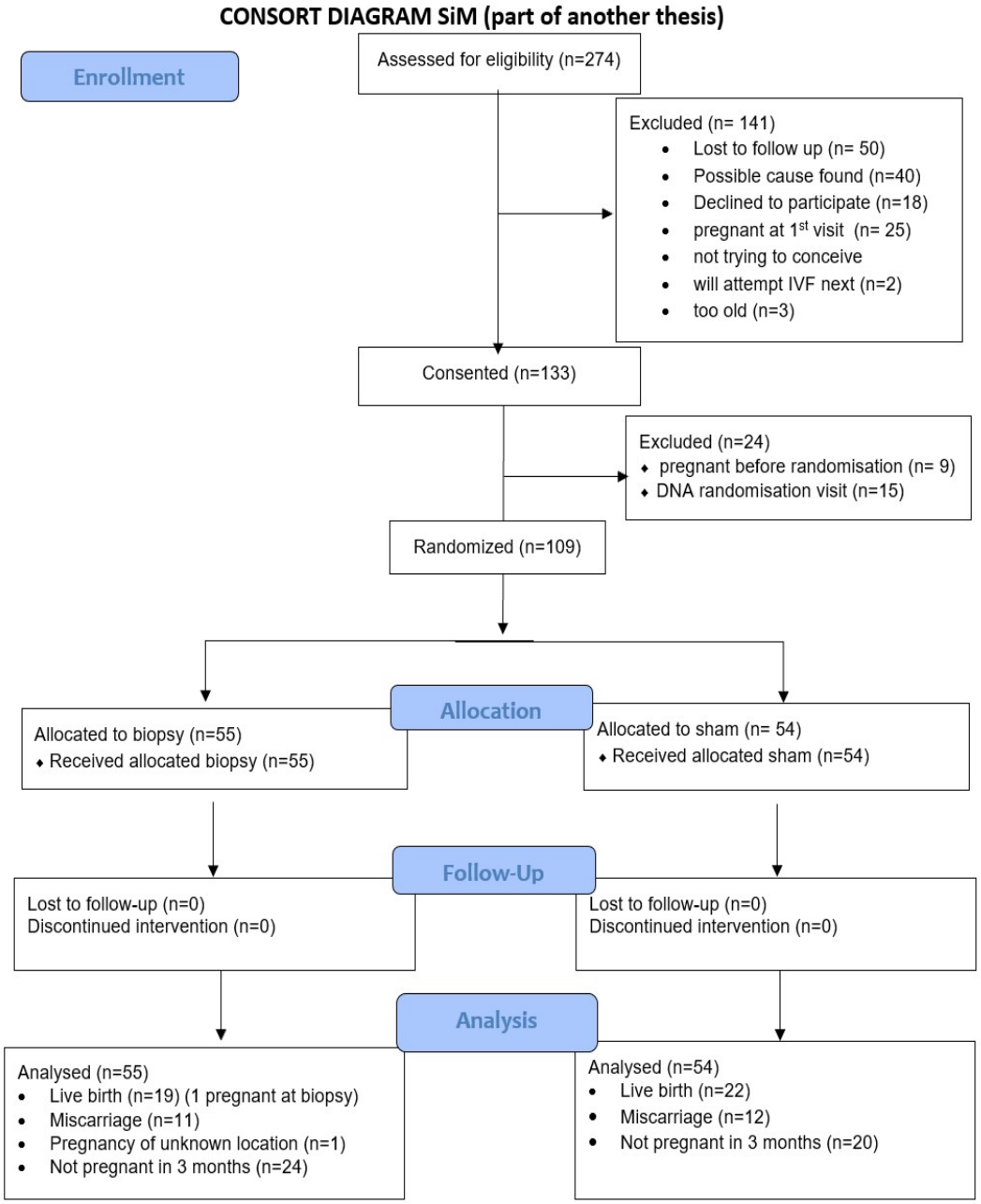

Figure 6. 1: CONSORT diagram for the SiM trial (unpublished). ***Reproduced with permission from Dr V Kandavel and Prof S Quenby (Biomedical research unit- UHCW/University of Warwick). In the process of recruiting and randomising women into the trial, 34 women were excluded due to being pregnant before we were able to recruit or randomise them.***

6.2.2 Demographics

In this study sample of 55 women, two were excluded from analysis as the first had a pregnancy of unknown location and the second was pregnant at time of biopsy, then proceeded to have a live birth. In the remaining 53 women, 18 had a live birth, 11 miscarried and 24 did not conceive in the time period for my study. Among the women who miscarried, 2 were found to have miscarried trisomy pregnancies (Trisomy 16, 22). The demographics of these women can be found in Table 6.1. There was significant difference between age of women with live birth or miscarriage and women who did not conceive within 3 months or had a karyotypically abnormal embryo (Kruskal-Wallis; p=<0.0001). However, there was no difference in number of previous miscarriages and parity between the groups (Kruskal-Wallis; p=0.59 and Chi-square; p=0.32 respectively).

