Uterine Natural Killer (uNK) Cells and Recurrent Miscarriage: A Pilot Randomised Controlled Trial of Prednisolone in Women with High uNK Cells and Recurrent Miscarriage

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By

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ABSTRACT

Recurrent miscarriage (RM) is stressful. One reason for this is because no causes can be found for the pregnancy loss in the majority of cases. Focus has been on the endometrium which undergoes decidualisation in preparation for implantation. Any problems in the finely organised interactions between the endometrium and invading trophoblast cells may contribute towards a miscarriage. Immunological mechanisms are thought to be one of the pathways involved as there is the need of maternal adaptation of her immune response to the semi-allogenic developing embryo. Uterine natural killer (uNK) cells are the most abundant in the endometrium during the window of implantation. They interact with trophoblast cells, and are involved in vascular remodelling, an important step in implantation. Hence, they have a biological plausibility of playing a major role in RM.
Both peripheral NK (pNK) and uNK cells tests have been developed as assessments of immunological causes of RM. A systematic review performed showed inadequate evidence for both pNK and uNK cells tests as markers for adverse pregnancy outcomes. There were only twelve studies, with 446 patients reporting pregnancy outcomes. There was no accepted consensus of normality and methodology for analysing NK cells. The conclusion was the need for well-designed studies to assess the role of NK cell tests as a clinically useful marker for screening.

This led to the conduct of the pilot phase of a RCT of prednisolone in early pregnancy in women with idiopathic RM and raised uNK cells density. The main aim of this trial was to assess feasibility of recruitment and tolerability of prednisolone. Secondary clinical outcomes included live birth, types of miscarriage, miscarriage karyotype, gestational age at delivery, birthweight and pregnancy complications (eg: pre-eclampsia, gestational diabetes, fetal abnormality, stillbirth, IUGR). 160 women were screened for uNK cells density and 40 were randomised, despite the majority (85%) desiring prednisolone if given a choice. There was a trend towards improved live birth rate with prednisolone treatment but this was not significant. There were equal numbers of biochemical, sac and fetal pregnancy losses in both groups. All completed treatment with main reported side effects in the prednisolone group of insomnia. There were no pregnancy complications. The analysis of uNK cells was found to be very time consuming. To accommodate potentially large numbers who will be screened in the definitive trial, an alternative, quicker and equally accurate method of analysing uNK cells was developed using the colour deconvolution and area measurement plug-ins of a public domain image analysis package, Image J.

Women supported this trial. Randomisation was acceptable. The prednisolone was safe. UNK cell density is a valid biomarker of severe outcomes. There was a trend towards improvement in live birth rates. This trial paves the way for the development of an endometrial based test to screen for the subgroup of women with RM that could potentially benefit from individualised treatment.

CONTENTS

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LIST OF ABBREVIATIONS

RM	Recurrent miscarriage
APS	Antiphospholipid syndrome
PCOS	Polycystic ovarian syndrome
UNK	Uterine natural killer
RCT	Randomised controlled trial
PGD	Pre-implantation genetic diagnosis
ART	Assisted reproductive techniques
LDA	Low dose aspirin
IVIG	Intravenous immunoglobulin
UFH	Unfractionated heparin
LMWH	Low molecular weight heparin
FVL	Factor V Leiden
APCR	activated protein C resistance
TEG	Thromboelastography
hCG	Human Chorionic Gonadotrophin
LH	Luteanising hormone
ACA	Anticardiolipin antibodies
LA	Lupus anticoagulant
ANA	Anti-nuclear antibodies
DNA	Deoxyribonucleic Acid
HLA	Human leucocyte antigens
ELISA	Enzyme-linked immunosorbent assay
IFN	Interferon
IL	Interleukin
TNF	Tumour necrosis factor

EVT	Extravillous trophoblastic
PNK	Peripheral blood natural killer
GM-CSF	Granulocyte-macrophage colony stimulating factor
CSF	Colony stimulating factor
TGF	Transforming growth factor
LIF	Leukemia-inhibitory factor
ER	Estrogen receptor
MIP	Macrophage inflammatory protein
VEGF	Vascular endothelial growth factor
KIR	Killer-cell immunoglobulin-like receptors
ILT	Immunoglobin-like transcripts
HPL	Human placental lactogen
MIF	Macrophage migration inhibitory factor
PAI	Plasminogen activator inhibitor
MMP	Matrix metalloproteinases
IVF	In-vitro fertilisation
Ig	Immunoglobulin
CI	Chief investigator
MA	Miscarriage association
EPU	Early pregnancy unit
PI	Principal investigator
HIER	Heat induced epitope retrieval
NaOH	Sodium hydroxide
TBS	Tris buffered saline
NaCl	Sodium chloride
HCl	Hydrochloric Acid
H_20_2	Hydrogen peroxide

BSA	Bovine serum albumin
DAB	Diaminobenzidine
NIH	National institutes of health
HPF	High powered field
MHRA	Medicines and healthcare products regulatory agency
NIHR	National institute for health research
SAE/AE	Serious adverse event or adverse event
SUSAR	Suspected unexpected serious adverse reaction
DMC	Data monitoring committee
TSC	Trial steering committee
TMG	Trial management group
CRF	Case report forms
POC	Products of conception
ROC	Receiver operating characteristic
SAIA	Semi-automated image analysis
CD	Colour deconvolution
AM	Area measurement
IHC	Immunohistochemistry
RGB	Red green blue
LOA	Limits of agreement

CHAPTER 1

INTRODUCTION

CHAPTER 1 – INTRODUCTION

1.1 Background

Miscarriage is the most common complication in human pregnancy. Recurrent miscarriage (RM), historically defined as the loss of three or more consecutive pregnancies before 24 weeks gestation, occurs in about 1% of fertile women trying to conceive, and is associated with significant psychological morbidity[1]. This definition is recommended by the European Society of Human Reproduction and Embryology (ESHRE), but is not accepted universally[2]. The definition should ideally be based on the knowledge of risk for a subsequent miscarriage and the probability of finding a treatable factor for the disorder after evaluation. The prevalence of aetiological factors was found to be similar in those after two or three miscarriages, suggesting that investigations should be initiated after two miscarriages, challenging the classical definition of RM[3]. The American Society of Reproductive Medicine (ASRM) defines RM as the loss of two or more pregnancies consecutively[4]. However, this definition would increase the prevalence of RM to 5%, and significantly impact on the numbers that need to be evaluated.

This heterogenous condition is associated with many pathologies, but yet none are found in more than 50% of couples after numerous investigations[5]. Recognized associations of RM include parental and foetal chromosomal abnormalities[6, 7], certain structural uterine abnormalities[8], antiphospholipid syndrome (APS)[9], some thrombophilia[10] and endocrinological disorders such as polycystic ovarian syndrome (PCOS)[11].

In cases where no known associations are diagnosed, women are termed to have idiopathic RM. This is frustrating for both patients and clinicians alike, as an incidence of 1% is higher than the calculated incidence of a spontaneous miscarriage (15%[12]) occurring 3 times consecutively due to chance alone ($15\%^3 = 0.3\%$), suggesting that there may be an underlying pathology for the miscarriages. Different aetiologies have been proposed, with the trial of various empirical treatments, in an attempt to prevent the occurrence of another miscarriage. However, research studies are compromised due to the inconsistencies in the definition of RM, leading to

variability in the initiating of evaluation and potential treatment across studies, creating heterogeneity.

An immune-based aetiology is one of the proposed theories, although the exact mechanisms are still not fully understood. The placenta and fetus are genetically different from the host mother. This would be expected to lead to immune incompatibility. Yet, in a normal pregnancy this developing 'unit' is protected from the maternal immune system. This led to the use of numerous immune-modulating therapies in women with RM, without high quality evidence of benefit. This thesis will focus on uterine natural killer (uNK) cells, the most abundant leucocytes found in the endometrium during the window of implantation. We will examine the feasibility of assessing the benefit of prednisolone, an immunosuppressive drug, to improve live birth rates in women with abnormal levels of uNK cells and no other recognised pathology associated with RM in a randomised controlled trial (RCT).

Initially, an overview of the recognised associations of RM, and evidence on the available management options for these women is discussed, followed by a discussion on idiopathic RM. The subsequent chapters will consider the immune system in the endometrium, the association of NK cells and reproductive problems, the pilot phase of the RCT to assess the use of prednisolone in women with idiopathic RM and abnormal levels of uNK cells, and an alternative method of quantifying uNK cells for screening in the definitive trial.

1.2 Structural genetic defects

1.2.1 Fetal chromosomal abnormality

Chromosomal abnormality such as trisomy, polyploidy or monosomy in the miscarried pregnancy is the most common cause of pregnancy loss. This especially holds true for women in the older reproductive age group. Chromosomal anomalies account for up to 70% of early pregnancy losses (<12 weeks gestation), falling to 20% when the pregnancy is lost between 13 and 20 weeks gestation[7]. For this

reason, products of conception ought to be sent for karyotyping, as an abnormal fetal karyotype is diagnostic for the cause of miscarriage, and is an important prognostic factor, suggesting a successful outcome of more than 75% in the next pregnancies[7].

1.2.2 Parental chromosomal abnormalities

Parental chromosomal abnormalities are found in about 2% of women with RM, with the most common being a balanced reciprocal translocation[13]. They could be referred to a clinical geneticist for counselling when an abnormality is found, and offered prenatal diagnosis. It is common practice to routinely perform karyotype on all couples with RM. However, large case series of couples with RM and balanced translocation have found the risk of unbalanced translocation in the offspring to be less than 1%, which is less than the miscarriage rate of normal pregnancies after invasive prenatal diagnosis[14]. Furthermore, these couples, if evaluated and treated for other related problems and offered supportive care[15], have encouraging pregnancy outcomes of over 70% live birth rates in the subsequent pregnancy. This is similar to couples with RM, but without chromosomal abnormalities. Thus, conservative management, may be optimal, especially if women are not keen to undergo invasive prenatal diagnosis[13].

There were expectations that preimplantation genetic diagnosis (PGD) and assisted reproductive techniques (ART) would further improve the live birth rate for women with RM and balanced translocations. However, the pregnancy rate and live birth rate from PGD-ART were found to be lower than natural conception[14, 16]. Natural conception involves the selection of normal oocytes, then the selection of normal pregnancy, allowing genetically abnormal pregnancies to miscarry inherently. These natural selection steps are circumvented in PGD-ART, creating large numbers of abnormal embryos. Furthermore, PGD is not completely accurate at predicting the pregnancy karyotype as not all the cells in a 4 or 8 cells embryo are genetically identical. Although ART with or without PGD is not recommended in couples with normal karyotype[17], consideration should be given for PGD in a situation if ART is needed, as observational studies show PGD-ART have better pregnancy outcomes, and shorter time to a successful pregnancy compared to ART without PGD[16].

1.3 Structural uterine abnormalities

1.3.1 Congenital uterine anomaly

The exact contribution of congenital uterine anomaly in causing RM is difficult to assess, due to the vast difference in criteria and techniques for diagnosing abnormal uterine morphology. However, the prevalence of congenital uterine anomaly, such as bicornuate, septate or arcuate uterus in women with RM is 16.7%, much higher compared to the general population at 6.7%[18]. The commonest of these is arcuate uterine anomaly. Advances in hysteroscopic surgery mean that these malformations can be corrected using a resectoscope, and laparotomy is avoided, reducing operative morbidity. Observational studies have indicated that surgery (hysteroscopic metroplasty) reduces subsequent miscarriages and improves pregnancy outcomes[19-21]. Furthermore, a systematic review of 17 observational studies (N=1501) assessing hysteroscopic metroplasty or septoplasty in women with reproductive problems (RM, 2nd trimester miscarriage, infertility or pre-term labour) showed a pooled pregnancy rate of 60% and LB rate of 45% overall, and appears safe with a complication rate of only 1.9%[22]. Thus, surgery could be considered after individualised counselling. However, the women in many of these studies served as their own controls. Therefore, the results of The Randomised Uterine Septum Transsection (TRUST) RCT evaluating the value of surgical treatment which was recruiting in Netherlands are awaited which may direct future management of congenital uterine anomalies (Netherlands Trial Register: NTR1676, Date of registration 18/2/2009).

1.3.2 Acquired uterine anomaly

Fibroids and intrauterine adhesions (Asherman's syndrome), are also associated with RM[8]. Observational studies of pregnancy outcomes after myomectomies have shown improvement in post-operative pregnancy rates, which is not significantly influenced by the location, size and number of fibroids[8, 23, 24]. Nonetheless, patients need to be adequately counselled regarding potential complications from surgical intervention. In Asherman's syndrome, surgical treatment is only considered after comprehensive counseling, as the benefit of

hysteroscopic adhesiolysis is uncertain. Some observational studies report similar pregnancy loss rate in women with Asherman's syndrome and the general population[25]. Furthermore, there is the risk of adhesions reforming after treatment.

1.4 Antiphospholipid syndrome

RM is one of the clinical components for the diagnosis of antiphospholipid syndrome (APS), which is prevalent in about 15% of women with RM[9, 26]. Even with adequate treatment, these are high risk pregnancies and should be monitored for complications in all three trimesters[27]. Low dose aspirin (LDA), heparin, prednisolone and intravenous immunoglobulin (IVIG) have all been investigated as treatment options for this condition. A systematic review showed that prednisolone and IVIG do not improve pregnancy outcomes and are associated with increased risk of diabetes and premature birth, but a combination of LDA and unfractionated heparin (UFH) reduced a subsequent pregnancy loss by 54%[28]. Thus, LDA and heparin became the recommended treatment for women with RM and APS[27]. However, in clinical practice, low molecular weight heparins (LMWH) are preferred as they have reduced risk of thombocytopaenia, only need once a day administration and levels do not need to be monitored. But, LMWH may not have the same effect compared to UFH in reducing risks of miscarriage in APS[28]. Two RCTs have shown similar live birth rates of 79% in both groups, in women treated with LDA and LMWH, compared with LDA alone[29, 30]. Therefore, the role of heparin in the treatment of APS in women with RM needs to be further investigated by looking into differences between UFH and LMWH to optimise treatment regimes.

1.5 Thrombophilia

A large group of conditions are included under the definitions of acquired and inherited thrombophilia, which predispose patients to thromboembolic events. This may partially explain the association of some of these conditions with RM. A

meta-analysis of 25 studies on early pregnancy loss showed that factor V Leiden (FVL) mutation, activated protein C resistance (APCR) and prothombin gene G20210A mutation were significantly associated with RM[10]. Treatment with LDA, with or without LMWH has been proposed as thromboprophylaxis to prevent putative placental infarcts or vascular thrombosis. Initial studies suggest there may be beneficial effects of improved live birth rates for women with thrombophilia and RM[31]. However, more recently, high quality, large RCTs failed to substantiate this[29, 32]. Thus, the evidence to support the use of LDA and LMWH treatment in women with thrombophilia associated RM for the sole purpose of improving pregnancy outcomes was weakened. However, anticoagulant treatment could be considered if there was high risk of a thromboembolic event, for example in women homozygous for FVL mutation. As there is still controversy about the prognostic implications of positive tests, and the lack of evidence based treatment, it is uncertain if all women with RM should be screened for thrombophilia[33].

1.6 Endocrine Disorders

1.6.1 Polycystic ovarian syndrome

The association of PCOS and RM is possibly through mechanisms of hyperandrogenism, obesity and insulin resistance[11]. But, the huge variation in criteria for diagnosing PCOS before the availability of the Rotterdam diagnostic criteria makes it difficult to assess the importance and prognostic value of this condition in relation to RM[4]. Small studies suggest there may be a role for metformin, now regarded as having low risks during pregnancy, in reducing miscarriage rates especially in the presence of abnormal glucose tolerance test[11, 34]. However, a RCT in infertile women indicated that clomiphene is superior to metformin in achieving live births[35]. Whether or not this trial can be extrapolated to women with RM is debatable, especially if ovulation is not a problem. Similarly, the benefit of ovarian drilling to improve pregnancy outcome is uncertain in the absence of anovulation[11]. Nevertheless, a simple, safe and cheap way to reduce subsequent

pregnancy loss in obese women with PCOS is weight loss, and should be recommended to all[36].

1.6.2 Thyroid and glucose metabolism abnormalities

Well controlled thyroid disorders and diabetes are not risk factors for RM. Thus, national guidelines do not recommend routine screening in the absence of symptoms[27]. However, these are easily treatable conditions with a potential to improve pregnancy outcome, and therefore have been commonly requested for in many hospitals as part of the investigations for RM. The association with thyroid antibodies is discussed later in the chapter.

1.7 Idiopathic Recurrent Miscarriage

Women are classified as 'idiopathic' or have 'unexplained' RM when none of the 'conventional' associations of RM are found after a series of investigations. This occurs in more than half of the women diagnosed with RM[3, 5]. They have an excellent prognosis, up to 75% success of a live birth in future pregnancies with supportive care of regular ultrasound scans for reassurance, and psychological support in a dedicated early pregnancy assessment unit (EPAU)[37]. There is still a lack of explanation for their recurring miscarriages and lack of treatment to prevent further miscarriages.

This has led to the development of different theories of potential pathways leading to a miscarriage. Various treatment options based on these theories have been attempted. They broadly fall into categories of thrombotic, endocrinological or immunological factors. The associations of these factors with RM are controversial as studies have shown contradictory results of association, and it is unclear if these relationships are causal or casual. Similarly, treatment options addressing these factors are still being examined, and there is the lack of unequivocal evidence of benefit in reducing the risk of miscarriage in a subsequent pregnancy.

1.7.1 Thrombotic factors

An explanation for the process leading to a miscarriage could possibly be of inflammation or thrombosis in the placental bed. This led to the empirical use of aspirin and/or heparin for their anti-inflammatory and antithrombotic properties. Although LDA was commonly prescribed for women with idiopathic RM, there is currently no evidence for its use. A recent systematic review and RCT both showed no benefit of LDA in improving live birth rates[32, 38]. Similarly for heparin, a systematic review of five studies concluded insufficient evidence to recommend this treatment, even in the presence of thrombophilia, to improve live birth rates[39]. This is consistent with the recent published results of two multicentre RCTs reporting that LMWH was not of therapeutic benefit in this group of women[32, 40].

Thromboelastography (TEG), a test of whole blood haemostasis, may identify a subgroup of women with idiopathic RM with thrombotic abnormalities. A prospective study showed that women with RM had abnormal TEG results, compared with parous controls, and were at an increased risk of subsequent miscarriage[41]. But, it is still unclear if correcting these TEG parameters with antiplatelet agents will improve live birth rate and needs to be investigated in a prospective placebo-controlled trial.

1.7.2 Endocrinological factors

Another potential factor is the malfunction of hormones, such as progesterone and human chorionic gonadotrophin (hCG), which are thought to create a suitable endometrial environment for implantation to occur. A condition known as luteal phase deficiency, diagnosed when two timed endometrial biopsy in consecutive cycles is 'out of phase' to the expected morphology of the endometrium, may be the result of this problem. The process may relate to either decreased progesterone production by the corpus luteum, abnormal luteanising hormone (LH) secretion, or poor response of the endometrium to available progesterone[42]. In view of this, progesterone has been empirically prescribed. A systematic review however showed that progesterone treatment did not significantly reduce the risk of miscarriage, though a subgroup analysis of 3 small studies on women with RM

showed a significant decrease in the miscarriage rate[43]. A large scale, multicentre RCT (Progesterone in Recurrent Miscarriages - PROMISE study) investigating women with idiopathic RM, to ascertain whether progesterone supplementation is beneficial in preventing subsequent miscarriages has recently completed recruitment and results are awaited[44] (ISRCTN Number: 92644181).

Treatments with clomiphene, with or without gonadotrophins and hCG injections, have also been attempted but there are no completed RCTs that have shown treatment efficacy[42]. A small RCT of hCG injections found no overall benefit, but a subgroup analysis found an improved live birth rate with hCG in women with oligomenorrhoea[45].

High levels of prolactin may inhibit progesterone secretion and lead to endometrial defects[42]. However, the role of hyperprolactinaemia in RM is unclear. One study showed no association of RM with hyperprolactinaemia[46] whereas the only small RCT published an improved pregnancy success when women with hyperprolactinemia were treated with bromocriptine[47]. So, it may be important to maintain normal levels of prolactin prior to pregnancy.

1.7.3 Immunological factors

Immunological mechanisms have been thought to play a role in RM as a successful pregnancy involves maternal adaptation of the immune response to the semi-allogenic developing embryo. Currently, the exact mechanisms of how the fetus is protected from the maternal immune system during pregnancy are unknown, but potential defects in the immune system have been investigated.

One aspect investigated is autoantibodies. Autoantibodies that have been studied in the RM population include antiphospholipid antibodies (primarily anticardiolipin antibodies (ACA) and lupus anticoagulant (LA), collectively known as APS), antithyroid antibodies, antinuclear antibodies (ANA), and anti-DNA antibodies[48]. However, the associations of most autoantibodies are weak apart for APS, which has been discussed earlier in the chapter.

Antithyroid antibodies (thyroglobulin Ab, thyroid peroxidase Ab) are more prevalent in women with RM compared to controls[49]. They potentially play a role which could be explained by mechanisms such as an underlying maternal autoimmune pathology or mild thyroid failure[42]. A systematic review of 12,126 women showed that the presence of maternal thyroid autoantibodies is strongly associated with miscarriage, and there is evidence that treatment with levothyroxine can attenuate the risk[50]. The results of the multicentre Randomised Controlled Trial of the Efficacy and Mechanism of Levothyroxine Treatment on Pregnancy and Neonatal Outcomes in Women with Thyroid Antibodies (TABLET RCT, ISRCTN Number: 15948785) evaluating the efficacy of levothyroxine treatment are awaited. Although the TABLET trial does not solely include women with RM, it may provide an indication whether treatment could improve pregnancy outcomes in women with antithyroid antibodies and previous miscarriages.

Alloimmune factors such as human leucocyte antigens (HLA) compatibility of couples, the maternal leucocytotoxic antibodies, or maternal blocking antibodies have also been postulated to play a role in RM. However, the correlation is controversial, and no definite associations to pregnancy outcomes have been found[51]. Yet, different immuno-modulatory therapies have been developed and tried on women with idiopathic RM, including steroids, IVIG and leucocyte immunization therapy (either paternal or third party donor). A systematic review of twenty trials of various immunotherapies, showed no significant beneficial effect over placebo in improving live birth rates[52].

A specialized immune system is thought to exist in the endometrium, hosting many immune cells such as T cells, macrophages and uNK cells, and a range of cytokines are produced by these cells[53, 54]. There has been significant interest in the interactions and functions of these cells and cytokines as potential contributing factors in the aetiology of RM, which will be discussed in further detail in the next chapter.

1.8 Conclusion

After reviewing all the recognised and potential associations of RM, it can be concluded that only fetal chromosomal abnormalities and APS have an established causal link with RM. Observational studies of other associations with RM such as uterine abnormalities, thrombophilia, and PCOS have shown contradictory results. It is also disappointing that more than 50% of women with RM continue to be termed 'idiopathic', although it is likely that there is an underlying pathology to their condition.

Ideally, assessment and investigation of a couple with RM would have the aim of guiding management options by finding contributory factors to the miscarriages, providing prognostic value in the subsequent pregnancy and directing treatment of proven benefit to improve live birth rates. However, there are only few observational studies that give prognostic implications for positive tests for conditions associated with RM. There are even fewer high quality, large scale RCTs that show unequivocal proven benefit of a reduction in risk of miscarriage in a subsequent pregnancy following treatment.

In women with idiopathic RM, there are numerous factors, as described above that could potentially play a role leading to a miscarriage. RM is a heterogeneous disorder. All these factors may relate to each other, contributing in different mechanisms to the miscarriage. At present, there is no conclusive evidence-based treatment to offer these women, and supportive care is all that can be recommended. The challenge is, to develop a prognostic test that predicts pregnancy outcome for one of these potential associations, and can be used as a screening tool or marker to identify the subgroup of women with idiopathic RM, who will respond to treatment, and benefit from it in preventing the recurrence of another miscarriage.

We aimed to achieve this goal by investigating the role of the endometrium, in the population of women with idiopathic RM. The endometrium is very important as it plays the key role for the establishment and maintenance of the pregnancy. All the factors discussed above may influence the growth and development of the endometrium, either in the pre-pregnancy phase when it decidualises and prepares for implantation, or in early pregnancy, when fetal trophoblast invasion occurs for

the establishment and nourishment of the pregnancy. I have focused on uNK cells, the most abundant leucocyte found in the window of implantation and early pregnancy, and their potential role in RM, which is described in detail in the next chapter.

CHAPTER 2

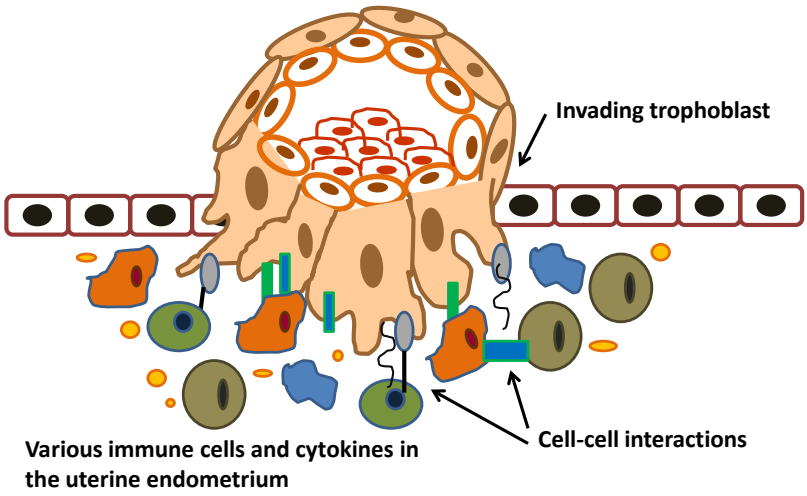
THE ENDOMETRIUM AND RECURRENT MISCARRIAGE

CHAPTER 2 – THE ENDOMETRIUM AND RECURRENT MISCARRIAGE

2.1 Introduction

A receptive endometrium is required for the implantation of the embryo, nurturing of the developing fetus and the success of a pregnancy. Although the exact pathways are still not fully understood, the implantation process involves a series of finely organised and regulated interactions between immune cells in the endometrium and the invading trophoblast cells of fetal origin (*Figure 2.1*). Defective implantation leading to inappropriate development of the feto-placental unit and miscarriage may partly be explained by some of the many associations of RM as discussed in the previous chapter, including an immune-based aetiology. The endometrium hosts a number of immune cells, and there is considerable evidence for the presence of a specialized immune system within the maternal part of the placenta which may play a role in regulating implantation[54-57].

Figure 2.1. The endometrial trophoblast interface



The selection failure hypothesis also exists. According to this hypothesis, there is the 'failure of prevention of poor quality embryos implanting, allowing embryos that are destined to fail to implant and present clinically as RM'[58]. Examples include embryos with lethal genetic or chromosomal abnormalities that implanted too easily into the endometrium. Therefore, a miscarriage can occur as a result of either an error in the implantation processes and developing feto-placental unit in the endometrium, rendering it 'too hostile', or 'too receptive'.

This chapter will discuss the association of the endometrium and RM from an immunological perspective, with emphasis on uNK cells, the most abundant leucocyte in the endometrium during the window of implantation.

2.2 Methods of Endometrial Investigations

It is difficult to study the endometrium as tissue is not easily accessible, especially in an ongoing pregnancy. Thus many studies are done on animals and there is a tendency to extrapolate results of these studies where knowledge of the process is incomplete, onto humans. There are important differences in the implantation process between mice, where most studies are carried out, and humans, and so careful interpretation of animal findings are needed. Attention is also needed on certain aspects of investigating the endometrium, as detailed below.

2.2.1 Timing of endometrial sampling

The endometrial structure and function regularly changes throughout the menstrual cycle[59]. This makes chronological dating of the endometrium very important when it is sampled for investigations and interpretation of any findings needs to be correlated to the cycle. Traditionally, the onset of menstruation was used as the reference point, but it is now known that a more precise reference point is either[60]

- the LH surge, established by daily monitoring of LH levels through plasma or urine samples, or
- follicular rupture, detected by daily follicle scanning

The period of interest in the menstrual cycle in investigations of women with RM is the 'window of implantation', also known as the mid-secretary phase. This is commonly taken as day 6 to day 9 after the LH surge[61].

2.2.2 Analysis of the endometrium

The endometrial sample for analysis is obtained either with a Pipelle endometrial sampler, or endometrial curettage. Pipelle endometrial sampling is more comfortable for women, but is a 'blind' procedure using an inner plunger to create a vacuum to suction out a sample of the endometrium[62]. The benefit of using curettage is knowing the exact site in the uterine cavity where the endometrial sample was taken from. However, no differences were observed in the endometrium sampled with a curette from different sites[61]. Hence, Pipelle endometrial sampling is acceptable in acquiring tissue for investigations.

Different techniques are available in the analysis of endometrium in women with RM such as morphological studies and quantitative studies through immunohistochemical, flow cytometric or enzyme-linked immunosorbent assay (ELISA) methods. Morphological studies on the endometrium are widely based on the dating criteria described by Noyes *et al*[59]. There are characteristic differences in the glands and stromal cells in the endometrium during the proliferative or secretory phase, timed to the menstrual cycle or LH surge[59, 60]. This method used in studies has shown that some women with RM have a delay in the maturation of the endometrium, also known as luteal phase defect described previously[46, 63].

Quantitative methods employing immunohistochemical staining or flow cytometry of the constituents of the endometrium have also been used to investigate women with RM. These methods are commonly used to quantify the leucocytes or expression of cytokines in the endometrium. Leucocytes in the endometrium of women with RM examined with immunohistochemical staining and two-colour flow cytometric analysis after enzymatic digestion of the endometrium were found to

have a different population when compared to women with normal reproductive histories[64, 65]. Immunohistochemistry is more time consuming, but it reveals the exact location of cells[66]. Analysis using flow cytometry is faster, but involves digesting the tissue, and thereby potentially losing cells and antigens. Furthermore, it needs a large sample of endometrium that may be difficult to obtain in some women.

Cytokines became of interest as murine models demonstrated that pregnancy rejection was mediated by Th1 cytokines such as interferon (IFN)-γ, interleukin (IL)-2, IL-12 and tumour necrosis factor (TNF)-β, while Th2 cytokines such as IL-4, IL-6, and IL-10 was important for successful implantation and pregnancy[67]. Cytokine profiling is commonly done using ELISA and studies have found different levels of cytokines in women with RM compared with fertile controls[68, 69]. The endometrium is cytokine rich, produced by the array of endometrial leucocytes, but the exact mechanism of their involvement in RM is still not fully understood[55].

2.3 Endometrial leucocytes and cytokines

The population of leucocytes in the endometrium has been extensively studied and differences were found between endometrium of women with RM and control women with normal reproductive histories[64, 65]. The majority consists of three cell types, T cells, macrophages and uNK cells, with very few neutrophils, and hardly any B cells. These cells vary in number and proportions throughout the menstrual cycle[70]. In the proliferative phase, leucocytes make up <10% of the stromal cells but they increase in numbers to comprise >20% of stromal cells towards the late secretory phase and to >30% in early pregnancy[66]. This observation of the large increase in numbers during the window of implantation is one of the reasons for implicating endometrial leucocytes in playing a key role in the implantation process and immunological maintenance of a pregnancy. Cells that are found and investigated in a non-pregnant cycle are commonly termed ‘endometrial’, while cells that are investigated in pregnancy are termed ‘decidual’.

The three major endometrial cell types will be described, with the emphasis on uNK cells. Uterine NK cells have the most dramatic rise in numbers to become the predominant leucocyte in the window of implantation and early pregnancy, and express receptors for the invading extravillous trophoblastic (EVT) tissue, with evidence of cell-cell interaction. Thus, of these three, we investigated the role of uNK cells in RM in the thesis.

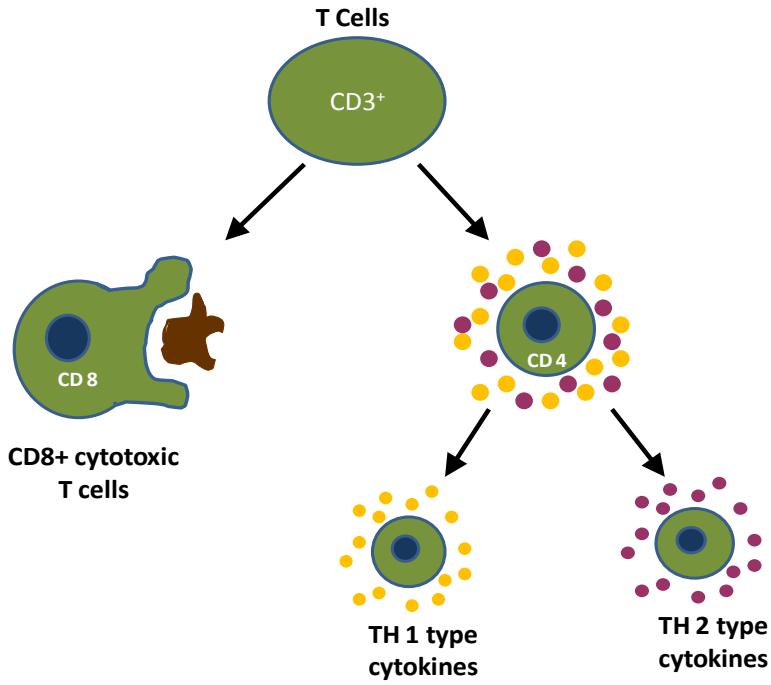
2.3.1 T cells

T cells, all $CD3^+$, comprise about 40% of leucocytes in the proliferative endometrium[66]. The cell numbers remain fairly constant through the menstrual cycle, but there is a relative decrease in proportion due to the remarkable rise in uNK cells numbers in the secretory phase[66, 70]. The numbers also hardly vary throughout pregnancy[71].

T cells express either the CD8 or CD4 antigen[71]. CD8+ T cells are cytotoxic while CD4+ T cells are more cytokine producing. CD4+ T cells are further divided into Th1 or Th2 type cells depending on the cytokines production profile (*Figure 2.2*). T cells are also classified according to their receptor expression, $\alpha\beta^+$ or $\gamma\delta^+$. Human endometrial T cells are mostly $\alpha\beta^+$, where only 5-10% are $\gamma\delta^+$[71].

Although studies in mice have shown important roles for T cells in the maintenance of pregnancy[67, 72], evidence for this is not as apparent in humans[55, 73]. There appears to be no difference in the absolute number of T cells in the endometrium between women with RM and control women[64, 65] in early pregnancy, deciduas between women with RM and women with normal fertility[74], and in deciduas of normal pregnancies and after spontaneous miscarriage[73]. It may be possible that the difference in the ratio of T cell subtypes, CD8 and CD4, or the balance of Th1 and Th2 cytokines that are produced by these cells, which play a role in the initiation or maintenance of a pregnancy.

Figure 2.2 Classification of T cells



2.3.2 Macrophages

Macrophages, identified either by $CD14^+$ or $CD68^+$, also account for a substantial proportion of endometrial leucocytes[66]. They appear to increase in numbers during the secretory phase and further rise in early pregnancy, although to a lesser extent compared to uNK cells[66]. They are present in both endometrium/decidua and myometrium, and are found near spiral arteries, glands and extravillous trophoblast[75].

Functional studies have proposed a role of phagocytosis of cell debris during the implantation process[76] and of immunosuppression by preventing maternal T cell activation in pregnancy[77]. Endometrial macrophages also produce Th2 type cytokines such as IL-10, TNF-α and IL-1β, which may play a role in the regulation of trophoblast invasion[77].

A difference in macrophage populations have been found in the endometrium of studies comparing women with RM and controls. More macrophages were found in women with RM who subsequently miscarried compared to those who subsequently have a live birth[64]. However, no differences in macrophages were found in deciduas of women with RM compared to controls[74], although a trend of increased macrophages was found in deciduas of women with sporadic miscarriages[78].

2.3.3 Uterine NK cells

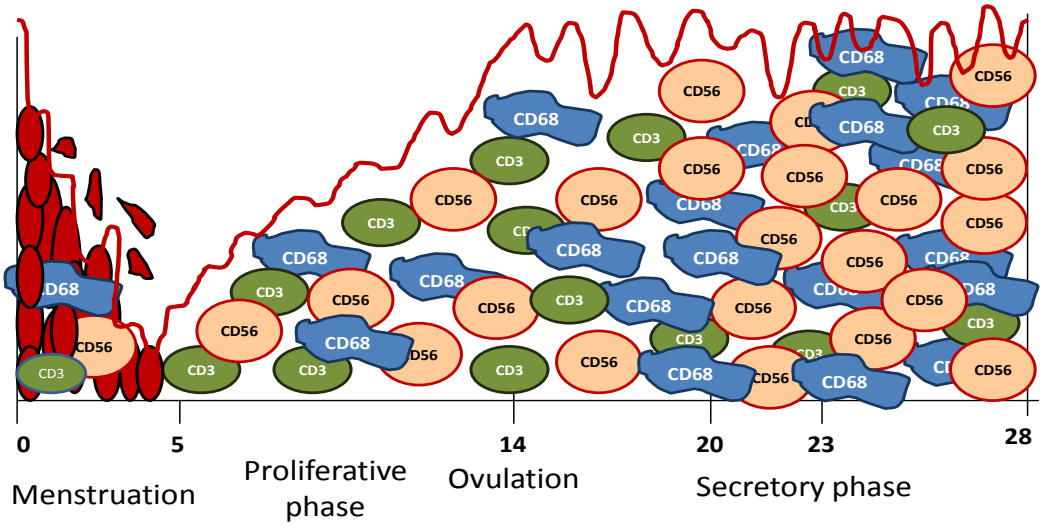
Uterine NK cells form the largest group of leucocytes in the endometrium and are characterised by the cytoplasmic granules. They previously had many names, including 'granular endometrial stromal cell', 'endometrial granulocytes', 'K cells', and 'large granulated lymphocyte'[79]. The name 'uterine Natural Killer' has been adopted as these cells share similar properties with NK cells in the peripheral blood, part of the innate immune system[79]. But, they are a unique subset of NK cells with its own distinct antigenic features. To date, similar cells have not been found in large numbers in other organs[54].

All uNK cells express the CD56 antigen. It is the intensity of CD56, and the lack of CD16 and CD57 antigens, typical NK cell markers that differentiate uterine from peripheral blood NK (pNK) cells[80]. The density of CD56 on uNK cells is 20 times that of the majority of pNK cells, and 80% of uNK cells are $CD56^{bright}CD16^{-}$ [80]. Despite their name, uNK cells display only weak cytotoxic capabilities against target cells and probably exert their function by producing high levels of angiogenic growth factors (VEGF-C, placental growth factor, angiopoietin-1 (Ang-1), Ang-2 and TGF-β) and cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF), colony stimulating factor (CSF)-1, TNF-α, IFN-γ, transforming growth factor (TGF)-β1, leukemia-inhibitory factor (LIF) and IL-2[81, 82].

There is a well-described fluctuation of uNK cells throughout the menstrual cycle. In the proliferative phase, both T cells and uNK cells are scanty and of equal proportion, about 40% each of all leucocytes (<10% of stromal cells). However, uNK cells, which are predominantly found in the stratum functionalis of the

endometrium, start to increase in numbers after ovulation, to encompass about 60% of leukocytes towards the late secretory phase, forming aggregates around spiral arteries and glands. They continue to peak to >75% of leukocytes (about 30-40% of all stromal cells) in early pregnancy when implantation occurs[66] (*Figure 2.3*). This fluctuation of numbers according to the menstrual cycle suggests that their existence depends on hormonal regulation[53]. In pregnancy, uNK cells are found in highest numbers in the decidua basalis, closest to the invading EVT at implantation. The level of uNK cells in the second trimester of pregnancy is not certain as there is difficulty in obtaining tissue for analysis of uNK cells in this gestation, but is still thought to form the majority of leucocyte population[79]. In the third trimester, uNK cell numbers generally decreases drastically but a substantial number remains in both deciduas basalis and parietalis[79].

Figure 2.3 Population of endometrial leucocytes in the menstrual cycle



The rise in uNK cells begin in the early secretary phase, regardless of the presence of trophoblast and before decidualisation occurs. UNK cells have been found to co-exist with decidua in normal pregnancy and also in 'ectopic decidua' in fallopian tube, cervix, ovary or foci of endometriosis[83]. Furthermore, persistent infiltration of uNK cells was also seen in pseudo-decidualised endometrium associated with levonorgestrel intra-uterine devices[84], implicating a hormonally regulated function of uNK cells in the initiation of the decidualisation process. Although exact pathways are not clear, uNK cells play important roles in early pregnancy of decidualisation, implantation and placentation (*discussed further in 2.3.3.2*).

There is also a role for uNK cells when implantation does not occur and the functional layer of the endometrium is shed at menstruation. UNK cells undergo characteristic nuclear changes of apoptosis before other morphological features of endometrial breakdown such as neutrophil infiltration, clumping of stromal cells and interstitial haemorrhage. These changes are only seen in late-secretory endometrium and not in normal deciduas. Therefore the death of uNK cells could be the initial triggering event for mucosal breakdown and menstruation[53]. This menstrual cycle is a tightly regulated ongoing process of growth and breakdown, and any changes to numbers or angiogenic growth factors and cytokines produced by endometrial immune cells, including uNK cells, can lead to benign endometrial disease such as menorrhagia and endometriosis[85].