	Live birth N=18	**Miscarriage** N=9	**Miscarried trisomy** N=2	**Did not conceive in 3 months** N=24
Age; mean (SD)	31 (4.1)	28 (4.3)	36 (0.7)	36 (4.5)
No previous miscarriages Median (IQR)	3 (2-3)	3 (3-3)	2 (2-2)	3 (2-5)
Nulliparous N (%)	10 (56%)	7 (78%)	1 (50%)	10 (42%)

Table 6. 1: Demographic details of women who underwent an endometrial biopsy in the mid-luteal phase of a menstrual cycle. There appeared to be a significant difference in maternal age in this demographic. The women who miscarried a trisomy pregnancy or did not conceive within 3 months of their endometrial biopsy, were older. There was no difference in number of previous miscarriages or parity. (Kruskal-Wallis test)

6.2.3 Test validation

I have used the same study protocol (Chapter 5) to investigate the distribution of uNK centile and glandular cell senescence with pregnancy outcome in the women identified in the RCT setting. The trial was designed to obtain a biopsy from women with recurrent miscarriage and collect pregnancy outcome prospectively. My aim was to investigate whether the 'uNK-glandular p16' centile test could predict subsequent pregnancy outcome by assessing endometrial stroma and glandular synchronicity. I applied the Kruskal-Wallis and Mann Whitney test to assess this association.

In Figure 6.2A, there was significant difference of uNK centile values between the 4 groups (Kruskal-Wallis; p=0.02). uNK centiles appear to most significantly discriminate women who will have a live birth to women who did not conceive within 3 months (Mann-Whitney; p=0.012). Women who did not conceive within the 3 months had higher uNK centile values with a median of 44th centile compared to a median of 18th centile in women with a live birth. However, the uNK centile values were similar in the women who had a miscarriage to those who did not conceive within 3 months (Mann-Whitney; p=0.74).

Glandular p16 centile did not show significant difference across all four groups (Kruskal-Wallis; p=0.28). However, unlike the uNK centile, glandular p16 centile was able to discriminate between women who had a miscarriage and those who did not conceive within 3 months (Mann-Whitney; p=0.029). This is illustrated in Figure 6.2B.

Combining the ability of both tests using 'uNK – glandular p16' showed that the combined test is able to predict pregnancy outcome and confirms findings in Chapter 5. There is a significant difference in 'uNK-glandular p16' centile values between women who had a live birth and women who had a further first trimester miscarriage (Mann-Whitney; p=0.036) (Figure 6.3).

Using the ‘uNK-glandular p16’ cut-off value of ≤ 13 as described in Chapter 5, I divided the study’s participants (n=53) into two groups to compare their subsequent pregnancy outcome. Group 1 had a ‘difference value’ of ≤13 (n=30) and Group 2 a ‘difference value’ of >13 (n=23). In Group 1, I was able to predict a live birth rate of 43% (13/30) in the 3 months cycle; compared to live birth rate of 22% (5/23) in Group 2. Furthermore, a ‘difference value’ > 13, predicted 8 out of 9 miscarriages not affected by known trisomy.

According to the hypothesis derived in Chapter 5, a ‘uNK – glandular p16’ centile value of ≤13 would have identified women with an endometrium that was within normal limits and preparing effectively for pregnancy. Interestingly, the uNK-glandular p16 test also correctly predicted both miscarriages of abnormal karyotype (difference value ≤13) and all 3 karyotypically normal embryos (difference value >13). However, the numbers are too small to make any conclusions.

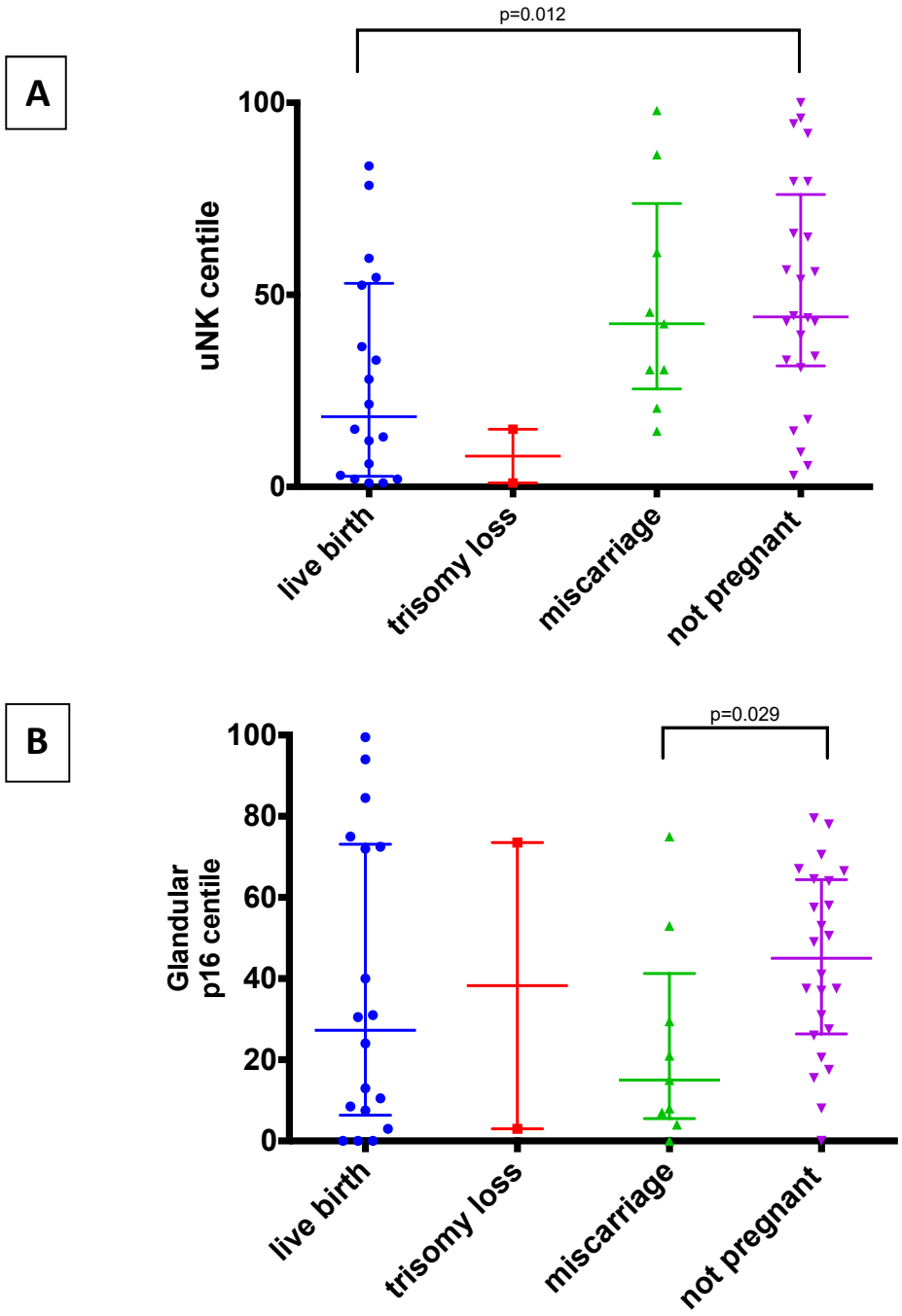

Figure 6. 2: uNK centile and glandular p16 centile in the outcome groups; (i) women with a live birth, (ii) women with a trisomy pregnancy loss, (iii) women with a further miscarriage and (iv) women who did not conceive in the specified timeframe.

Graph A demonstrates that there was significantly higher uNK centile value in women who did not conceive when compared to women with a live birth (p= 0.012; Mann-Whitney U test). Graph B illustrates that there was significantly less glandular p16 expression in women who miscarried compared to those who did not conceive (p= 0.029; Mann-Whitney U test).

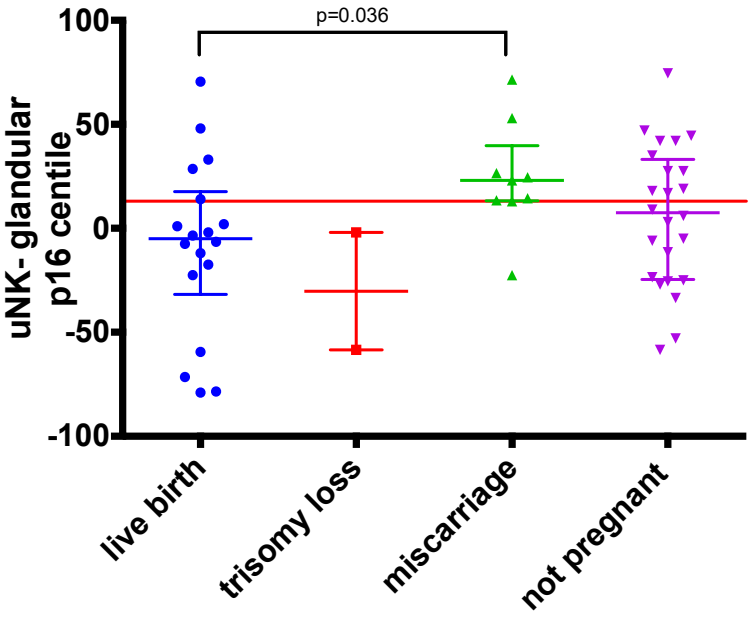

Figure 6. 3: The 'uNK – glandular p16' centile test and SiM trial outcome groups. The combined test did predict women who miscarried from the women who conceived in the 3-month period. The test was significantly different in women who miscarried and women who had a live birth (p= 0.036; Mann-Whitney U test). The red line depicts the 'difference value' of 13, which was determined as a useful cut-off value in Chapter 5. A 'uNK – glandular p16' centile test value of ≤ 13, predicted a live birth rate of 43% (13/30) within the 3-month period. This is compared to a live birth rate of 22% (5/23) with a value of >13. All but one woman in the miscarriage group had a value above 13.