2.3.3.1 Source of Uterine NK cells

The process of how uNK cells arrive in large numbers into the endometrium during the late secretory phase of the menstrual cycle is still uncertain, but two main theories exists:

- In-utero proliferation and differentiation of stem cells or indigenous NK cells in the endometrium, or
- Recruitment of haematopoietic stem cells or NK cells from peripheral blood which subsequently differentiate in the uterine microenvironment into uNK cells phenotype

In-utero proliferation and differentiation

Though $CD56^{bright}$ cells are few in the proliferative phase, they are still present. Therefore, there could be local proliferation of residual uNK cells present in the stratum basalis that is not shed during menstruation[79]. Furthermore, there is an increase in the expression of Ki-67, a proliferative marker on uNK cells in both secretory phase endometrium and decidua of early pregnancy which supports this theory[80]. When comparing both tissue types, maximum proliferation was seen in secretory phase endometrium and there was a downward trend of proliferation in deciduas as gestation proceeds[80]. Although the exact pathways are unknown, prolactin and IL-15, under the influence of progesterone, are thought to regulate uNK cells proliferation and maturation in the endometrium[86].

The endometrium sheds every month and thus is highly regenerative. There is emerging evidence for both the presence and potential functional capability of endometrial stem cells[87]. Although the exact markers and roles of these stem cells are still under investigations, there is potential for these cells to differentiate and proliferate into uNK cells in the uterus.

Trafficking from peripheral blood

The alternate theory proposes the recruitment of peripheral blood cells into the endometrium through hormonally regulated methods via chemokines and cytokines. This theory is supported by population of uNK cells that are commonly found to form aggregates in large numbers around spiral arteries and glands[66]. Once peripheral cells are recruited, they could then differentiate into uNK cells.

The precise mechanism of recruitment of cells from peripheral blood is still unknown. UNK cells express estrogen receptor (ER)-β1 and glucocorticoid receptors but not progesterone receptors[88], which is the main hormone during the secretory phase of the menstrual cycle. Thus, recruitment could be potentially mediated directly through actions of oestrogen via the existing ER-β1 receptor, directly through action of progesterone via yet an unidentified receptor, or through progesterone action on endometrial T cells and stromal cells via cytokines and

chemokines such as IL-15, macrophage inflammatory protein-1β (MIP-1β), vascular endothelial growth factor (VEGF) or CXCL10 and CXCL11[79, 86].

For example, IL-15, secreted by endometrial stromal cells, are distinctly expressed in the vascular and perivascular areas in the secretory phase endometrium at a higher concentration compared to the proliferative phase, and thus may serve as chemoattractants for recruitment of peripheral cells[89]. There is also a strong correlation between cytokine levels and the number of uNK cells[89]. Once recruited, IL-15 can continue to assist in its proliferation and differentiation into unique uNK cells[89]. There is also a possibility that precursor immature NK cells are recruited from the peripheral circulation and subsequently differentiate into uNK cells in the endometrium[90].

2.3.3.2 Functions of Uterine NK cells

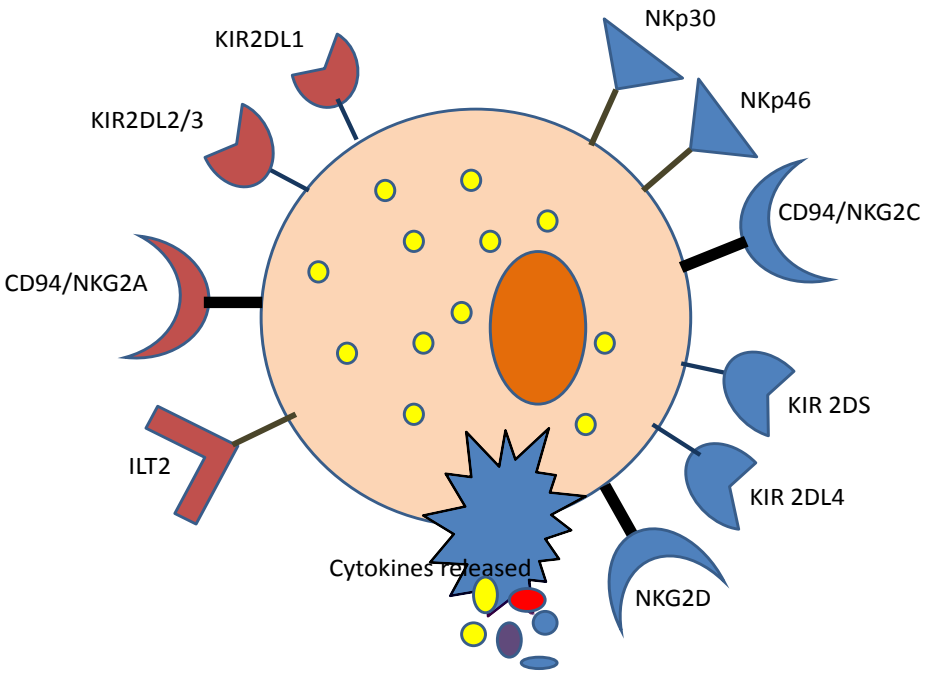
Despite extensive studies into uNK cells, their exact roles remain undefined. Their temporal distribution may indicate a function in the establishment and maintenance of early pregnancy, as uNK cells peak during the window of implantation. Human species have an invasive placenta. When implantation occurs, there needs to be adequate invasion of the trophoblast to allow for good maternal blood supply to support the developing fetal-placental unit[53]. There is evidence to imply that uNK cells play an important role in initiating decidualisation and regulating trophoblast invasion. These cells are hormonally dependant and accumulate as a dense infiltrate at the implantation site near stromal cells, glands, blood vessels and trophoblast cells in early pregnancy[80].

Regulation of trophoblast invasion and growth though direct cell-cell interactions

Trophoblast cells (consisting of syncytiotrophoblast and cytotrophoblast layers) constitute the fetal side of the interface between fetal and maternal tissue, and mediate the implantation of the embryo into the endometrium. In-vitro studies have shown that EVT and uNK cells interactions occur and may regulate the maternal immune response to the fetal allograft[56].

Both syncytiotrophoblast (outermost layer implanting into maternal tissue) and cytotrophoblast (inner layer of trophoblast) cells do not express classical class I major histocompatibility complex (MHC) alloantigens HLA-A and HLA-B, or class II HLA-DP, HLA-DQ and HLA-DR involved in graft rejection. Instead, the invasive EVT cells express an unusual combination of non-classical class I MHC HLA-E and HLA-G with low expression of HLA-C[56]. Uterine NK cells are found to express receptors such as killer-cell immunoglobulin-like receptors (KIR), immunoglobin-like transcripts (ILT), NKp and CD94/NKG2 proteins, belonging to two main families, the NKC (NK complex of lectin-related genes) (CD94/NKG2) and the LRC (leukocyte receptor complex of immunoglobulin-related genes) (KIR, ILT, NKp) families, which recognise all these non-classical MHC molecules expressed by EVT cells[56] *(Figure 2.4).* Thus, there are potential molecular interactions for maternal recognition of trophoblast, which results in either activating or inhibitory mechanisms.

Figure 2.4 Activating and inhibitory receptors on uNK cells



The receptors on uNK cells for HLA-C are members of the KIR multigene family. All women express KIRs for both group of HLA-C alleles and because HLA-C is polymorphic, maternal uNK cells can encounter paternal (non-self) HLA-C alleles on trophoblast. Each pregnancy may present a different combination of KIRs and HLA-C[91]. The percentage of KIR expressed and the density of receptor expression also differs between individuals. This interaction plays a physiological role related to immune regulation and placental development. Obvious differences were observed with different combinations of polymorphic ligand-receptor pairs and have been associated with pre-eclampsia, a condition that is known to be secondary to poor trophoblast invasion[92]. These specific fetal HLA-C/maternal KIR genotype combinations have also been identified in RM[93].

HLA-E has high affinity for CD94/NKG2 dimers on uNK cells. The overall effect is inhibition of cytolysis of either maternal or fetal tissues by uNK cells[56]. However, uNK cells are unable to kill trophoblast even when these receptors were blocked by antibodies, which suggest that this interaction may regulate other functions besides cytolysis during implantation. It could be that other inhibitory pathways exist or trophoblast lacks specific surface molecules to initiate killing[56].

The expression of HLA-G is almost entirely by EVT cells. Although specific receptors for it are yet to be defined, HLA-G is recognised by CD94/NKG2 via co-expression with HLA-E or by ILT-2[56]. HLA-G has also been shown to bind to KIR2DL4, ILT 2 and ILT4 receptors[94], and potentially lead to decreased sensitivity to NK cell mediated cytotoxicity[55, 95]. Three polymorphisms of HLA-G have been investigated and found to be associated with women with RM[55]. HLA-G interaction has also been shown to stimulate proliferation of uNK cells and increased production of IFN-γ and VEGF[96]. Although there is evidence for uNK cells and EVT interaction through these MHC molecules and receptors, the final consequences of these interactions are still unclear.

Regulation of trophoblast invasion and growth though cytokine production

Control of trophoblast invasion was initially thought to be via cell-mediated cytotoxicty as uNK cells were capable of cytolysis, although at a lesser extent than

their peripheral equivalent[81]. However, as previously described, trophoblast cells are resistant to lysis by uNK cells. They express non-classical HLA class I antigen, unless stimulated by IL-2, which is not present in the endometrium in large amounts in normal pregnancy. Thus, a different mechanism had been proposed, where uNK cells and EVT interactions alter the profile of cytokine production, ultimately resulting in a change in the invasive behaviour of trophoblast[53].

Uterine NK cells are known to produce many cytokines such as GM-CSF, CSF-1, TNF-α, IFN-γ, TGF-β1, LIF, IL-2, and IL-10, some of which trophoblast cells have receptors for[82, 97]. Thus, there could be a role for these uNK cell derived cytokines on trophoblast growth and differentiation, or apoptosis and defective invasion of the endometrium. For example, GM-CSF has been shown to stimulate DNA synthesis in culture of murine trophoblast and CSF-1 increases production of hCG and human placental lactogen (hPL) by trophoblast. Both these cytokines have also been shown to cause placental cell proliferation in mouse models[86]. Similarly, IL-4, IL-6 and LIF stimulate hCG secretion by trophoblast cells[55]. Another cytokine, macrophage migration inhibitory factor (MIF), produced by uNK cells and expressed highly in endometrium and human placenta, reduces the cytolytic capabilities of uNK cells[98].

On the other hand, IFN-γ has been shown to inhibit EVT invasion within early human pregnancy decidua both by increased EVT apoptosis and reduced levels of active proteases[99]. Similarly, TNF-α impairs trophoblast invasion through elevation of plasminogen activator inhibitor-1 (PAI-1)[100]. TGF-β is also known to affect growth and differentiation of first trimester trophoblast by inhibiting intergrin expression, hPL and hCG secretion[101]. Some of these cytokines also regulate production of matrix metalloproteinases (MMP) -2 and MMP-9 that plays a role in trophoblast invasion[102]. A recent study demonstrated that granulysin, a cytotoxic granule protein produced by uNK cells causes apoptosis of EVT and granulysin-positive uNK cells can attack EVT[103]. Therefore, any alternations in the production of cytokines could contribute to the imbalance of this unique fetal-maternal interface immune phenomena leading to abnormal implantation and the clinical presentation of a miscarriage.

In addition to direct effects of individual cytokines on trophoblast invasion, the patterns of cytokine release may be important. Animal models have provided evidence that Th2 cytokines are favourable to pregnancy, and Th1 cytokines are detrimental to implantation[67]. Although studies in humans have proved that this is true in normal pregnancy, the mechanisms leading to a miscarriage are more complicated than a simple shift in the Th1-Th2 cytokines balance[55]. In our unit, when peripheral cytokines were examined before the miscarriage occurred, a lack of Th1-Th2 switch was not found to predict miscarriage[69].

Regulation of angiogenesis and decidual vascular remodelling

High numbers of uNK cells are found around blood vessels. Whether their location is a reflection of trafficking cells from peripheral circulation or due to the possible function of uNK cells in development and remodelling of uterine spiral arteries is still not known. However, early structural changes that occur in decidual spiral arteries, including dilatation and disorganisation, happen at the time when uNK cells are present, and are generally completed after 20 weeks gestation when uNK cells reduce in number. The variation in numbers and time line implicate uNK cells function in vascular remodelling[79].

Uterine NK cells are found to secrete high levels of angiogenic growth factors important for vascular remodelling, such as VEGF-C, placental growth factor, angiopoietin-1 (Ang-1), Ang-2 and TGF-β, in both non-pregnant endometrium and early pregnancy deciduas[104, 105]. Their roles include mediating spiral artery transformation and destabilisation of vessel structure. The modulation of vascular growth in early pregnancy is a fine interaction between these angiogenic growth factors and other cytokines, such as IFN-γ, also secreted by uNK cells[105]. Studies in mice models, where a lot of evidence for uNK cells' involvement in spiral artery transformation has come from, show that mice deficient in uNK cells or IFN-γ signalling have implantation site abnormalities and failure of decidual artery remodelling[106]. Uterine NK cells density was also positively correlated with the formation of blood vessels, lymphatics, spiral arterial smooth muscle differentiation and endometrial oedema in humans[107].

2.3.4 Uterine NK cells and Recurrent Miscarriage

As discussed above, it is likely that uNK cells are essential for decidualisation, implantation and the maintenance of pregnancy and it may be changes in their numbers or function that results in miscarriages. The association between high uNK cell density and RM has been repeatedly reported in pre-pregnancy endometrium[64, 108, 109], with a further association of higher density in women with RM of severe phenotype[110]. A likely function of uNK cells was controlling angiogenesis, and their density was found to be positively correlated with endometrial angiogenesis and uterine artery blood flow in women with RM[79, 107]. Similarly, sub-endometrial vascular flow was also shown to increase with higher uNK cells density in women with unexplained recurrent failure of IVF[111]. Embryo implantation and early pregnancy development occur in a relatively hypoxic environment (2-3% O_2)[112]. Therefore, inappropriate blood flow to the intervillous space could be associated with oxidative stress damage to the developing placenta[113]. One theory is that increased uNK cell density is associated with increased number of spiral arteries which may lead to inappropriate blood flow to the developing foetal-placental unit, causing oxidative stress and consequent miscarriage[107].

Studies comparing normal and miscarried early pregnancy decidua have also implicated uNK cells in the aetiology of miscarriage by being phenotypically different in these 2 groups[74, 114, 115]. Uterine NK cells were found to be more numerous in the decidua of chromosomally abnormal miscarriages compared with chromosomally normal miscarriages[115]. This may be due to uNK cells facilitating implantation of abnormal blastocysts ('selection failure' theory), and leading to the clinical presentation of miscarriage[58].

Although an increased density of uNK cells in pre-implantation endometrium has been found in women with RM compared to fertile controls[64, 108-110], there are also studies that have shown no difference in the population of uNK cells in women with RM and controls. However these included women with only 2 consecutive miscarriages[116, 117]. It has been suggested that the significantly decreased number of uNK cells in controls who all have had previous live births were due to the effect of a previous term normal pregnancy as pregnancy and birth involved extensive changes in size and vascularisation of the uterus. However, a study in our unit

showed that five women who had a previous birth had high uNK cells density, excluding the possibility that a live birth reduces uNK cells to a normal range in all women[110].

The endometrium is shed every cycle and these observations of increased uNK cells density were seen in one menstrual cycle. It is uncertain if the elevated result is a transient or persistent phenomenon, and there is currently only one study which has investigated the cycle to cycle variability of uNK cells density[118]. Although they investigated only 10 women with reproductive problems, they found an overall poor agreement of uNK cells density (κ=0.348) in repeated endometrial samples. However, there was an agreement in the classification of abnormal uNK results (high or low) of repeated endometrial samples 70% of the time. This significant variation in different menstrual cycles needs further investigation and is a factor to be considered when the test is done.

Furthermore, numbers of uNK cells may not correlate directly to function and the significance of these observations are still not known. Whether high uNK cells density is indeed harmful to the invading trophoblast is still uncertain. Abnormal uNK cells density may however serve as a marker of a reproductive pathology that is yet to be identified. There is one study which suggested that high density of uNK cells in the mid-luteal phase predict subsequent miscarriage[64] but a slightly larger study refuted this[109]. This relationship was further explored in a systematic review of NK cells and pregnancy outcomes in the next chapter.

2.4 Immunolodulation Therapy for Immunological Endometrial Pathology

2.4.1 Glucocorticoids

Glucocorticoids may influence several aspects of implantation through its effect on endometrial cellular proliferation, apoptosis, remodelling and trophoblast invasion[119]. The subgroup analysis of a Cochrane review of peri-implantation steroid use in women undergoing standard in-vitro fertilisation (IVF) suggested that

pregnancy outcomes were improved[120]. There have also been published case reports of live births after treatment with steroids in women with RM. In Japan, a patient with 10 previous miscarriages had a live birth after receiving preconceptual steroids[121]. In our unit, a patient with 19 previous miscarriages, and found to have high uNK cells density, had a successful live birth after steroid therapy[122].

Although uNK cells are found in high numbers during a progesterone rich environment for decidualisation in the late secretary phase, progesterone receptors have not been found on uNK cells. Instead, uNK cells express ER-β1, and glucocorticoid receptors which could be directly pharmacologically manipulated with estrogen or steroid treatment, such as prednisolone[88]. Indirectly, progesterone has been reported to exert effects on uNK cells by modulating their cytokine production via the glucocorticoid receptor[123]. This is important for further understanding the role of uNK cells and potential treatment mechanisms during the implantation window. Progesterone has also been postulated to exert its effect on uNK cell function through the cytokines such as IL-15, or stromal cells.

A prospective study using 20mg prednisolone from day 1 to day 21 of the menstrual cycle demonstrated a reduction in uNK cells in preimplantation endometrium of patients with RM[110]. In addition to reducing uNK cell numbers, prednisolone has been shown to reduce the degree of blood vessels maturation, which could be advantageous for the implanting embryo that favours a low oxygen environment[124]. In the same study, prednisolone also decreased some angiogenic growth factor expression, important for angiogenesis, a vital process in decidualisation and implantation.

The effect of glucocorticoids on uNK cells may not only be regulated by glucocorticoid receptors but also by the expression of steroid metabolising enzymes such as the 11-β hydroxyl steroid dehydrogenase (HSD) family[88]. Recently, laboratory studies have shown defective decidualisation correlating with high uNK cells and a lack of 11-β hydroxyl steroid dehydrogenase type 1 in stromal cells[125]. 11-β hydroxyl steroid dehydrogenase type 1 activates cortisol. Hence, administration of prednisolone may overcome this deficit. Prednisolone could exert its therapeutic effect through either one or a combination of the mechanisms

discussed and modify the endometrial environment to one more favourable for maintaining a pregnancy.

It is however unclear, if reducing uNK cells density to normality with steroid therapy will improve pregnancy outcomes. This could be examined in a double blinded, placebo controlled, randomised trial. We described a pilot trial of this nature in detail in Chapter 4 of this thesis.

2.4.2 Intravenous Immunoglobulin (IVIG)

IVIG is a costly treatment of fractionated blood product of pooled immunoglobulin (Ig) G extracted from plasma of blood donors, which has in the recent decade been used to treat women with reproductive problems, including RM. Although the exact mechanisms of action are unknown, IVIG is thought to suppress and neutralize autoantibodies, reduce NK cell activity, modify cytokine production and inhibit complement binding and activation[126]. A few systematic reviews have been performed to assess the beneficial effect of IVIG treatment in improving live birth rates. Although one of the systematic reviews demonstrated a significant benefit in improving subsequent live births in women with secondary RM[127], a larger RCT carried out thereafter challenged this conclusion as the trial found no benefit of IVIG treatment in idiopathic secondary RM[128]. This was further confirmed by the latest systematic review addressing this topic[129]. Thus, consideration should be given to seek out a marker to identify the subgroup of women with RM and immunological pathology, where the efficacy of IVIG treatment in these women subgroups can be further assessed through RCTs. Although there are potentially significant side effects such as sepsis and venous thromboembolism from this treatment, the reviews have shown a good safety profile of its use.

2.4.3 Other immunomodulation therapies

Therapies such as 3rd party donor cell immunization, paternal cell immunization and trophoblast membrane infusion have previously been proposed and used on women with RM. However, there is conflicting evidence as to their

efficacy. A meta-analysis of trials comparing these immunotherapies has found no evidence of a beneficial effect over placebo in preventing further miscarriages[52]. These treatments are less commonly prescribed now, with the availability of newer treatments such as IVIG, described above, and intralipid.

Intralipid fat emulsion infusion is a standard component of parenteral nutrition. A number of studies have reported that intralipid infusion can modulate the immune system through suppression of NK cells cytotoxicity and pro-inflammatory cytokines formation, and has been shown to reduce abnormal NK cells activity[130]. This has led to its empiric use to improve pregnancy outcomes for women with abnormal NK cell parameters and reproductive problems. It is considerably cheaper, and thought to be safer than IVIG as it is not a blood product. However, its use is still in the experimental state and there is minimal evidence about its efficacy in recent literature.

2.5 Conclusion

There is considerable evidence for a specialized immune system in the endometrium that potentially plays a major role in the initiation and maintenance of a successful pregnancy. Uterine NK cells are the predominant immune cells during the window of implantation and early pregnancy, implicating an importance in the implantation process. This is further strengthened by evidence of cell-cell interactions between uNK cells and EVT, and possible functions of uNK cells that play direct important roles in the process of implantation. Alternately, uNK cell density may serve as a marker of an underlying endometrial or immunological phenomenon that leads to miscarriage.

Immunotherapy has been empirically prescribed in an attempt to improve pregnancy outcomes in women with RM but systematic reviews of its efficacy have not shown significant benefit. It is possible that the apparent absence of an effect of immunotherapy reflects the lack of a screening test to identify the subgroup of women that may benefit from immunotherapy. Immunomodulation may not be a suitable treatment for all women with idiopathic RM. In-vitro and in-vivo studies

have shown these therapies to normalise NK cell density and function. Therefore, NK testing may be a suitable screening tool.

There are studies investigating peripheral blood NK (pNK) cell and endometrium NK cell density and function in relation to reproductive problems as both have been associated with RM. The next chapter will discuss the association of both pNK and uNK cells and subsequent pregnancy outcomes in women with reproductive problems using the method of a systematic review, to assess if a NK cell test can be utilized as a screening tool for poor pregnancy outcomes.

CHAPTER 3

NATURAL KILLER CELLS AND PREGNANCY OUTCOMES IN WOMEN WITH RECURRENT MISCARRIAGE AND INFERTILITY: *A SYSTEMATIC REVIEW*

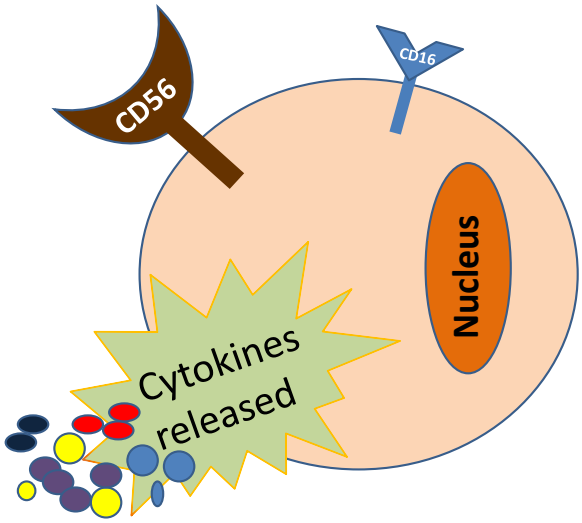
CHAPTER 3: NATURAL KILLER CELLS AND PREGNANCY OUTCOMES IN WOMEN WITH RECURRENT MISCARRIAGE AND INFERTILITY: *A SYSTEMATIC REVIEW*

3.1 Introduction

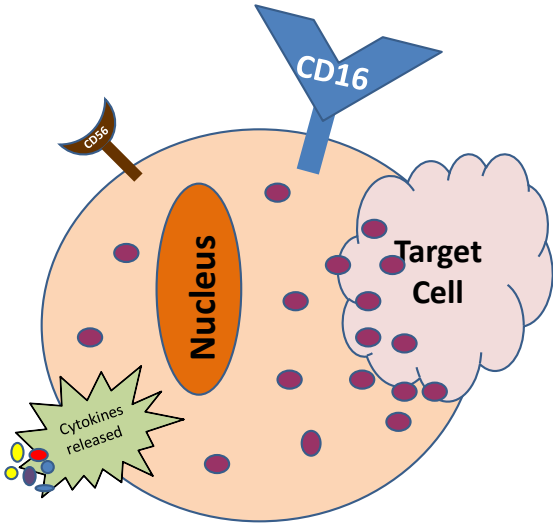
There are two types of NK cells. One found in the endometrium, known as uNK cells, has been extensively discussed, and the other is found in peripheral blood, known as pNK cells. Although both pNK and uNK cells express the surface antigen CD56, there are significant differences between them and thus should be considered separate entities[86] (*Figure 3.1*). 90% of pNK cells are $CD56^{dim}$ and $CD16^{+}$, while 80% of uNK cells are $CD56^{bright}$ and $CD16^{-}$. PNK cells have been demonstrated to show significant cytotoxic activity with well established anti-viral and anti-neoplastic functions. In contrast, the exact functions of uNK cells are still under investigations as described in detail in the previous chapter.

Reproductive failure includes women with RM or infertility and both may have similar underlying pathology with endometrial receptivity. In general, only 30% of conceptions result in live births. Of the other 70% where pregnancy losses occur, about 30% are loss before implantation, another 30% after implantation but before a missed period, and 10% is loss after a missed period[131]. Pregnancy losses before a missed period are often not known. It is thus possible that couples who are thought to be infertile have repeated conceptions lost prior to missing a period, either before or after implantation. A non-receptive endometrium, manifesting as infertility could be one end of the spectrum of reproductive failure, compared to implantation then miscarrying consecutively, manifesting as RM. Hence, both RM and infertility have been investigated for their association with NK cells.

Figure 3.1 Differences in uterine NK (uNK) and peripheral blood NK (pNK) cells



Uterine NK cells



Peripheral Blood NK cells

3.1.1 Association of NK cells and reproductive failure

The association of NK cells and women with reproductive problems is complex as some studies show elevated NK cells number and activity in women with reproductive problems[64, 108-110, 132-137] while others do not[116, 117, 138-142]. There have been a series of case-controlled studies reporting an association between pNK cells number[132-134] and activity[135, 136] with RM and some studies that showed no difference in pNK cells parameters between RM and controls[138-140]. Similarly, there have been case-controlled studies reporting an association between uNK cells and RM[64, 108-110], but others that included women with only two miscarriages failed to find this association[116, 117].

There is also inconsistency in the association of pNK cells and uNK cells with infertility. Some groups found an association of pNK cells and infertility[133, 143, 144] while some did not[141]. Likewise for uNK cells, one group reported an association with infertility[137] and another found no difference[142].

3.1.2 The rationale for pNK and uNK tests

Tests examining both pNK and uNK cells number and activity have been offered to women with reproductive failure, occasionally as a screening method to identify women who may benefit from manipulation of their immune system, although the association between pNK and uNK cells and reproductive failure has not been conclusive.

Peripheral NK cells may not reflect the condition of the endometrium where implantation occurs and the mechanism of how these cells can be associated with miscarriage is unclear. However, how large numbers of uNK cells appear in the mid-secretory phase is still not known. There are two theories: recruitment from pNK cells which subsequently differentiate in the uterine microenvironment into uNK cell phenotype through a series of organised processes; or in-utero proliferation and differentiation of stem cells or endogenous NK cells in the endometrium[79, 145]. The former is the rationale for testing pNK cells.

In contrast, uNK cells are resident in the endometrium and constitute 70% of endometrial leucocytes during the time of implantation and early pregnancy[66]. They are adjacent to fetal trophoblast cells in the maternal-fetal interface and express receptors that recognise antigens on trophoblast cells, where cell-cell interactions occur[56]. Thus, there is a biological plausibility for the role of uNK cells in reproductive failure.

The strength of the association between NK cells test and reproductive problems would be considerably improved if the tests of pre-pregnancy NK cells number or activity correlate to subsequent pregnancy outcome. Thus, we performed a systematic review of the current literature to ascertain the relationship of pre-pregnancy NK cell tests results and outcomes of miscarriage, live births and implantation failure in women with RM and those undergoing ART.

3.2 Methods

3.2.1 Search criteria

We searched the electronic MEDLINE database through OvidSP from 1950 to April 2010 for published literature in all languages *(Appendix 1)*. The MeSH terms 'Natural Killer cells', 'Reproduction', and 'Pregnancy Complications' were exploded. To identify citations relevant to NK cells, we used terms 'CD56', 'uterus', 'uterine', 'endometrial', 'decidual' and 'peripheral'. Search terms such as 'abortion/miscarriage', 'ectopic pregnancy', 'fetal death', 'fertilisation', 'insemination', 'livebirth', 'pregnancy', 'pregnancy outcome' and 'stillbirth', in relation to pregnancy outcomes were under the MeSH tree of 'Reproduction' and 'Pregnancy Complications', to retrieve all papers relevant to NK cells and reproductive outcomes. The search was then limited to humans and females. Advice was sought from the trials search coordinator in the Cochrane Collaboration Pregnancy and Childbirth Group with regards to the development of the search strategy protocol, who advised on the terminology and methods of searching. The trials search co-ordinator was not involved in the study selection of the articles, or data extraction.

The abstracts for all the citations were retrieved and assessed for their suitability for inclusion. Papers that were published in other languages all had abstracts in English. Original articles for abstracts that identified NK cells using the CD56 marker, either $CD56^{+}$, $CD56^{bright}$ or $CD56^{dim}$, CD69 activation marker and NK cells activity measured by ^{51}Chromium release cytotoxicity assay, in women with RM (defined as two or more consecutive miscarriages) and women with infertility seeking ART, and for those where relevance could not be judged from the abstract alone were obtained for analysis. Additionally, the reference lists of these publications identified were examined for possible studies not included in the initial search. The search was updated in March 2014 and the findings discussed in section 3.6.

3.2.2 Study Selection

Inclusion criteria were studies that identified NK cells using the CD56 marker, either $CD56^{+}$, $CD56^{bright}$ or $CD56^{dim}$, CD69 activation marker and NK cells activity measured by ^{51}Chromium release cytotoxicity assay, and investigated women with RM (defined as two or more consecutive miscarriages) and women with infertility seeking ART.

Review articles, letters, and studies with no pregnancy outcomes reported were excluded after reading the abstracts by myself. The list of publications for inclusion and exclusion were then reviewed by one of my supervisor SQ. We also excluded studies that reported on treating women with immunotherapy such as prednisolone or IVIG as prednisolone reduces both uNK[110] and pNK[146] cells and IVIG alters pNK cell parameters[147], which may either positively or negatively affect the pregnancy outcome, and distort the results of this review. Furthermore, these treatments are still experimental without evidence from methodologically sound RCTs.

All the papers selected for detailed evaluation were read and analysed by myself and SQ. Study quality was assessed using The Guidelines Manual 2009 published by National Institute for Health and Clinical Excellence (NICE)[148]. Information was obtained for the inclusion and exclusion criteria of women in the

study, the source and method of analysing NK cells, the percentage/number and activity levels of NK cells, the level of normality of NK cells in the unit, and pregnancy outcomes.

Publications were divided into four groups according to the source of NK cells and type of reproductive failure:

- pNK cells test in RM,
- pNK cells test in infertility,
- uNK cells test in RM, and
- uNK cells test in infertility.

Pregnancy outcomes assessed were:

- failure to get pregnant (defined as no positive pregnancy test after ART)
- miscarriage <24 weeks gestation,
- livebirths
- implantation success leading to either miscarriage of <24 weeks gestation
- implantation success leading to livebirths

Data were extracted from texts, tables and graphs of each of the included studies. Original data in our unit were reexamined by classifying the pregnancy outcomes according to the cut-off of 5% published in a later article[110]. When appropriate, meta-analyses were performed using Review Manager (RevMan) Version 5.0 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration 2008) on studies reporting pregnancy outcomes according to pre-determined cut-offs for normality of NK cell parameters in similar patient groups. Odds ratio were used to present the results of meta-analyses as this is the common statistical method used for systematic reviews. In the presence of significant heterogeneity, random effects were used to pool the results. The random effect model would take into account that the effects being estimated in each study were not identical and would consider these effects as if they were random. Heterogeneity was evaluated statistically using I^2, and the causes for these explored from the aspect

of population studied, methods of NK cells testing and outcomes measured. We also wrote to the authors of the studies in an attempt to obtain data that were not published. The results were reported according to the Meta-analysis of Observational Studies in Epidemiology (MOOSE) Guidelines[149].

3.3 Results

There were altogether 783 citations identified (*Figure 3.2*). 780 were from the search terms mentioned above, and three were from the assessment of reference lists of these publications. There were no non-English language publications that were found to be relevant to this review. After reading the abstracts, 756 articles were excluded, and 27 full text articles regarding pNK and uNK cells were retrieved. After detailed analysis, fifteen publications were excluded: three were letters, six did not report relevant outcomes[133, 134, 142, 150-152], four reported on studies using the same group of women for slightly different aspects of NK cells[153-156] and two reported immunotherapy use in some women[143, 157]. A total of twelve publications met the criteria for analysis. There were seven studies reporting on pNK cells (*Table 3.1)*, six studies reporting on uNK cells *(Table 3.2)*, as one study investigated both pNK and uNK cells. Five publications reporting on pNK cells and two publications on uNK cells presented pregnancy outcomes, dichotomized into groups of high and normal levels of NK cell parameters, established from controls as described in the tables.

Only three studies[64, 65, 109], all investigating uNK cells, fit the definition of idiopathic RM where women were included after three consecutive miscarriages with no causes for the miscarriages found after routine investigations in their hospital. Three studies on pNK cells[132, 136, 140] and one study on uNK cells[117] included women after only two miscarriages, and one[132] included women with known associations of RM such as endocrine disorders, APS and thrombophilia. The group for infertility included all women who underwent IVF treatment, regardless of the cause for infertility apart from one study[137] which included women after three cycles of implantation failure.

Figure 3.2 Flow chart of the selection process of publications included in this review

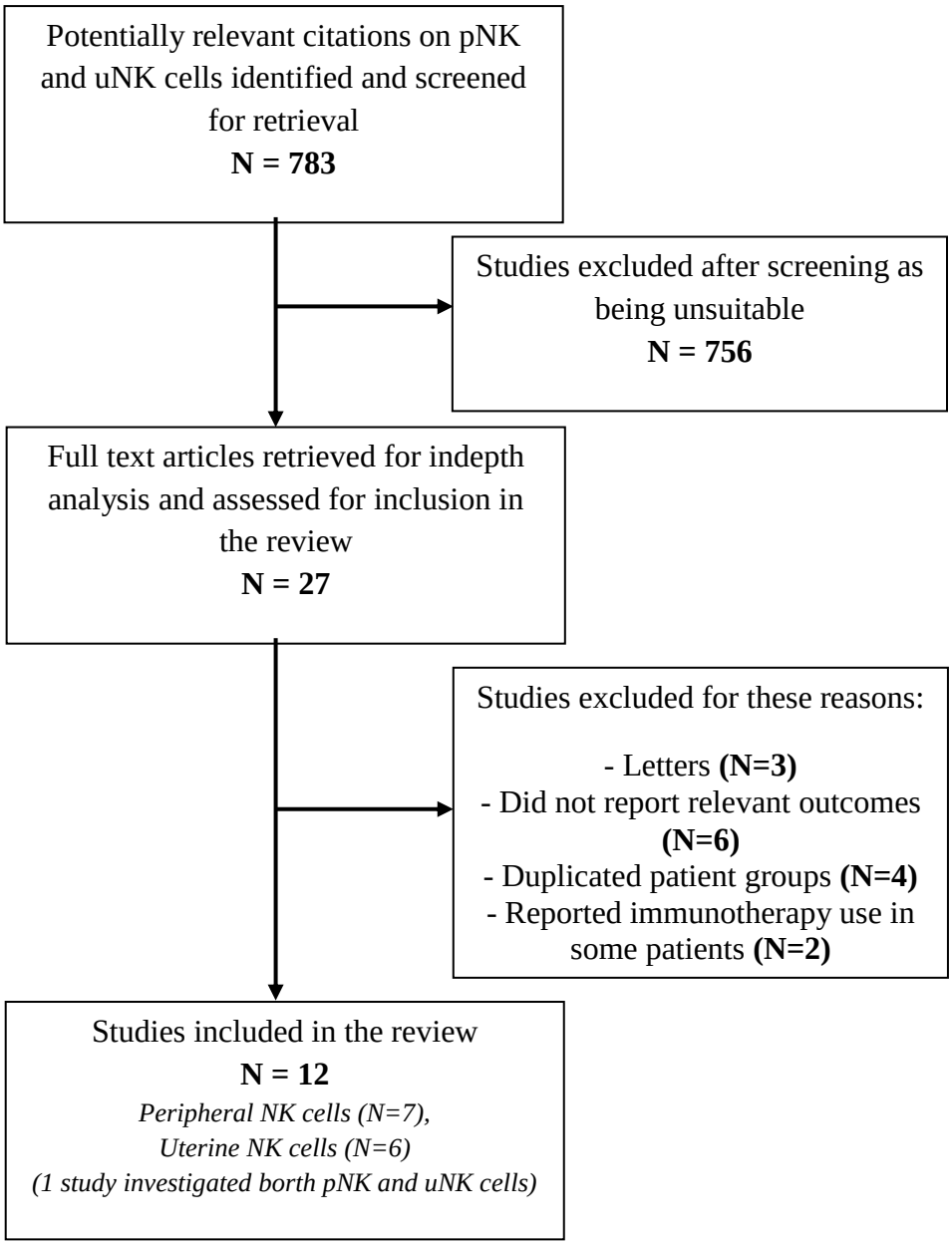


Table 3.1 Included studies investigating peripheral NK cells (7 studies)

Study	N	Inclusion criteria	Method of analysis	Results
Recurrent Miscarriage				
Aoki *et al* (1995)	68	≥2 miscarriages; idiopathic RM	^{51}Cr release assay	Higher % NK activity in patients who miscarried Normal range – <41.8% NK activity determined by the mean +1 SD of 47 healthy controls with no history of miscarriages with pregnancy outcomes reported according to normal range
Emmer *et al* (2000)	142	≥2 miscarriages; idiopathic RM	Flow cytometry, ^{51}Cr release assay	Normal range – <12% NK cells taken from publications by other groups and <322 lytic units for NK cells activity (*no mention of how this was obtained*) with pregnancy outcomes reported according to normal range
Yamada *et al* (2003)	113	≥2 miscarriages; all RM	Flow cytometry, ^{51}Cr release assay	Higher % NK cells in miscarriage of normal karyotype and biochemical pregnancy compared with live birth Normal range – <16.4% $CD56^{+}$ cells and <46% NK activity determined by ROC curve for optimal discrimination between miscarriage (normal karyotype or biochemical pregnancy) and live birth

Infertility				
Fukui *et al* (1999)	85 NK cells 297 NK activity	Patients undergoing IVF	Flow cytometry ^{51}Cr release assay	**% NK cells** *Pre-IVF cycle* – No difference in % NK cell and subpopulation in women with infertility who failed to get pregnant and those who became pregnant after ART **% NK cell activity** *Pre-IVF cycle* – No difference in % NK cell activity in women with infertility who failed to get pregnant and those who became pregnant after ART; and between miscarriage and LB in those who were pregnant No normal range reported
Thum *et al* (2005)	138	Patients undergoing IVF	Flow cytometry	No difference in % NK cell and NK cell subpopulation in women with infertility who failed to get pregnant and those who became pregnant after ART, and between miscarriage and LB in those who were pregnant Normal range – <12% taken from publications by other groups with pregnancy outcome reported according to this normal range
Matsubayashi *et al* (2005)	94	Patients undergoing IVF	^{51}Cr release assay	Higher % NK cell activity in women with infertility who failed to get pregnant and those who became pregnant after ART No difference in % NK cell activity between miscarriage and LB in those who were pregnant

				Normal range – <44% as determined by the mean +1 SD of 94 healthy, age-matched controls with pregnancy outcomes reported according to normal range
Baczkowski *et al* (2007)	58	Patients undergoing IVF	Flow cytometry	No difference in % NK cell in women with infertility who failed to get pregnant and those who became pregnant after ART No normal range reported

Table 3.2 Included studies investigating uterine NK cells (6 studies)

Study	N	Inclusion criteria	Method of analysis	Results
Recurrent Miscarriage				
LaChapelle *et al* (1996)	20	≥3 miscarriages; idiopathic RM	Flow cytometry	No difference in % total NK cells in women with miscarriage and ongoing pregnancy No normal range reported
Quenby *et al* (1999)	22	≥3 miscarriages; idiopathic RM	Immuno-histochemistry	Higher % NK cells in women with miscarriage compared with LB Normal range – <5% determined by 75th percentile of 18 control women who were attending for sterilization with two or more pregnancies and no miscarriages
Michimata *et al* (2002)	17	≥2 miscarriages; idiopathic RM	Immuno-histochemistry	No difference in number of NK cells/10 high power fields in women with miscarriage and LB No normal range reported
Tuckerman *et al* (2007)	87	≥3 miscarriages; idiopathic RM	Immuno-histochemistry	No difference in % NK cells in women with miscarriage and LB Normal range – <13.8% determined by the 90th centile of 10 control women with regular menstrual cycle not on hormonal contraception

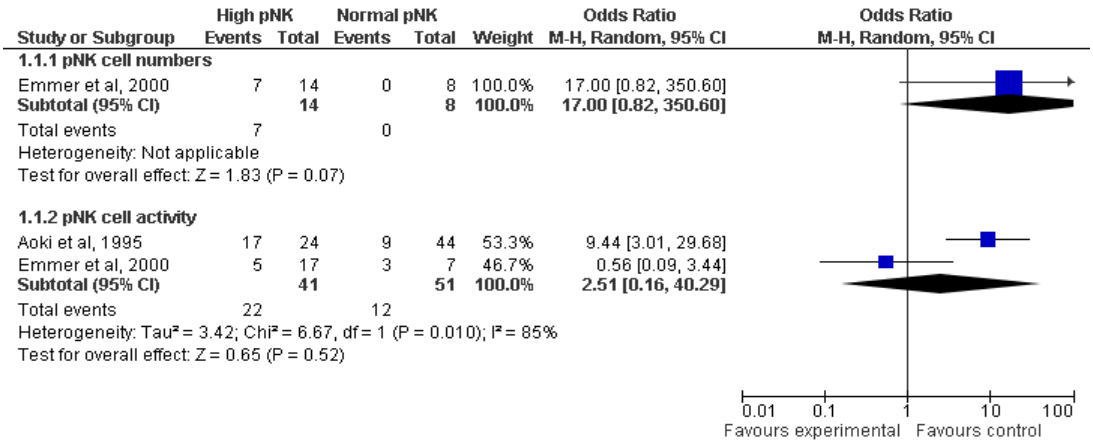
Infertility				
Fukui *et al* (1999)	76	Patients undergoing IVF	Flow cytometry	No difference in % NK cells in women with infertility who failed to get pregnant and those who became pregnant after ART; and between miscarriage and LB in those who were pregnant Higher subpopulation of %$CD56^{bright}$ NK cells in women who had LB compared to miscarriage No normal range reported
Ledee-Bataille *et al* (2004)	15	≥3 IVF cycles failures	Immuno-histochemistry	No difference in number of NK cells/100x fields in natural IVF cycle in women with infertility who failed to get pregnant and those who became pregnant after ART No normal range reported

3.3.1 Peripheral NK cells in reproductive failure

All studies on pNK cells used flow cytometry to investigate the number of pNK cells as a percentage of lymphocytes or leucocytes depending on the panel of monoclonal antibodies used to identify these cells, and ^{51}Cr release cytotoxicity assay to assess NK cell activity.