6.3 Discussion

6.3.1 'uNK – glandular p16' centile test

The evaluation of the combined pre-conceptual endometrial test ('uNK – glandular p16' centile test) showed that it was reproducible in the context of the pilot 'Scratch in Miscarriage' RCT study conducted in our research unit. The SiM trial aim is to assess the effect of endometrial scratch (biopsy) against 'sham-procedure' in recurrent miscarriage. We chose to evaluate the combined test on women in the intervention arm of this trial to reduce bias; as it was a prospective study, had strict inclusion criteria and the participants were not offered any other therapy.

Only 29 of the 53 participants had a pregnancy event within 3 months of their biopsy. The 29 women were divided into groups based on their pregnancy outcome. The first group had a live birth (n=18). The second group had a miscarriage of a trisomy fetus (n=2) and therefore were put in a separate group because miscarriage of trisomy pregnancies is considered normal. The third group had a further first trimester miscarriage (n=9). Of the third group 3 women miscarried karyotypically normal embryos and we were unable to obtain karyotyping for the other 6 women.

The statistical analysis showed a difference between those that miscarried and those that had a live birth. Therefore, we have been able to validate the 'uNK – glandular p16' centile test with pregnancy outcomes conceived within 3 months. The period of 3 months was our pre-specified outcome measure from the SiM trial. I did re-analyse the data with one year's follow up and there was no difference in the 'uNK-glandular p16' centile test between the two groups. The problem was that some who miscarried in the first pregnancy after the biopsy conceived again quickly and had a live birth. This is a good outcome for the patients and clinical trial perspective but is less useful in our understanding of the endometrium. In addition, some women conceived more than 6 months after the biopsy and it is unlikely the test performed would reflect the process of implantation for these women.

Limitations of this evaluation study

One of the limitations of the evaluation study is the small numbers that were studied as 24 women did not conceive in the 3 months following their biopsy. The range of uNK centile and glandular p16 centile values were large and overlapped affecting the accuracy of the test (Figure 6.2). We also aimed to test all further miscarriage with cytogenetics. However, we found testing for cytogenetics on further pregnancy loss was not straightforward. Only 5 of 11 miscarriages had a cytogenetic result due to reasons of insufficient or no pregnancy tissue obtained.

Another aspect that will be explored next, is the presence of inter-cycle variation. We know that the endometrium rejuvenates each menstrual cycle. This invokes the question of whether this ultimately limits the pregnancy outcome predictive ability of a static test, which evaluates endometrial preparation for pregnancy in only one menstrual cycle. Hence, limiting the value of this combined test for pregnancies conceived beyond the 3-month period as discussed above.

CHAPTER 7:
INTER-CYCLE VARIATION

Chapter 7: Inter-cycle Variation

In our research team, information from repeat biopsies from the same patient, have been analysed to investigate inter-cycle variation in uNK centile values. Some patients have shown a change in response to an endometrial biopsy, while others do not.

I extracted data from the repeat biopsies for a 3-year retrospective study (2014 – 2017) of women with recurrent reproductive failure, who had repeat biopsies within a 6-month duration and had a subsequent pregnancy outcome within a year of the second biopsy. This study of 60 women with recurrent reproductive failure, were divided into two groups. Group 1 had a subsequent ongoing pregnancy beyond 12 weeks' gestation and Group 2 had a further first trimester miscarriage. The aim was to investigate uNK centile variation between cycles and association to pregnancy outcomes.

7.1 Results

Table 7.1 shows the demographic details of this study. There is no significant difference in the characteristics of the women in both groups. The mean age is 35.7 years and mean BMI is 23.9 for the whole study sample of 60 women. More women had infertility issues 56.7% and most were nulliparous 75%. The majority of women had an interval between biopsy of ≤ 3 months (80%). The median number of embryo transfers for the infertility patients was 4 (IQR: 2-7) and median number of previous miscarriages for women with recurrent pregnancy loss was 5 (IQR: 3-7).

	All subjects; n=60	Pregnancy beyond 12 weeks; n=29 (48.3%)	Further IVF failure or miscarriage; n=31 (51.7%)	P value (unadjusted; adjusted)
Age; Mean age (±SD)	35.7 (4.1)	34.3 (4.1)	36.9 (3.8)	0.02; 0.10
BMI; Mean BMI (±SD)	23.9 (4.6)	24.1 (4.9)	23.7 (4.4)	0.79; 0.64
Fertility (%)				0.21; 0.44
Infertility	34 (56.7)	14 (48.3)	20 (64.5)	
RPL	26 (43.3)	15 (51.7)	11 (35.5)	
Parity (%)				0.30; 0.61
Nulliparous	45 (75)	20 (69)	25 (80.6)	
Multiparous	15 (25)	9 (31)	6 (19.4)	
Interval between biopsy				0.61; 0.89
≤ 3 months	48 (80)	24 (82.8)	24 (77.4)	
3-6 months	12 (20)	5 (17.2)	7 (22.6)	

Table 7. 1: Demographic data of women with repeat biopsies and a pregnancy event within 1 year of second biopsy (n=60). There was no significant difference in the two groups in all the demographic data above; maternal age, BMI, fertility, parity and interval between biopsy. Most women were nulliparous (75%) and had an interval between biopsy within 3 months (80%).