There were three studies[132, 136, 140] investigating pNK cells (number and activity) in women with RM, but only two studies included women with idiopathic RM[136, 140]. Emmer *et al*[140] reported pregnancy outcomes in relation to both number and activity of NK cells. It is likely that the same women were tested for both parameters, and therefore, the results were not pooled to avoid double counting. In addition, both tests are significantly different in the laboratory where cell numbers alone may not reflect the function or activity of pNK cells. There was significant heterogeneity between the studies of pNK cell activity with I^2=85%. Therefore, random effects were used. High pNK cells number or activity did not predict miscarriage in a subsequent pregnancy in women with idiopathic RM as the meta-analysis of these studies did not reach statistical significance, although positive OR were found (*n*=22, OR 17, 95% CI 0.82-350.60; *n*=92, OR 2.51, 95% CI 0.16-40.29) (*Figure 3.3*).

Figure 3.3 Odds of miscarriage with high pre-pregnancy peripheral NK cell parameters in women with idiopathic RM



Of the four studies[158-161] that investigated pNK cells in women with infertility, only two[159, 161] reported outcomes of implantation failure after ART, dichotomized into high and normal levels of pNK cells, but used different parameters of pNK cells number and activity. The effect of high pNK cells number and activity on subsequent implantation failure is unclear (*n*=126, OR 0.63, 95% CI 0.30-1.33; *n*=77, OR 3.13, 95% CI 1.12-8.69) (*Figure 3.4*). The same two studies[159, 161] also reported outcomes of miscarriage after implantation success, and abnormal pNK cell test results did not predict subsequent miscarriage after implantation success (*n*=51, OR 1.31, 95% CI 0.37-4.68; *n*=28, OR 7.13, 95% CI 0.88-57.55) (*Figure 3.5*). Results were not pooled due to the differences in techniques used to obtain the pNK cells test result.

Figure 3.4 Odds of implantation failure after ART with high levels of pre-pregnancy peripheral NK cell numbers and activity in women with infertility

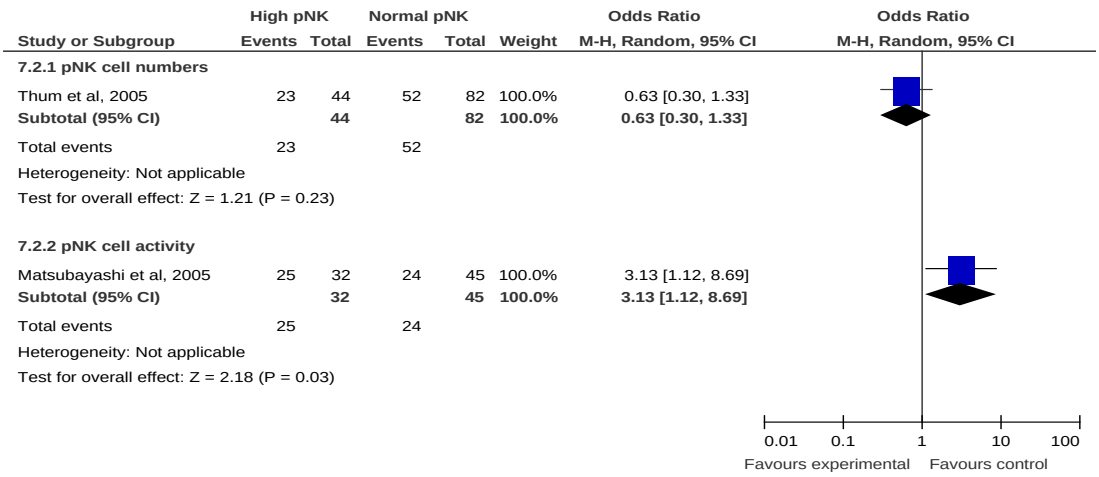
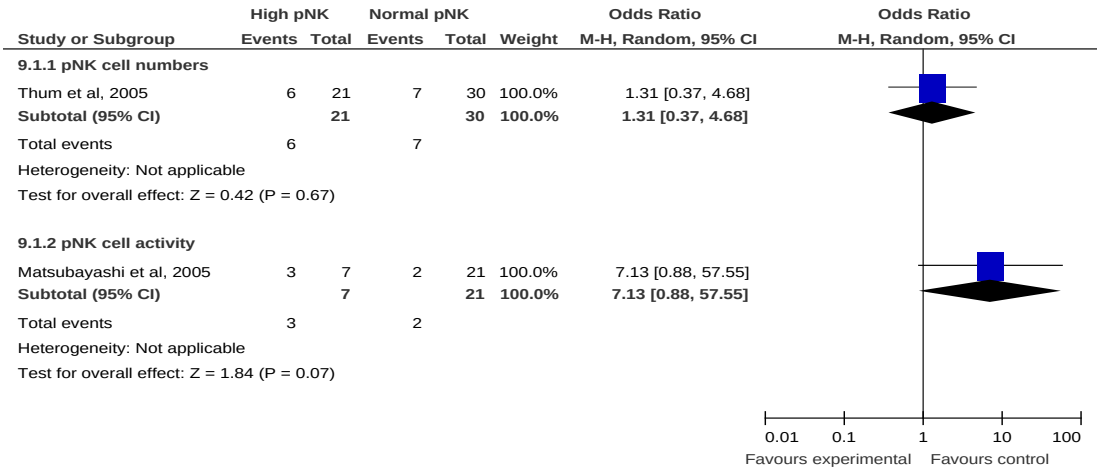


Figure 3.5 Odds of miscarriage (after implantation success from ART) with high levels of pre-pregnancy pNK cell parameters in women with infertility



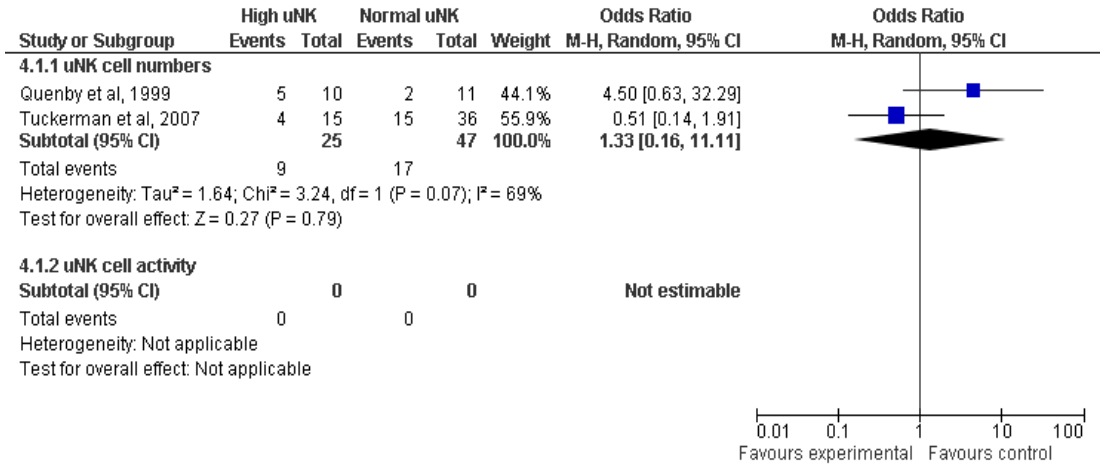
Of all the studies investigating pNK cell parameters, two studies[158, 160] did not report pregnancy outcomes according to a pre-determined cut-off of normality. Both were investigating women with infertility. Fukui *et al*[158] reported no difference in pre-pregnancy pNK cells number and activity between women who failed to get pregnant and those who became pregnant after ART, and between miscarriage and live birth in those who were pregnant after ART. Baczkowski *et al*[160] also found no difference in percentage pNK cells in these two groups of women

3.3.2 Uterine NK cells in reproductive failure

All the samples in the studies were timed from the LH surge and thus examined the endometrium in the implantation window of a non-conception cycle. Four studies[64, 109, 117, 162] investigating uNK cells used immunohistochemistry of frozen or paraffin fixed sections with antibodies to CD56 to identify NK cells staining, and two studies[65, 158] used enzymatic digestion of endometrium and then flow cytometry to identify $C56^{bright\ or\ dim}$ cells. Uterine NK cells results were presented either as an absolute count of NK cells per three[162] and ten high power fields[117] or as a percentage of total stromal cells[64, 109]. There were no studies that reported pregnancy outcomes according to uNK cell activity.

There were four studies investigating uNK cells in women with idiopathic RM but only two[64, 109] reported pregnancy outcomes according to high and normal levels of uNK cells. A meta-analysis, with high heterogeneity (I^2=69%) did not provide adequate evidence that high levels of uNK cells was correlated to subsequent miscarriage in women with RM (*n*=72, OR 1.33, 95% CI 0.16-11.11) *(Figure 3.6).* The two studies that did not report dichotomized pregnancy outcomes found no differences in mean percentage of uNK cells in women who subsequently miscarry and those who had live births.

Figure 3.6 Odds of miscarriage with high levels of pre-pregnancy uterine NK cell numbers in women with idiopathic RM



Two studies[158, 162], differing in methodology to each other, investigated uNK cells in women with infertility. Both did not report pregnancy outcomes according to a pre-determined cut-off of normality. One included women who underwent IVF treatment reported NK cells as a percentage of lymphocytes. The other included women with implantation failure after three cycles of IVF and reported NK cells as mean absolute numbers in three 100x fields. Both reported no differences in uNK cells between women who failed to get pregnant and who became pregnant after ART. However, the study by Fukui *et al*[158] which analysed NK cells by flow cytometry showed a higher percentage of the subpopulation $CD56^{bright}$ NK cells in women who had live births compared with women who miscarried.

3.4 Discussion

This systematic review did not demonstrate sufficient evidence for the association of abnormal pNK and uNK cell parameters of numbers and activity with adverse pregnancy outcomes of miscarriage or implantation failure, in women with RM and infertility. The observational studies were individually and collectively underpowered to answer this important question. Assuming that, irrespective of the reason for NK testing, women with a normal NK cell count have a miscarriage rate of around 30%, one would expect an increase in miscarriage rate by at least 10% (33% miscarriage) when NK cells are high. At least 376 women per group would have to be followed until delivery (752 in total) to test this hypothesis with 80% power (α=5%). This is considerably more than the largest individual study available, which investigated 126 women[159].

It was possible that only studies suggesting an association of NK cells testing and poor pregnancy outcomes were published as this was the indication for offering these tests to women with reproductive disorders, leading to publication bias. However, NK cells testing were only carried out regularly in a few centres, and we addressed this by writing to all the authors of published literature, asking for further information of unpublished data. Unfortunately, there were no replies from these requests. It was not possible to carry out a funnel plot analysis to assess for publication or related bias in view of the small number of studies. However, it was unlikely there were any unpublished research that would significantly change the findings of this review as a recent review published last year on this subject reported similar conclusions[163].

Odds ratio from individual studies were analysed using a random effects model. In this situation, the use of either risk ratio or odds ratio, and random or fixed effects model would result in the same findings due to the small effect size and small number of studies. However, we need to be cautious when interpreting odds ratio as they could overestimate or underestimate an effect size, especially when the effect is large.

There was significant heterogeneity between studies in terms of variation in the inclusion criteria, methodology of NK cells analysis, and outcome

measurements. As discussed in the introduction, there is inconsistency in the definition of RM, leading to differences in study population. There were some studies that included women after two miscarriages and some included women after three miscarriages. Similarly for infertility, there were studies that included all women undergoing ART, while some included women after three implantation failures. Heterogeneity could also be explained by differences in NK cells analysis, which could be by flow cytometry or immunohistochemistry. Furthermore, every study had a different cut-off of normality for NK cells as discussed further below. There were also variations in the outcomes measured with differences in definition of implantation failure and miscarriages which led to the high heterogeneity.

3.4.1 Peripheral NK cells

It has been postulated that uNK cells originate from pNK cells, which subsequently differentiate in the uterine microenvironment into uNK cell phenotype[145]. Testing for pNK cells involves venous blood sampling at any time during the menstrual cycle as they have not been shown to fluctuate throughout the cycle[164], or with sampling in pregnancy. All studies used flow cytometry, the best method to analyse and quantify lymphocyte subsets[86]. Investigations have been reported in the pre-pregnancy period, during pregnancy or on the day of embryo transfer in infertility patients undergoing ART. However, only results from investigations done prior to pregnancy were analysed in this study as the pre-specified aim was to assess the association of pre-pregnancy NK testing and subsequent pregnancy outcomes. Further studies were planned to determine if there was a potential for using NK cell tests as a pre-conception screening test to identify and select a subgroup of women with reproductive problems for treatment.

All three studies of pNK cells and RM included women after only two miscarriages which do not fit the ESHRE definition of RM[2]. Yamada *et al*[132] and Emmer *et al*[140] investigated both pNK cells number and activity, while Aoki *et al*[136] only studied pNK activity. Emmer *et al*[140] and Aoki *et al*[136] both studied women with idiopathic RM whereas Yamada *et al*[132], the study with the largest sample size, included women with known associations of RM such as endocrine disorders, APS and thrombophilia. Although they reported that high pNK cells number and activity

predicted subsequent biochemical miscarriage and miscarriage of normal karyotype, more than half of the women investigated had another contributing factor to their miscarriage, creating potential bias in the results.

There is significant heterogeneity between studies which is not surprising given potentially important differences in the analysis and interpretation. The determination for the cut-off of normality in different studies was through different methods with different control groups. Emmer *et al*[140] used <12% pNK cells number as normal range set by Beer *et al*[143] with no explanation of how this level was calculated. Yamada *et al*[132] used <16.4% pNK cells number and <46% pNK cell activity as the normal range determined by ROC curve for optimal discrimination between miscarriage (normal karyotype or biochemical pregnancy) and live birth. However, Aoki *et al*[136] set <41.8% pNK cell activity measurement as normal range determined by the mean +1 SD of 47 healthy controls with no history of miscarriage and Emmer *et al*[140] set <322 lytic unit as normal with no mention of how this was obtained. The inconsistency was also seen in studies of women with infertility where Matsubayashi *et al*[161] determined <44% pNK activity as normal range with controls, although age-matched, not all of proven fertility whilst Thum *et al*[159] used <12% pNK cells number as set by Beer *et al*[143]. Therefore, it is clear that there is lack of a commonly accepted normal range for pNK cells number and activity, or generally accepted type of pNK cells testing. In addition, there were also differences in IVF protocols in different units and the definition for implantation failure or success was not mentioned in some studies[160, 161].

Peripheral NK cells are phenotypically and functionally different from uNK cells and less than 10% of pNK cells resemble uNK cells[56]. Thus, pNK cells tests to gauge the state of endometrium is questionable as it may not reflect the condition of the endometrium where implantation occurs, and the mechanism of how these cells can be associated with miscarriage remains unclear. Two recent studies have attempted to answer these by studying pNK and uNK cells at the same in the same women. However, the results were contradictory. The study by Park *et al* investigated pNK cells and NK cells in miscarried endometrium (decidua) in 22 women with two miscarriages[165]. They reported a correlation (r=0.5) between pNK cells and decidual NK cells. In contrast, the other study by Laird *et al*[166] investigating 25 women with three or more miscarriages did not show a correlation

between pNK and uNK cells number. Therefore, the correlation of pNK and uNK cells is still contentious. With the low numbers investigated in these studies, it is difficult to draw meaningful conclusions.

It is also known that pNK cells increase significantly with stress and exercise and this was not taken into account when blood was taken for investigation[167, 168]. Furthermore, the value of an abnormal test for pNK cells activity is also unknown as it may be a reflection of a transient stress response at the time of blood withdrawal, or a representation of the response to other stresses in daily life[169]. It is unclear if these phenomena exist for uNK cells. Hence, more studies investigating pNK cell population and function in women with reproductive problems, including pregnancy outcomes, are needed to assess the utility of pNK cells tests. Currently, pNK cells tests should not be used to identify women for treatment except in a well-designed study.

3.4.2 Uterine NK cells

Uterine NK cells constitute 70% of endometrial leucocytes during the window of implantation in the menstrual cycle and are thought to play a role in the regulation of trophoblast invasion and angiogenesis[56, 79]. Testing of uNK cells involves an endometrial biopsy that can only be carried out in the pre-pregnancy period. All the samples in the studies were timed from the luteinizing hormone (LH) surge as the number of uNK cells vary through the menstrual cycle. They are scanty in the proliferative phase, increasing in numbers after ovulation and through the secretory phase[79]. Thus, the mid-luteal biopsy examines the endometrium in the implantation window, of a non-conception cycle. Immunohistochemistry was the method used in most of the studies of uNK cells. This is more time consuming than flow cytometry but it reveals the location of the uNK cells[66]. Analysis using flow cytometry involves digesting the tissue, and thereby potentially losing cells and antigens. Furthermore, it needs a large sample of endometrium that may be difficult to obtain in some women.

The evidence for the association between uNK cells and adverse outcomes in a subsequent pregnancy is limited as two studies reported no difference in uNK cells

between outcomes of miscarriage and live births while the other two studies which reported pregnancy outcomes according to high and normal uNK cells had contradictory results[64, 109]. The studies that reported no difference in uNK cell numbers were different to each other and are not comparable. Michimata *et al*[117] included women after two miscarriages and used immunohistochemistry for analysis while LaChapelle *et al*[65] included women with three miscarriages and analysed NK cells using flow cytometry. Although a recent study did show a significant correlation (r=0.497, p=0.026) between measurements of uNK cells by methods of immunohistochemistry and flow cytometry, the percentages are only comparable if measurements of CD56+ cells by flow cytometry are expressed as a percentage of CD45+ cells, and not a percentage of all cells[166].

There was again considerable heterogeneity between the two studies that reported dichotomized pregnancy outcomes. Similarly to pNK cell parameters, the normal ranges were obtained with different control women. Quenby *et al*[64, 110] used <5% uNK cells as a normal range based on the 75th percentile of 18 control women while Tuckerman *et al*[109] defined <13.8% as normal determined by the 90th percentile of ten control women. For analysis, Quenby *et al*[64] used frozen sections while Tuckerman *et al*[109] used waxed embedded specimens with possibly different methods of counting the stained sections. Uterine NK cells are not evenly distributed through the tissues. Hence their density varies depending on where the cells are counted, either along the epithelial edge or deeper into the section, and whether they are assessed near to or away from glands and blood vessels.

Both studies investigating uNK cells in women with infertility reported no difference in uNK cell numbers and percentage between women who failed to get pregnant and those who became pregnant after ART. However, the women included in the studies were different as Fukui *et al*[158] included all women undergoing ART while Ledee-Bataille *et al*[162] included women without pregnancies after three cycles of ART. In addition, the method of analysis was different where one employed flow cytometry and the other immunohistochemistry. Thus, the studies are not comparable enough to draw conclusions about the implications of uNK cells tests in women with infertility.

There is a biological plausibility for a role for uNK cells in reproductive failure as discussed in the previous chapter. Uterine NK cells are most numerous in the implantation window and in early pregnancy[79], they are adjacent to and interact with EVT cells by expressing antigens that are recognized by the receptors of these cells[56] and different uNK cells population have been described in deciduas of normal and miscarried early pregnancy[74]. Furthermore, uNK cells have been shown to regulate angiogenesis[107], an important factor in implantation. Hence, the rationale for testing uNK cell numbers. There is controversy about the origin of uNK cells as some suggest they are recruited from peripheral blood. However, a study by Park *et al*[165] investigated chemoattractants CCL3 and CXCL12 for NK cells in the decidua and reported that staining intensity for these cells were not correlated to the number of decidual NK cells, thereby suggesting that the origin of uNK cells are not solely from peripheral blood.

3.5 Conclusion

This review suggests that the prognostic value of measuring pNK and uNK cells number or activity remains uncertain as these parameters have not been shown to be associated with subsequent pregnancy outcome. This finding is similar to that of the many conditions that have been associated with RM such as thrombophilia and structural uterine anomalies, none of which have been shown to clearly relate to pregnancy outcome.

This may be because of the disappointingly small number of studies reporting clinical outcomes, on small number of women. The inclusion criteria were also inappropriate in studies that investigated women after only two miscarriages. Furthermore, there is still no consensus on what an abnormal NK cell test result is, as the normal ranges in different studies are derived from different controls. There is a need for more studies to investigate pNK and uNK cells population and function, to assess these tests as clinically useful markers in screening women with reproductive problems. Ideally, future studies should be prospective, with appropriate inclusion criteria and have a standard methodology of analyzing and

reporting pNK and uNK tests results. Sample size should also be calculated to avoid lack of power in the study.

Before the availability of the results from these larger, more methodologically sound evaluation of prognostic value, or RCT of therapeutic modalities on specifically selected women with potential immunological pathology, women with reproductive problems should not be routinely offered NK testing[170]. The Cochrane review on immunotherapy for RM quotes '*a specific assay to diagnose immune-mediated early pregnancy loss and a reliable method to determine which women might benefit from manipulation of the maternal immune system are urgently needed*'[52].

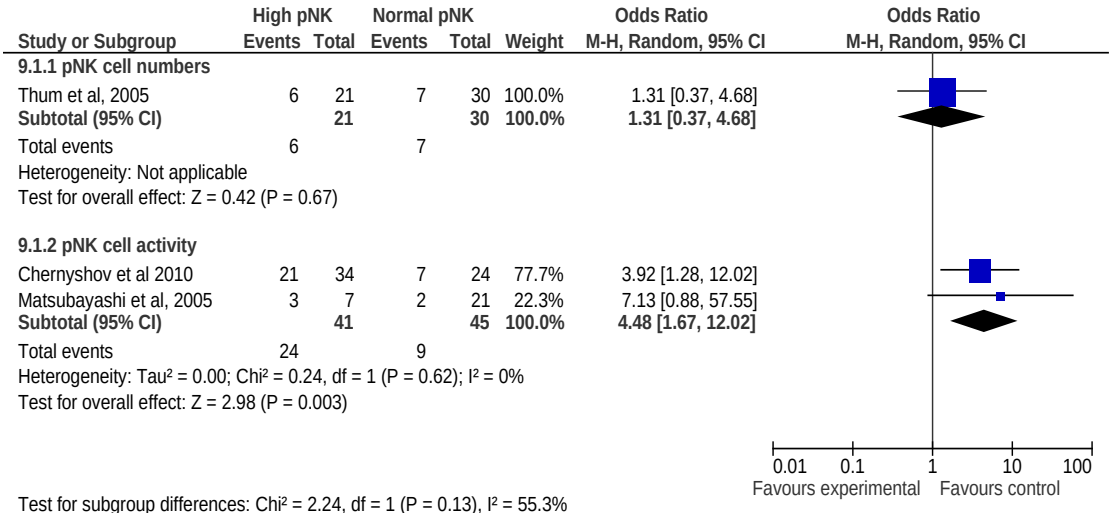
Although the review showed that the role of uNK cells testing was uncertain, there were biological plausibility for a role of uNK cells in RM as discussed in chapter 2, and the aim of the thesis was to investigate the endometrium in women with RM. Furthermore, in some units, empirical treatment was being prescribed for women with RM according to NK cells test results without evidence from RCTs. Thus, we carried out the pilot phase of a RCT investigating the value of prednisolone therapy in women with idiopathic RM and found to have high levels of uNK cells, to further assess the role of uNK cells in RM, and to explore the feasibility of a screen and treat trial of this nature in women with RM. This is described in the next chapter.

3.6 Updated literature review

The updated search to March 2014 found another 241 citations. After reading the abstracts, four full text articles, which investigated on pNK cells were analysed in detailed[171-174]. They were no abstracts on uNK cells which were relevant. After reading the full text of the four publications, one was excluded as it did not report pregnancy outcomes[173] and three were found to be relevant. Of these, two reported on women with infertility and one was on RM.

Zhou J *et al*[171] investigated women underdoing ART treatment and reported on the numbers of a subset of pNK cells called NKT cells. They concluded that high NKT cell numbers were associated with favourable pregnancy outcomes of increased implantation rate and live birth rate, contradicting previous reports of poor obstetric outcomes in abnormally high results. The other relevant article by Chernyshov *et al* reported that high pNK cell activity (cytotoxicity) was associated with poor pregnancy outcomes of miscarriage[174]. When meta-analysed together with the study by Matsubayashi *et al*, high pNK cells activity is significantly associated with the poor pregnancy outcome of miscarriage in women with infertility (*n*=86, OR 4.48, 95% CI 1.67-12.01) (*Figure 3.7*). The only report on RM was the largest study of this nature which investigated 552 women with idiopathic RM[172]. Of these, 124 (22%) women miscarried and they concluded that there was no linear relationship between pNK cell activity and adverse pregnancy outcomes.

Figure 3.7 Odds of miscarriage (after implantation success from ART) with high levels of pre-pregnancy pNK cell parameters in women with infertility including updated study



The updated search showed that measurements of pNK cells activity could possibly be an important marker of adverse outcomes in women undergoing ART and thus should be an area of important research. There remains a scarcity of studies of uNK cells in women with RM in the published literature. It is admirable that there was a large study (N=552) of pNK cells in women with RM which gives a stronger conclusion that pNK cells analysis is unlikely to have a role as a marker of poor outcome for women with RM. A study of similar size should be carried out for uNK cells to ascertain any utility for this test.

CHAPTER 4

A PILOT RANDOMISED CONTROLLED TRIAL OF PREDNISOLONE FOR WOMEN WITH IDIOPATHIC RECURRENT MISCARRIAGE AND HIGH LEVELS OF NK CELLS IN THE ENDOMETRIUM

CHAPTER 4 – A PILOT RANDOMISED CONTROLLED TRIAL OF PREDNISOLONE FOR WOMEN WITH IDIOPATHIC RECURRENT MISCARRIAGE AND HIGH LEVELS OF NK CELLS IN THE ENDOMETRIUM

4.1 Introduction

Immunomodulation therapies such as steroids, IVIG, 3rd party donor cell immunization, paternal cell immunization and trophoblast membrane infusion have been proposed as treatment for women with idiopathic RM. However, evidence of their efficacies has been conflicting as previously described. It may be the lack of a test that selects the subgroup of women with RM that could benefit from such treatment.

From discussions in previous chapters, uNK cells may possibly play a direct role in the pathophysiology of RM. The systematic review carried out to assess the prognostic value of uNK cells tests in RM found only four studies where two studies reported no difference in uNK cells between outcomes of miscarriage and live births, and the other two studies which reported pregnancy outcomes according to high and normal uNK cells had contradictory results[64, 109]. There is a need for more studies assessing uNK cells as clinically useful markers in screening women with reproductive problems for initiating treatment.

4.1.1 The effect of prednisolone on uNK cells

Steroids were chosen as the immunomodulator agent as uNK cells express both glucocorticoid and ER-β receptors[88] and therapeutic manipulation of these cells is possible. IVIG were not considered as a systematic review has shown that this treatment is not beneficial in improving live births in women with RM[129]. Although intralipid is also known to modulate NK cells function, they are only available as an infusion[130], which is not as convenient. Prednisolone was chosen as the steroid of choice as it is metabolised by the placenta to inactive prednisone and thus very little of the active drug (only about 10%) reaches the fetus[175]. Prednisolone is thought to

be safe as it has been commonly used to treat medical conditions in pregnancy and has been given in the first trimester to women with asthma[176], rheumatoid arthritis[177] and hyperemesis gravidarum[178] with minimal side effects. Although there are concerns of possible complications of intrauterine growth restriction and an association with cleft palate from animal studies, more recent studies, even with postnatal follow-up, have not shown such complications with prednisolone use[178, 179]. Furthermore, prednisolone is orally available.

There were also case reports of prednisolone use in the situation of RM and yielding successful pregnancies. A team in Japan reported prednisolone use directly in the uterine cavity in a woman with 10 idiopathic RM, and achieved a successful pregnancy[121]. In Liverpool, one woman with 17 miscarriages was found to have significantly raised uNK cells. She was given 5 mg of prednisolone pre-conceptually. Unfortunately, she had a further 2 miscarriages. Subsequently, she was prescribed 20 mgs of prednisolone once a day both pre-conceptually and in early pregnancy. On this regimen, she had a live birth[122].

This led to the preliminary study which assessed the normal range of uNK cells, and the effect of prednisolone on uNK cells[110]. This study carried out 7 years ago investigated 85 women with RM and 18 women with 2 or more normal pregnancies (controls) for uNK cells density via a mid-luteal phase endometrial biopsy. The normal range of uNK cells was defined using the upper end of the inter-quartile range for the 18 control women. Thus, women with more than 5% uNK cells were considered to have high levels. 32 (38%) women with RM had high levels of uNK cells. 29 agreed to take 20mg prednisolone from day 1 to day 21 of the menstrual cycle for 3 weeks and have a second biopsy. In 23 (79%) women, the number of uNK cells decreased. The reduction in uNK cells density was significant with a mean level of 14% before treatment to 9% after treatment (p=0.0004, CI 2.3-12). Furthermore, 3 women requested a third biopsy after a further month of prednisolone and in each case, the uNK cells level had fallen further suggesting that prolonging the steroid therapy for longer than 3 weeks further reduces the level of uNK cells. None of these women reported side effects significant enough to stop the medication. The next step was hence to assess if the reduction in uNK cells in the pre-implantation endometrium of women with idiopathic RM with 20mg of prednsiolone led to an improved pregnancy outcome.

A mode of mechanism of how prednisolone may be therapeutic is through the function of uNK cells in regulating blood vessel formation. A recent study showed that prednisolone treatment not only reduced uNK cells density, but also reduced the number of matured endometrial blood vessels, and decreased some angiogenic growth factor expression[124]. This may modify the endometrial environment to one of low oxygen tension that is more favourable for maintaining a pregnancy.

4.2 Objectives of the trial

The rationale for carrying out a double blind, RCT of prednisolone use in women with idiopathic RM and found to have high uNK cells density (≥5%) was to assess if the reduction of uNK cells density with 20mg of prednisolone therapy would impact on the subsequent pregnancy outcome. This trial was intended to make progress towards meeting the challenge of diagnosing and treating endometrial immune-mediated early pregnancy loss and RM, with the primary aim to investigate if prednisolone use during the first trimester of pregnancy was able to reduce the risk of miscarriage and improve live birth rates.

A large trial with adequate power calculation would be needed to show significance for an impact in clinical practice. For this, it was essential to demonstrate that women were in support of attending for an endometrial biopsy to assess for a potential cause of RM, and their willingness for randomisation, potentially to placebo, when pregnant. Prednisolone was also a medication known to have side effects, and it is unclear if women would comply in completing the course of treatment. In view of the above, we conducted the pilot phase of the RCT to assess the feasibility of recruitment and randomisation, integrity of trial methodology and procedures, including women's perspective of the trial. Clinical aims of this pilot phase would include live birth rates, miscarriage rates, pregnancy complications and acceptability and safety of prednisolone treatment. This pilot phase should also provide results of preliminary data for accurate power calculations for the definitive trial.

4.3 Methodology

4.3.1 Overview of pilot trial design (*Figure 4.1*)

The trial protocol was designed by my supervisor (SQ) for submission for ethical approval and to source for funding. When funding was available to start the trial, I took up post and conducted the trial as the principal investigator, contributing to every step in the trial, discussed below. Women were recruited primarily from Liverpool Women Hospital's RM clinic (tertiary referral centre for Cheshire and Merseyside) and an endometrial research clinic *(details in section 4.3.3)*. Patient information leaflets regarding the trial were given or sent to them for consideration of their participation in this study when these women were first seen in the RM clinic, or if a referral letter to the research clinic was received *(Appendix 2)*. Women willing to participate were encouraged to ring for an appointment 6-9 days after their luteinising hormone (LH) surge in a cycle where they have not tried to conceive (patient information leaflet advised patients to get an ovulation kit and to use non-hormonal/barrier contraception). A trial recruitment clinic was set up in hospital to allow me to review women suitable for the trial. At this appointment, a full history was taken and the results of previous investigations noted to ensure that there was no cause found for RM. They were given a questionnaire to assess whether they thought they had an endometrial cause to the miscarriages, and their preference to medication allocation if they had an option to choose their medication rather than be randomised *(Appendix 2)*. If suitable, consent was obtained to perform an endometrial biopsy, which was taken in clinic with a Wallach endocell sampling device. This was stored in formalin prior to being processed for analysis.

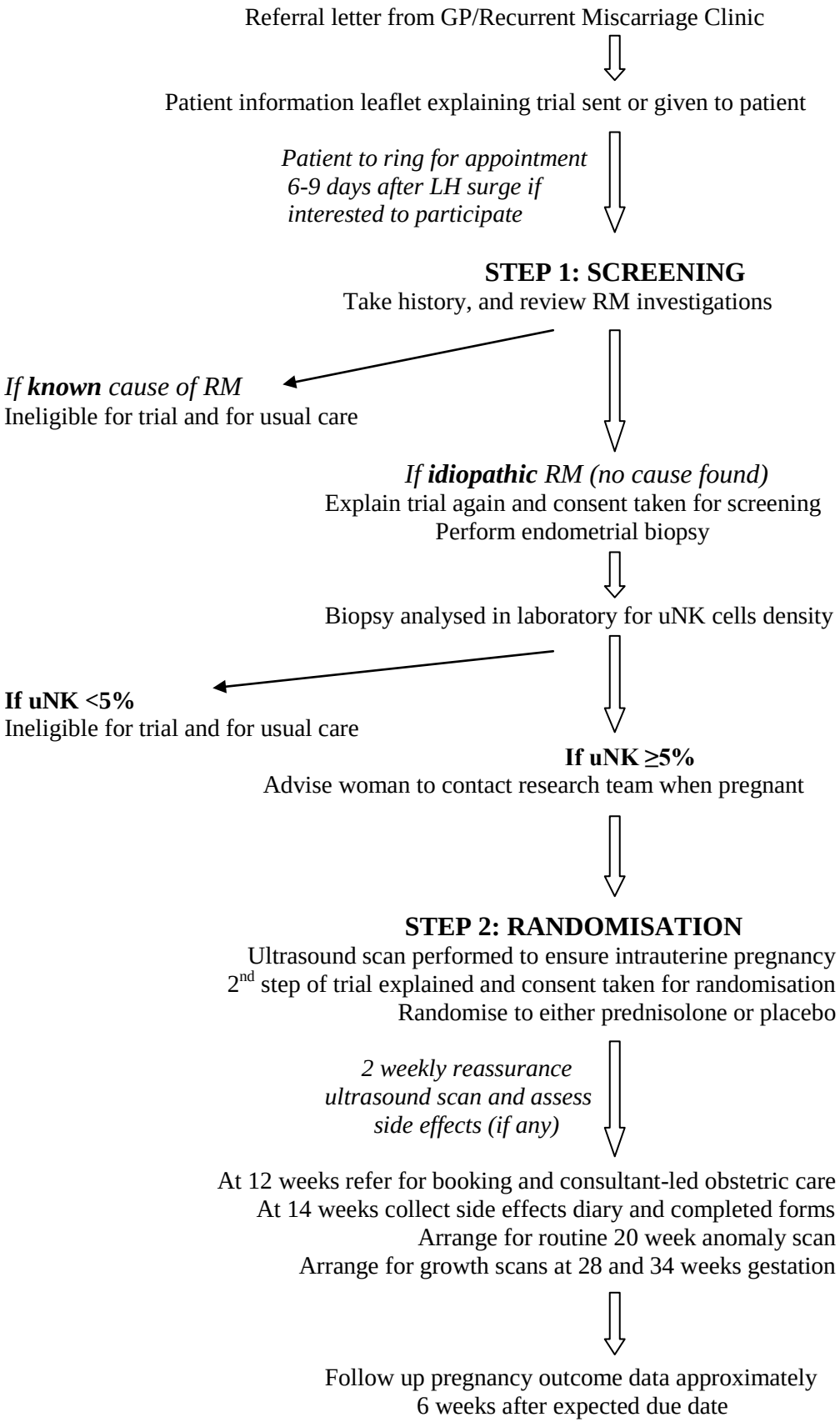
The endometrial biopsy was analysed for uNK cell density in the laboratory through immunohistochemistry and image analysis *(details in section 4.3.5)*. The normal range was defined as <5% uNK cells per stromal cell using the upper end of the interquartile range of control patients from the preliminary study conducted previously. Results of the uNK cell density were communicated to the women by letter and followed up with a telephone consultation. Those with normal uNK cell density were advised to follow standard management, and those with uNK cell counts of ≥5% were advised to contact us as soon as they were pregnant to be considered for randomisation. They were reviewed again when pregnant by myself

or SQ to ensure they fit all the inclusion criteria and to consent for randomisation into the trial.

Once randomised, women were dispensed a bottle of tablets and advised to take four tablets a day for six weeks, then two tablets a day for one week, and one tablet a day in the final one week *(details in section 4.3.3.2 and 4.3.4)*. They were also given a chart to remind them of the number of tablets to take each day and marked off each day as they took the medication *(Appendix 2)*. They completed a diary and reported any unusual symptoms, hospital admissions or side effects that were severe *(Appendix 2)*. Both we and the women were blinded to the treatment allocation.

After randomisation, women were offered an ultrasound scan every 2 weeks for reassurance and clinic consultations to assess side effects of treatment. At 12 weeks gestation, women were referred to the hospital of their choice for booking and consultant-led antenatal care. At 14 weeks gestation, they were reviewed again by us, and all case report forms (CRF) were collected *(Appendix 2).* Completed CRFs were given to an independent research administrator who entered the information on a database and kept them as confidential trial documentation. Reports were generated to the data monitoring committee (DMC) as necessary. A routine anomaly scan at 20 weeks gestation and growth scans at 28 and 34 weeks gestation were then performed in the hospital where the women have booked to deliver. The final follow-up occurred 6 weeks after the delivery of the baby to assess the pregnancy and secondary outcomes through a clinic appointment or telephone consultation.

Figure 4.1 Patient flow during trial



4.3.1.1 Inclusion and exclusion criteria

The inclusion criteria for the trial were:

- 3 or more consecutive miscarriages with no cause found after routine investigations (idiopathic)
- Age less than 40 years old
- ≥5% uNK cells density at 6 to 9 days after urinary LH surge

The exclusion criteria for the trial were:

- A known cause for recurrent miscarriage:
    - Parental chromosomal abnormalities
    - Uterine anomaly (bicornuate or septate uterus from ultrasound or cervical weakness diagnosed at hysteroscopy)
    - Antiphospholipid syndrome (positive anticardiolipin antibody or lupus anticoagulant on 2 separate occasion at least 6 weeks apart)
    - Thrombophilia (FVL heterozygous or homozygous mutation, APCR, protein C or S deficiency, prothrombin G20210A mutation, antithrombin III deficiency)
    - Abnormal thyroid function tests
- Contraindications to steroid therapy such as:
    - Chronic hypertension on regular treatment
    - Diabetes on regular medication
    - Serious mental health problems requiring psychiatric services
    - Obesity with BMI ≥35
- Decline consent to randomisation

4.3.1.2 Feasibility outcome

The main outcome for the pilot phase was feasibility of recruitment, assessed by the willingness of women with RM to be randomised into either placebo or treatment arm of the trial

4.3.1.3 Clinical outcomes

Secondary outcomes for the trial included:

- Number of babies born alive after the age of viability (24^{+0} weeks gestation)
- First trimester (including number of empty gestational sacs or fetal losses) and second trimester miscarriages
- Results of karyotype of miscarried pregnancies
- Stillbirths
- Gestational age at delivery
- Any fetal abnormality
- Intrauterine growth restriction (IUGR) defined as birth weight $<5^{th}$ centile according to customised birth weight charts
- Pregnancy complications such as pre-eclampsia or gestational diabetes
- Side effect of steroids (eg: mood changes, weight gain, increased appetite, indigestion, or hyperglycemia) and patient acceptability of medication defined as compliance to completing the course of tablets

4.3.2 Sample size calculation

Advice on pilot and feasibility studies recommended that more than 30 was a reasonable number[180]. Thus, we continued recruiting women for uNK cells density analysis and stopped after randomising 40 women. This was done for pragmatic reasons as funding allocation was limited and the randomisation list was stratified to

groups of 20. Results of this pilot phase was intended to assess feasibility of recruitment and allow for more accurate power calculation for the definitive trial as preliminary power calculations were based on multiple presumptions.

4.3.3 Patient recruitment into the trial

This trial involved a two-step process where first, women need to fit the inclusion criteria for screening, and then further needed to have high uNK cells density (≥5%), before they can be considered for randomisation when pregnant. Thus, a large group of women need to be recruited for screening as only a small proportion of all women screened will eventually be randomised. Therefore, many methods were used in recruitment for screening.

4.3.3.1 Recruitment for screening

Endometrial Research Clinic

The endometrial research clinic was established by my supervisor and Chief Investigator (CI) of the trial, SQ. It was designed to review women, with either idiopathic RM or idiopathic RIF after ART, for further management. It was also a platform to inform women about relevant research projects and recruit them if they wished to participate. Recruitment for screening into this trial began with women from this clinic a few months before I took up this post. These women were referred from all over UK and Europe following national presentations, media publicity surrounding the publication of the case report about a successful pregnancy with steroid use after 19 consecutive miscarriages[122] and evidence that prednisolone reduces the density of uNK cells in the endometrium[110].

After the commencement of the trial, the endometrial research clinic was expanded to a trial recruitment clinic, and operated three times a week on a Monday, Wednesday and Friday to review anybody interested in the trial. This ensured that an appointment was always available during the mid-luteal phase (*day 6-9 after LH surge from urinary testing*) of the menstrual cycle for any women who wished to have the endometrial biopsy taken and be screened for the trial.

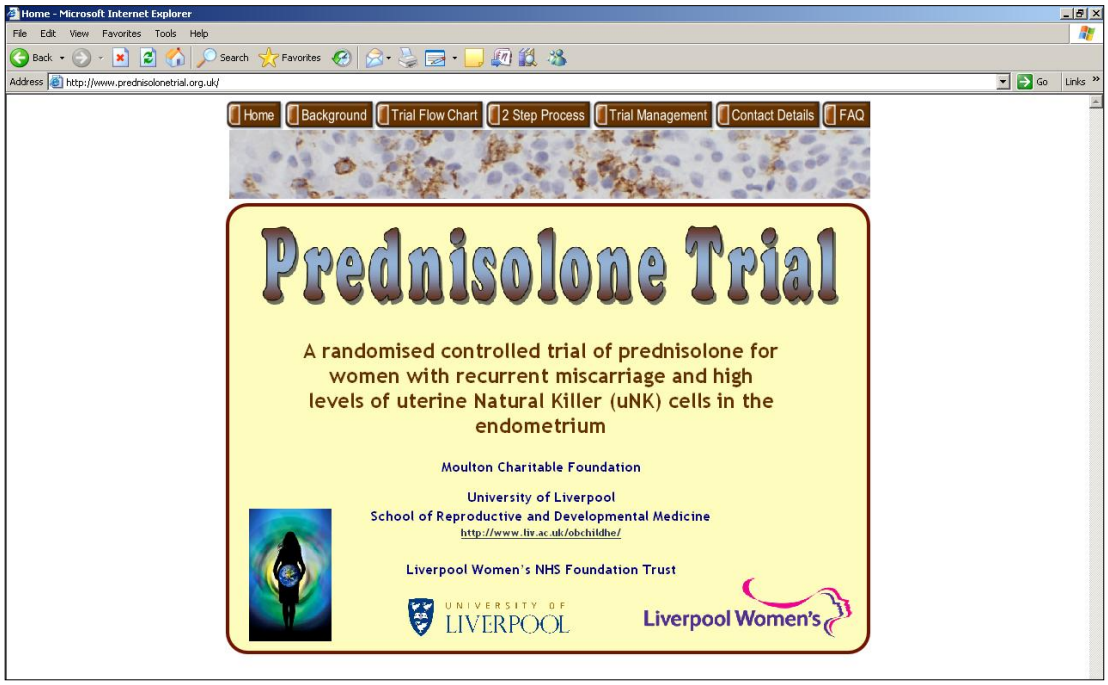
Liverpool Women Hospital's (LWH) Recurrent Miscarriage Clinic

Women were also recruited from the LWH's Recurrent Miscarriage Clinic (RMC), the tertiary referral centre for the region. The referral letter and hospital notes of every woman attending the clinic were examined to identify suitable women for trial screening. Approval was obtained from consultants conducting the clinic to provide information to these women after clinic consultations.

Trial Website – www.prednisolonetrial.org.uk

I designed and launched an internet webpage to further improve patient recruitment and to provide regular updates and information on the trial (*Figure 4.2*). The availability of a trial website allowed women to have constant access to information relating to the trial and links to trial support groups from relevant websites such as the Miscarriage Association (MA). Information available on the website included the rationale for the trial, process for recruitment, and contact details for women who were interested to participate. The website was maintained until the pilot phase of the trial was completed.

Figure 4.2 Homepage of the trial website



The Miscarriage Association (MA) website and forum

The President of the MA of UK acted as the trial patient representative. The Association showed their support by advertising the trial regularly on their website and newsletter. There was a link from the MA website to the Prednisolone Trial website for women to get more information and contact details if they were interested to participate. They also agreed to be independent advisors to women during the recruitment phase and throughout the trial. There was a forum associated to RM (not accessible to myself or SQ) to provide support for women who had been recruited and those who were thinking of participating in the trial. The forum also allowed for women to discuss the trial confidentially among themselves, with support from the MA.

Early Pregnancy Units (EPU)

The EPU was where many miscarriages are first diagnosed, and a cohort of these women may have RM. In some cases, women request an alternative plan of 'what next'. Thus if suitable, information leaflets about the trial were given to these women. They were not expected to make any decisions, but were welcomed to make further enquiries through the contact details on the information leaflet. Therefore, appointments were made to meet up with the person-in-charge of the EPU in all the surrounding hospitals in the Northwest to explain about the trial and to leave trial leaflets with them. The EPU in the following hospitals were visited: Warrington Hospital, Macclesfield Hospital, Leigh Infirmary, Salford Hope Hospital, North Manchester General Hospital, Royal Oldham Hospital, Royal Bolton Hospital, Leighton Hospital, Whiston Hospital, Tameside Hospital, Ormskirk Hospital, Chester Hospital, Arrowe Park Hospital, and St Mary's Manchester.

A Northwest Early Pregnancy Regional Meeting was held annually. This was organised for the 13th of July 2009 and was taken as an opportunity for an educational update on NK cells, a chance to inform all the attendees about the existence of this RCT, answer any queries relating to it, and to encourage recruitment.

Media Publicity

Although there were no intentions for it, there was massive media hype after the publication of the case report and journal article about the effect of prednisolone in reducing uNK cells. This was followed by a surge of telephone calls of interest from women with idiopathic RM and demonstrated the power of media services. It was inevitable that media publicity became one of the means for informing the public about the existence of this RCT.

SQ presented about the potential use of steroid in idiopathic RM and the existence of this on-going RCT in the British Association (BA) Festival of Science Conference in September 2008, which was reported as a news article in The Times newspaper. This was followed by an increase in recruitment for screening, and October 2008 saw the highest number of women screened per month in the entire recruitment period of the pilot phase of this trial. Similarly a few months later, recruitment for screening increased after an appearance of SQ and a trial participant on Granada News on ITV, highlighting the importance for more studies into treatment for idiopathic RM.

4.3.3.2 Recruitment for randomisation

Women meeting the inclusion criteria for randomisation had a transvaginal ultrasound performed in the trial clinic as soon as possible after a positive pregnancy test. If a viable intrauterine pregnancy between 4-8 weeks gestation was found, then a second consent was obtained for randomisation to either 20mg of prednisolone or placebo. 20mg was chosen as this was the dose that resulted in a live birth for a previous patient[122], and the dose that demonstrated a reduction in uNK cells density in a previous study[110]. They were then dispensed the tablets accordingly from pharmacy and reviewed in the clinic every 2 weeks for reassurance scans. Tablets were taken in the first trimester for 8 weeks after a confirmed pregnancy, as the aim was to improve the implantation process which was completed after the first trimester. If the initial ultrasound scan was inconclusive, women had serial serum β-hCG to exclude an ectopic pregnancy, and were recruited for randomisation only after β-hCG results has confirmed an ongoing intrauterine pregnancy. Prednisolone

was tapered off at the end of the treatment regime to allow for the adrenal glands to readjust and prevent steroid withdrawal symptoms.

4.3.4 Randomisation and Blinding

4.3.4.1 Sequence generation

Once consent was obtained, women were assigned consecutive study numbers. A dedicated pharmacist for the trial dispensed the tablets, either the active treatment (prednisolone) or placebo to the women according to her study number. This study number was paired to a pre-constructed randomisation list by a computerised random number generator in blocks of 20. Stratified sampling was not considered in the randomisation process as the population studied were already a selected group (uNK cells ≥5%) of women with RM, and sample size was small. Stratification would create difficulties in interpreting results as there would be smaller numbers in each group, and possibly make recruitment even more difficult. The randomisation list was only available to the dedicated pharmacist for the trial.

4.3.4.2 Blinding

This was designed as a double blinded RCT where both we and women did not know whether they were on the active or placebo tablets. Both prednisolone and placebo tablets were similar in size, colour and dispensed in identical packaging. The active tablets consisted of 5 mgs of prednisolone (manufactured by Wockhardt UK Ltd), and the placebo (manufactured by Quay Pharmaceuticals) was an inert substance specially made to be identical to the placebo. Each bottle given to the women were identical and contained 189 tablets of either 5mg prednisolone or placebo.

4.3.4.3 Mechanism of unblinding

All women randomised in the trial kept a trial ID card where contact details of the hospital were available, to get in touch with either the CI or principal investigator (PI) in the event of an emergency where the trial group allocation needed to be identified. The CI or PI could then authorise the pharmacy department to unblind the group in severe illness where the attending physician needed to know whether the women had been allocated steroids. The DMC was also able to unblind these groups if necessary.

4.3.5 Laboratory methods for processing and analysing endometrial biopsies

The laboratory methods for processing and analysing of uNK cells density were already established from previous studies investigating the function of uNK cells[107]. The normal range of uNK cells for our laboratory had also been determined to be <5% in a previous study[110]. Currently, there is no universally accepted method for investigating uNK cells although ideally, laboratory methods should be standardised. While I was not involved in the development of the method used to analyse uNK cells density, I performed nearly all the laboratory analysis on the endometrial samples for the trial, with the assistance of 2 other laboratory personnel.

4.3.5.1 Tissue preparation

The endometrial biopsy taken from the women was immediately transferred to the laboratory to be fixed in formalin. The specimen was then dehydrated, cleared and impregnated with paraffin wax using the automated Shandon Citadel 1000 processing machine (manufactured by Thermo Electron Corporation, Runcorn, UK). The total processing time was 18¾ hours, and therefore was always run overnight. Processing involved a rotation through these solutions for the duration:

- 4% formalin in neutral buffer (45 minutes), 60% Ethanol (1 hour), 70% Ethanol (1 hour), 90% Ethanol (1 hour), 100% Ethanol (1 hour), 100%

Ethanol (1 ½ hours), 100% Ethanol (2 hours), Xylene 1 (1 hour), Xylene 2 (1 ½ hours), Xylene 3 (2 hours), Wax 1 (2 ½ hours), Wax 2 (3 ½ hours)

The cassette with the endometrial biopsy was then filled with wax, cooled and stored as paraffin blocks in the freezer.

3 µm sections from the paraffin blocks were cut, mounted on APES coated glass slides and air-dried overnight. Each slide consisted of 2 endometrial samples from the same women. The sample on top was for incubating with the antibody for analysis, and the sample at the bottom served as a negative control. They were then baked overnight at 37^{o}C in racks. The next day, samples were then dewaxed and rehydrated through xylene followed by alcohol in the following manner:

- Xylene (10 minutes)
- Xylene (10 minutes)
- 100% alcohol (5 minutes)
- 100% alcohol (5 minutes)
- 90% alcohol (1 minute)
- 70% alcohol (1 minute)

The slides in racks were then submerged in water prior to antigen retrieval.

4.3.5.2 Antigen retrieval

Heat induced epitope retrieval (HIER) was carried out by boiling the slides in citric acid buffer to break any protein cross-links, and unmasking the antigens and epitopes in the tissue sections. The buffer was prepared fresh with 3.15g of citric acid in 1500mls of distilled water. The pH was adjusted to 6 by adding in 2M Sodium Hydroxide (NaOH).

The citric acid buffer was then boiled in a pressure cooker. Once the buffer was bubbling, the slides were inserted and pressure cooked for 1 minute. After that, they were immediately cooled under running tap water.

4.3.5.3 Immunohistochemical staining with CD56 antibody

The slides were then transferred to a staining dish and washed in TBS (Tris buffered saline) solution prepared either on the day of immunohistochemical staining or the day before, for 5 minutes. TBS was made by mixing 12g Trizma base and 17.4g Sodium Chloride (NaCl) in 2000mls of distilled water. The pH was adjusted to 7.6 by adding in Hydrochloric Acid (HCl).

Endogenous peroxidase activity was quenched by incubating the slides in 0.3% peroxidase block (30% H_20_2 diluted to 1:100 with TBS) for 10 minutes. Thereafter, the slides were washed twice (5 minutes x2) in TBS solution. The endometrial specimens on the slides were then circled using a DAKO hydrophobic marker to define the staining area containing the antibody. All slides were then washed in TBS for another 5 minutes. Then, slides were removed from the staining dish and placed in a humidified chamber.

All the antibodies used for incubation were diluted using 0.5% BSA solution (10% BSA diluted to 1:20 with TBS). The primary antibody used to detect uNK cells was CD56 (diluted to 1:50, NCL-L-CD56-1B6, Novocastra, Milton Keynes, UK). IgG mouse antibody (diluted to 1:100, MCA928, Serotech, Kidlington, UK) was used as the negative control. A positive control slide from a patient known to have high uNK cells, and an IgG mouse negative control was used for every slide undergoing immunohistochemistry to ensure no false negative or false positive staining. 50μl of CD56 antibody was pipetted onto each section on the top, and 50μl of IgG mouse antibody was pipette onto each section at the bottom (negative control), and incubated in the humidified chamber for 60 minutes at room temperature.

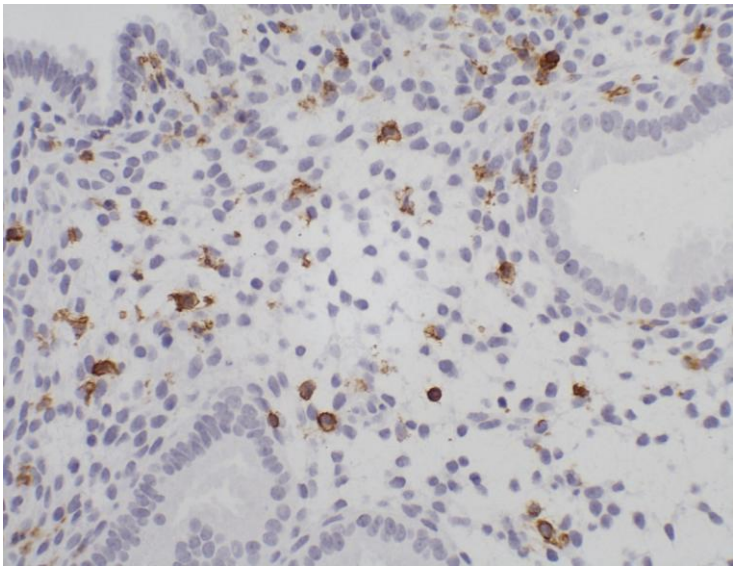
After 60 minutes, the slides were washed twice (5 minutes x2) in TBS solution. Next, a drop of HRP-labelled polymer (supplied with EnVision System reagent kit) which was conjugated with secondary antibodies was applied onto the sections and incubated for 30 minutes in the humidified chamber at room temperature. The slides were then washed twice (5 minutes x2) again in TBS to remove excess polymer. Staining for CD56 (uNK cells) was completed with incubation of sections in 3'3-Diaminobenzidine (DAB) chromagen solution for 10 minutes at room temperature to give a brown coloured reaction. After 10 minutes,

the slides were then immediately immersed in tap water to stop further reaction. The nuclei were then counterstained with Gill 2 haematoxylin for 90 seconds, and rinsed in tap water until clear. The slides were then dipped in acid alcohol and rinsed again under running water until cells were blue. The intensity of haematoxylin was checked, and the slides were re-counterstained if staining was found to be too faint. The sections were then dehydrated through alcohol and xylene in the following:

- 70% alcohol (1 minute)
- 90% alcohol (1 minute)
- 100% alcohol (3 minutes)
- 100% alcohol (3 minutes)
- Xylene (5 minutes)
- Xylene (10 minutes)

Finally, the slides were mounted with Consul mount (Thermo Fisher Scientific). The positive control slide was checked under a wet microscope for DAB colour development *(Figure 4.3).* The slides were then left to dry overnight in a fume hood. They were all then checked for adequate staining intensity the next day. If DAB staining was too faint, the endometrial sample was prepared again for repeat staining.

Figure 4.3 DAB staining of CD56 positive uNK cells under the microscope



4.3.6 Image analysis for determination of uNK cell density

The method for image analysis for uNK cells density had also already been developed for previous studies on uNK cells. However, I was involved in the validation of digital image analysis for uNK cells density. Digital image analysis was shown to markedly reduce the inter-observer error as compared with manual counting[181]. Thus, a combination of manual and digital counting was used to determine uNK cells density for all the samples analysed in the trial. Digital counting was performed by Image J, an image analysis software developed by National Institutes of Health (NIH) which is freely available for public use (http://www.rsbweb.nih.gov/ij/download.html)[182].

UNK cells density was established by averaging the results of 10 images of endometrial stained sections for each woman. Each result was the manual calculation of CD56 positive stained cells (uNK cells) in proportion to digital calculation of total stromal cells ($CD56^+$ and $CD56^-$ cells) using the formula:

$$\frac{\text{Number of uNK cells}}{\text{Total number of stromal cells}} \times 100\%$$

4.3.6.1 Image capture and editing

10 high-powered fields (HPF) (x40 magnification) images were captured from different areas along the epithelial edge of the stained endometrial sections for analysis. Images were captured using a Nikon DS-Fi1 digital camera Head 5M pixel, and Nikon Eclipse 50-i Microscope with the software NIS-Elements-F, developed for Nikon instruments. All images were captured on the following settings:

Mode – Normal

Resolution – 640 x 480 (fast focus)

1280 x 960 (quality capture)

AE lock on

Exposure – Mode – Auto exposure

AE comp +1.0 EV - +1.6 EV

Colour – Contrast – High

Sharpness – High

These images were then edited with Adobe Photoshop CS2 or CS3 (Adobe, San Jose, USA). First, the glandular epithelial cells and blood vessels were removed electronically by encircling these structures with the 'Magic Lasso' tool and deleted. Next, the brown DAB and non-specific background staining were removed by selecting them using the 'Eyedropper' or 'Magic Wand' tool. The threshold for stain intensity and area to be removed were adjusted according to the area and darkness of DAB stain in each image, and deleted. These steps were done to ensure that the edited image consisted only of nuclei of stromal cells, which were used as the denominator for assessing uNK cells density (*Figure 4.4*).

4.3.6.2 Counting of uNK and stromal cells

Uterine NK cells were manually counted by totalling the number of blue-stained nuclei surrounded by cytoplasmic brown-stained DAB to the CD56 antibody. Positively identified uNK cells were digitally marked with a cross using the 'point picker' function on Image J, and summed at the end *(Figure 4.5).* This was more reproducible and accurate as compared with the conventional method of counting across the microscope[181].

Figure 4.4 Edited image after removing, glands, blood vessels and DAB

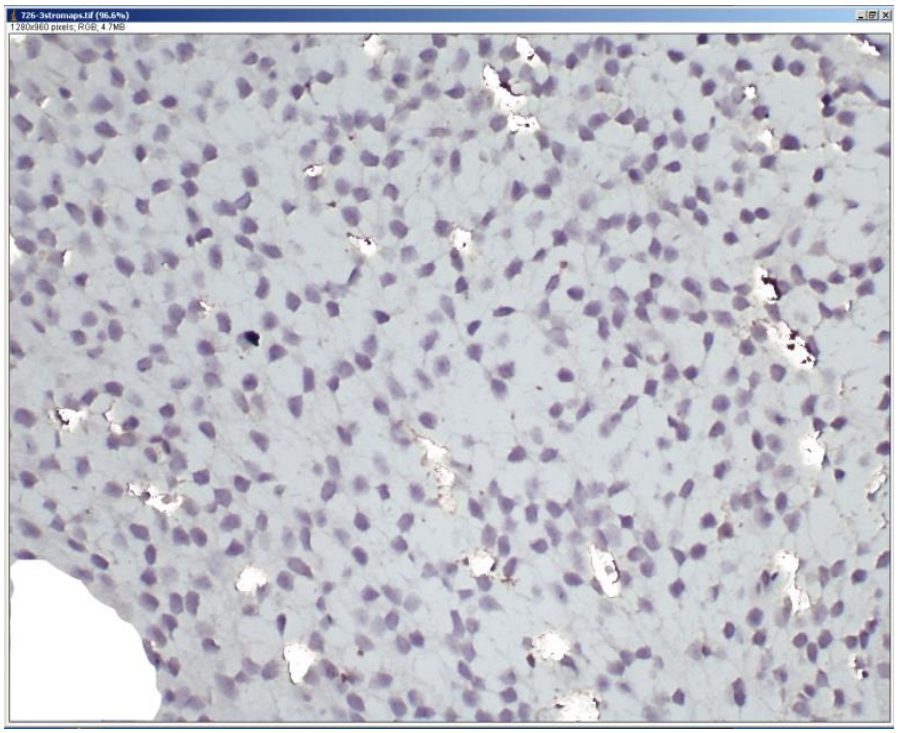
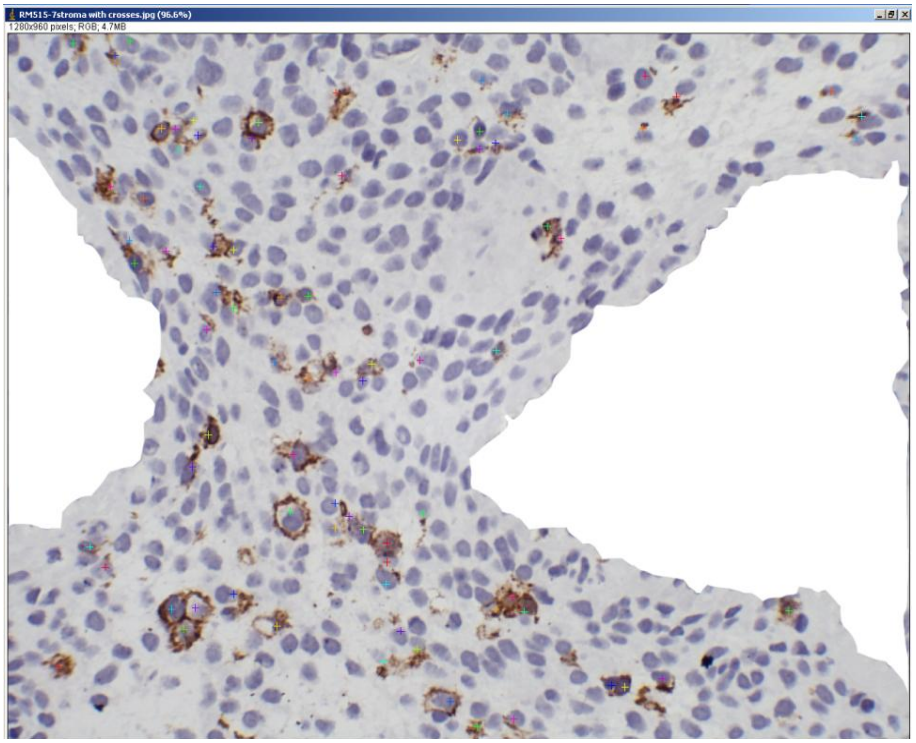


Figure 4.5 UNK cells marked with a ‘point picker’



The counting of total stromal cells was performed using the 'Analyze Particles' tool in Image J on the edited image of only blue nuclei. The plugin was set at the following parameters for each nucleus to be digitally counted *(Figure 4.6)*:

Particle size: 36-2250

Circularity: 0.3-1

This edited image was then merged with the corresponding original image for visually checking that outlines of analysed particles matched with cell nuclei, clusters of cells were split properly, DAB and background staining were not calculated as cells and large cells were not split into multiple cells *(Figure 4.7*).

The total stromal cells count was then recorded, and used as the denominator for the total uNK cells count obtained manually and applied to the formula above. The above steps were repeated 10 times for each woman and averaged, to obtain the uNK cells density for that woman.

Intermittently, the images were double counted by another observer to ensure that the uNK results were consistent, especially when there was borderline result. For each observer, Bland-Altman plots were produced to ensure that there were no systematic differences in uNK or stromal cell counts between observers.

Figure 4.6 Edited image of outlines of nuclei digitally counted

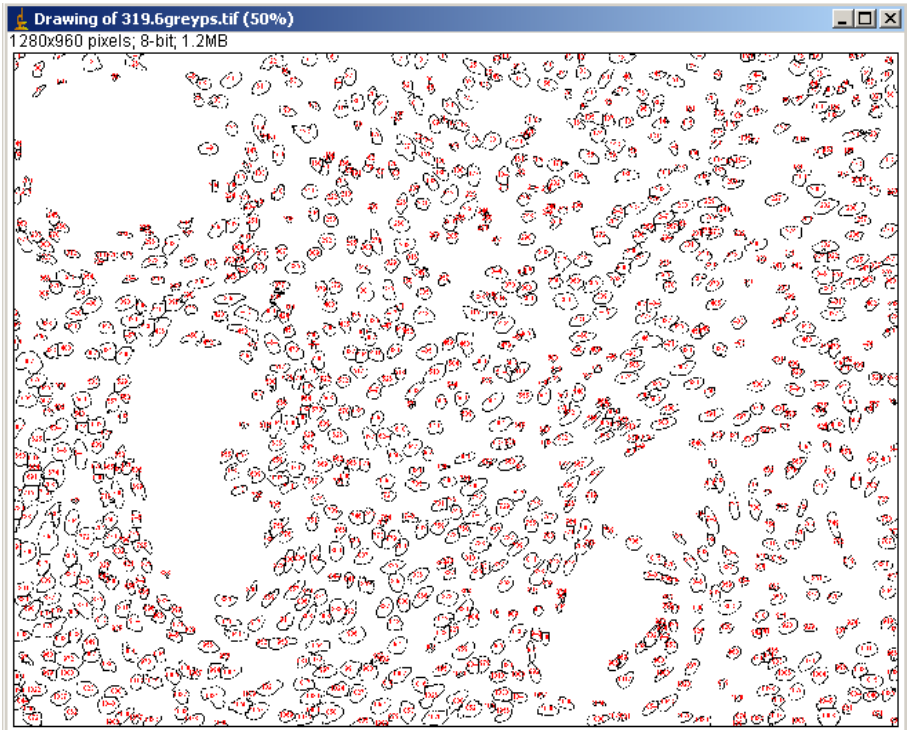
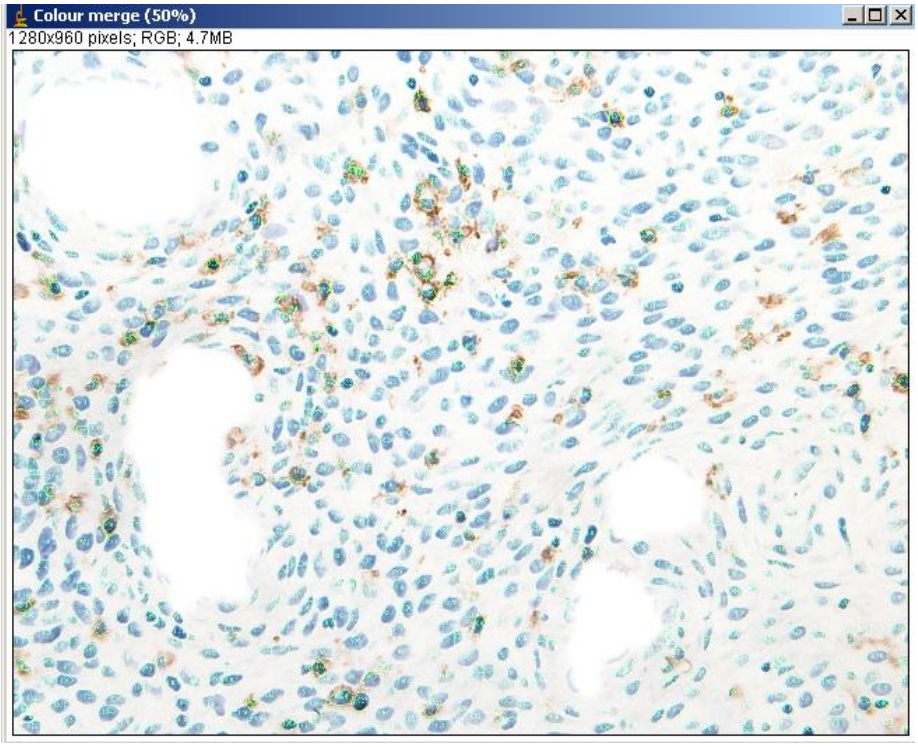


Figure 4.7 Merged image of nuclei outlines and original image



4.3.7 Trial management and monitoring structure

4.3.7.1 Ethical and Regulatory Issues

Approval to conduct the trial was granted from the Liverpool Local Research Ethics Committee and MHRA. The trial was included in the National Institute for Health Research (NIHR) Clinical Research Portfolio (NIHR CRN ID: 6567) and was registered with the European and international clinical trials database (EUDRACT No: 2005-003307-36, ISRCTN28090716).

Apart from the monitoring of pregnancy with ultrasound and side effects of medication as described above, any events reported were assessed in terms of their seriousness, causality and expectedness. A serious adverse event or adverse event (SAE/AE) report form was completed by the CI or PI and submitted to the relevant committees if the side effect was deemed serious *(Appendix 2)*. If it was a suspected unexpected serious adverse reaction (SUSAR), then reporting was expedited accordingly

4.3.7.2 Trial management bodies

A data monitoring committee (DMC) independent of the trial investigators was appointed for the trial to provide independent review of unblinded data at agreed intervals to ensure that no harm was being caused by the treatment. Particular emphasis was placed upon monitoring the side effects of steroids, fertility rate and pregnancy complications. They also addressed issues such as:

- Significant problems with trial design or methodology
- Recruitment rate
- Patient's acceptance of the possibility of randomisation to placebo

The first DMC meeting occurred after 36 women were randomised into the trial in May 2010. The chair of the DMC then reported to the Trial Steering Committee (TSC) approximately 2 weeks after the meeting in accordance with recommended trial oversight.

A TSC and trial management group (TMG) supervised and managed the trial and was responsible for approving the core protocol and any subsequent amendments. The TMG met every 4-6 months to discuss:

- recruitment rate and patient acceptability of the trial
- side effect of steroids
- case report forms (CRF)
- problems arising from the trial

The TMG reported the above and presented possible solutions to problems or strategies to improve recruitment, to the TSC who met when deemed necessary by the CI. The accuracy of CRFs to the case notes were intermittently checked by the sponsors of the trial at random. There was also double entry data management to minimise human error.

4.3.8 Data Analysis

Data was cleaned prior to any analysis. As this was the feasibility phase of the trial, the proportion of women who screened positive with high uNK cells density and returned for randomisation into the trial was recorded. Their willingness for randomisation was assessed by the questionnaire and proportion randomised. Demographic information for all women screened was tabulated. Similarly, summary statistics for information relating to the allocation groups of women who were randomised were tabulated and examined to assess whether both treatment and placebo groups were similar.

Clinical outcomes of live birth rate in each group were expressed as a risk ratio with 95% confidence intervals. Other outcomes such as the rates of miscarriage, type of miscarriage (biochemical, sac or fetal loss), karyotype of miscarried pregnancies, gestational age at delivery, mode of delivery and pregnancy complications were tabulated. Compliance to medication and side effects of steroids were evaluated. All adverse events were also noted according to allocation group.

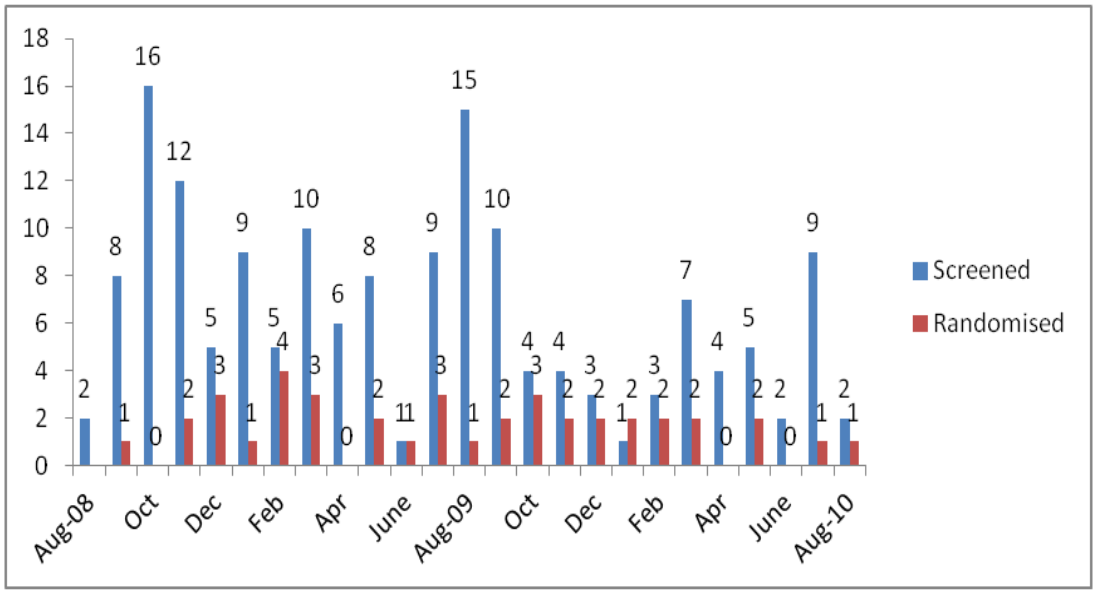
4.4 Results

4.4.1 Feasibility of patient recruitment

In the two year period, a total of 160 women were screened for the trial and 72 (45%) women had ≥5% uNK cells and were eligible for randomisation. It was not possible to assess the true recruitment rate as information of the trial were widely disseminated and it is unknown how many women were approached. The majority of women who attended for screening were informed of the trial through the consultant who was in charge of their care for RM, and thus referred to the endometrial research clinic. It was also clear that publicity of the trial through public events impacted on the recruitment for screening. October 2008 saw the highest number of women screened per month after publication of a news article in The Times which reported on the presentation by SQ about the potential use of steroid in idiopathic RM and the existence of the on-going RCT, in the British Association (BA) Festival of Science Conference in September 2008 *(Figure 4.8)*.

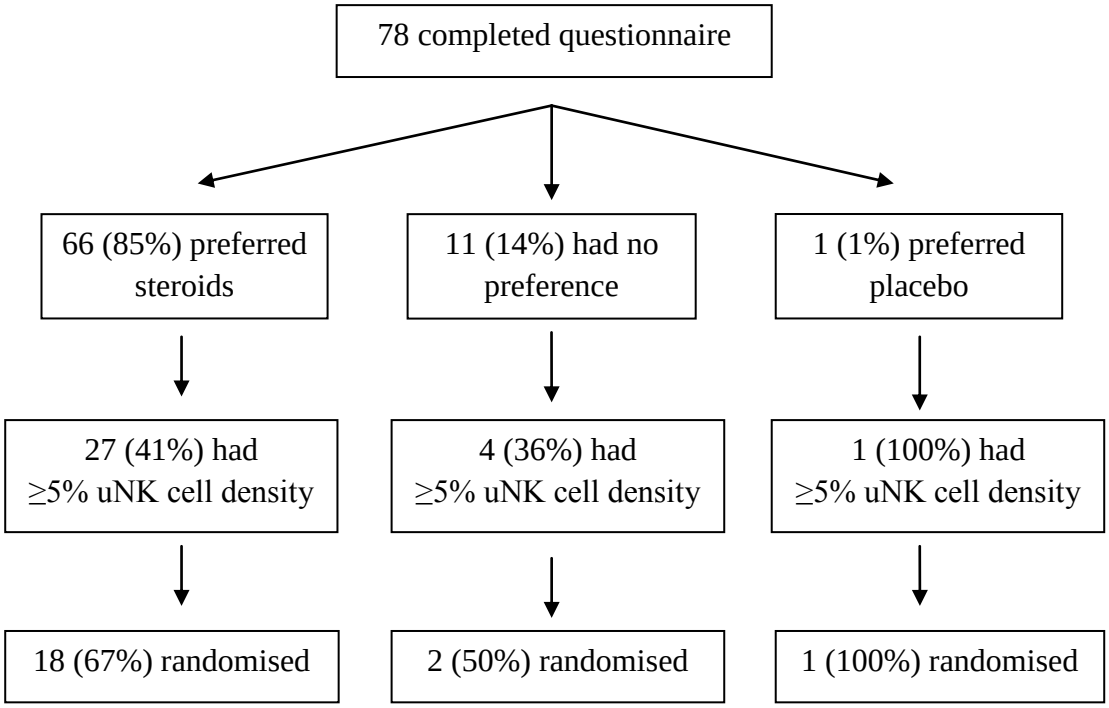
About half (78/160, 49%) of women screened were given and completed a questionnaire to ask if they think they had the pathology, and if given a choice whether they wanted the active medication, placebo or had no preference. 53 (68%) women thought they had an endometrial immune cause for the RM, and thus attended for assessment. There was little relationship between the patient's perception of their result and their actual result: 23 of 53 (43%) who thought they had high uNK cells density did so and 7 of 17 (41%) who thought they had normal uNK cells density did so.

Figure 4.8 Number of women screened and randomised each month



Overall, 85% (66/78) of women wanted prednisolone treatment if they were given an option (*Figure 4.9*). This finding was not unexpected as these women commented on the questionnaire that their desire for a live birth far outweighed side effects and potential risk from any empirical treatment. Of those wanting active medication, 27 (41%) were found to have high uNK cells and 18 (67%) were randomised. 11 (14%) women had no preference for either group and 4 (36%) were found to have high uNK cells. Of these, 2 (50%) were randomised. Only one woman preferred to have placebo, due to fear of side effects from prednisolone. She was found to have high uNK cells density, and returned for randomisation when pregnant. Thus, the response to randomisation was good (66% overall of women eligible for the trial), despite the wish for active treatment in the majority of women screened.