The two groups were analysed separately, to investigate uNK cell density changes following endometrial tissue injury and whether this relates to subsequent pregnancy outcome in women with recurrent reproductive failure. Each patient's biopsy was processed using IHC to calculate a uNK centile value. The first biopsy's uNK centile was compared to that of the paired second biopsy to assess inter-cycle variation for each group. The aim was to assess whether the endometrium behaved differently between menstrual cycles in women who had a positive pregnancy outcome and those who did not. I used the Wilcoxon statistical test to compare the paired biopsies and $p < 0.05$ was significant. Results are reported as (Median difference (95% CI); p value).

The results showed that women with a subsequent pregnancy beyond 12 weeks had a significant fall in their uNK centile value in the second biopsy (-13 (-26.7 to -4.6); $p = 0.0073$). In comparison, the uNK centile values did not change significantly in women with a further IVF failure or miscarriage (-1.5 (-16.6 to 10.8); $p = 0.585$). This is illustrated in Figure 7.1.

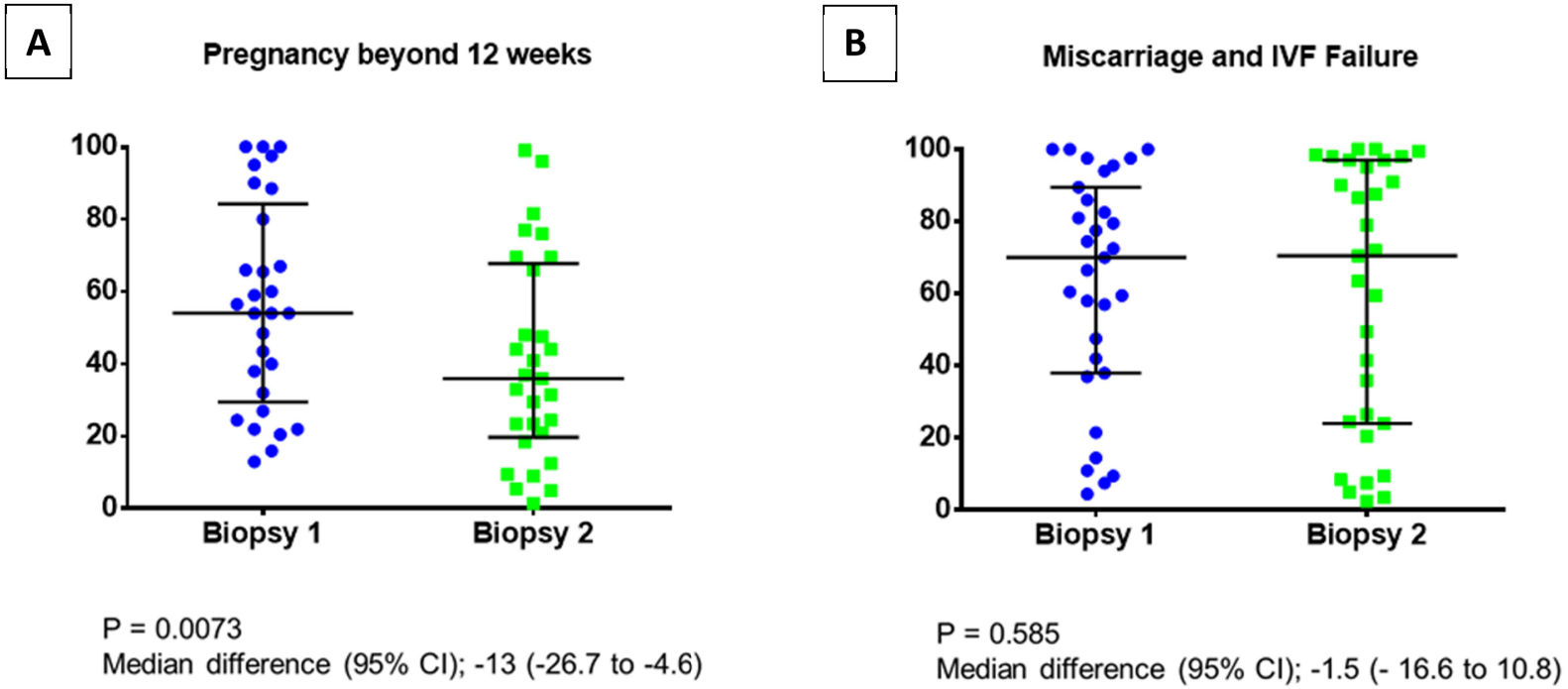

Figure 7. 1: Inter-cycle variation of uNK centile test and pregnancy outcome. Graph A demonstrates the change in uNK centile between two endometrial biopsies (within 6 months interval) in women who had an ongoing pregnancy beyond 12 weeks gestation. There was a significant downward trend in uNK centile associated with good pregnancy outcome (p= 0.0073; Wilcoxon test). Graph B illustrates that there is no significant change in uNK centile between endometrial biopsies (within 6 months interval) in women with a further miscarriage or failed IVF/ICSI (p=0.585; Wilcoxon test).

7.2: Advantages and limitations

This study is one of the first examining repeat endometrial biopsies and subsequent pregnancy outcomes in reference to uNK cell activity. It provides insight into the difference in the decidual process between menstrual cycles and raises two theories. The first is the concept that the repeat samples show the variation present in menstrual cycles as the endometrium undergoes regeneration and adaption as a crucial role in human reproductive success. The second theory is that the endometrial biopsy is a source of endometrial injury and the difference in uNK centile values found are a direct reflection of the biopsy. Therefore, the difference recorded is the ability of the endometrium to respond to the stimuli and not just a passive reflection of decidual processes.

Another advantage of this study is the enrichment of its study population. As previously stated, the median number of embryo transfers for the infertility patients was 4 (IQR: 2-7) and median number of previous miscarriages for women with recurrent pregnancy loss was 5 (IQR: 3-7). This reflects a more severe phenotype, which confers a higher likelihood of aberrant decidualisation and hence is more demonstrable when studied.

The limitation of this study is the bias introduced as a retrospective study, which includes data collection bias. This type of study can be run efficiently in this setting because it is less time-consuming and is low cost. Due to the proportion of women who do not conceive and are loss to follow up following an intervention, as seen in the prospective RCT study (SiM trial), the number that would need to be entered into a prospective cohort study would be large.

This study is also limited by the unknown proportion of miscarriages or implantation failures which will be due to embryo factors. Furthermore, due to the very nature of variation between menstrual cycles, it is difficult to determine the duration in which the results would still be reflective of the patient's endometrial status and the

optimum time between repeat endometrial biopsies. The more commonly accepted time-frame that would still reflect endometrial status, is a conception event within 3 months of an endometrial biopsy test.

Another factor to be considered, is the results of this study are still not yet robust as confidence intervals are wide.

7.3: Impact of study

This is likely the most important finding in my research study as a whole. The results give credence to scientific thought that each menstrual cycle must differ as it continues to move through its regenerative and adaptive phases (Lucas *et al.*, 2016; Lucas *et al.*, 2013). As evidence emerges that uNK cells and senescent cells form part of the mechanisms of decidualisation, it is reasonable to suggest that they're activity changes between cycles as the endometrium aims to balance its receptive and selective traits, which are important for implantation and reproductive success.

This study suggests that a reduction in uNK cell density in response to endometrial tissue injury (endometrial biopsy) appears to be associated with higher rates of ongoing pregnancies beyond 12 weeks gestation. These findings add to current evidence that endometrial plasticity (the ability of the endometrium to adapt from cycle to cycle in response to stress signals) is paramount to endometrial preparation for pregnancy (Lucas *et al.*, 2016).

Concept of endometrial plasticity

The Warwick group has explored the concept of endometrial plasticity and its importance in reproductive fitness. It is hypothesised that reproductive success is dependent on a constant homeostatic rebalancing of embryonic and maternal traits (Chuong *et al.*, 2013; Crespi & Semeniuk, 2004; Emera *et al.*, 2012). This is particularly important due to the invasive nature of human embryos and its intrinsic

chromosomal instability. The cyclical regeneration of the endometrium is remarkable and is associated with an abundance of adult stem cells, which are mainly found around the spiral arteries (Du *et al.*, 2012; Du & Taylor, 2010; Gargett *et al.*, 2012; Gargett & Ye, 2012; Lucas *et al.*, 2016). The underlying mechanism for recruitment of these mesenchymal stem cells which differentiate into mature stromal cells is still unclear but is likely to differ following 'endometrial injury'; such as menstruation, miscarriage or parturition (Lucas *et al.*, 2016). This innate adaptability may explain the high rate of successful pregnancy outcome in women who suffer from repeated pregnancy loss.

Patient-specific response

In addition, the patient-specific response to endometrial injury is highlighted in this study. It appears that the endometrium behaves differently for women who have a subsequent successful pregnancy, in the months preceding conception. This variation between patient's response to the endometrial biopsy likely determines the clinical benefit of the scratch procedure. These findings also support the limitations of a static test taken in one menstrual cycle in predicting subsequent pregnancy outcomes, as it does not assess each patient's individual endometrial response.

Ideally, the repeat endometrial biopsy test would identify women with an adaptive endometrium, who will have a high likelihood of positive pregnancy outcome. Conversely, those where this is aberrant would be stratified to having a poorer outcome prediction, as their decidualisation process is disordered. If the validity of this identification process is confirmed in future research, it may be a measure to focus recruitment of women into trials and enable targeted therapies.

Future challenge

The future challenge is to confirm or refute the findings that a fall in uNK cell density in response to 'endometrial injury' predicts improved subsequent pregnancy outcome using larger, prospective cohort studies. It would then be essential to understand whether there are specific signature patterns that discriminate women who have RPL, are 'superfertile' or have RIF. If there is credence to these findings, future research would be able to use these signature uNK density changes to focus recruitment to trials and target therapies.