Figure 4.9 Women's perspective of the trial



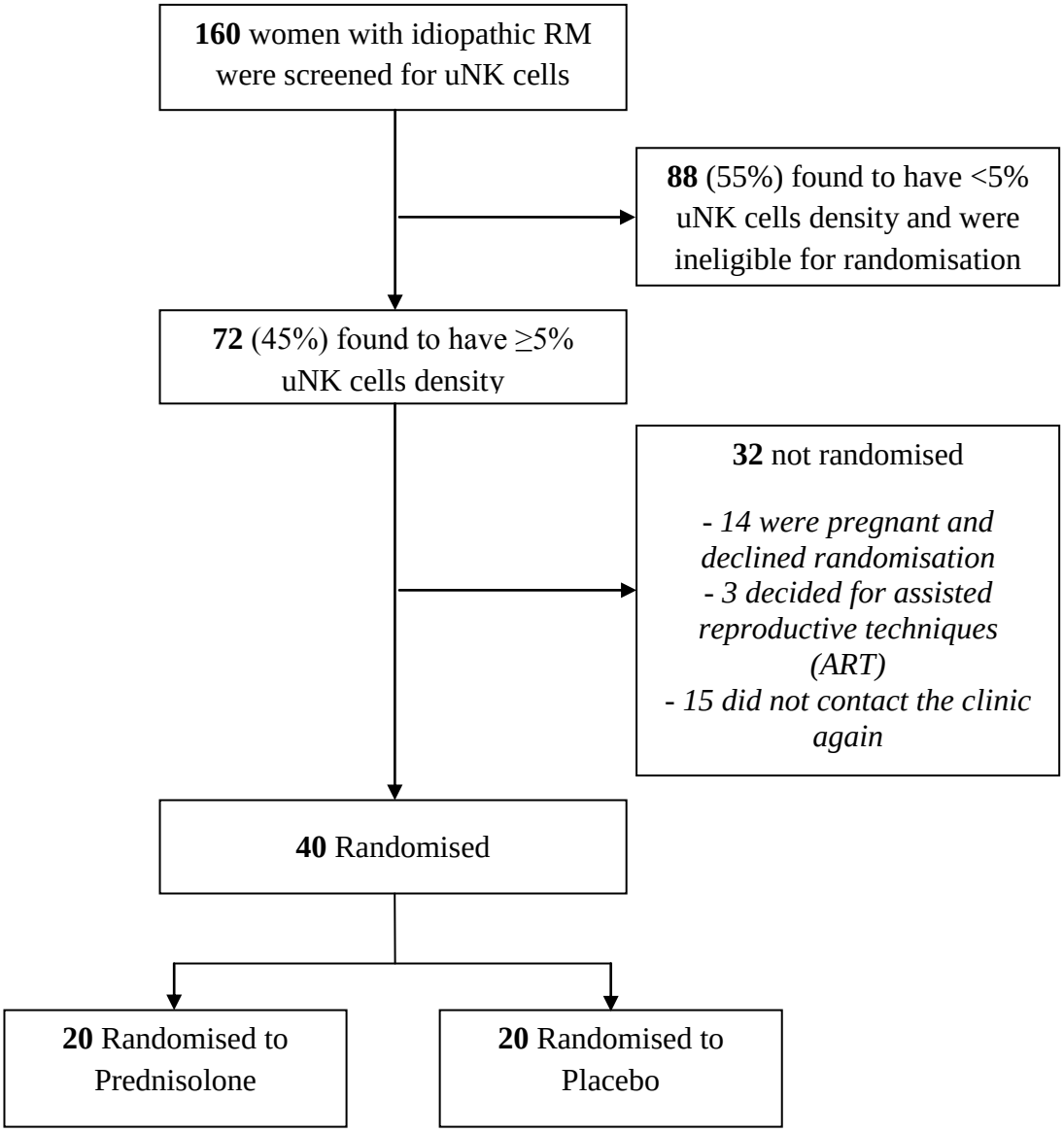
4.4.1.1 Study population of women screened for the trial

160 eligible women were screened for uNK cells density *(Table 4.1)*. Of these, 72 (45%) were found to have ≥5% uNK cells, and were suitable to be randomised. Eighty-eight women were informed of the normal results and discharged back to the referring clinician for standard care. Of the 32 women with ≥5% uNK cells and not randomised, 14 conceived but declined randomisation and 3 decided to seek assisted reproduction. 15 women did not contact the clinic again pregnant before the trial was stopped (*Figure 4.10*). The demographic characteristics of women were similar in those screen positive (≥5% uNK) and those screen negative. It was interesting to note that there were no difference in the miscarriage rate in women with normal or high levels of uNK cells *(Table 4.1).*

Table 4.1 Baseline characteristics of women screened for the trial (N=160)

Characteristics	**<5% (N = 88)**	**≥5% (N = 72)**
Age in years (Mean (SD))	34 (3.8)	34 (4.5)
% UNK (Median (Range))	2.8 (0.4-4.7)	7.1 (5-23)
Previous live birth (Number of women (%))	26 (30%)	15 (21%)
Number of previous miscarriages (Median (range))	4 (3-14)	4 (3-15)
Number of previous fetal loses (Median (range))	1 (0-5)	1 (0-5)
Number of previous losses of empty sac (Median (range))	3 (0-13)	3 (0-12)
Previous 2nd trimester miscarriage (Number of women (%))	2 (2.3%)	5 (6.9%)
Previous cctopic pregnancy (Number of women (%))	7 (8%)	6 (8.3%)

Figure 4.10 Trial flow chart



4.4.1.2 Study population of women randomised into the trial

The baseline characteristics of all women randomised were similar in both groups (*Table 4.2*). The mean age at randomisation was 33 years, with a mean BMI of 26. The mean uNK cells density for all randomised women was 7.7%. The characteristics of the pregnancy at randomisation were also similar.

4.4.2 Clinical outcomes

The live birth rate was 60% in the prednisolone group and 40% in the placebo group, but this did not reach statistical significance (RR 1.5, 95% CI 0.79-2.86) (*Table 4.3*). The gestational age at delivery was similar between both groups (272 days (prednisolone) and 279 days (placebo)). Similarly, the mean birth weight was comparable (3522g vs 3547g), with no pregnancy complications of IUGR (<5th centile) or macrosomia. There were also no reports of gestational hypertension, pre-eclampsia or gestational diabetes in either group. There were no adverse fetal outcomes. One baby in the prednisolone group had post-natally detected mild hearing problems and one baby in the placebo group had ante-natally detected renal tract abnormalities. Both the admissions to neonatal unit (NNU) were for observation. The baby from the prednisolone group had mild jaundice and hypoglycaemia and the baby in the placebo group was treated with antibiotics due to the renal tract abnormality. Both babies were discharged home with the mother.

There were 8 (40%) miscarriages in the prednisolone group and 12 (60%) miscarriages in the placebo group (*Table 4.3*). They were all first trimester miscarriages, with similar number of biochemical, sac and fetal losses in both groups. Karyotypes of 6 miscarriages were obtained, 3 in each group, and 67% were normal. Products of conception (POC) were not readily available for the other miscarriages as most women chose to have either conservative or medical management of the miscarriage.

Table 4.2 Baseline characteristics of women randomised (N=40)

Patients randomised	**Prednisolone (N=20)**	**Placebo (N=20)**	**Total (N=40)**
Age in years (Mean (SD))	34 (4.6)	33 (3.6)	33 (4.1)
% UNK cells (Mean (SD))	8.3 (4.2)	7.2 (3.5)	7.7 (3.8)
BMI (kg/m^2) (Mean (SD))	26.1 (4.3)	25.6 (3.4)	25.8 (3.8)
Previous live birth (Count (%))	4 (20)	3 (15)	7 (17.5)
Number of previous miscarriages (Median (Range))	4 (3-8)	4 (3-15)	4 (3-15)
Fetal Losses (Count (%))	20	20	40
0	10 (50)	5 (25)	15 (37.5)
1	4 (20)	8 (40)	12 (30)
2	2 (10)	4 (20)	6 (15)
> 2	4 (20)	3 (15)	7 (17.5)
Biochemical/Sac Losses (Count (%))	20	20	40
0	0	0	0
1	2 (10)	2 (10)	4 (10)
2	5 (25)	5 (25)	10 (25)
>2	13 (65)	13 (65)	26 (65)
2^{nd} T Misc (Count (%))	0	2 (10)	2 (5)
Previous Ectopics (Count (%))	1 (5)	2 (10)	3 (7.5)
Current pregnancy in trial	20	20	40
Folic Acid intake	20	19	39
-400 mcg	15	17	32
-5 mg	5	2	7
Aspirin intake	4	5	9
Sac present at randomisation	15	17	32
FH present at randomisation	3	1	4

Table 4.3 Clinical outcomes of women randomised into the trial

	Prednisolone (N = 20)	**Placebo (N = 20)**	**Relative Risk (95% CI)**
Live Birth (%)	**12 (60)**	**8 (40)**	**1.5 (0.79-2.86)**
Delivery at <37 weeks (%)	1 (8.3)	0	3.00 (0.13-69.52)
Vaginal Delivery (%)	3 (25)	4 (50)	0.75 (0.19-2.93)
Caesarean Section Delivery (%)	9 (75)	4 (50)	2.25 (0.83-6.13)
- Elective CS	2	1	
- Emergency CS	7	3	
Birth Weight (g) (mean)	3522	3547	-
Admission to Neonatal Unit (%)	1 (8.3)	1 (8.3)	1.00 (0.07-14.90)
Miscarriages (%)	**8 (40)**	**12 (60)**	**0.67 (0.35-1.27)**
- Biochemical Loss	2	1	
- Sac Loss	2	3	
- Fetal Losses	4	6	
Normal karyotype	2	2	
T22	1	1	
- Pregnancy of unknown location	0	1	
- Ectopic (tx with methotrexate)	0	1	

4.4.3 Acceptability of trial medication

Side effects were more commonly reported in women in the prednisolone group compared with the placebo group (*Table 4.4*). However, none were severe enough for women to stop taking medication and all completed treatment. Women were advised to discontinue treatment when a miscarriage was confirmed. Despite the fact that women were advised to take the tablet early in the morning, the most common side effect was insomnia (RR 7, 95% CI 0.9-51.0). There were no reports of bruising or infection. In the diaries that were kept, 8 women in the prednisolone group reported additional side effects. 2 women reported increased appetite, 1 headache and hallucinations, 1 reported palpitations, 1 reported hirsutism, 1 had worsening of her irritable bowel syndrome, and 2 complained of nausea. In the placebo group, 4 complained of additional side effects (2 headache and 2 bloating).

Table 4.4 Side effects profile of women randomised in the trial

Side effects (Count (%))	**Prednisolone (N=20)**	**Placebo (N=20)**	**Relative Risk (95% CI)**
Acne	5 (25%)	2 (10%)	2.5 (0.5-11.4)
Bruising	0	0	-
Flushing	4 (20%)	3 (15%)	1.3 (0.3-5.2)
GI problems (Reflux)	5 (25%)	3 (15%)	1.6 (0.4-6)
Infections	0	0	-
Insomnia	7 (35%)	1 (5%)	7.0 (0.9-51.0)
Joint pain	0	2 (10%)	0.2 (0.01-3.9)
Mood changes	3 (15%)	2 (10%)	1.3 (0.3-8)
Others	8 (40%)	4 (20%)	2.0 (0.7-5.9)

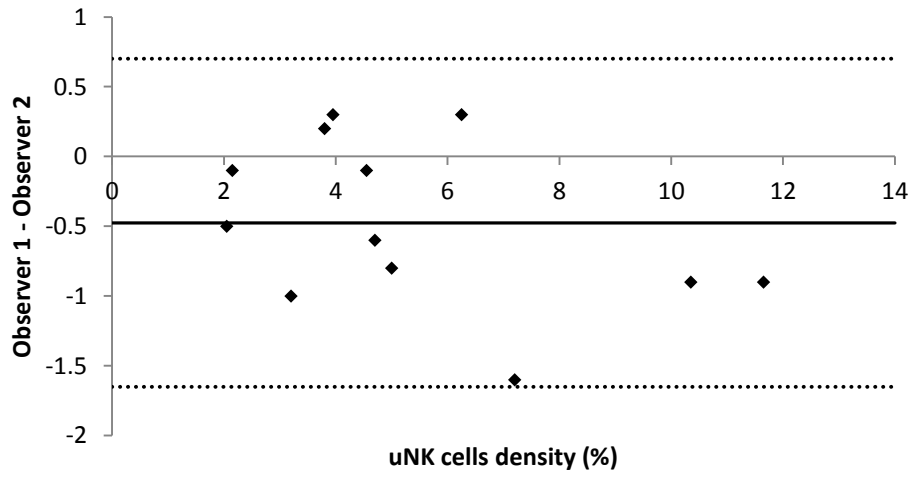
4.4.4 Adverse events

There were no unexpected events that led to the need for unblinding of trial medication. Both ectopic pregnancies that required methotrexate management were reported as serious adverse events and file notes were recorded. Both completed treatment and had no other complications.

4.4.5 Laboratory analysis

It was reassuring to know that there has been consistently about 40% of women with RM that were found to have uNK cells density of ≥5% in different cohorts of women with RM recruited for different studies in this laboratory[110]. This demonstrated the reliability and reproducibility of our method of analysis. We have also intermittently performed double counts on random samples to ensure consistency in the analysis for the trial. The mean difference was <0.5% uNK cells density between myself and another observer in the laboratory, and there were no systematic errors in the analysis (*Figure 4.11*).

Figure 4.11 Bland-Altman plot of uNK cells density between myself and another observer with a mean difference of <0.5%



4.4.6 Post-hoc analysis

It was observed that women who miscarried in the trial appeared to have higher uNK cells density, regardless of trial medication (*Figure 4.12*). This was however not statistically significant (p=0.3) (*Figure 4.13*).

Figure 4.12 Distribution of uNK cells density of women randomised

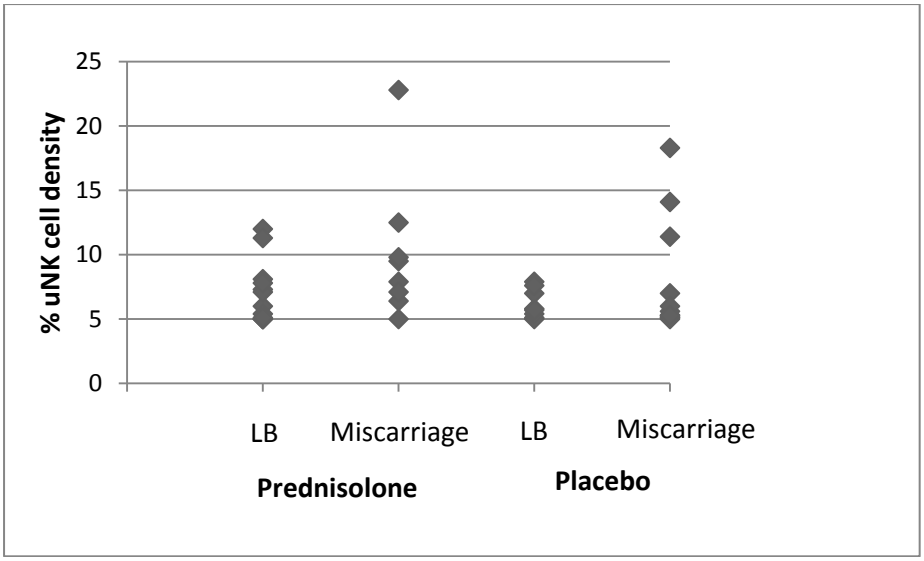
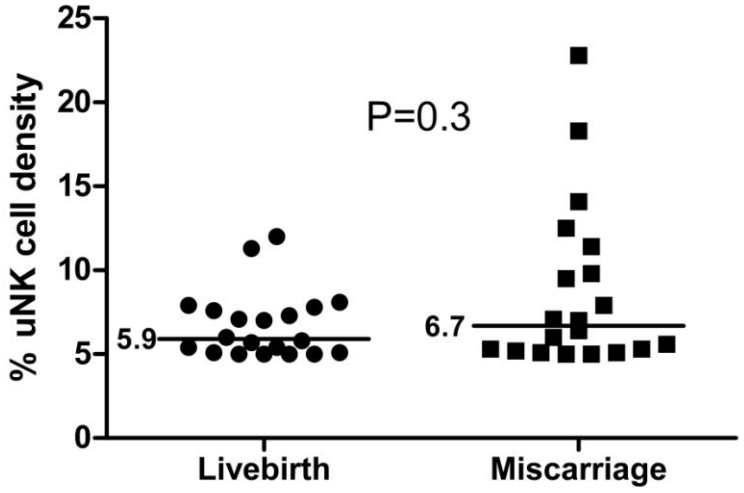


Figure 4.13 UNK cell density and pregnancy outcomes of women on the trial



4.5 DISCUSSION

4.5.1 Feasibility of recruitment and acceptability of trial medication

This pilot trial has demonstrated that it was possible and acceptable to women to conduct a stratification and treatment trial based upon uNK cell density in a reasonable time period, allowing for new approaches to the management of RM to be developed. We did not seek for a particular sample size but advice on pilot studies recommended that more than 30 was a reasonable number[180]. Thus, we stopped after randomising 40 women for pragmatic reasons as the randomisation list was stratified to groups of 20 and funding was limited. The limitation of funding also meant that it was not possible to follow up the obstetric outcomes of all the women screened, and important information was lost.

From the questionnaire that was completed by the women who attended for screening, 68% (53/78) thought they had an underlying endometrial immune pathology, which could have been the reason for them to participate in screening. There were however no correlation between the women's perception of immune pathology and the result of the uNK cell density. Of those who completed the questionnaire, 32 (41%) were screened positive. 84% (27/32) of them desired active medication if given an option. Despite this, the majority (66%) self-referred when pregnant and consented to randomisation. Furthermore, the women in the trial travelled from all over the UK and funded their own transport for both the screening and randomisation visits. This showed the women's commitment and support of a study that looked for a test that could identify the subgroup of women where targeted treatment could be beneficial. This concept is analogous to the trend of personalised clinical practice, where treatment should be tailored to the individual patient.

Tertiary referral centres review high numbers of women with RM but many of these present with complicated histories, and may not be suitable for inclusion into trials. With the development of national guidance in the management of RM, many women with idiopathic RM are now managed in district general hospitals and the community. Thus, recruitment of trials should not solely be targeted at tertiary referral centres, but also involve smaller hospitals and GPs. Health care providers also need to support recruitment into trials by avoiding empiric prescribing,

especially if women have attended for screening. A large trial will need extensive planning and communication with many centres to encourage participation.

It was encouraging to find that prednisolone was well accepted, as it was important to assess if women will tolerate the side effects of prednisolone for 8 weeks. Although side effects were reported, no women stopped the medications because of its severity, and all completed treatment. Compliance may have been improved because this treatment was targeted towards a plausible cause of their previous miscarriages. Reassuringly, there were no serious adverse effects or SUSAR reported from treatment with prednisolone. Although the safety of prednisolone were only examined in 20 women in this study, there are other published studies of its safety profile in the first trimester of pregnancy where prednisolone was used to treat asthma, hyperemesis or rheumatoid arthritis[176-178]. Furthermore, studies with post-natal follow-up have also not shown complications of prednisolone use[178, 179]. Thus, prednisolone treatment was thought to be safe.

4.5.2 Karyotype of miscarriages

The most common cause of miscarriage is chromosomal abnormalities in the conception. These happen in about 70% of all miscarriages[7]. As discussed in previous chapters, endometrial receptivity plays a role in recognising and selecting the embryo for implantation[183]. Thus, karyotype of miscarriage is important in making progress towards the management of idiopathic RM. Therapeutic endometrial manipulation should prevent miscarriages of pregnancies with normal chromosomes, and not encourage implantation and support pregnancies that have genetic abnormalities. Results of an abnormal karyotype also provide the reason for the miscarriage, and helps in counselling the couple. The development of new microarray-based technologies have revealed a much higher rate of chromosomal abnormalities than previously thought in the developing embryo, and when possible, should be the method used for assessing genetic abnormalities in all miscarriages[184]. Further studies should be targeted towards women with idiopathic RM of normal karyotype, where the miscarriage was not due to chromosomal abnormalities.

Unfortunately in this trial, results of karyotype were only available for 30% of miscarriages. Reassuringly, the percentage of normal and abnormal karyotype in both groups was similar. Although the numbers were small, this indicated that prednisolone therapy was not improving implantation of abnormal pregnancies. We could not determine what type of pregnancy loss prednisolone could potentially prevent as the number of losses in each category was too small. The reason for not having many results of karyotype may be that women either had biochemical miscarriages, with no tissue for karyotyping; or were counselled towards medical and conservative management to avoid general anaesthesia, where complications could occur if the trial medication allocation was unknown. In many occasions, women who miscarried in other hospitals or at home did not have POC sent for karyotype. In further studies, the importance of sending POC for karyotyping need to be emphasised to both health care providers and women to improve the rate of chromosome analysis.

4.5.3 Uterine NK cell density as a screening test

It is reported in the literature that women with idiopathic RM have live birth rates of >70% with supportive care and reassurance scans[37]. For the uNK cells density test to be able to select women who need treatment, the live birth rate in the screen positive and placebo group need to be lower than expected in trials involving similar women. In this pilot trial, the overall live birth rate was 50%, and in the placebo group, only 40%. This was significantly lower than the live birth rates in the placebo group of other recently published RCTs in women with RM (*Table 4.5*). Furthermore, it was also observed that women who miscarried in the trial appeared to have higher uNK cell density, regardless of trial medication (*Figure 4.12*). This finding, alongside the biological plausibility of the role uNK cells play in implantation as discussed in previous chapters, would support raised uNK cells density as a clinical marker to identify a subgroup of women with an increased risk for adverse pregnancy outcomes for potential treatment.

It is possible that 5% may not be the accurate threshold of normality, or the correct inclusion criteria for randomisation. There is currently no universally accepted normal level of uNK cell density as the method of assessment varies from

laboratory to laboratory as discussed in Chapter 3. 5% was determined by using the upper end of the interquartile range of control women. This may not be the best method of obtaining a cut-off as this is based on a normal population and not in the population of interest, women with RM. An alternative method would be to obtain pregnancy outcomes of all women with untreated idiopathic RM and perform a ROC analysis of uNK cells density between the live birth and miscarriage groups to obtain an optimal cut-off value. In the context of a RCT, it would be to randomise all women screened when they fell pregnant. In this alternative study, uNK cells analysis would be performed at the end of the study when the pregnancy outcomes were known. This would allow for a ROC analysis to determine the optimum cut off for adverse pregnancy outcomes (miscarriage) in the placebo group. This method will improve recruitment as every woman who had an endometrial biopsy taken can be randomised, but it may not be ethically right to withhold results of her uNK cells density. This study would involve an entirely different methodology, but if adequately powered, may be the best technique to identify the optimal cut-off of normality for uNK cells density. But even though the level of normality is known, there is significant cycle-to-cycle variability of uNK cells density which needs to be further investigated and addressed in the process of developing uNK cells density as a screening test[118].

Table 4.5 Live birth rates in the control group in similar recent RCTs

Authors	Year of study	Population studied	Control group	Live birth rate in control group
Visser et al[185]	2011	≥3 RM, Idiopathic	Aspirin	61%
Stephenson et al[128]	2010	Previous LB followed by ≥3 RM, Idiopathic	Placebo	63%
Kaandorp et al[32]	2010	≥2 RM, Idiopathic	Placebo	67%
Clark et al[40]	2010	≥2 RM, Idiopathic	USS surveillance	80%
Laskin et al[29]	2009	≥2 RM with APS, Thrombophilia or ANA	Aspirin	79%
El-Zibdeh[186]	2005	≥3 RM, Idiopahtic	Placebo	71%

4.5.4 Laboratory standardisation and analysis

The method of immunohistochemistry and image analysis to assess uNK cells, and the cut-off threshold of 5% was determined and validated from previous studies in our laboratory[107, 110, 181]. The same method of analysis was applied to all endometrial samples in a single laboratory in the trial for consistency.

The mid-luteal phase of the menstrual cycle were assessed through the urinary surge of LH as women were advised to use ovulation kits and contacted us on the day of ovulation for an appointment 6-9 days later for an endometrial biopsy. In the laboratory, these biopsies were further assessed for accuracy of dating according to the Noyes criteria[59]. We could have further improved our accuracy of dating through serum progesterone but we considered two methods of assessment of mid-luteal phase as adequate. Women were advised to reuse the ovulation kit and reattend if their endometrial biopsy was found not to meet the Noyes criteria for mid-

secretary phase the first time. All but 4 (2.5%) women have results of their uNK cells density.

Unfortunately, the current method of analysis was very time consuming. Thus, there is a need for an alternative method for image analysis to produce results quickly to accommodate the potentially large number of women in a bigger trial or if uNK cells test was to be developed as a screening marker for adverse pregnancy outcomes. A more time efficient method for uNK cells analysis will be further discussed in Chapter 5.

4.5.5 Mechanism of action of prednisolone

Prednisolone treatment may improve the live birth rate from 40% to 60% (absolute risk reduction of 20%) in this group of women with high uNK cells density. This is a preliminary observation that needs to be interpreted with caution as it does not demonstrate efficacy. The exact mechanism of how prednisolone could work in improving outcome is unknown. It is unlikely through immunosuppression as a systematic review of RCTs on IVIG therapy (arguably a more powerful immunomodulator compared to prednisolone) in both primary and secondary RM, has not shown to be of benefit in improving outcomes[128, 129]. Prednisolone reduces the number of uNK cells, and also affects angiogenesis, a function of uNK cells, and an important factor in implantation[107, 124, 187]. This mechanism through altering the angiogenic growth factors production of uNK cells was thought to be one of the therapeutic effects of prednisolone[124]. More recently, laboratory studies have shown defective decidualisation correlating with uNK cell density of >5% and a lack of 11-β hydroxyl steroid dehydrogenase type 1 in stromal cells[125]. 11-β hydroxyl steroid dehydrogenase type 1 activates cortisol. Hence, administration of the glucocorticoid prednisolone may overcome this deficit and modify the endometrial environment to one more favourable for maintaining a pregnancy.

The dose of 20mg was used as this was the dose that resulted in a live birth in a previously published case report[122], and the dose that demonstrated a reduction in uNK cells density after treatment[110]. It is unclear if this is the optimum dose. It is

also uncertain if the effect of prednisolone on uNK cells and subsequent outcomes are dose-dependent. But, an increase in the dose would need to be balanced with the increase in side effects, which can be investigated in a dose finding study. Efficacy of different doses could perhaps be examined by assessing the effect of different dosages of prednisolone on uNK cell number and function in the laboratory, prior to clinical assessment in influencing pregnancy outcomes. This could lead to different therapeutic dosage of prednisolone for different results of uNK cell density.

In this trial, prednisolone was only commenced at a positive pregnancy test and it is known that the endometrium decidualises in preparation for implantation, before any interaction with the blastocyst[188]. Thus, it was possible that starting prednisolone at about 4 weeks gestation was too late, as there was inadequate time to modify the endometrium to a presumed more favourable one for implantation. Efficacy could be further improved if it was commenced prior to the cycle of conception. However, this could subject the women to repeated cycles of high dose prednisolone if they do not conceive easily, which may result in unwanted side effects such as immunosuppresion, hypertension or weight gain.

The endometrium sampled was only a single point in the cycle, and may not be representative of every cycle or the cycle when implantation occurred. As it is ethically wrong to sample conception endometrium, any results of the effect of prednisolone on endometrium when implantation occurred were extrapolated from in-vitro studies of endometrium in a pre-conception cycle, or on deciduas of miscarriages. Therefore, the clinical effect of prednisolone on improving pregnancy outcomes is best confirmed or refuted in a clinical trial.

4.5.6 Sample size calculation for a definitive trial

Preliminary power calculation was based on multiple presumptions informed by minimal data. With the results of this pilot trial, we performed a more precise sample size calculation. The live birth rate of women with uNK cells ≥5% in the placebo group was 40% and 60% in the prednisolone group. This pilot trial has also confirmed that about 40% of all women screened had raised uNK cells and of these, about 60% of women returned for randomisation. These proportions would mean

that in excess of 890 endometrial biopsies would need to be sampled, to randomise 214 women (107 in each arm) in the definitive trial to significantly detect the benefit of prednisolone with 80% power (α 0.05) (*Figure 4.14*) (Stats Direct v2.6.8).

The results from this pilot trial also allowed us to estimate the sample size in a definitive trial if the expected benefit of prednisolone in improving live birth rates was more or less than anticipated (*Table 4.6*). It is important to consider that women randomised were only about a quarter (25%) of all women that will need to be screened with endometrial biopsies. Thus, it may not be feasible to recruit the large numbers for screening to detect a significant difference if the expected benefit of prednisolone was less than this pilot trial. The most optimistic view would be an improvement in live birth rates with prednisolone to one commonly quoted for women with idiopathic RM at 70-75% from the rate of 40% live birth in the current placebo group. If the recruitment rate and benefit was equivalent to this pilot trial, it would take about 10 years to complete the definitive trial with adequate power. However, the recruitment time would shorten in a multi-centre trial.

Figure 4.14 The power calculation for the definitive RCT

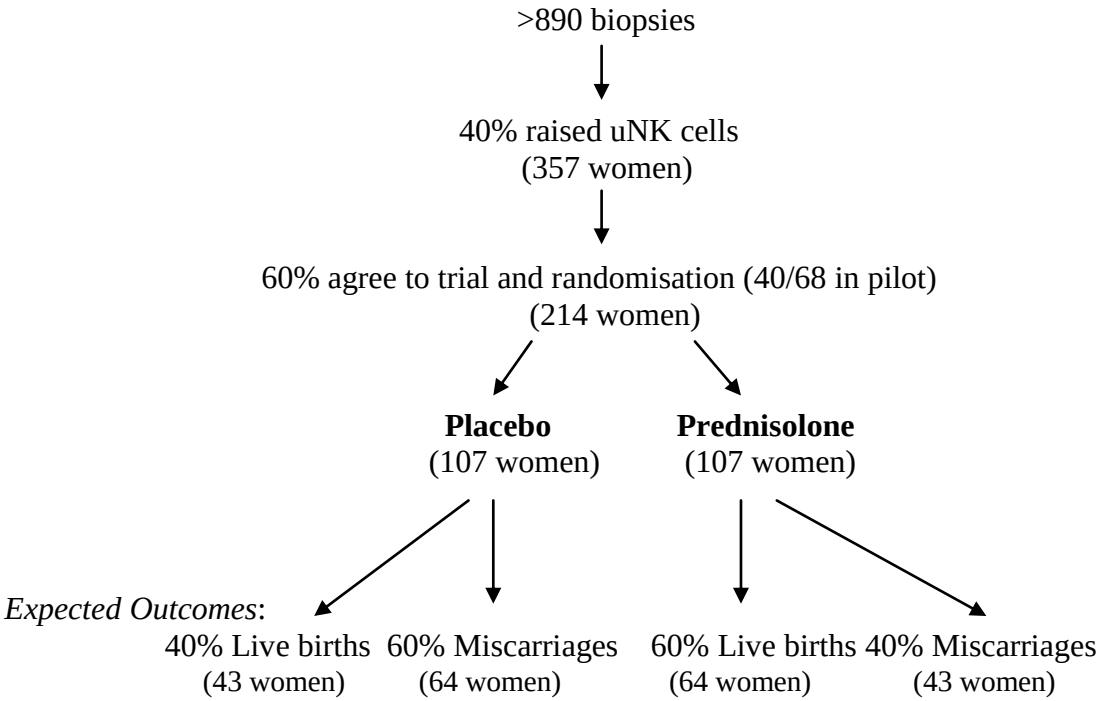


Table 4.6 Numbers needed for randomisation with different expected LB rate

Expected live birth rate		**Women needed to be randomised**	
Placebo group	**Prednisolone group**	**80% Power**	**90% Power**
40%	50%	816	1078
40%	55%	374	590
40%	**60%**	**214**	**280**
40%	65%	140	180
40%	70%	98	126
40%	75%	74	92

4.6 CONCLUSION

This pilot trial has demonstrated that it is feasible to conduct screen and treat trials of selecting women through an endometrial based investigation for randomisation into a double-blind, placebo-controlled trial. Women were in support of a trial where a test is used to select the subgroup of women whom may benefit from treatment. They appear to tolerate the active medication prednisolone well with minimal side effects and all completed treatment. Reassuringly, there were no adverse effects from prednisolone. Support will be needed from both tertiary referral centres and health care providers in the community to ensure adequate recruitment to meet the sample size needed to assess for a significant difference in the live birth rate in a similar trial, prior to consideration for implementation into clinical practice.

In planning the definitive trial or future trials of similar nature, it is of importance that karyotype results are available for all the miscarriages, as therapy is aimed at supporting pregnancies of normal chromosomes. Hence, measures such as good communication between centres of the trial and written information to patients should be taken to ensure POC are available for cytogenetics analysis. There should also be consistency in the entry into trials to allow for uniformity in starting of trial medication.

It is still unclear how prednisolone could work in improving pregnancy outcomes. Proposals include regulating angiogenesis, a function of uNK cells and an important factor in implantation[107], or through improving steroid metabolism in stromal cells[125]. Further laboratory studies are needed to explore these different mechanisms. Additional studies could also consider investigating the optimal dosage of prednisolone, whether effects are dose dependent, and timing of commencing prednisolone. All these may then path the way for uNK cells density to be developed as a clinical marker of endometrial cause of RM.

Analysis of uNK cells is currently time consuming and will need to be more efficient to accommodate the large numbers for screening in a definitive trial. The development of an alternative, quicker method for assessing uNK cells density will be discussed in the next chapter.

CHAPTER 5

COLOUR DECONVOLUTION AND AREA MEASUREMENT AS AN ALTERNATIVE METHOD FOR ESTIMATING UTERINE NK CELLS DENSITY

CHAPTER 5: COLOUR DECONVOLUTION AND AREA MEASUREMENT AS AN ALTERNATIVE METHOD FOR ESTIMATING UTERINE NK CELLS DENSITY

5.1 Introduction

As described in the previous chapters, there is interest in uNK cells density being used as a diagnostic or screening tool for women with RM or other reproductive problems. Unfortunately, the lack of standardisation in the methodology between laboratories in the analysis of uNK cells density has made comparisons of results between units difficult.

The current method of assessing uNK cells density in our laboratory, described in the last chapter, is by a combination of manually counting the number of uNK cells stained positive with DAB staining, divided by the number of total stromal cells calculated through the application of 'particle analysis' function of Image J. This method of semi-automated image analysis (SAIA) has been validated and is reproducible[181], but was too time consuming. The feasibility of conducting the definitive trial would involve the ability to inform a large number of women in a reasonable time whether they were screened positive or negative for suitability of randomisation. The method of uNK analysis should also be easily reproducible and standardised to allow for other units to replicate, to permit synthesis and meta-analysis of results from different departments. The development of an alternative method for uNK cells counting that is quick, reliable and reproducible is needed to further develop uNK cells density as a screening test to select women with reproductive problems for targeted treatment.

5.1.1 Image J – Colour Deconvolution plug-in and Area Measurement (CDAM)

Image analysis in IHC by means of different computer assisted image processing programmes has been widely used in biologic research[189-191]. In our experience, digital image analysis has shown to be more objective, more

reproducible with low inter and intra-observer errors, and served as a good teaching tool[181].

Image J, the Java-based, freely available software that has been used in the analysis of uNK cells density for the RCT, has other plug-ins contributed by different authors that are used in different types of image analysis[182]. One of the plug-in is colour deconvolution (CD), a technique used to separate an image with different colour stains into the individual colour component based on the stain specific RGB (red, green, blue) absorption[192]. Vectors for several commonly used stains such as Haematoylin and Eosin (H & E), and Haematoxylin and DAB (H DAB) have been developed and were freely available for use on Image J. The H DAB vector would be suitable as uNK cells were stained with DAB and all nuclei were counterstained with haematoxylin. Therefore, this plug-in could be used with the area measurement (AM) tool as an alternative method to estimate uNK cells density. Although editing of the image was still needed to remove epithelial edge, glands and blood vessels from the captured image, there was no need for further steps of removing DAB staining, digitally counting the stromal cells and manually counting the uNK cells. Therefore, this method would hopefully be more time efficient.

5.1.2 Inconsistency in reporting of uterine NK cells

There is currently inconsistency in the reporting of uNK cells analaysed through IHC. Clifford *et al*[108] reported the number of uNK cells per 10 HPF, and did not take into consideration the stromal cells count, which would affect the density. Michimata *et al*[117] on the other hand reported uNK cells density as uNK cells number per $CD45^+$ cells and not over all stromal cells. In our unit, similar to Tuckerman *et al*[109], uNK cells density were reported as total uNK cells over the total of stromal cells.

It would be particular useful to standardised the method of analysing and reporting uNK cells density, to allow for comparison and reproducibility of uNK cells density between different laboratories. With Image J being freely available on

public domain, and the vector for haematoxylin and DAB set, the method of CDAM could potentially be used as a universal method of reporting uNK cell density.

5.2 Objective

The aim was to develop a new method of estimating uNK cell density using both the colour deconvolution (CD) and area measurements (AM) function available on Image J. Subsequently, the new method was validated, and assessed for its feasibility to be used as a method to quantify uNK cells density in endometrial samples from women for the definitive RCT of prednisolone in women with RM and high uNK cells, by determining the optimal CDAM cut-off equivalent to the 5% uNK cell density with the current combined method.

5.3 Methodology

5.3.1 Sample selection

Images for analysis were captured from mid-luteal phase endometrial biopsies processed from 100 consecutive women who consented for screening into the RCT as described in Chapter 4. Each woman had an average of 10 images taken from the stained slide, and thus 997 images were examined with this new method. As with any IHC staining procedure, the staining was checked with the positive control slide to ensure adequate colour development from DAB. If it was found to be too faintly stained (when compared with the control slide), the process of IHC staining was repeated on these samples.

After ensuring that this method was feasible, images from 40 women (400 images) with uNK cells density ranging from 2-16% were randomly selected to test for intra and inter-observer errors for this new CDAM method.

5.3.2 Preparation of images for analysis

Images of the selected samples were captured using Eclipsenet software as described in the previous chapter and were saved in a compression free format (TIFF), and edited prior to being analysed. First, the background colour was changed to white. Similarly to editing images for the trial, all the epithelial edges, glands and blood vessels in the images were removed. In this study, they were removed using the Image J Freehand selection tool *(Figure 5.1)*, rather than using Adobe Photoshop CS2 or CS3 as with the SAIA method used in the pilot trial. Once the selected area was surrounded, the mouse button was released and the chosen area cleared *(Figure 5.2)*. The image was ready to be analysed when all the luminal epithelium, glands and blood vessels have been removed *(Figure 5.3)*.