CHAPTER 8:
SUMMARY OF CONCLUSIONS AND FUTURE WORK

Chapter 8: Summary of conclusions and future work

The overall aim of this research project is to design a pre-conceptual endometrial test that can improve prediction of subsequent pregnancy outcome when compared to the uNK test in recurrent pregnancy loss. There is a dearth of strong evidence in predicting future pregnancy outcomes in couples with recurrent reproductive failure. This is due to the highly complex and varied factors that influence the outcome of care in these couples. It makes for a difficult clinical situation fraught with controversy in its management and causes significant stress for couples. The ability to predict outcome and profile or identify women with poorer prognosis will help individualise care and focus research more effectively.

The uNK cell test has been widely adopted because they have consistently been found to be higher in reproductive failure (Quenby *et al.*, 1999; Tuckerman *et al.*, 2007), correlates negatively with markers of decidualisation (Kuroda *et al.*, 2013) and been used to identify women who may benefit from steroid therapy to prevent repeated pregnancy loss (Tang *et al.*, 2011). To date, efforts to design or improve prediction tests include work combining uNK test and histological dating using Noyes' criteria (Liu *et al.*, 2014) and assessing activity of uNK cells by measuring released interleukin levels (IL-15, IL-18) (Lédée *et al.*, 2016; Lédée *et al.*, 2017). They show limited predictive ability. The number of previous miscarriages or implantation failures and maternal age remain factors that outweigh other parameters in predicting outcome but are themselves unchangeable. My aim was to translate novel high-quality laboratory science into a clinical application to improve prediction of pregnancy outcome and highlight therapies by understanding underlying pathophysiology in recurrent pregnancy loss. If results were encouraging, future work would involve a predictive test for women with recurrent implantation failure.

The scientific hypotheses that formed the structure of this research project included:

1. uNK cell density on its own is not a good predictor of pregnancy outcome (Liu *et al.*, 2014)
2. When uNK cell density is normalised for day of cycle creating the uNK centile, its ability in predicting pregnancy outcome improves (Brighton *et al.*, 2017)
3. Histological dating includes assessment of endometrial glandular development in decidualisation therefore improves predictive ability of uNK test (Liu *et al.*, 2014)
4. Increased rate of proliferation indicated by Ki67 in uNK cells indicates active uNK cells and is associated with recurrent pregnancy loss (Lédée *et al.*, 2016; Lédée *et al.*, 2017)
5. Endometrial stromal senescence (HMGB2 loss or p16 presence) promotes senescence, impairs decidualisation and is associated with recurrent pregnancy loss (Lucas *et al.*, 2016).

In assessing the predictive ability of the uNK test, I have demonstrated that the uNK test on its own does not predict pregnancy outcome based on 281 women with recurrent reproductive failure (Chapter 3). I did find that there was improvement in prediction using uNK centile (uNK cell density normalised for day of cycle) but this was still not statistically significant. In the randomised controlled trial, it was interesting that uNK centile did appear to predict which women were slower to conceive.

In my research, I have found a pre-conceptual endometrial test that incorporates the endometrial stromal and glandular components (uNK – glandular p16 centile test) which demonstrated improved prediction of pregnancy outcome when compared to the uNK test (Chapter 5). The improvement is illustrated in the ROC curve produced but is not robust and still has poor predictive ability (Figure 5.8). These findings showed some promise, as it suggested synchronicity between the two compartments reflected an ordered decidualisation process and was important

for a successful pregnancy. It supported findings by our research team that demonstrated the uNK cells' role in clearing endometrial senescent cells and invoking human endometrial stem cell proliferation (Brighton *et al.*, 2017).

I have shown a corroboration of the above findings relating to the 'uNK – glandular p16' centile test's predictive ability, in the setting of a prospective Randomised-Controlled Trial (RCT) setting (Chapter 6). A strong conclusion could not be made as this was limited by the sample size of 53 women. It was found that the most fertile women with recurrent pregnancy loss conceived before randomisation and a possibly less severe phenotype was recruited into the trial. In addition, only a small proportion of miscarriages had cytogenetic results to limit the confounding factor of abnormal embryos. However, this setting provided the best option to limit bias.

Technically, I have been able to design an endometrial pre-conceptual test, that is easy to perform albeit not yet robust. The results in conducting this research have strengthened and confirmed hypotheses formed in our research unit concerning the underlying pathophysiological processes in recurrent reproductive failure. We have shown it is possible to assess stromal and glandular development in the endometrium using immunohistochemistry (IHC) staining. The study protocol developed enables this process to be performed on a large scale and could be adopted in future research work analysing pathophysiology.

This research has also invoked the question of whether the concept of a one-off endometrial test is sufficient to provide prognostic information that is strong enough to use in a clinical setting. Alternatively, the 'uNK – glandular p16' centile test could be used to enter women into a randomised controlled trial and would be far better than the uNK test. This is because a value >13 selects nearly all those who miscarry (89%) with 22% of live births receiving unnecessary treatment, as seen in the RCT setting (Chapter 6). In contrast the uNK centile test at >75th centile would have only selected 22% of the miscarriages but suggested 11% of the live births received unnecessary treatment. It may be possible to further enrich the study population by

incorporating the measure of each participants' endometrial plasticity denoted by inter-cycle variation (Chapter 7).