Figure 5.1 The freehand selection icon on Image J

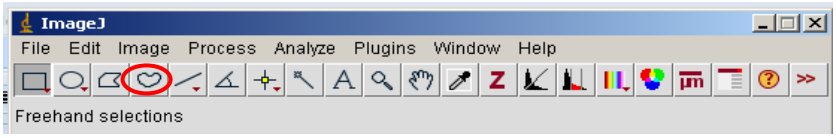


Figure 5.2 The selection of area to be removed prior to image analysis

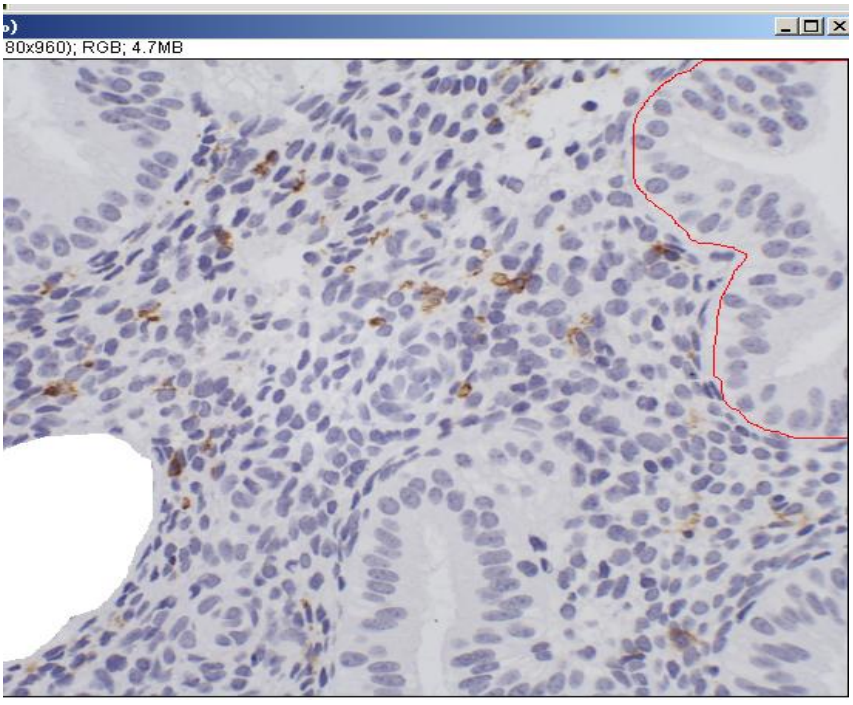
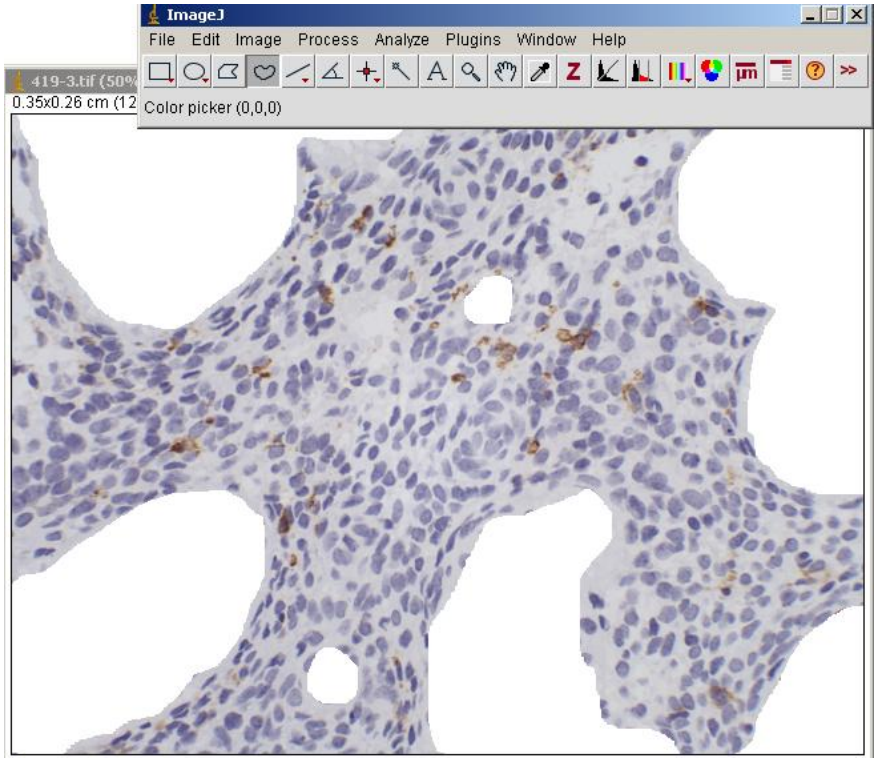


Figure 5.3 The edited image ready for CDAM analysis for uNK cells density



5.3.3 Method for colour deconvolution and area measurement (CDAM)

The background of the image was ensured to be neutral with respect to colour intensity prior to using the CD plug-in. This was checked by clicking on 'point selection' (arrow) and ensuring that the RGB values were similar (circled values) (*Figure 5.4*). Next, the 'colour deconvolution' function was selected and built-in vectors for haematoxylin and DAB chosen to separate the colours for DAB (brown) and haematoxylin (blue). This resulted in one image of each, corresponding to the individual stain and another which was white, and the original image (*Figure 5.5*). The haematoxylin stained image (colour 1) and the DAB stained image (colour 2) were individually analysed for area measurements. An estimate of UNK cell density would be the total area occupied by DAB stain of uNK cells (brown), divided by the area occupied by stromal cell nuclei (blue).

Figure 5.4 Neutralising the background of the image

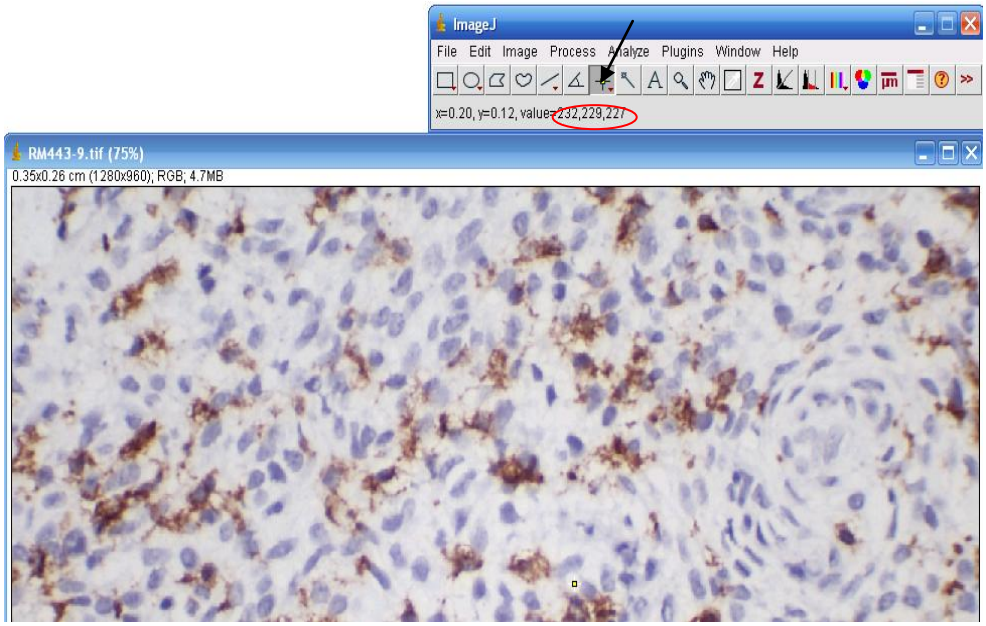
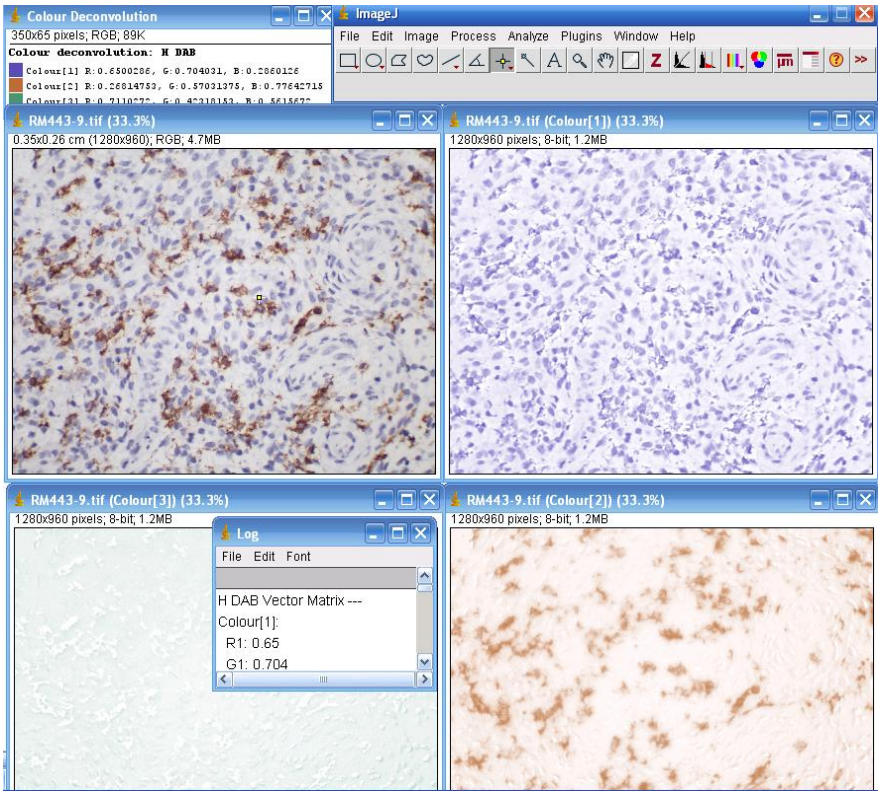


Figure 5.5 The resulting images after utilising CD function on Image J



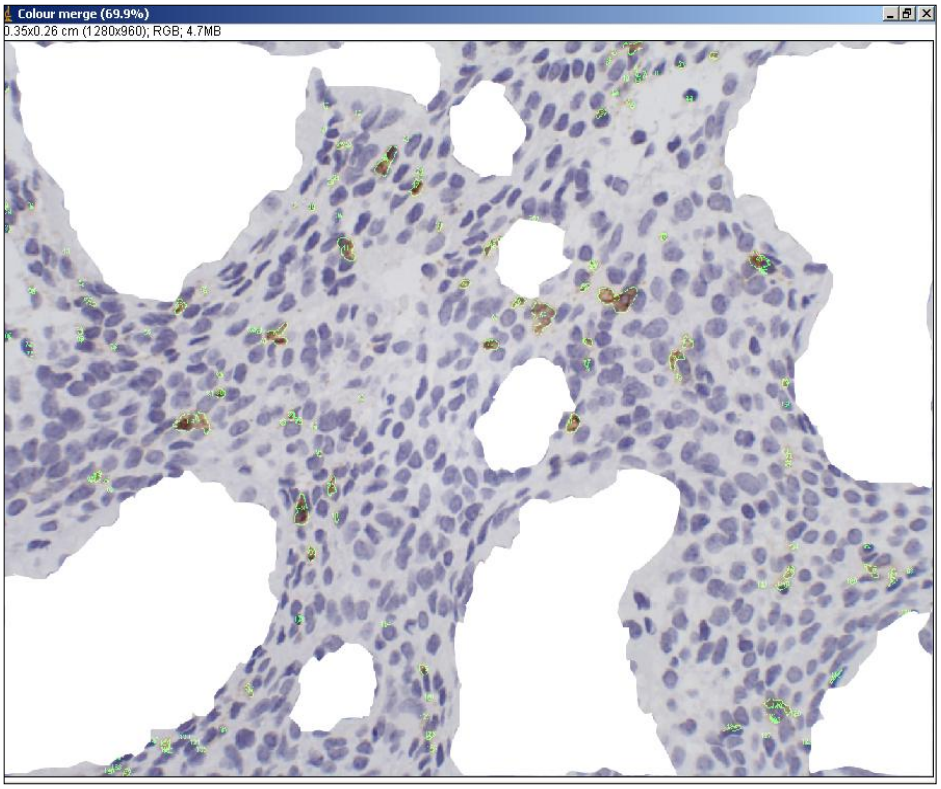
First, a threshold was applied to the haematoxylin stained nuclei (colour 1). This was adjusted to ensure the selection of area matched the original blue stained nuclei. The manual threshold was compared with the auto threshold available in the plug-in for 10 women (100 images) and was found to yield similar results. Thus, the auto threshold for haematoxylin was used in the rest of the study and further adjustments were not required. Auto threshold had the benefit of reducing subjectivity. The total area thresholded was measured using the Image J 'area measurement' (AM) function. The values for the threshold, total area and area fraction were recorded.

These steps were repeated for the DAB stained image (colour 2). The threshold for this image needed to be adjusted manually as DAB cytoplasmic staining was not as clearly demarcated as haematoxylin nuclei staining. A few methods to manually threshold for DAB staining were tested on images from 10 women (100 images) prior to deciding on the best method that was consistent, reproducible, and easily explained to and understood by another user. The methods tried were:

- threshold for the brown to match as closely as possible to the DAB staining in the original image
- threshold to ensure that every uNK cell was accounted for, while minimising background staining
- threshold to match the strongest area of brown stain with the DAB staining in the original image, regardless of background staining

The best, most easily taught and understood method was found to be to match as closely as possible the threshold brown area to the DAB staining on the original image. The accuracy could be checked by merging the DAB stained image with the original image file and checking that the outlines matched the DAB staining *(Figure 5.6)* (*steps explained in section 4.3.6.2*).

Figure 5.6 A correctly measured and matched DAB area



If the merged image did not match well, then, the image was reanalysed by using a different threshold. The total area thresholded for DAB was measured using the Image J 'area measurement' (AM) function, and values for the threshold, total area and area fraction were recorded.

The final result for this method using CDAM was calculated by dividing the DAB total area (brown stain) with the haematoxylin total area (blue stromal nuclei), multiplied by 100. This would be an estimate of the uNK cells density.

$$\textit{Estimate of \% uNK cells density} = \frac{\textit{Area of brown in 10 HPF}}{\textit{Area of blue in 10 HPF}} \times 100$$

5.3.4 Validation of CDAM method

We estimated uNK cells density from 100 consecutive women (997 images) that were screened for the trial with the new CDAM method. The results of the uNK cells density from the current SAIA ('gold standard') were blinded. The time taken for analysis with CDAM method was recorded.

The correlation between the results of the current SAIA method and new CDAM method was assessed by Pearson's correlation coefficient. Repeated analysis of images from 40 women (400 images) with a range of uNK cells density that spanned across the whole spectrum was done by myself and another independent trained observer to assess the reproducibility of the CDAM method. The repeat analysis was done after a period of one week. The time for analysis of each of these samples were recorded. As both methods required the editing of glands, blood vessels and epithelial edge removal, time was recorded for the steps of:

- Removal of DAB staining
- Manual counting of uNK cells
- Digital quantification of total stromal cells with Image J

The total time was recorded and compared with the time taken for the CDAM method. Mann-Whitney U test was used to compare the differences in the analysis time. From the results of these 400 images, inter and intra observer errors were calculated, and Bland-Altman plots were used to evaluate the relationship between both methods and between observers.

Results of raised uNK cells density of ≥5% was used as entry criteria into the RCT, as discussed in Chapter 4. Thus, we needed to determine the optimal CDAM cut-off equivalent to the current 5% uNK cells from the SAIA method, for CDAM to be used to analyse the images. We calculated the number of misclassifications (classified 'normal' with current SAIA method but 'high' with CDAM, and vice versa) for different percentages with the CDAM.

5.4 Results

5.4.1 Correlation and agreement between current SAIA method and CDAM

The analysis of 997 images from 100 women showed good rank order agreement between both methods of quantifying uNK cells density (r^2 = 0.92) centred around the line of equality (*Figure 5.7*). There was also good agreement between both methods with no systematic differences in the measurements. The mean difference between both methods was 0.12%, with upper and lower limits of agreement (LOA) of 1.5% and -1.7% respectively and no evidence of systematic error (*Figure 5.8*).

Figure 5.7 Correlation between CDAM and SAIA

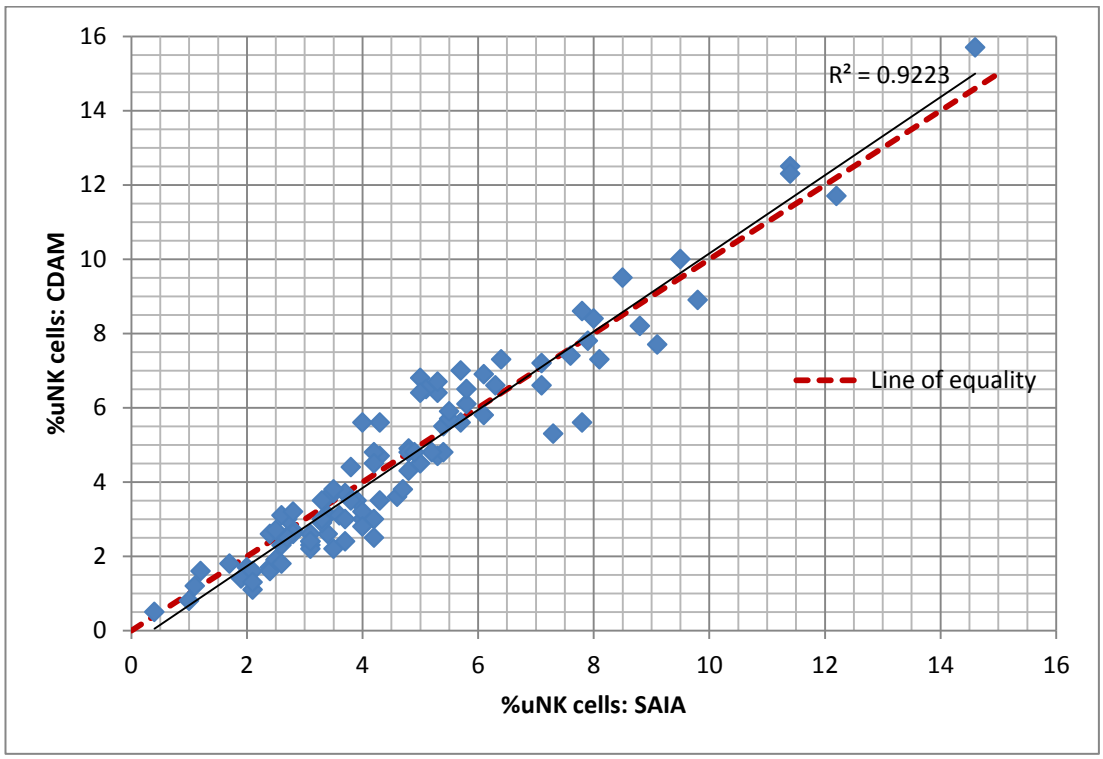
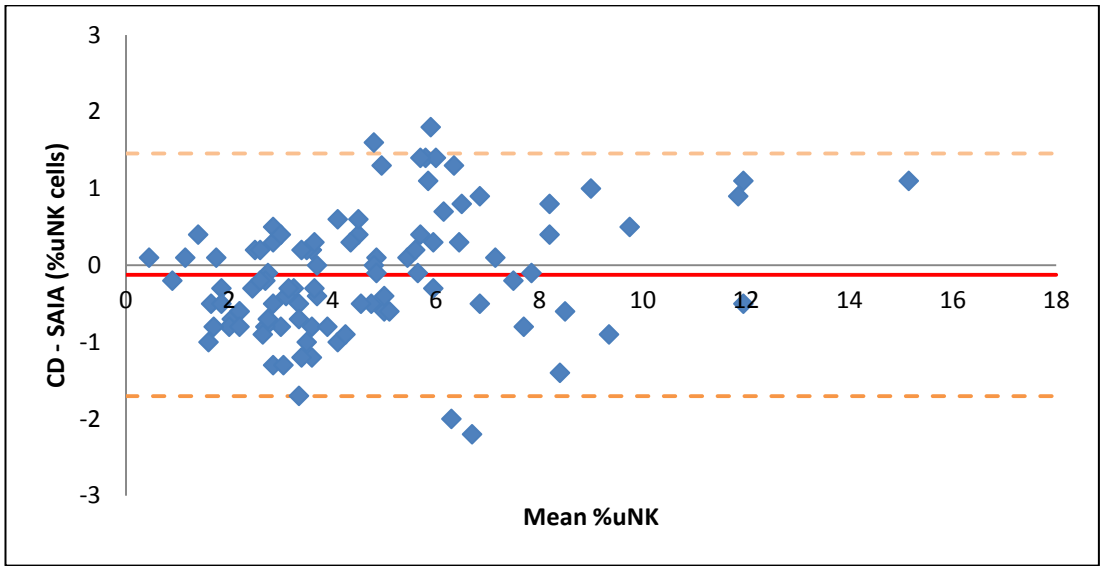


Figure 5.8 Bland-Altman plot showing agreement between CDAM and SAIA



The mean time taken to provide uNK cells density results for one woman (from 10 images) was significantly shorter with the CDAM method, compared with the current SAIA method (mean time 10 mins (range 7-13 mins) vs 28 mins (range 16-47 mins, $p<0.0001$) (*Figure 5.9*). With the SAIA method, there was a great variability in the time taken in analysing each sample, largely dependent on the density of uNK cells (*Table 5.1*).

Figure 5.9 Chart showing significant difference in time taken for uNK analysis

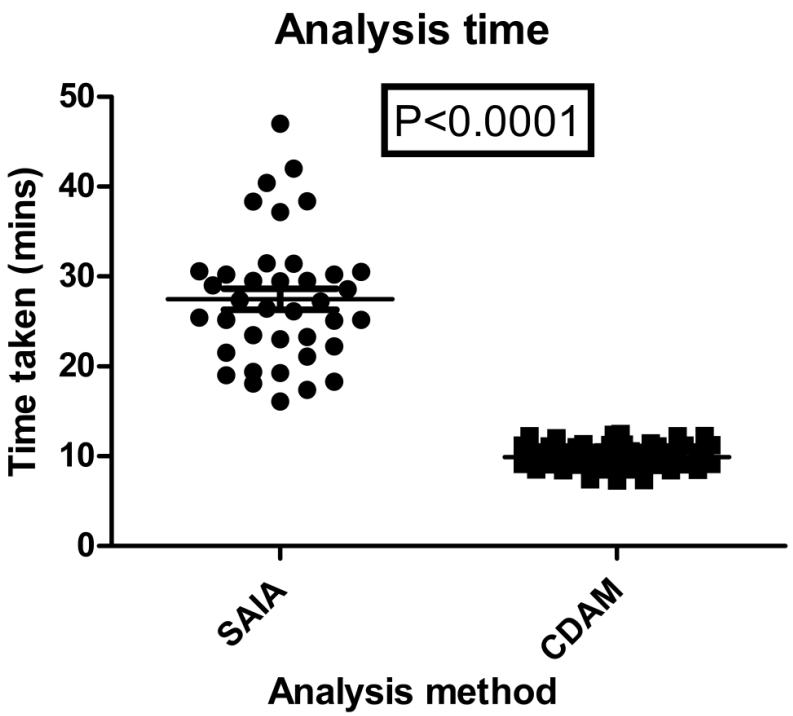


Table 5.1 Time taken for each step in uNK cells analysis with the SAIA method

Steps	**Mean time taken (range) (mins)**
Remove of DAB	6 mins (3-16)
Manual counting of uNK cells	12 mins (3-34)
Digital counting of stromal cells	13 mins (9-20)

5.4.2 Reproducibility of CDAM method

Repeated analysis of 400 images from 40 women by myself showed good reproducibility with a mean difference of 0.04% and standard deviation of 0.6. This provided an upper LOA of 1.1% and lower LOA of -1.2% (*Figure 5.10*). Bland-Altman plots of the results from both observers showed no systematic differences in the method of analysis. The mean difference was 0.51%, with upper LOA and lower LOA of 2.2% and -1.2% respectively (*Figure 5.11*). There was also good correlation between observers where $r^2 = 0.96$ (*Figure 5.12*).

Figure 5.10 Bland-Altman plot showing intra-observer agreement with CDAM

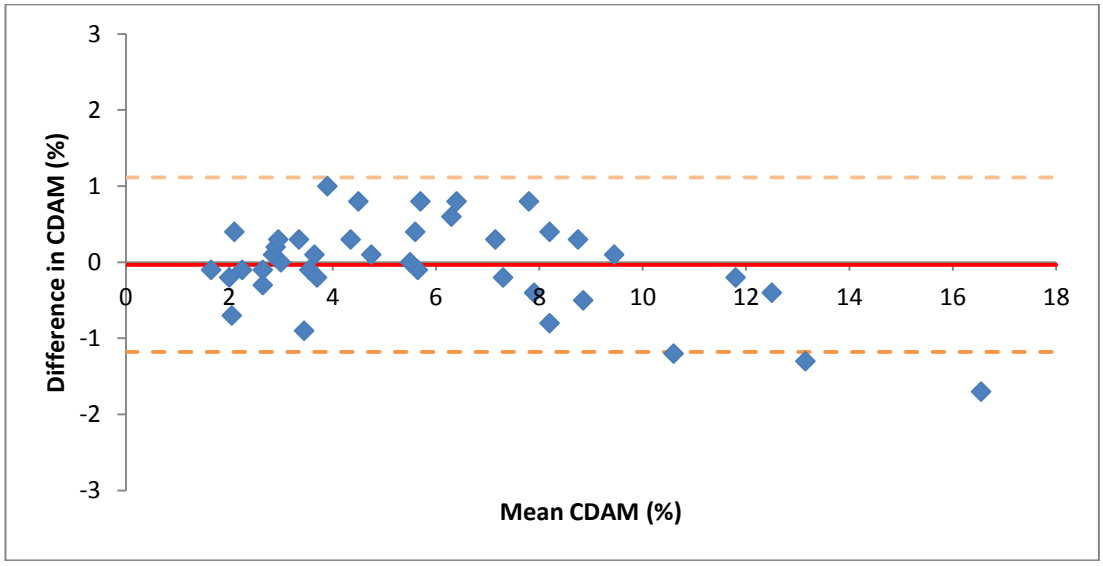


Figure 5.11 Bland-Altman plot showing inter-observer agreement with CDAM

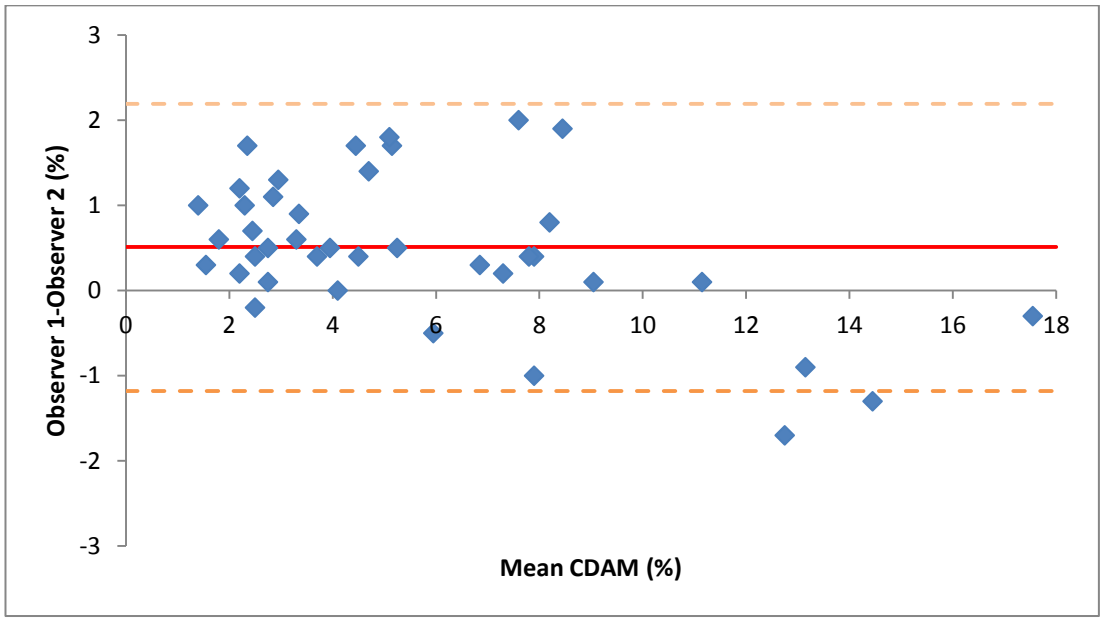
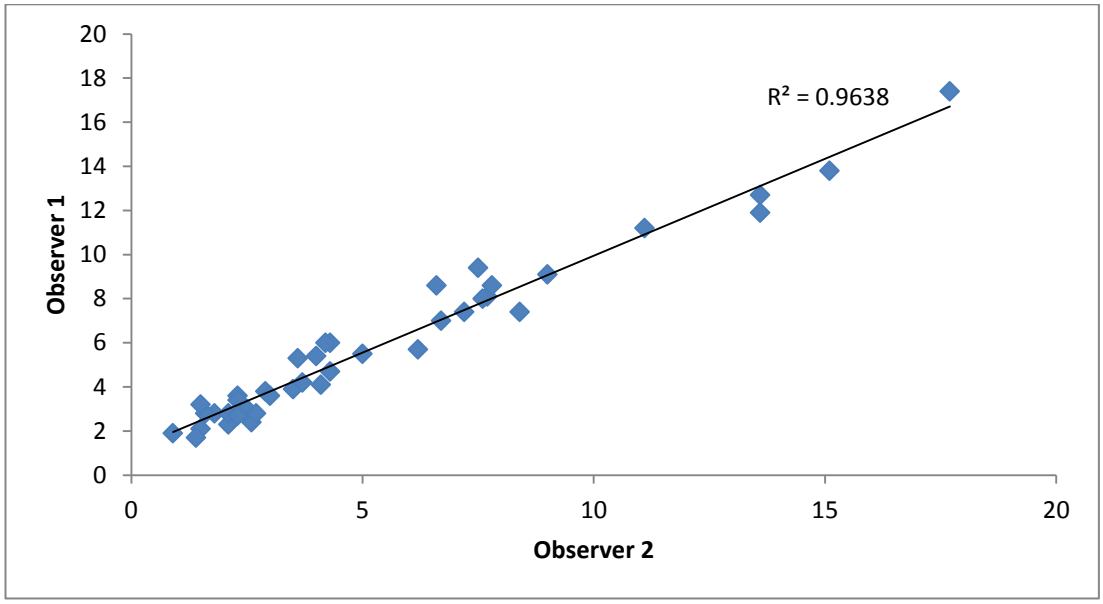


Figure 5.12 Correlation of uNK cells density (%) between observers with CDAM



5.4.3 Validating the new method CDAM as a screening tool

The total number of misclassifications was compared with different cut-off percentage of uNK cells density. Misclassifications of false positive was defined as categorizing the women as having a raised result from CDAM, when her uNK cells density test from the SAIA method was normal (<5%), and the definition of false negative was defined as categorising the women as having a normal result from CDAM, when current SAIA method of uNK cells assessment gave her a raised uNK cells result of ≥5%. The implications of a false negative test would be the women missing out from participating in the trial. Misclassifications were least with 5% as a cut-off for high uNK cell density for CDAM, similar to the current SAIA method (*Table 5.2 and Figure 5.13a,b*).

Table 5.2 The optimal CDAM cut-off equivalent of the SAIA method

uNK cells density cut-off with CDAM	**Misclas-sifications**	**False +ve (*normal results with SAIA method*)**	**False –ve (*missing the trial*)**	**Sensitivity**	**Specificity**
4.5%	8	8	0	100%	87.1%
5%	**6**	**2**	**4**	**89.5%**	**96.8%**
5.5%	7	2	5	86.8%	96.8%
6%	11	0	11	71.1%	100%

Figure 5.13a The number of misclassifications at different CDAM cut-offs

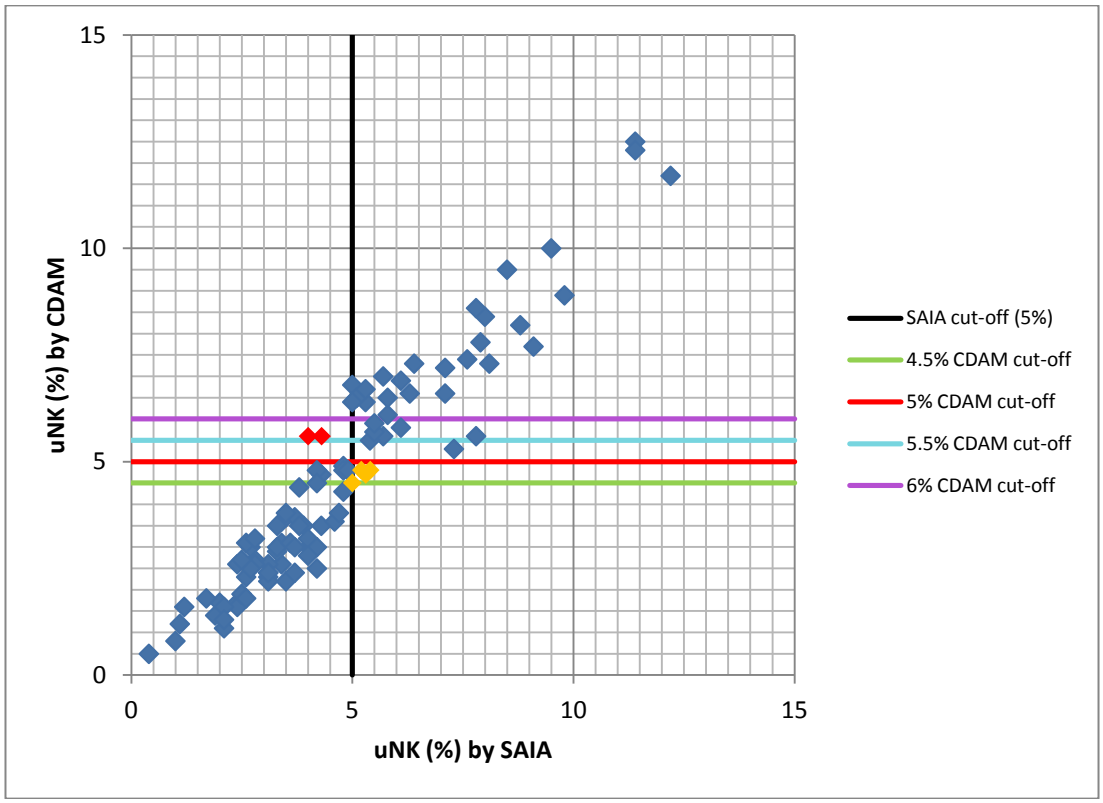
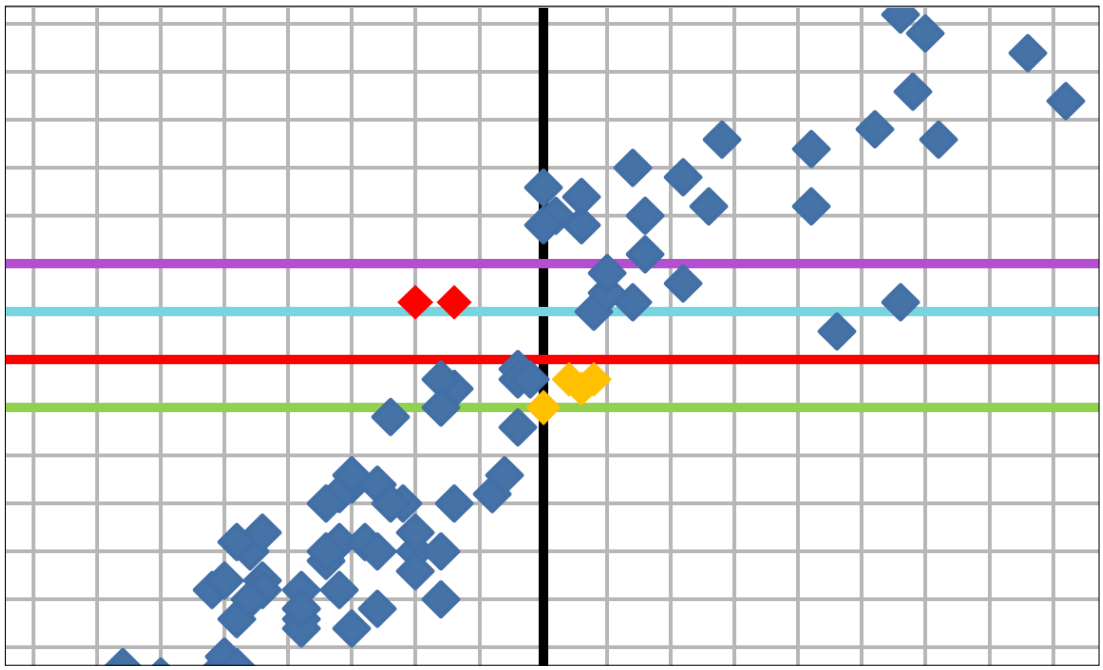


Figure 5.13b The magnified area of interest for misclassifications



5.4 Discussion

The results show that it is possible to use this new method of estimating uNK cells density with the 'colour deconvolution' and 'area measurements' functions on Image J. This concept of utilising area measurements to estimate cell density removes the need for manual counting, which may have more errors compared with computer aided automated analysis. It is important to remember that this result is an estimation of the uNK cells density according to the area occupied by uNK cells and stromal nuclei, and not the exact percentage of uNK cells density. However, validation has shown that the correlation was very strong and results were similar. This may be because the cytoplasmic staining of DAB equated to the area occupied by the nuclei of cells overall. As the function of the results was for screening women who may have high uNK counts, an estimate of the uNK cells density may be adequate. The estimate of uNK cells density was achieved with a significantly faster turnaround compared with the current SAIA method. In borderline cases, the combined method of manually counting uNK cells and digitally calculating stromal cells could be used to obtain the exact percentage. To maintain the standard between observers, results from the CDAM method should be intermittently confirmed with the SAIA method, to ensure that they remain similar to each other.

This CDAM method has the advantage of being significantly faster, while maintaining similar intraobserver errors of 0.5%, with similar 95% confidence intervals. It is important for the analysis of uNK cells density to be time efficient, if this becomes a universally available screening test in the future. Furthermore, the CDAM method was also more cost effective as the whole process involved only the utility of Image J, a freely downloadable software, and removed the need to purchase specialist image processing programmes such as Adobe photoshop.

A limitation of this new method is the dependence on the staining intensity of the DAB and haematoxylin on uNK cells and nuclei. However, this holds true for any analysis of IHC staining. Thus, it is vitally important to have a positive control slide for each staining run, to check the consistency of stain intensity in each IHC cycle. There are also other variables that affect uNK cells density measurements such as number of days since LH surge, fixation method, antibody clone and detection system used, depth of endometrium where counting is performed and

exclusion of other structures including glands, vessels and clusters. However, these other variables could be standardised between laboratories and quality control is an achievable objective. Similarly to any other analysis and new skills, this method would require training. But, the review of results by a new observer can be readily achieved as the threshold results are documented and can be reevaluated and reassessed.

5.5 Conclusion

We have shown that this new method of CDAM is a high through-put, reliable and accurate estimation of uNK cells density that can be used as the alternative method of screening for women with RM for selection into the pilot RCT. Apart from utility in the trial, it could potentially be used, subsequent to individual validation to the 'gold standard' method currently used, for the quantification of uNK cells in other studies. Although this method gives an estimation of uNK cells density rather than the exact percentage, results have shown that they are both strongly correlated. If there were uncertainty with the results, then the manual or gold-standard method could be used to get the exact percentage to confirm or disprove the results.

Future studies can be designed to further develop a fully automated and reliable method for analysis of uNK cells density using this concept. This will move towards a standardised, reproducible method of uNK cells analysis for comparison and evaluation of different studies from different institutions, if uNK cells test become important for screening in women with RM in the future.

CHAPTER 6

CONCLUSION OF THESIS

CHAPTER 6 – CONCLUSION OF THESIS

6.1 Endometrial pathology in recurrent miscarriage

The endometrium is a rich source of cells and cytokines that are involved in the essential processes such as implantation of the conception and nurturing of the developing fetus for the outcome of a successful pregnancy. The signalling pathways in the endometrium could render the endometrium unreceptive to implantation or lead to defective implantation. Many of these processes are not yet fully understood and more studies are needed to comprehend the endometrial physiology and pathologies in RM, and hopefully lead to development of further treatment for these women.

Understanding the decidualisation process in the endometrium is difficult as it is ethically wrong to obtain endometrium sample at the cycle of implantation for that index pregnancy. Currently, much evidence is extrapolated from studies of endometrium prior to the conception cycle or from animal studies. There is a need to further develop invitro models to simulate the decidualisation and implantation process to have better understanding of interactions between the embryo and endometrium. Focus could be on endometrial immune cells (uNK cells, macrophages and T cells), and stromal cells which are in abundance in the endometrium, and their relationship to RM. Endometrial explants of receptive and non-receptive endometrium can also be developed to compare and comprehend differences between them.

Uterine NK cells are the most abundant leucocyte in the endometrium during the critical period for implantation and there is evidence for biological plausibility that uNK cells play a role in this intricate process. UNK cells have been shown to contribute to the endometrial and decidual environment during menstrual cycles and pregnancy. However, they are difficult to study due to the transient nature and cycling of these cells with the menstrual cycle. But, experiments are improved with development in co-culture models. Further investigations into uNK cells biology, their role in angiogenesis and vascular remodelling, including the array and pattern of growth factors and cytokines released, and interaction with the other cells in the

endometrium is needed. Much focus has been on their role in reproduction but investigations need to also target potential roles of uNK cells in the non-pregnant endometrium. Recent studies have also shown inter-cycle variability in uNK cells density which needs to be further explored[118]. The study was carried out in a small number of women with reproductive problems and it needs to be known if this variation also exists in the normal population, and whether this variation is consistent in every cycle. The degree of biological variation in women with reproductive problems compared to normal fertile women needs further analysis as this may have implications to the differences of uNK cell density reported in both these groups of women. The endometrial pipelle biopsy samples the superficial functional layer of the endometrium and studies needs to examine differences in the uNK cell density, and variation in different layers of the endometrium. Greater understanding of the biology of uNK cells will provide better insight into whether they serve as a marker of endometrial immune pathology.

6.2 Clinical implications of results

Our systematic review, updated to March 2014, found insufficient evidence for the association of abnormal uNK cells density with adverse pregnancy outcomes in women with RM. There were only 2 studies that were found to have investigated this and they had contradictory results where one study reported that high uNK cells were predictive of further miscarriage while the other did not. There is also currently no consensus on the definition of an abnormal test result. Similarly for pNK cell tests, there is still inadequate evidence for its utility as a marker to select women with RM for treatment. This conclusion was echoed in another systematic review on NK cells performed by a group in London[163]. In the feasibility trial, women with uNK cells density of more or less than 5% had similar number of previous miscarriages, further questioning the meaning of an abnormal test result. Therefore, women with RM should not be offered any uNK or pNK cell assessment as part of a diagnostic work-up in a clinical setting. More importantly, they should not be offered any treatment based on test results of uNK or pNK cells in an attempt for a positive outcome as there is currently no adequately powered, conclusive RCT

that has proven efficacious. Any NK cells analysis and immunotherapy should only be performed in the context of clinical research.

In the pilot trial although 85% of women expressed that they preferred the active medication if given a choice, 66% returned voluntarily for randomisation into the trial when pregnant. This demonstrated that women were in favour and in support of trials to look for a test that can identify pathology for targeting treatment. Hence, they should be encouraged to participate in well-designed research studies and be supported throughout these trials, rather than be prescribed empirical treatment.

6.3 Feasibility of future clinical trials

There is a call for '*a specific assay to diagnose immune-mediated early pregnancy loss and a reliable method to determine which women might benefit from manipulation of the maternal immune system*'[52]. This pilot phase assessing patient acceptability of the trial has shown that it is feasible to recruit for a 'screen and treat' trial as the majority of women returned for randomisation despite wanting the active medication. A weakness of this thesis was that the trial was designed before the results of our systematic review. A suggestion of a future trial would be a change in methodology to include assessment and analysis of both uNK and pNK cells (numbers and activity), and to randomise all women regardless of the NK cell results to either prednisolone or placebo when pregnant. Every woman recruited into the trial should be followed up to get details on whether they get pregnant, and if they do, what the pregnancy outcome is. A trial design of this nature would also allow one to obtain the optimal cut-off of uNK or pNK cells tests by using the ROC analysis to discriminate between outcomes of live birth or miscarriage. This would add to the literature about levels of normality of NK cells which is currently limited. Furthermore, it will also show if any of these tests are useful, and if they are, which one is ideal. Reassuringly, there were minimal side effects from prednisolone treatment and all completed treatment. Research should also examine the mechanisms of how prednisolone could exert its effect on the pregnancy outcome.

This pilot trial showed that the analysis of uNK cells density was time consuming, with a wide variability depending on the uNK density (16-47 minutes). For uNK cells test to be developed to allow for a quick turnover of results when conducting a large trial, analysis need to be efficient and reliable. Thus, an alternative method for quantifying uNK cells was validated using the freely available Image J. This new CDAM method may also be used as a standardised method for the analysis and reporting of uNK cells in other studies, to allow for comparison between different institutions.

6.4 Final Conclusion

With current progress in the field of medicine, it is difficult to accept that more than half of women with RM are informed that there is no explanation for the miscarriages, and counseled about the lack of available evidence in most treatments. There is good evidence that the endometrium plays an important role in the regulation of implantation, with biological plausibility that uNK cells participates in this process. Research should be targeted at further understanding of uNK cell distribution, function and regulation. It is possible that uNK cells function as a marker of poor decidualisation, but the definition of normality is first needed. The systematic review on the association of uNK cells and adverse pregnancy outcomes revealed insufficient studies with small numbers of women included in each study, demonstrating the need for more studies in this field.

The best verification of effectiveness of treatment is results from adequately powered RCTs. This pilot RCT has demonstrated that women with RM are in favour of studies looking for a cause for their condition prior to initiating treatment. Although time consuming, it is realistic and feasible to recruit for screen and treat trials as suggested above. This may pave the way towards finding another treatment for RM with an endometrial immune cause, and women with RM should be encouraged to participate. Future trials will hopefully be able to confirm or refute the role of uNK cells, or pNK cells as a clinically useful marker for screening women with RM, and the efficacy of prednisolone as a new treatment option.

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APPENDICES

Ai-Wei Tang, University of Liverpool / LWH

Search History (12 searches) (Click to close)

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	# ▲	Searches	Results	Search Type	Actions
☐	1	exp Killer Cells, Natural/	31514	Advanced	Display More >>
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☐	3	(natural adj killer).ab,hw,ot,ti,nm.	25858	Advanced	Display More >>
☐	4	(nk adj cell$).ab,ti,ot,hw,nm.	21206	Advanced	Display More >>
☐	5	4 or 1 or 3 or 2	44759	Advanced	Display More >>
☐	6	(uterus or uterine or peripheral or endometrial or decidual).ab,ti,ot,hw,nm.	575141	Advanced	Display More >>
☐	7	6 and 5	10804	Advanced	Display More >>
☐	8	exp Reproduction/	820531	Advanced	Display More >>
☐	9	exp Pregnancy Complications/	290116	Advanced	Display More >>
☐	10	8 or 9	847981	Advanced	Display More >>
☐	11	7 and 10	1046	Advanced	Display More >>
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Remove Selected | Save Selected | Combine selections with: And | Or

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Liverpool Women's NHS
NHS Foundation Trust

A randomised controlled trial of prednisolone for women with recurrent miscarriage and high levels of uNK cells in the endometrium. (Version 6, 1/05/08)

You are being invited to take part in a research project. Here is some information to help you decide whether or not to take part. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything you do not understand or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study?

Recurrent Miscarriage (RM) can be a very distressing condition to the woman and her partner. In half of the cases we do not know the reasons why it happens. We would like to find out if certain cells that are called the Natural Killer (NK) cells from the lining of the womb (endometrium) contribute to the miscarriage. Natural killer cells are white blood cells that help to fight against infections, viruses and cancer. However, we have discovered that some women with recurrent miscarriage have high levels of NK cells in their womb. Furthermore, we have found that a steroid drug, known as prednisolone decreases the number of these NK cells. We now want to do a further trial to see if the steroid treatment increases the chance of you having a live baby.

Why have I been chosen?

You have been chosen because you have a history of recurrent miscarriage where the cause has not been found. A maximum of 700 patients will be studied over the period of 10 years.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?

Step 1 screening with endometrial biopsy

If you decide to take part, a small sample of the lining of your womb will be taken on Day 21 of the menstrual cycle. The sample will be taken in a similar manner to taking a cervical smear. You may experience some mild discomfort whilst the biopsy is being taken. The risk of any damage to your womb during the procedure is absolutely minimal and if any damage should occur it will heal spontaneously. If we took the biopsy whilst you are pregnant, it could possibly cause a miscarriage but the risk is very small. ***Therefore, you need to use barrier method of contraception in the month that you have your biopsy (for example use condoms).*** The biopsy will be analysed for the NK cells and various other cells that line your womb. You have a 30% chance of having high levels of NK cells.

Step 2 trial of prednisolone therapy against placebo

If you have high levels of NK cells then, you will be asked to ring the NK cell study answering machine when you are pregnant. You will be telephoned back within one day and given an appointment for a scan. If the scan shows that the pregnancy is in the uterus, you will be asked if you would agree to enter the trial. That is, you will be entered into one of two groups randomly. One group will receive a packet containing prednisolone daily for 8 weeks, and the other group will be given a packet containing identical dummy tablets. You will not know which drug you are taking and neither will your doctor (although if your doctor wants to find out he/she can do so). You may experience minor side effects of steroids such as mood changes, weight gain, increased appetite, indigestion, high blood pressure and high blood sugar levels and a rare complication called avascular

necrosis (This causes severe pains on your hip joint). You will be kept under regular review whilst you are on the medication. You will be contacted via telephone at 14 weeks gestation, and asked about any side effects you had.

The prednisolone is thought to cause the developing fetus very little harm. However, there is some evidence to suggest that it could be associated with cleft lip and poor growth in the uterus.

What are the possible benefits of taking part?
The information we get from this study will help us to find out if prednisolone is an effective treatment for women with recurrent miscarriage and high levels of NK cells.

We would like to keep your sample for examination of other factors in the lining of your womb that may be important in miscarriage. This part of the research is at an early stage hence we will not be able to give you the results. If you agree to this you can sign an extra box on the consent form. We would like to keep this sample for up to ten years.

What if something goes wrong?
If taking part in this research project harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

Will my taking part in this study be kept confidential?
All information collected about you during the course of the research will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognised from it. Dr Quenby or another doctor involved in the research project may look at your medical notes and those of any baby born, to obtain information relevant to this study. Because you may be taking this medication, your GP will be notified of your participation in the study and a medical alert card will be given to you stating that you may be taking prednisolone or placebo drug.

What will happen to the results of the research study?
The results will be published in scientific journals and presented at conferences to further our understanding of recurrent miscarriage. All published data will be anonymous.

Who is organising and funding the research? Liverpool Women's Hospital. The Moulton Charitable Trust

Who has reviewed the study? The Liverpool Research Ethics Committee

Contact for Further Information
Dr. S. Quenby, Liverpool Women's Hospital, Crown Street, Liverpool L8 7SS
Tel: Dr Quenby's Secretary 0151 702 4271

The Miscarriage Association: http://www.miscarriageassociation.org.uk/
helpline: 01924 200799 (Mon-Fri, 9am - 4pm)

Thank you for reading this. You will be given a copy of this information and a signed consent form to keep should you decide to take part.

What to do:

GP/Consultant letter

⇩

Letter with information to patient

⇩

Need to get ovulation kit

⇩

Ring when ovulate 0151 702 4180

⇩

- **See patient 7 days later in Liverpool**
- **Take medical history**
- **Discuss uNK cells investigation via endometrial biopsy**

⇩

- **Transvaginal ultrasound scan**
- **Explain test and consent**
- **Take endometrial biopsy**

⇩

- **Letter sent to patient, with the result**
- **Telephone consultation to discuss the results with Dr Quenby or Dr Tang (Research Fellow)**

Version 4

Liverpool Women's NHS

NHS Foundation Trust

uNK CELL CLINIC

PREDNISOLONE TRIAL

FOR RECURRENT MISCARRIAGE

Dr Siobhan Quenby
Reader/ Consultant Obstetrician
Liverpool Women's Hospital
Crown Street
Liverpool
L8 7SS
0151 702 4271

Information

- Recurrent miscarriages are very distressing and in most cases there is no cause found for these.
- We have found that some women who have miscarriages have too many uterine Natural Killer cells (uNK) in the lining of their womb.
- We have found that a steroid drug, known as Prednisolone can decrease the number of uNK cells. However, we do not know whether Prednisolone stops miscarriage.
- We have developed an accurate method of counting uterine NK cells using a computer generated image analysis.
- We are offering a service where you can get your uNK cells tested by performing a biopsy from the lining of your womb.

Who will I see?

- You will be seen by Dr Quenby or Dr Tang (Research Fellow) at your visit.

What will happen at my appointment?

- Consultation with Dr Quenby or Dr Tang (Research Fellow).
- Transvaginal ultrasound scan (need empty bladder)
- Endometrial biopsy taken.

How is the test done?

- The sample is taken in a similar way to a cervical smear, you may experience some mild discomfort whilst your biopsy is being taken.
- It is important that you are not pregnant when the procedure is performed, therefore you need to use barrier methods, such as **condoms, 4 weeks prior to having your sample taken.**

Possible complications

- The risk of any damage to your womb during the procedure is absolutely minimal. If any damage should occur, it should heal without further treatment. If the sample is taken whilst you are pregnant, it could possibly cause a miscarriage, but the risk is very small.

How long do the results take?

- The results should be available within 5-6 weeks.

If I want to have the test done, what should I do next?

- Buy an ovulation kit
- Use barrier method of contraception
- Contact patient services on 0151 702 4180 when you ovulate to make an appointment.

Cost

There is no cost involved if you fit the criteria for inclusion in the trial

Criteria for inclusion in the trial

- Between the ages of 20 – 40 years
- 3 consecutive miscarriages, with no cause found
- Referral from either your GP/ Consultant
- You have no contraindications to taking Prednisolone

For more information:

Trial Website: www.prednisolonetrial.org.uk
Diane- secretary to Dr Quenby on 0151 702 4271

PREDNISOLONE TRIAL

FOR RECURRENT MISCARRIAGE WITH HIGH NK CELLS

Liverpool Women's Hospital is in the process of recruiting patients with recurrent miscarriage who have high levels of uterine NK cells to a Randomised Control Trial of Prednisolone vs Placebo.

To qualify for the trial, patients must first be screened to assess if they have a high level of NK cells in the endometrium. This is done by taking a pipelle endometrial sample 7 days after the patient ovulates.

Thus, we hope that you will help in our recruitment by contacting us with patients who are interested to be screened so that we can make arrangements to see the patient.

Entry Criteria for screening:

- Age 20-40 years old
- 3 consecutive 1st Trimester miscarriages with no cause found
- Has no contraindications to taking steroids (eg: BMI>35, Diabetic, Hypertension, Severe mental illness)

Thank you very much.

Sincerely,

Dr. Ai-Wei Tang
Cinical Research Fellow
Contact details: 0151-7089988 (Bleep 115)

Dr. Siobhan Quenby
Consultant Obstetrician
Contact details: Secretary (Diane - 0151-7024271)

Liverpool Women's NHS
NHS Foundation Trust

Study Number: LWH0606
EudraCT Number: 2005-003307-36
Patient Identification Number for this trial:

CONSENT FORM 1 screening

Title of Project: A randomised controlled trial of prednisolone for women with recurrent miscarriage and high levels of uNK cells in the endometrium.

Name of Researcher: **Dr Siobhan Quenby**

Please initial box

1. I confirm that I have read and understand the information sheet dated 01/5/8 (version 6) for the above study. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my or my child's medical notes may be looked at by responsible individuals from Liverpool Women's NHS Foundation Trust or from regulatory authorities. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the screening investigation (endometrial biopsy) for above study. ☐

5. I agree for my endometrial sample to be stored for further miscarriage research ☐

Name of Patient	Date	Signature
Name of Person taking consent (If different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient. 1 copy for research. 1copy to be kept with hospital notes

Contact for Further Information
Dr. S. Quenby, Liverpool Women's Hospital, Crown Street, Liverpool L8 7SS
Tel: Dr Quenby's Secretary 0151 702 4271

Liverpool Women's NHS
NHS Foundation Trust

Study Number: LWH0606
EudraCT Number: 2005-003307-36
Patient Identification Number for this trial:

CONSENT FORM 2 randomisation

Title of Project: A randomised controlled trial of prednisolone for women with recurrent miscarriage and high levels of uNK cells in the endometrium.

Name of Researcher: **Dr Siobhan Quenby**

Please initial box

1. I confirm that I have read and understand the information sheet dated 01/5/8 (version 6) for the above study. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my or my child's medical notes may be looked at by responsible individuals from Liverpool Women's NHS Foundation Trust or from regulatory authorities. I give permission for these individuals to have access to my records. ☐

4. I agree to take part and be allocated to prednisolone or placebo in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent (If different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient. 1 copy for research. 1copy to be kept with hospital notes

Contact for Further Information
Dr. S. Quenby, Liverpool Women's Hospital, Crown Street, Liverpool L8 7SS
Tel: Dr Quenby's Secretary 0151 702 4271

PREDNISOLONE TRIAL

A randomised controlled trial of prednisolone for women with recurrent miscarriage and high levels of uNK cells in the endometrium

Lab ID: ☐

You will first be screened to assess the level of uNK cells. If you are found to have high levels of uNK cells, you will then be randomised to either prednisolone or placebo for the trial.

Do you think you will have high uNK cells (>5%)? Yes ☐ No ☐

IF you had a choice, which group would you prefer to be in?

- Pregnancy support with Prednisolone ☐
- Pregnancy support with Placebo ☐
- No preference ☐

Thank you very much for your help.

Kind regards,

Dr. Siobhan Quenby / Dr. Ai-Wei Tang
Prednisolone Trial Researchers

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Section 1: Randomisation

Lab biopsy number_____

Patient ID Affix ID (hospital unit) sticker here:

Inclusion/exclusion criteria

Inclusion	**Yes**	**No**
pregnant		
Aged between 20 and 40 years		
uNK level more than 5%		
More than 2 consecutive miscarriages?		
Signed consent form		
Exclusion	**Yes**	**No**
Cause for miscarriage found?		
Contraindication to prednisolone*?		

*contraindications include obesity (BMI>35); mental illness; diabetes; hypertension.

If you have ticked ANY of the shaded boxes, the woman is not eligible for the trial (please file this sheet in her notes), otherwise please continue to randomise:

Study number:

Agree to randomise Y/N

Steroid card given? Y/N

If NO: Steroids unacceptable Y/N

Placebo unacceptable Y/N

Other Y/N

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Section 2: Demographics

Lab biopsy number_____

Patient ID Affix ID (hospital unit) sticker here:

Patient demographics

Age:
BMI:
uNK level (%):
Smoking status:
Height (cm):
Ethnicity:
Weight (kilos):
Parity:
No. live births:
No. 1st empty sacs:
No. fetal losses:
No. second trimester miscarriages:
No. ectopics:
No. top:
No. moles:
No. SB/NND:

Current pregnancy

LMP:

Date pregnancy test positive:

Regularly taking folic acid? **Y/N** Dose: **400mcg / 5mg**
Regularly taking aspirin? **Y/N**

Date randomised:

Ultrasound findings: Sac diameter_____ mm FH: **Y/N** Yolk sac: **Y/N**

Date started trial tablets:

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Section 3: 14 week gestation follow up

Miscarried: Y/N

If miscarried:

Date:

Ultrasound findings:

Karyotype:

Took the whole course of tablets? **Y/N**
If no, how many missed? _____
Why missed tablet? _____

If discontinued, why? _____

Is tablet bottle returned to pharmacy? **Y/N**

Weight: BMI: BP: Urinanalysis:

If not miscarried, please ask the woman about the following side effects:

Side effects	Yes	No
1. Insomnia		
2. Mood changes		
3. Infections		
4. General stomach problems (e.g. indigestion)		
5. Joint pain		
6. Bruising		
7. Flushing		
8. Acne		

Other side effects:

_____

_____

Any pregnancy complications?

_____

_____

Please explain to the woman the need for a growth scan at 28 and 34 weeks gestation, respectively.

Obstetric Consultant where pregnancy will be booked

Hospital:
Contact number:

Section 4: After delivery

Please complete 6 weeks after expected date of delivery (EDD: _____)

Patient ID:

Obstetric Consultant: _____

Hospital: _____

Hospital contact: _____

Patient Contact: _____

Growth scan findings 28 weeks:

34 weeks:

Did the woman think she had prednisolone: **Y/N**

Delivery

Date:	Gestation:	Mode of delivery:
Hospital where baby delivered:		
Alive? **Y/N**	Weight (g):	Sex: **M/F**
Any abnormalities (please explain):		
Admission to NICU? **Y/N**		
Did the baby have a routine baby check/neonatal exam? **Y/N** If yes, by whom? Date: Findings:		
Has the baby had any problems since being born? If yes, what (please explain)?		
Any hospital visits? **Y/N**	If yes, reasons & dates (approximate)?	
Any hospital admissions? **Y/N**	If yes, reasons & dates (approximate)?	
If there are any problems, who is the best doctor to contact for further information (e.g. GP or Paediatrician etc)? Contact details:		

Participant diary

A randomised controlled trial of prednisolone for women with recurrent miscarriage and high levels of uNK cells in the endometrium

Please place woman's unit sticker below:

To the participant: Please use this diary to record details of any symptoms/illness/adverse reactions you might experience during your participation in the prednisolone trial. This booklet should be taken with you to any consultations or appointments you have with a doctor (your GP or at hospital) and shown to your doctor for their information.

At the end of your participation in the trial, this diary should be returned to Siobhan Quenby at Liverpool Women's NHS foundation Trust.

To the medical practioner: this woman is participating in a clinical trial of prednisolone versus placebo for recurrent miscarriage. Contact Siobhan Quenby at Liverpool Women's NHS Foundation Trust if you require further details (0151 702 4271)

Date	Description of symptoms	Medical treatment or advice sought (e.g. GP or hospital visit?)	Diagnosis (if applicable)	Outcome (e.g. end date, or treatment ongoing)

Liverpool Womens NHS
NHS Foundation Trust

PREDNISOLONE TRIAL

Medication Record Chart

Directions on how to take your medication please tick each day on your chart to remind you that you have taken your tablets.

Day number and quantity	√	Day number and quantity	√
Day 1 Four tablets		Day 29 Four tablets	
Day 2 Four tablets		Day 30 Four tablets	
Day 3 Four tablets		Day 31 Four tablets	
Day 4 Four tablets		Day 32 Four tablets	
Day 5 Four tablets		Day 33 Four tablets	
Day 6 Four tablets		Day 34 Four tablets	
Day 7 Four tablets		Day 35 Four tablets	
Day 8 Four tablets		Day 36 Four tablets	
Day 9 Four tablets		Day 37 Four tablets	
Day 10 Four tablets		Day 38 Four tablets	
Day 11 Four tablets		Day 39 Four tablets	
Day 12 Four tablets		Day 40 Four tablets	
Day 13 Four tablets		Day 41 Four tablets	
Day 14 Four tablets		Day 42 Four tablets	
Day 15 Four tablets		Day 43 Two tablets	
Day 16 Four tablets		Day 44 Two tablets	
Day 17 Four tablets		Day 45 Two tablets	
Day 18 Four tablets		Day 46 Two tablets	
Day 19 Four tablets		Day 47 Two tablets	
Day 20 Four tablets		Day 48 Two tablets	
Day 21 Four tablets		Day 49 Two tablets	
Day 22 Four tablets		Day 50 One Tablet	
Day 23 Four tablets		Day 51 One tablet	
Day 24 Four tablets		Day 52 One tablet	
Day 25 Four tablets		Day 53 One tablet	
Day 25 Four tablets		Day 54 One tablet	
Day 27 Four tablets		Day 55 One tablet	
Day 28 Four tablets		Day 56 One tablet	

VIABILITY SCAN RECORD

Date randomised:

Date	Sac Size	CRL	FH	Yolk Sac	Gestation	?Problems

Human Reproduction, Vol.26, No.8 pp. 1971–1980, 2011
Advanced Access publication on May 25, 2011 doi:10.1093/humrep/der164

human reproduction

META-ANALYSIS ***Early pregnancy***

Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: a systematic review

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BACKGROUND: Peripheral natural killer (pNK) and uterine NK (uNK) cells have been associated with reproductive failure. We systematically reviewed the literature to assess whether numbers or activity of pNK or uNK cells predicted subsequent pregnancy and outcome.

METHODS: We searched the electronic MEDLINE database from 1950 to April 2010 for relevant publications by using MeSH terms 'natural killer cells', 'reproduction' and 'pregnancy complications'. We included studies that measured pre-pregnancy pNK and uNK cell numbers or activity in women with recurrent miscarriage (RM) or infertility, and reported subsequent pregnancy outcomes of miscarriage or failure to conceive after assisted reproductive technology (ART).

RESULTS: The search identified 783 publications and 12 fulfilled the inclusion criteria. There were too few women entered into the observational studies to assess whether high pNK cell percentages or activity predicted subsequent miscarriage in women with idiopathic RM (numbers: $n = 32$, OR 17, 95% CI 0.82–350.6, activity: $n = 92$, OR 2.51, 95% CI 0.16–40.29), or implantation failure ($n = 203$, OR 1.35, 95% CI 0.28–6.46), or miscarriage in infertile women after ART ($n = 79$, OR 2.48, 95% CI 0.50–12.32). Similarly, the studies of uNK cells were not large enough to assess whether abnormal uNK cell density predicted subsequent miscarriage in women with idiopathic RM ($n = 72$, OR 1.33, 95% CI 0.16–11.11). None of the uNK cell studies in women with infertility reported pregnancy outcomes dichotomized for uNK cell numbers.

CONCLUSIONS: The prognostic value of measuring pNK or uNK cell parameters remains uncertain. More studies are needed to confirm or refute the role of NK cell assessments as a predictive test for screening women who may benefit from immunotherapy.

Key words: systematic review / natural killer cells / recurrent miscarriage / infertility / pregnancy outcomes

Introduction

Immunological mechanisms have been thought to play a role in reproductive problems such as recurrent miscarriage (RM) (defined as three or more consecutive miscarriages), infertility and implantation failure. This implies that a successful pregnancy involves maternal adaptation of the immune response to the semi-allogenic developing embryo.

Natural killer (NK) cells are part of the innate immune system, and are found in both peripheral blood and endometrium. Although both peripheral NK (pNK) and uterine NK (uNK) cells express the surface antigen CD56, pNK cells are phenotypically and functionally different from uNK cells and <10% of pNK cells resemble uNK cells (Moffett-King, 2002). Furthermore 90% of pNK cells are $CD56^{dim}$ and $CD16^{+}$ whereas 80% of uNK cells are $CD56^{bright}$ and $CD16^{-}$ (Nagler *et al.*, 1989; King *et al.*, 1991).

Peripheral NK cells ($CD56^{dim}$) have been demonstrated to show significant cytotoxic activity with well-established antiviral and antineoplastic functions, while uNK cells have little cytotoxic activity, but are a rich source of cytokines, particularly angiogenic ones, with possible roles in regulation of trophoblast invasion and angiogenesis (Bulmer and Lash, 2005; Dosiou and Giudice, 2005). A high proportion of peripheral NK cells may not reflect the condition of the endometrium where implantation occurs and the mechanism of how these cells can be associated with miscarriage is unclear. In contrast large numbers of uNK cells appear in the mid-secretory phase although the mechanism is still not known. There are two theories:

recruitment from pNK cells which subsequently differentiate in the uterine microenvironment into uNK cell phenotype through a series of organized processes, or uNK cells come from the *in utero* proliferation and differentiation of stem cells or endogenous NK cells in the endometrium (Bulmer and Lash, 2005; Kitaya *et al*., 2007). The former theory is the rationale for testing pNK cells although the latter is the more widely held view. In contrast to pNK cells, uNK cells are resident in the endometrium and constitute 70% of endometrial leucocytes, the most predominant leucocyte population during the time of implantation and early pregnancy (Bulmer *et al*., 1991). They are adjacent to fetal trophoblast cells in the maternal–fetal interface and express receptors such as killer-cell immunoglobulin-like receptor (KIR), immunoglobulin-like transcript-2 (ILT2) and NKG2, for fetal trophoblast antigens, human leucocyte antigen-C (HLA-C), HLA-E and HLA-G (Moffett-King, 2002). Thus, pNK and uNK cells have different surface antigens, functions and receptors and hence, should be considered separate entities.

There have been a series of case–control studies reporting an association between pNK cell numbers (Kwak *et al*., 1995; Ntrivalas *et al*., 2001; Yamada *et al*., 2003) or activity (Aoki *et al*., 1995; Shakhar *et al*., 2003) with RM, as well as some studies that have shown no difference in pNK cells parameters between RM and controls (Emmer *et al*., 2000; Souza *et al*., 2002; Wang *et al*., 2008). Similarly, there have been case–control studies reporting an association between uNK cells and RM (Clifford *et al*., 1999; Quenby *et al*., 1999, 2005; Tuckerman *et al*., 2007), but others, that have included women with only two miscarriages, have failed to find this association (Michimata *et al*., 2002; Shimada *et al*., 2004).

There is also inconsistency in the association of pNK cells and uNK cells with infertility. Some groups have found an association of pNK cells and infertility (Beer *et al*., 1996; Matsubayashi *et al*., 2001; Ntrivalas *et al*., 2001) while some have not (Vujisic *et al*., 2004). Likewise for uNK cells, one group has reported an association with infertility (Ledee-Bataille *et al*., 2005) and another has found no difference (Matteo *et al*., 2007).

Evidence for a causative role for NK cells test in reproductive problems would be considerably improved if the tests of pre-pregnancy NK cell numbers or activity predicted subsequent pregnancy outcome. Thus, our aim was to perform a systematic review of the current literature to ascertain the relationship between pre-pregnancy NK cell tests results and outcomes of miscarriage, live births or implantation failure in women with RM or infertility requiring assisted reproductive technology (ART).

Materials and Methods

Data sources

We searched the electronic MEDLINE database through OvidSP from 1950 to April 2010 for published literature in all languages. The MeSH terms 'natural killer cells', 'reproduction' and 'pregnancy complications' were exploded. To identify relevant citations about NK cells, we used terms 'CD56', 'uterus', 'uterine', 'endometrial', 'decidual' and 'peripheral', in addition to 'NK cells'. Search terms such as 'abortion/miscarriage', 'ectopic pregnancy', 'fetal death', 'fertilization', 'insemination', 'live birth', 'pregnancy', 'pregnancy outcome' and 'stillbirth', in relation to pregnancy outcomes were under the MeSH tree of 'reproduction' and 'pregnancy complications', to retrieve all papers relevant to NK cells and reproductive outcomes. The search was then limited to humans and females. Advice was sought from the Trials search coordinator of the Cochrane Collaboration Pregnancy and Childbirth Group with regards to the development of the search strategy protocol, who advised on the terminology and methods of searching. The Trials search coordinator was not involved in the study selection of the articles, or data extraction.

The abstracts for all the citations were retrieved and assessed for their suitability for inclusion. Papers that were published in other languages all had abstracts in English. Original articles of abstracts where relevance could not be judged from the abstract alone were obtained for detailed analysis. Additionally, the reference lists of the publications identified were examined for possible studies not included in the initial search.

Study selection

Review articles, letters and studies with no pregnancy outcomes reported were excluded after reading the abstracts. This review also excluded studies that reported on treating women with immunotherapy such as prednisolone or intravenous immunoglobulin (IVIG) as prednisolone reduces both uNK (Quenby *et al*., 2005) and pNK (Thum *et al*., 2008) cells and IVIG alters pNK cell parameters (Morikawa *et al*., 2001b), which may either positively or negatively affect the pregnancy outcome, and distort the results of this review. Furthermore, these treatments are still experimental without evidence from methodologically sound randomized controlled trials (RCTs).

Inclusion criteria were studies that identified NK cells using the CD56 marker, either $CD56^{+}$, $CD56^{bright}$ or $CD56^{dim}$, the CD69 activation marker or NK cells activity measured by $Chromium^{51}$ release cytotoxicity assay, and investigated women with RM (defined as two or more consecutive miscarriages) or women with infertility seeking ART.

Two reviewers (A.T. and S.Q.) read through all the papers selected for detailed evaluation. Study quality was assessed using The Guidelines Manual 2009 published by National Institute for Health and Clinical Excellence (NICE). Information was obtained for the inclusion and exclusion criteria of women in the study, the source and method of analysing NK cells, the percentage/number and activity levels of NK cells, the level of normality of NK cells in the unit, and pregnancy outcomes.

Publications were divided into four groups according to the source of NK cells and type of reproductive failure: pNK cells test in RM, pNK cells test in infertility, uNK cells test in RM and uNK cells test in infertility. Pregnancy outcomes of implantation failure (defined as no positive pregnancy test after ART), miscarriage <24 weeks gestation, live births or implantation success (positive pregnancy test) leading to either miscarriage of <24 weeks gestation or live births were collected for women in all these groups. Data were extracted from texts, tables and graphs of each of the included studies. Original data in our unit were re-examined by classifying the pregnancy outcomes according to the cut-off of 5%, published in a later article (Quenby *et al*., 2005). When appropriate, meta-analyses were performed using Review Manager (RevMan) Version 5.0 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration 2008) on studies reporting pregnancy outcomes according to predetermined cut-offs for normality of NK cell parameters in similar patient groups. In the presence of significant heterogeneity, random effects have been used to pool the results. The results were reported according to the Meta-analysis of Observational Studies in Epidemiology (MOOSE) Guidelines (Stroup *et al*., 2000).

Results

There were altogether 783 citations identified. Seven hundred and eighty were from the search terms mentioned above and three were from the assessment of reference lists of these publications.

There were no non-English language publications that were found to be relevant to this review. The selection process leading to the included publications in the review are presented in Fig. 1. After reading the abstracts, 756 articles were excluded and 27 full text articles regarding pNK and uNK cells were retrieved for evaluation. After detailed analysis, 15 publications were excluded: three were letters, 6 did not report relevant outcomes (Kwak *et al.*, 1995; Ntrivalas *et al.*, 2001; Michou *et al.*, 2003; Putowski *et al.*, 2004; Matteo *et al.*, 2007; Thum *et al.*, 2007), 4 reported on studies using the same group of women for slightly different aspects of NK cells (Emmer *et al.*, 1999; Yamada *et al.*, 2001; Morikawa *et al.*, 2001a; Thum *et al.*, 2004) and 2 reported immunotherapy use in some women (Coulam *et al.*, 1995; Beer *et al.*, 1996). A total of 12 publications met the criteria for analysis. There were seven studies reporting on pNK cells (Table I), and six studies reporting on uNK cells (Table II), as one study investigated both pNK and uNK cells. Five publications reporting on pNK cells and two publications on uNK cells presented pregnancy outcomes, dichotomized into groups of high and normal levels of NK cell parameters, established from controls, as described in Tables I and II.

Only three studies (Lachapelle *et al.*, 1996; Quenby *et al.*, 1999; Tuckerman *et al.*, 2007), all investigating uNK cells, fit the definition of idiopathic RM where women were included after three consecutive miscarriages with no causes for the miscarriages found after routine investigations in their hospital. Three studies on pNK cells (Aoki *et al.*, 1995; Emmer *et al.*, 2000; Yamada *et al.*, 2003) and one study on uNK cells (Michimata *et al.*, 2002) included women after only two miscarriages, and one (Yamada *et al.*, 2003) included women with known associations of RM such as endocrine disorders, antiphospholipid syndrome (APS) and thrombophilia. The group for infertility included all women who underwent IVF treatment, regardless of the cause for infertility, apart from one study (Ledee-Bataille *et al.*, 2005), which included women after three cycles of implantation failure.

Peripheral NK cells

All studies on pNK cells used flow cytometry to investigate the numbers of pNK cells as a percentage of lymphocytes or leucocytes depending on the panel of monoclonal antibodies used to identify these cells, or ^{51}Cr release cytotoxicity assay to assess NK cells activity.

There were three studies (Aoki *et al.*, 1995; Emmer *et al.*, 2000; Yamada *et al.*, 2003) investigating pNK cells (number or activity) in women with RM, but only two studies included women with idiopathic RM (Aoki *et al.*, 1995; Emmer *et al.*, 2000). Emmer *et al.* (2000) reported pregnancy outcomes in relation to both number and activity of NK cells. It is likely that the same women were tested for both parameters and, therefore, these results were not pooled to avoid double counting. When the results of the different studies were pooled, there was significant heterogeneity between the studies with $I^2 = 85\%$. Therefore, random effects were used. High pNK cell numbers or activity did not predict miscarriage in a subsequent pregnancy in women with idiopathic RM as the meta-analysis of these studies did not reach statistical significance, although positive OR were found ($n = 22$, OR 17, 95% CI 0.82–350.60; $n = 92$, OR 2.51 95% CI 0.16–40.29; Fig. 2).

Of the four studies (Fukui *et al.*, 1999; Matsubayashi *et al.*, 2005; Thum *et al.*, 2005; Baczkowski and Kurzawa, 2007) that investigated pNK cells in women with infertility, two (Matsubayashi *et al.*, 2005; Thum *et al.*, 2005) reported outcomes of implantation failure after ART, dichotomized into high and normal levels of pNK cells, but used different parameters of pNK cell numbers and activity. High pNK cell parameters did not predict subsequent implantation failure ($n = 203$, OR 1.35, 95% CI 0.28–6.46; Fig. 3). The same two studies (Matsubayashi *et al.*, 2005; Thum *et al.*, 2005) also reported outcomes of miscarriage after implantation success, and the effect of abnormal pNK cell test results on the risk of subsequent miscarriage after implantation success was also uncertain as the meta-analysis was not statistically significant ($n = 79$, OR 2.48, 95% CI 0.50–12.32;

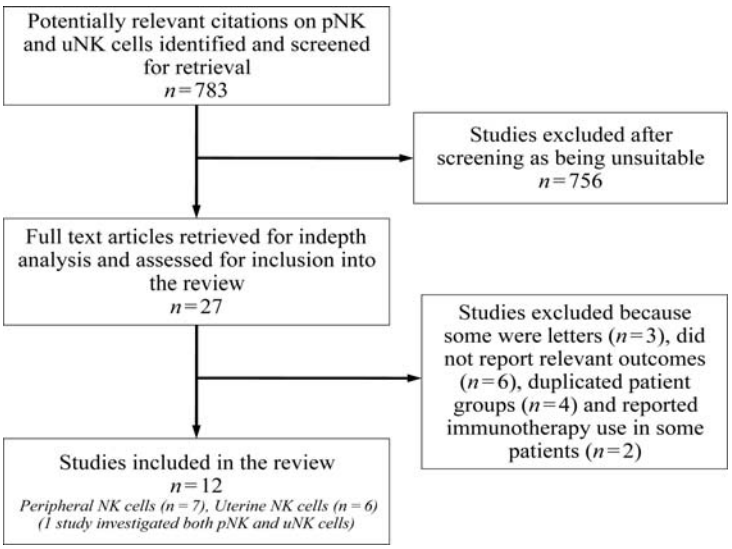

Figure 1 The selection process of the included studies.

Table I Included studies investigating peripheral NK cells (seven studies).

Study	*n*	Inclusion criteria	Method of analysis	Results
Recurrent miscarriage				
Aoki *et al.* (1995)	68	≥2 miscarriages; idiopathic RM	^{51}Cr release assay	Higher % NK activity in patients who miscarried
				Normal range—<41.8% NK activity determined by the mean +1 SD of 47 healthy controls with no history of miscarriages with pregnancy outcomes reported according to normal range
Emmer *et al.* (2000)	142	≥2 miscarriages; idiopathic RM	Flow cytometry, ^{51}Cr release assay	Normal range—<12% NK cells taken from publications by other groups and <322 lytic units for NK cells activity (no mention of how this was obtained) with pregnancy outcomes reported according to normal range
Yamada *et al.* (2003)	113	≥2 miscarriages; all RM	Flow cytometry, ^{51}Cr release assay	Higher % NK cells in miscarriage of normal karyotype and biochemical pregnancy compared with live birth
				Normal range—<16.4% $CD56^+$ cells and <46% NK activity determined by ROC curve for optimal discrimination between miscarriage (normal karyotype or biochemical pregnancy) and live birth
Infertility				
Fukui *et al.* (1999)	85 NK cells	Patients undergoing IVF	Flow cytometry	% NK cells
	297 NK activity		^{51}Cr release assay	Pre-IVF cycle—No difference in % NK cell and subpopulation in women with infertility who failed to get pregnant and those who became pregnant after ART
				% NK cell activity
				Pre-IVF cycle—No difference in % NK cell activity in women with infertility who failed to get pregnant and those who became pregnant after ART, and between miscarriage and live birth in those who were pregnant
				No normal range reported
Thum *et al.* (2005)	138	Patients undergoing IVF	Flow cytometry	No difference in % NK cell and NK cell subpopulation in women with infertility who failed to get pregnant and those who became pregnant after ART, and between miscarriage and live birth in those who were pregnant
				Normal range—<12% taken from publications by other groups with pregnancy outcome reported according to this normal range
Matsubayashi *et al.* (2005)	94	Patients undergoing IVF	^{51}Cr release assay	Higher % NK cell activity in women with infertility who failed to get pregnant and those who became pregnant after ART
				No difference in % NK cell activity between miscarriage and live birth in those who were pregnant
				Normal range—<44% as determined by the mean +1 SD of 94 healthy, age-matched controls with pregnancy outcomes reported according to normal range
Baczkowski and Kurzawa (2007)	58	Patients undergoing IVF	Flow cytometry	No difference in % NK cell in women with infertility who failed to get pregnant and those who became pregnant after ART
				No normal range reported

Fig. 4). There was again significant heterogeneity with I^2 of 84% between studies reporting implantation failure and I^2 of 46% for studies reporting miscarriage after implantation success, in women with infertility.

The other two studies (Fukui *et al.*, 1999; Baczkowski and Kurzawa, 2007) that investigated women with infertility, did not report pregnancy outcomes according to a predetermined cut-off of normality. Fukui *et al.* (1999) reported no difference in pre-pregnancy pNK cells number and activity between women who failed to get pregnant and those who became pregnant after ART, and between miscarriage and live birth in those who were pregnant after ART. Baczkowski and Kurzawa (2007) also found no difference in the percentage of pNK cells in these two groups of women.

uNK cells

All the samples in the studies were timed from the LH surge as uNK cells population vary throughout the menstrual cycle: scanty in the proliferative phase then increasing in numbers after ovulation and through the secretary phase (Bulmer and Lash, 2005). Thus, the mid-

Table II Included studies investigating uNK cells (six studies).