This combined endometrial pre-conceptual test still requires a significant amount of scientific exploration and should only be performed in a research setting.

8.1 Advantages and limitations of this research study

The advantage of this research project is its systematic approach to investigating the predictive ability of a combined pre-conceptual endometrial test. This enabled me to assess effectiveness of the uNK cell test as a predictor of pregnancy outcome, develop IHC protocol for cell proliferation and cell senescence in specific localised areas within the endometrium (stromal, glandular and luminal), describe possible relationship between these markers and pregnancy outcome, and evaluate the developed combined pre-conceptual endometrial test in a prospective RCT setting.

I was able to source data from an established 'Implantation Clinic' where women with recurrent reproductive failure attend for consultation and are offered the uNK test. This enabled me to derive information and knowledge from a considerable number of couples with more severe phenotype as a population, due to this centre being a tertiary referral unit.

Working closely with our research team examining scientific evidence and hypotheses of endometrial factor in recurrent reproductive failure has ensured my research project remained relevant to emerging concepts and evidence. It has given me insight into how research moves forward and increased my understanding on the subject.

There are limitations to my research project. These can be divided into data collection, inherent error in the uNK or IHC test, miscarriage of abnormal karyotype embryos and inter-cycle variation.

Data collection in the early stages of my research project assessing the uNK cell density test (Chapter 3) included retrospective data that would introduce bias. There was also bias in recording patient's pregnancy outcome due to patient self-reporting and practice of discharging patients from the 'Implantation Clinic' following a 10 to 12 weeks' gestation early pregnancy scan. In an ideal situation, live birth instead of ongoing pregnancy beyond 10 to 12 weeks' gestation would be the main outcome measure. We were able to mitigate this in the final evaluation test in the prospective RCT setting, as all final pregnancy outcomes were known (Chapter 6).

The combined endometrial test developed ('uNK – glandular p16' centile test) was based on IHC protocols used for many years in uNK testing. The inherent test errors include inter-observer error of 20% as seen in our laboratory and possible error in patient reported ovulation test ± 48 hours. This would affect calculated centile values for both markers.

Another limitation to any recurrent pregnancy loss study, is the proportion of first trimester miscarriages due to abnormal embryo karyotype. We attempted to address this limitation in the final evaluation test, by offering cytogenetics testing for all first trimester miscarriages. Despite our best efforts, we managed to gain cytogenetic results of only 5 out of 11 first trimester miscarriages in the final evaluation test (Chapter 6). This highlights the difficulties in obtaining this information for future studies.

In developing this combined endometrial predictive test, it has revealed the presence and importance of inter-cycle variation. It has been shown that women's endometrium reacts differently to endometrial injury (endometrial scratch). Some women's uNK cell levels differ between menstrual cycles and others stay at very similar levels. It appears that women whose uNK cell levels fall have more positive pregnancy outcomes (Chapter 6). Therefore, it suggests a one-off, static endometrial test may not be sufficient to predict pregnancy outcomes as it does not consider the variation between cycles.

8.2 Future work

An important area that needs further research is endometrial plasticity, which is the ability of the endometrium to respond to stressful stimuli. Understanding how the endometrium rejuvenates and how it varies between menstrual cycles, may be key to developing a test with a more robust ability to predict pregnancy outcome in recurrent reproductive failure.

This could take the form of repeat biopsies measuring changes in uNK cell or senescent markers; which could identify women with poorer prognosis. Research could focus on these women with the aim of identifying appropriate, individualised therapies. On the other hand, a marker of stem cell response that is important in endometrial rejuvenation, could be investigated and developed into a prognostic test.

Another area that I have not explored is the development of angiogenesis and spiral arteries during decidualisation. If a relationship between spiral artery development and pregnancy outcome can be established, it may further improve our combined pre-conceptual test (uNK – glandular p16 centile test) by including a vascular component. This would then measure the synchronicity between the stromal, glandular and vascular components of the endometrium.

There is also the question of how the endometrium of women with recurrent implantation failure differs from women with recurrent pregnancy loss. Do these clinical presentations sit on opposite sides of a clinical scale? The first having an excessively selective endometrium and the latter an excessively receptive endometrium. Would any future predictive test, work in the same way for both presentations?

Crucially, the recurring theme for any future research into pregnancy outcome for couples with recurrent reproductive failure has been the quality of pregnancy

outcome data and proportion of miscarriages due to abnormal embryo karyotype. It is accepted that the measures needed to address these issues are complex, time-consuming and carry costs. However, to have meaningful results it is essential. A comprehensive database to collect such information has been set up by my supervisor and will greatly improve our resources for excellent research. I would also suggest that research investigating pathophysiology of endometrial factor or designing a test should begin in women with more severe phenotype (≥ 5 previous miscarriage or ≥ 3 failed IVF/ICSI) to identify the abnormal processes. This is because results may be diluted by data from less severe phenotype which may represent chance or embryo abnormality instead of endometrial pathology. Once these processes are identified, we would need a way to identify affected women earlier in their reproductive history to implement measures and avoid preventable miscarriages.

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