Study	*n*	Inclusion criteria	Method of analysis	Results
Recurrent miscarriage				
LaChapelle *et al.* (1996)	20	≥3 miscarriages; idiopathic RM	Flow cytometry	No difference in % total NK cells in women with miscarriage and ongoing pregnancy No normal range reported
Quenby *et al.* (1999)	22	≥3 miscarriages; idiopathic RM	Immunohistochemistry	Higher % NK cells in women with miscarriage compared with LB Normal range—<5% determined by 75th percentile of 18 control women who were attending for sterilization with two or more pregnancies and no miscarriages
Michimata *et al.* (2002)	17	≥2 miscarriages; idiopathic RM	Immunohistochemistry	No difference in number of NK cells/10 high power fields in women with miscarriage and LB No normal range reported
Tuckerman *et al.* (2007)	87	≥3 miscarriages; idiopathic RM	Immunohistochemistry	No difference in % NK cells in women with miscarriage and LB Normal range—<13.8% determined by the 90th percentile of 10 control women with regular menstrual cycle not on hormonal contraception
Infertility				
Fukui *et al.* (1999)	76	Patients undergoing IVF	Flow cytometry	No difference in % NK cells in women with infertility who failed to get pregnant and those who became pregnant after ART; and between miscarriage and LB in those who were pregnant Higher subpopulation of %CD56^{bright} NK cells in women who had LB compared with miscarriage No normal range reported
Ledee-Bataille *et al.* (2004)	15	≥3 IVF cycles failures	Immunohistochemistry	No difference in number of NK cells/100x fields in natural IVF cycle in women with infertility who failed to get pregnant and those who b ecame pregnant after ART No normal range reported

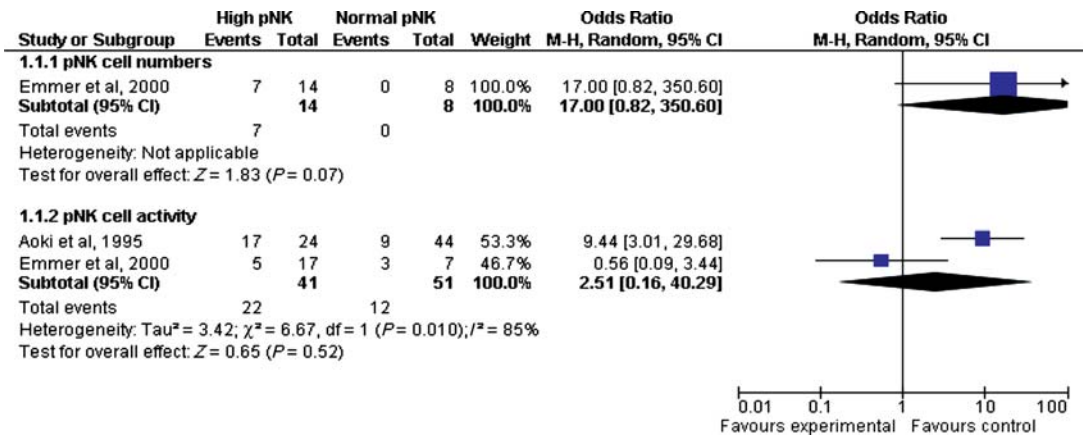

Figure 2 Odds of miscarriage with high pre-pregnancy peripheral NK cell parameters in women with idiopathic RM.

luteal biopsy examines the endometrium in the implantation window of a non-conception cycle.

Four studies (Quenby *et al.*, 1999; Michimata *et al.*, 2002; Ledee-Bataille *et al.*, 2004; Tuckerman *et al.*, 2007) investigating uNK cells used immunohistochemistry of frozen or paraffin fixed sections with antibodies to CD56 to identify NK cells staining, and two studies (Lachapelle *et al.*, 1996; Fukui *et al.*, 1999) used enzymatic digestion of endometrium and then flow cytometry to identify C56$^{bright\ or\ dim}$ cells. uNK cells results were presented either as an absolute count of NK cells per 3 (Ledee-Bataille *et al.*, 2004) and 10 high power fields (Michimata *et al.*, 2002) or as a percentage of total stromal cells (Quenby *et al.*, 1999; Tuckerman *et al.*, 2007). There were no

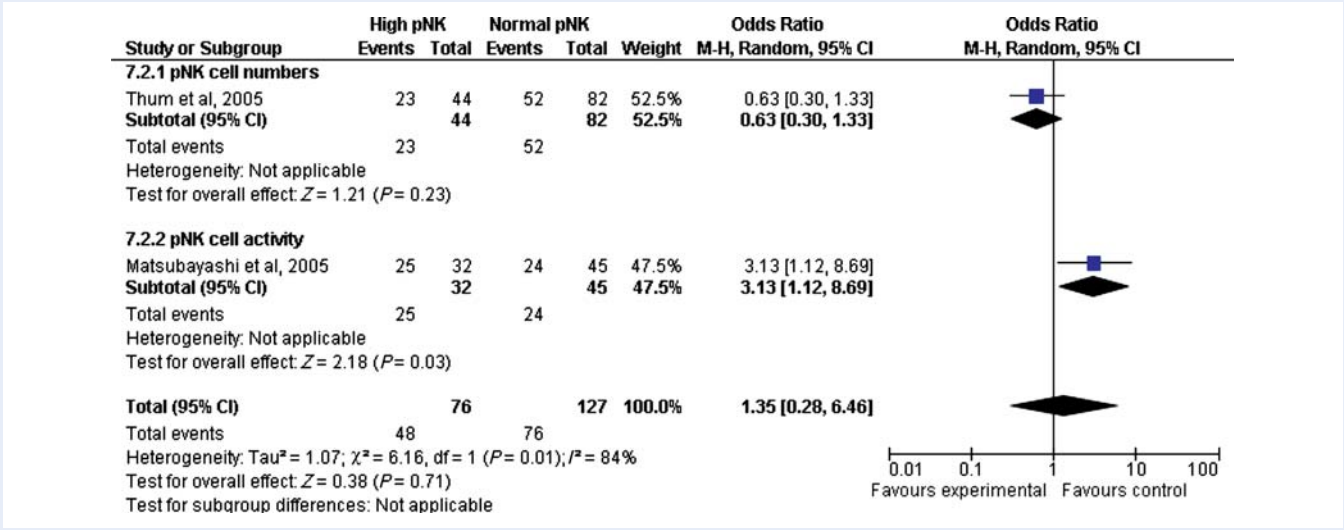

Figure 3 Odds of implantation failure after ART with high levels of pre-pregnancy peripheral NK cell parameters in women with infertility.

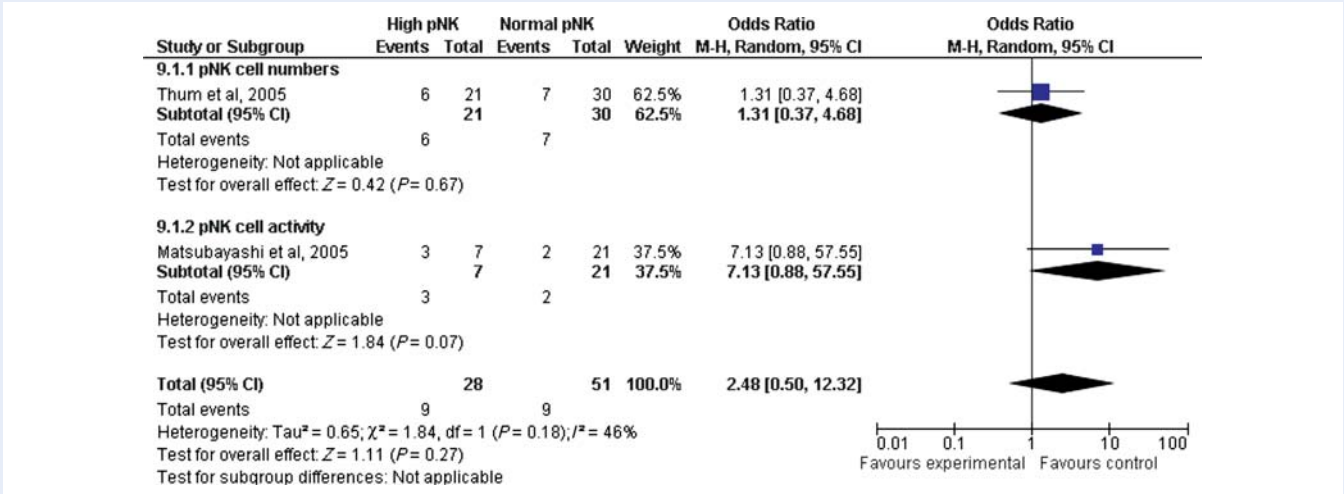

Figure 4 Odds of miscarriage (after implantation success from ART) with high levels of pre-pregnancy peripheral NK cell parameters in women with infertility.

studies that reported pregnancy outcomes according to uNK cells activity.

There were four studies investigating uNK cells in women with idiopathic RM but only two (Quenby *et al.*, 1999; Tuckerman *et al.*, 2007) reported pregnancy outcomes according to high and normal levels of uNK cells. A meta-analysis, with high heterogeneity ($I^2 = 69\%$), found that uNK cell density did not predict pregnancy outcome ($n = 72$, OR 1.33, 95% CI 0.16–11.11; Fig. 5). The two studies that did not report dichotomized pregnancy outcomes found no differences in mean percentage of uNK cells in women who subsequently miscarried and those who had live births.

Two studies (Fukui *et al.*, 1999; Ledee-Bataille *et al.*, 2004), differing in study design to each other, investigated uNK cells in women with infertility, and both did not report pregnancy outcomes according to a predetermined cut-off of normality. One included women who underwent IVF treatment and reported NK cells as a percentage of lymphocytes. The other included women with implantation failure after three cycles of IVF, and reported NK cells as mean absolute numbers in three 100x fields. Both report no differences in uNK cells between women who failed to get pregnant and who became pregnant after ART. However, the study by Fukui *et al.* (1999) which analysed NK cells by flow cytometry, showed a higher percentage of the subpopulation $CD56^{bright}$ NK cells in women who had live births compared with women who miscarried.

Discussion

This systematic review did not demonstrate that abnormal pNK and uNK cell parameters predicted adverse pregnancy outcomes of miscarriage or implantation failure, in women with RM or infertility. The

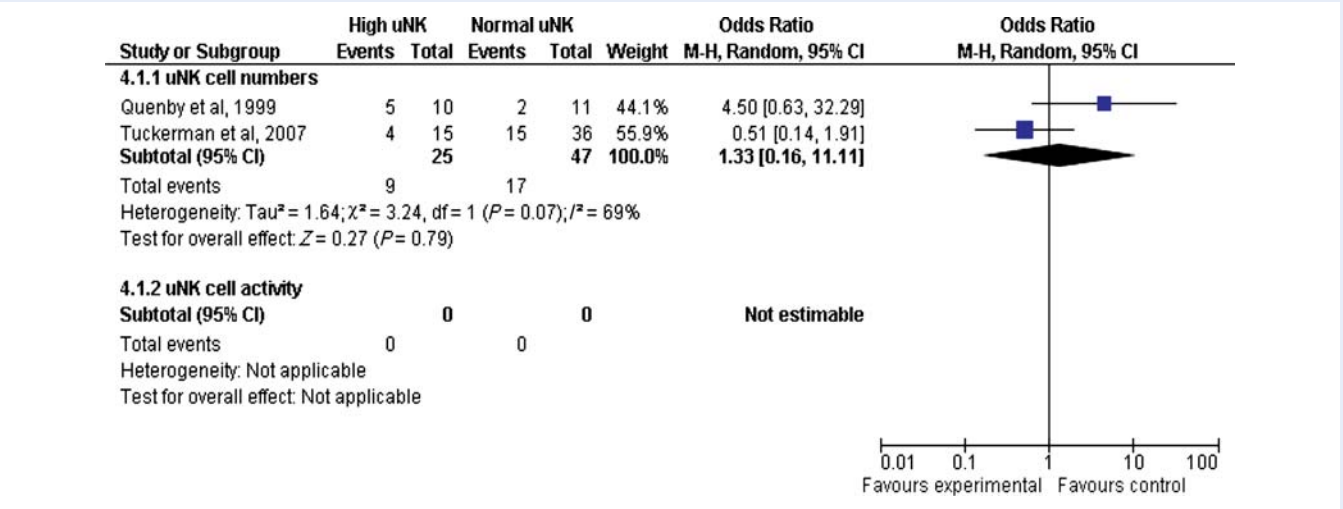

Figure 5 Odds of miscarriage with high levels of pre-pregnancy uNK cell numbers in women with idiopathic RM.

observational studies were individually and collectively underpowered to answer this important question. Assuming that, irrespective of the reason for NK testing, women with a normal NK cell count have miscarriage rate of around 30%, one would expect an increase in miscarriage rate by at least 10% (33% increase) when NK cells are high. At least 376 women per group would have to be followed until delivery (752 in total) to test this hypothesis with 80% power ($\alpha = 5\%$). This is considerably more than the largest individual study available, which investigated 126 women (Thum *et al.*, 2005). There was also significant heterogeneity between studies in terms on inclusion criteria, methodology of NK cells analysis and outcome measurements.

Peripheral NK cells

It has been postulated that uNK cells originate from pNK cells, which subsequently differentiate in the uterine microenvironment into the uNK cell phenotype (Kitaya *et al.*, 2007). Testing for pNK cells involves venous blood sampling at any time during the menstrual cycle as they have not been shown to fluctuate throughout the cycle (Pantazi *et al.*, 2010), or with sampling in pregnancy. All studies used flow cytometry, the best method to analyse and quantify lymphocyte subsets (Dosiou and Giudice, 2005). Investigations have been reported in the pre-pregnancy period, during pregnancy or on the day of embryo transfer in infertility patients undergoing ART. However, only results from investigations done prior to pregnancy were analysed in this study as the prespecified aim of the study was to assess the association of pre-pregnancy NK testing and subsequent pregnancy outcomes.

All three studies of pNK cells and RM included women after only two miscarriages which does not fit the ESHRE (European Society of Human Reproduction and Embryology) definition of RM (Farquharson *et al.*, 2005). Yamada *et al.* (2003) and Emmer *et al.* (2000) investigated both pNK cells number and activity, while Aoki *et al.* (1995) only studied pNK activity. Emmer *et al.* (2000) and Aoki *et al.* (1995) both studied women with idiopathic RM whereas Yamada *et al.* (2003), the study with the largest sample size in these group, included women with known associations of RM such as endocrine disorders, APS and thrombophilia. Although they reported that high pNK cells number and activity predicted subsequent biochemical miscarriage and miscarriage of normal karyotype, more than half of the women investigated had another possible contributing factor to their miscarriage, creating potential bias in the results.

There is also significant heterogeneity between studies which is not surprising given potentially important differences in the analysis and interpretation. The determination for the cut-off of normality in different studies was through different methods with different control groups. Emmer *et al.* (2000) used <12% pNK cell numbers as normal range set by Beer *et al.* (1996) with no explanation of how this level was calculated. Yamada *et al.* (2003) used <16.4% pNK cell numbers and <46% pNK cells activity as the normal range determined by receiver operating characteristic (ROC) curve for optimal discrimination between miscarriage (normal karyotype or biochemical pregnancy) and live birth. However, Aoki *et al.* (1995) set <41.8% pNK cells activity measurement as normal range determined by the mean +1 SD of 47 healthy controls with no history of miscarriages and Emmer *et al.* (2000) set <322 lytic units as normal with no mention of how this was obtained. The inconsistency was also seen in studies of women with infertility where Matsubayashi *et al.* (2005) determined <44% pNK activity as normal range with controls, although age-matched, not all of proven fertility, whilst Thum *et al.* (2005) used <12% pNK cell numbers as set by Beer *et al.* (1996). Therefore, it is clear that there is a lack of a commonly accepted normal range for pNK cells number and activity, or generally accepted type of pNK cells testing. In addition, there were also differences in IVF protocols in different units and the definition for implantation failure or success was not mentioned in some studies (Matsubayashi *et al.*, 2005; Baczkowski and Kurzawa, 2007).

It is also known that pNK cells increase significantly with stress and exercise and this was not taken into account when blood was taken for investigation (Benschop *et al.*, 1998; Timmons and Cieslak, 2008). Furthermore, the value of an abnormal test for pNK cells activity is also unknown as it may be a reflection of a transient stress response at the time of blood withdrawal, or a representation

of the response to other stresses in daily life (Shakhar et al., 2006). It is unclear if these phenomena exist for uNK cells.

uNK cells

Testing of uNK cells involves an endometrial biopsy that can only be carried out in the pre-pregnancy period. Immunohistochemistry was the method used in most of the studies of uNK cells. This is more time consuming than flow cytometry but it reveals the location of the uNK cells (Bulmer et al., 1991). Analysis using flow cytometry involves digesting the tissue, and thereby potentially losing cells and antigens. Furthermore, it needs a large sample of endometrium that may be difficult to obtain in some women.

The evidence for the association between preimplantation uNK cell density and miscarriage in a subsequent pregnancy is limited. Two studies reported no difference in uNK cell density between women who subsequently miscarried and those who had live births while two other studies reported pregnancy outcomes according to high and normal uNK cell density had contradictory results (Quenby et al., 1999; Tuckerman et al., 2007). The studies that reported no difference in uNK cell density were different to each other and are not comparable. Michimata et al. (2002) included women after two miscarriages and used immunohistochemistry for analysis while LaChapelle et al. (1996) included women with three miscarriages and analysed NK cells using flow cytometry.

There was again considerable heterogeneity between the two studies that reported dichotomized pregnancy outcomes. Similarly to pNK cell parameters, the normal ranges were obtained with different control women. Quenby et al. (1999, 2005) used <5% uNK cells as a normal range based on the upper quartile of 18 control women while Tuckerman et al. (2007) defined <13.8% as normal determined by the 90th percentile of 10 control women. For analysis, Quenby et al. (1999) used frozen sections and pressure cooker for antigen retrieval while Tuckerman et al. (2007) used waxed embedded specimens and microwave for antigen retrieval. Furthermore, uNK cells are not evenly distributed through the tissues and their density varies depending on where the cells are counted. One study counted cells near the epithelial edge (Quenby et al., 1999) and the other at random, including deeper into the section (Tuckerman et al., 2007).

Both studies investigating uNK cells in women with infertility reported no difference in uNK cell density and percentage between women who failed to get pregnant and those who became pregnant after ART. However, the population of women in both studies was different as Fukui et al. (1999) included all women undergoing ART while Ledee-Bataille et al. (2004) included women without pregnancies after three cycles of ART. In addition, the method of analysis was different where one employed flow cytometry and the other immunohistochemistry. Thus, the studies are not comparable enough to draw conclusions about the implications of uNK cells tests in women with infertility.

Despite the lack of clinical evidence, there is a biological plausibility for a role for uNK cells in reproductive failure. uNK cells are most numerous in the implantation window and in early pregnancy (Bulmer and Lash, 2005), and they are adjacent to and interact with extravillous trophoblast cells (Moffett-King, 2002). Different uNK cell populations have been found in the deciduas of normal and miscarried early pregnancy (Quack et al., 2001). Furthermore, uNK cells have been shown to regulate angiogenesis (Hanna et al., 2006; Kalkunte et al., 2009; Quenby et al., 2009), an important factor in implantation, and trophoblast cells express antigens that are recognized by the receptors on uNK cells, resulting in changes during the implantation process which may affect pregnancy outcome (Hiby et al., 2010).

Conclusions

This review suggests that the prognostic value of measuring pNK and uNK cell numbers or activity remains uncertain as these parameters have not been shown to be associated with subsequent pregnancy outcome. This finding is similar to that of the many conditions that have been associated with RM such as thrombophilia and structural uterine anomalies, none of which have been shown to predict pregnancy outcome.

This may be because of the disappointingly small number of studies reporting clinical outcomes on small numbers of women. The inclusion criteria were also inappropriate in studies that investigated women after only two miscarriages. Furthermore, there is still no consensus on what an abnormal NK cell test result is, as the normal ranges in different studies are derived from different controls. There is a need for more studies investigating pNK and uNK cells population and function in women with reproductive problems. Ideally, future studies should be prospective, with appropriate inclusion criteria and have a standardized methodology of analysing and reporting pNK and uNK test results. Sample size should also be calculated to avoid lack of power in the study.

Before the availability of results from these larger, more methodologically sound evaluations of prognostic value or RCTs of therapeutic modalities on specifically selected women with potential immunological pathology, women with reproductive problems should not be offered NK testing in routine clinical practice, and prescribed empirical immunotherapy, without clear evidence of benefit. An alternative would be to counsel them about the lack of available evidence, and encourage them to participate in well-designed studies, to confirm or refute the role of NK cells as a clinically useful marker for screening. We echo the Cochrane review on immunotherapy for RM that quotes 'a specific assay to diagnose immune-mediated early pregnancy loss and a reliable method to determine which women might benefit from manipulation of the maternal immune system are urgently needed' (Porter et al., 2006).

Authors' roles

A.T., S.Q. and Z.A. conceived the idea, developed the methodology and analysed and interpreted the data. A.T. and S.Q. reviewed all the original papers for inclusion and exclusion. A.T. performed the searches, read the abstracts and wrote the initial draft. S.Q. and Z.A. critically revised the manuscript. All the authors approved the final version of the manuscript.

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Trials

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Study protocol

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Prednisolone Trial: Study protocol for a randomised controlled trial of prednisolone for women with idiopathic recurrent miscarriage and raised levels of uterine natural killer (uNK) cells in the endometrium

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Abstract

Background: Idiopathic recurrent miscarriage is defined as 3 consecutive pregnancy losses with no contributing features found on investigations. At present there are no treatments of proven efficacy for idiopathic recurrent miscarriage. Uterine natural killer (uNK) cells, the most predominant leucocyte in the endometrium are adjacent to foetal trophoblast cells and thought to be involved in implantation. The exact mechanisms of how uNK cells affect implantation are not clear but are probably through the regulation of angiogenesis. Multiple studies have shown an association between high density of uterine natural killer cells and recurrent miscarriage. We have shown that prednisolone reduces the number of uNK cells in the endometrium. The question remains as to whether reducing the number of uNK cells improves pregnancy outcome.

Methods: We propose a randomised, double-blind, placebo controlled trial of prednisolone with a pilot phase to assess feasibility of recruitment, integrity of trial procedures, and to generate data to base future power calculations. The primary aim is to investigate whether prednisolone therapy during the first trimester of pregnancy is able to improve live birth rates in patients with idiopathic recurrent miscarriage and raised uNK cells in the endometrium. Secondary outcomes include conception rate, karyotype of miscarriage, miscarriages (first and second trimester), stillbirths, pregnancy complications, gestational age at delivery, congenital abnormality and side effects of steroids. The trial has 2 stages: i) screening of non-pregnant women and ii) randomisation of the pregnant cohort. All patients who fit the inclusion criteria (<40 years old, ≥3 consecutive miscarriages with no cause found and no contraindications to prednisolone therapy) will be asked to consent to an endometrial biopsy in the mid-luteal phase to assess their levels of uNK cells. Women with high levels of uNK cells (≥5%), will be randomised to either prednisolone or placebo when a pregnancy is confirmed. Follow-up includes 2 weekly ultrasound scans in the first trimester, an anomaly scan at 20 weeks gestation, growth scans at 28 and 34 weeks gestation and a postnatal follow-up at 6 weeks.

Trial Registration: Current Controlled Trials ISRCTN28090716

Background

Recurrent miscarriage (RM) is defined as the loss of 3 or more consecutive pregnancies and is a stressful condition for both patients and clinicians alike. It affects about 1% of all fertile couples trying to conceive [1,2]. Despite a wide range of investigations, no apparent cause is found in more that 50% of cases and they are categorized as idiopathic recurrent miscarriage [2,3]. Apart from supportive care in a dedicated early pregnancy unit with regular reassurance scans and psychological support, empirical treatment in this group of women is not recommended [1,4]. Immunomodulation therapies such as steroids, intravenous immunoglobulin (IVIG), 3rd party donor cell immunization, paternal cell immunization and trophoblast membrane infusion have been proposed with conflicting evidence as to their efficacies. A meta-analysis of trials evaluating IVIG, 3rd party donor cell immunization, paternal cell immunization and trophoblast membrane infusion found no evidence of a beneficial effect over placebo in preventing further miscarriages [5]. Porter *et al* concluded that "a specific assay to diagnose immune-mediated early pregnancy loss and a reliable method to determine which women might benefit from manipulation of the maternal immune system are urgently needed" [5]. This trial is intended to make progress towards meeting the challenge of diagnosing and treating immune-mediated early pregnancy loss.

Uterine Natural Killer Cells

The putative association of recurrent miscarriage and immunological phenomenon has been present for many years. Natural killer (NK) cells which form part of the innate immune system are found in peripheral blood and in the endometrium. Although sharing some similar properties, peripheral and uterine natural killer (uNK) cells are unique cell types with distinct antigenic features and functional markers. UNK cells in peripheral circulation differ in many respects to those in the endometrium [6-8]. It is the intensity of CD56 antigen expression and the lack of two typical NK cell markers - CD16 and CD 57 antigens, that differentiate uterine from peripheral NK cells. Around 80% of uNK cells are $CD56^{bright}$ and $CD16^{-}$ whereas 90% of peripheral NK cells show opposite characteristics; they are $CD56^{dim}$ and $CD16^{+}$ [7,9].

Uterine NK cells are the most predominant leucocytes in the endometrium and their density varies throughout the menstrual cycle. UNK cell density increases in number towards the mid-luteal phase and peaks in early pregnancy if implantation occurs [8]. UNK cells accumulate as a dense infiltrate at the implantation site near stromal cells, glands, blood vessels and trophoblast cells in early pregnancy [7]. In-vitro studies have also shown that extravillous trophoblast and uNK cells interaction occurs [10]. These interactions may have an effect on trophoblast invasion. However, more recent evidence points to the role of uNK cells as one of controlling angiogenesis [11].

Embryo implantation and early pregnancy development occur in a relatively hypoxic environment (2-3% O_2) [12]. Inappropriate blood flow to the intervillous space has been associated with oxidative stress damage to the developing placenta and thus miscarriage [13]. UNK cell density in women with recurrent miscarriage was found to be positively correlated with endometrial angiogenesis and uterine artery blood flow [14]. A similar positive correlation was also found in women with unexplained recurrent failure of in-vitro fertilisation (IVF) [15]. Thus, we have proposed that increased uNK cell density is associated with increased number of spiral arteries which may lead to inappropriate blood flow to the developing foetal-placental unit causing oxidative stress and consequent miscarriage [14].

Uterine NK cells and Recurrent Miscarriage

Increased density of uNK cells in pre-implantation endometrium has been found in women with RM compared to fertile controls [16-19]. However, there are also studies that have shown no difference in the population of uNK cells in patients with RM and controls but these included women with only 2 consecutive miscarriages [20,21]. Whether high numbers of uNK cells in the mid-luteal phase predict subsequent miscarriage is controversial. One study suggested that they do [17] but a more recent slightly larger study refuted this [19]. However, both studies were small in number and were retrospectively analysed.

Studies on normal and miscarried early pregnancy deciduas have also implicated uNK cells in the aetiology of idiopathic RM [22,23].

The effect of prednisolone on uNK cells

UNK cells express both glucocorticoid and ER-β receptors [24]. Thus, therapeutic manipulation of these cells may be possible. Prednisolone was chosen as the steroid for manipulation as it is metabolised by the placenta and very little of the drug reaches the fetus [25]. One woman with significantly raised uNK cells had 17 miscarriages and was given 5 mg of prednisolone pre-conceptually. She had a further 2 miscarriages and then had 20 mgs of prednisolone once a day both pre-conceptually and in early pregnancy. On this regime, she had a live birth [26].

Next, a prospective study was carried out with 20 mg prednisolone from day 1 to day 21 of the menstrual cycle which demonstrated a reduction in uNK cells in the pre-implantation endometrium of patients with idiopathic RM [18]. This study, carried out in Liverpool investigated 85 women with RM and 18 women with 2 or more nor-

mal pregnancies for uNK cells density via mid-luteal phase endometrial biopsy. The normal range of uNK cells was defined using the upper end of the inter-quartile range for the 18 control women. Thus, women with more than 5% uNK cells per stromal cell were considered to have high levels. 32 women with RM had high levels of uNK cells and 29 agreed to take 20 mg prednisolone for 3 weeks and have a second biopsy. In 23 women, the number of uNK cells decreased. The reduction in uNK cell density was significant with a mean level of 14% before treatment to 9% after treatment (p = 0.0004, CI 2.3-12). Furthermore, 3 women requested a third biopsy after a further month of prednisolone and in each case, the uNK cells level had fallen further suggesting that prolonging the steroid therapy for longer than 3 weeks further reduces the level of uNK cells. None of these women reported side effects significant enough to stop the medication.

Aims of trial

The primary aim of this trial is to investigate whether prednisolone therapy during the first trimester of pregnancy is able to reduce the risk of miscarriage and improve live birth rates in patients with idiopathic RM and raised uNK cells in the endometrium.

Primary Outcome

The primary outcome is the number of babies born alive.

Secondary Outcomes

They will include:

- Conception rate

- First and second trimester miscarriages

- Number of losses of empty gestation sacs and foetal losses

- Karyotype of miscarried pregnancies

- Stillbirths

- Intrauterine growth restriction (IUGR) defined as birth weight below the 5th centile according to customised birth weight charts

- Pregnancy complications such as pre-eclampsia or gestational diabetes

- Gestational age at delivery

- Foetal abnormality

- Side effect of steroids (eg: mood changes, weight gain, increased appetite, indigestion, avascular necrosis of the hip, hypertension or hyperglycemia)

Methodology

Design

This will be a randomised, double-blind, placebo controlled trial with an initial pilot phase to assess feasibility of recruitment and randomisation. Women are recruited from Liverpool Women's Hospital's recurrent miscarriage clinic which is the tertiary referral centre for the region and an endometrial research clinic. Referrals for the research clinic are from all over UK and Europe following media publicity surrounding the publication of the case report about a successful pregnancy with steroid use after 19 consecutive miscarriages [26] and national presentations.

When these women are first seen in the RM clinic, or if a referral letter to the research clinic is received, patient information leaflets regarding the trial are given or sent to them for consideration of their participation in this study (Figure 1). Women willing to participate are encouraged to ring for an appointment 6-9 days after their luteinising hormone (LH) surge in a cycle where they have not tried to conceive (patient information leaflet advises patients to get an ovulation kit and to use contraception). At this appointment, a full history is taken and the results of previous investigations noted to ensure that there is no cause found for RM. The trial is then explained further and consent obtained to perform a transvaginal scan and an endometrial biopsy.

The endometrial biopsy is then taken and the sample fixed in formalin, processed and embedded in paraffin wax. 3 μm sections are prepared and stained for CD56 using immunohistochemistry. A positive control slide from a patient known to have high uNK cells and an IgG mouse negative control is used for every slide undergoing immunohistochemistry to ensure no false negative or false positive staining. Next, 10 high-powered fields from different areas along the epithelial edge of the sample are selected and photographed. Then, the glandular epithelial cells and blood vessel are removed electronically. Subsequently, the number of CD56 positive and negative cells in each field are counted using image analysis software. Image analysis has markedly reduced the inter-observer error as compared with manual counting [27]. For each observer, Bland-Altman plots were produced to ensure that there were no systematic differences in uNK or stromal cell counts between observers. The inter- and intra-observer co-efficients of variation were 12.2 (SD 6.53) and 6.8 (SD 4.29) respectively. The normal range was defined as <5% uNK cells per stromal cell using the upper end of the interquartile range of control patients from a previous study [18]. The positive and negative predictive

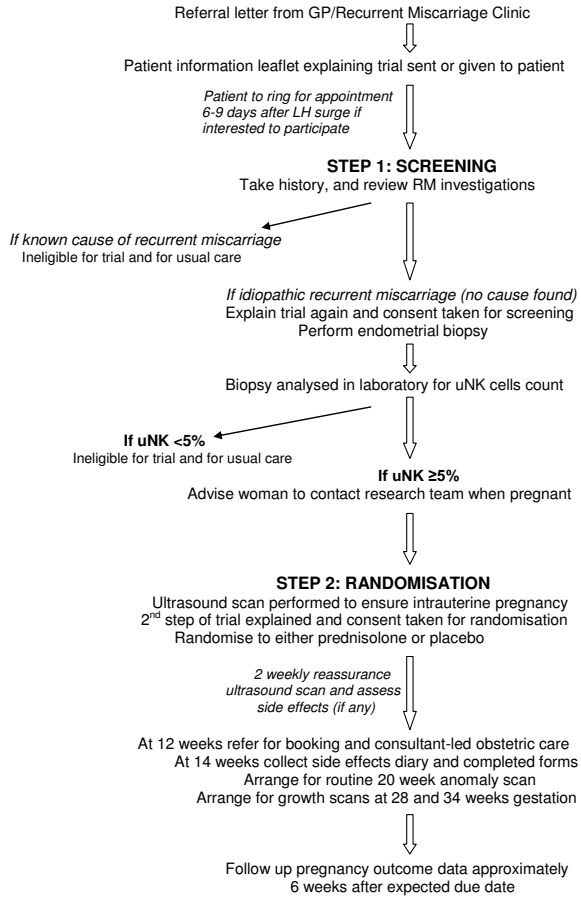

Figure 1
Patient flow during trial.

values will be calculated with the results of the trial. Results of the uNK cell counts are communicated to the women by letter with a follow up telephone consultation. Those with normal uNK cell density are advised to follow standard management. Those with uNK cell counts of ≥5% are advised to contact the research team as soon as they are pregnant to be considered for randomisation.

Inclusion Criteria

- 3 or more consecutive miscarriages with no cause found (idiopathic)

- Less than 40 years old

- ≥5% uNK cells at day LH +6 to +9

Exclusion Criteria

- Known cause for recurrent miscarriage: antiphospholipid syndrome (positive anticardiolipin antibody or lupus anticoagulant on 2 separate occasion at least 6 weeks apart), thrombophilia (factor V Leiden mutation, APCR resistance, protein C or S deficiency, prothrombin G20210A mutation, antithrombin III deficiency), abnormal thyroid function tests, parental balanced translocation or uterine anomaly (known subseptate uterus or cervical weakness diagnosed at hysteroscopy).

- Contraindications to steroid therapy: hypertension, diabetes, mental health problems or obesity with BMI >35

- Decline consent to randomisation

Randomisation and blinding

Women meeting the inclusion criteria are scanned after a positive pregnancy test. If a viable intrauterine pregnancy between 4-8 weeks gestation is found, then a second consent is obtained for randomisation to either prednisolone or placebo. Once consent if obtained, women are given consecutive study numbers then sent to pharmacy. A dedicated pharmacist then allocates to the treatment or placebo group the women using her study number. This study number allocation is performed by using a randomisation list that was pre-constructed with a computerised random number generator in blocks of 20. All women are given a pack of tablets by the pharmacist and advised to take 4 tablets for 6 weeks, 2 tablets for 1 week and 1 tablet for 1 week. Both prednisolone and placebo tablets are similar in size and colour and dispensed in identical packaging. The active tablets consist of 5 mgs of prednisolone and the placebo is an inert substance specially made to be identical to the placebo. Women are also given a chart that tells them how many tablets to take each day and instructions to mark off each day they take the study medication. They are then asked to return this chart to the investigators at 14 weeks gestation so that compliance is monitored. Thus, both the trial investigators and women are blind to the treatment allocation.

Case report forms (CRF) are then completed and given to an independent research administrator who enters the information on a database and keeps them as confidential trial documentation. The research administrator will generate reports to the data monitoring committee (DMC) as necessary.

All women participating in the trial are given a trial ID card where contact details of the hospital are available to get in touch with either the chief investigator (CI) or principal investigator (PI) in the event of an emergency where the trial group allocation needs to be identified. The CI or PI will then authorise the pharmacy department to unblind the group only if severe illness occurs and the attending physician needs to know whether the women had been allocated steroids. The DMC will also be able to unblind these groups if necessary.

Monitoring in pregnancy

After randomisation, women will be offered a 2 weekly ultrasound scan for reassurance and a clinic consultation to assess side effects of treatment. This will be done by asking them about side effects, reviewing the side effect diary, and measuring the blood pressure until 12 weeks gestation. If hypertension or any significant side effects occurred, they will be asked to stop the medication. Any side effects reported will be assessed in terms of their seriousness, causality and expectedness. If the event was deemed serious, a serious adverse event or adverse event (SAE/AE) report form will be completed by the CI or PI and submitted to the relevant committees. If it was a suspected unexpected serious adverse reaction (SUSAR), then reporting will be expedited accordingly.

At 12 weeks gestation, women are referred to the hospital of their choice for booking and consultant-led antenatal care. At 14 weeks gestation, they are reviewed again and all the CRF are collected. A routine anomaly scan at 20 weeks gestation and growth scans at 28 and 34 weeks gestation will then be arranged. Another telephone follow-up consultation will be arranged 6 weeks after the delivery of the baby to assess the pregnancy and secondary outcomes.

Safety considerations

Prednisolone is commonly used to treat medical conditions in pregnancy and has been given in the first trimester to women with asthma [28], rheumatoid arthritis [29] and hyperemesis gravidarum [30] with minimal side effects. It is metabolised by the placenta to inactive prednisone and thus only about 10% of active drug reaches the foetus [25]. Although there are concerns of possible complications of intrauterine growth restriction and an association with cleft palate from animal studies, more recent studies, even with postnatal follow-up, have not shown such complications with prednisolone use [30,31].

Sample size calculation

Preliminary power calculation was based on multiple presumptions informed by minimal data. The live birth rate on placebo, is assumed from a previous study where 12 women with >5% uNK cells conceived and 6 subsequently miscarried (50%) [17]. We believe an increase in pregnancy success from 50% to 79% would be clinically important. At present, there is an 80% pregnancy rate in the RM clinic in LWH. Thus, to reach statistical significance, 68 women will need to be recruited in each group (80% power, 5% 2-sided alpha). Assuming a 70% acceptance rate, we will need to ask 136 women to be randomised. Assuming an 80% conception rate after the biopsy and a 35% rate of high uNK cells, 694 patients with idiopathic RM will need to be recruited for endometrial biopsies (Figure 2). The current on-going pilot study can confirm the presumptions made above and provide more information to perform a definitive power calculation in the future.

Data Analysis

Data analysis will be performed on an intention to treat basis using a data analysis plan that will be finalised before data analysis starts. The outline of the data analysis plan is as follows.

Figure 1: Power calculation diagram

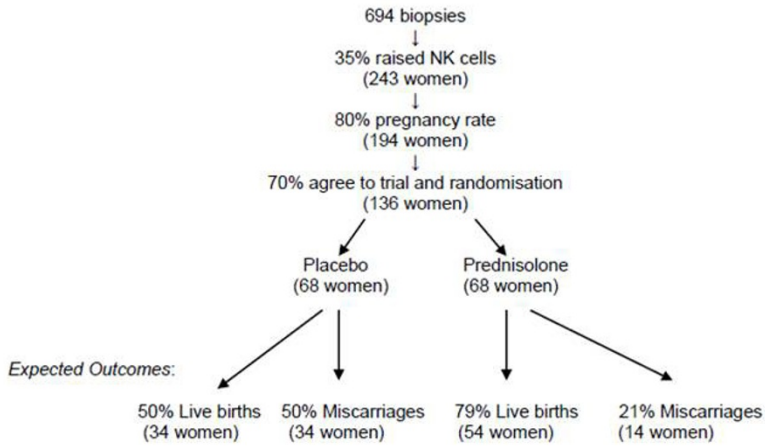

Figure 2
Power calculation diagram.

Data will be cleaned prior to any analysis.

The results of the trial, including patient flow during the trial, will be reported in accordance with the guidelines from the CONSORT (Consolidated Standards of Reporting Trials) statement [32].

Analysis will proceed in the following steps:

1. Summary statistics for demographic information relating to the allocation groups will be tabulated. The data will be examined to determine the extent to which the treatment and placebo groups are similar.

2. The primary outcome will be assessed. The live birth rate in each group will be expressed as a risk ratio with 95% Confidence Intervals. Statistical significance will be calculated using the Fisher's exact test. A significance value of $p < 0.05$ will be considered significant.

3. Secondary outcomes will be assessed. The rates of miscarriage, type of miscarriage (sac loss or fetal loss), karyotype of miscarried pregnancies, conception rate, gestational age at delivery and pregnancy complications will be tabulated. For dichotomous categorical variables, risk ratios and 95% Confidence Intervals will be calculated. For other categorical variables, a chi-squared test will be performed. For continuous variables, non-parametric tests will be used

4. Pre-specified subgroup analyses will include comparisons of women with primary and secondary recurrent miscarriage.

5. Adverse events. All adverse events will be tabulated according to allocation group.

6. Trial process measures. The following will be tabulated: how women come to know of the trial, the rate of acceptance for screening and randomisation, proportion of women who screened high for uNK cells and compliance to medication will be evaluated.

Trial Management and Monitoring Structure

Ethical and Regulatory Issues

Approval to conduct the trial has been granted from the Liverpool Local Research Ethics Committee.

The trial is included on the National Institute for Health Research (NIHR) Clinical Research Portfolio (NIHR CRN ID: 6567) and is registered with European and international clinical trials database (EUDRACT No: 2005-003307-36, ISRCTN28090716). MHRA approval was also granted.

Patient Acceptability and Consent

The trial has been discussed with the President of the Miscarriage Association, as the patient representative. We were advised that women who have had recurrent miscarriages would be suitable to be randomised to a placebo. The Miscarriage Association have also agreed to be independent advisors to women during the recruitment phase and throughout the trial. Additionally, women are informed that if a miscarriage occurred while they are on the trial, they can choose to have prednisolone in the subsequent pregnancy with the understanding that this is yet to be proven as an effective treatment.

Women who fit the inclusion criteria will be informed about the objectives of the study and be given written information about it. They will then have time to consider the trial and contact the investigators for a consultation appointment when they ovulate if interested to participate. Consent will be obtained by the trial investigators prior to screening for levels of uNK cells. A separate consent for randomisation into the study is taken when the woman is found to have raised uNK cells and is pregnant.

Data Monitoring Committee (DMC)

A data monitoring committee (DMC) independent of the trial investigators has been appointed for the trial to provide independent review of unblinded data at agreed intervals to ensure that no harm is being cause by the treatment. The first DMC meeting will be planned for after 20 patients have been randomised into the trial and passed 14 weeks gestation. Particular emphasis will be placed upon monitoring the side effect of steroids, fertility rate and pregnancy complications.

This committee will also address issues such as:

- Significant problems with trial design or methodology

- Recruitment rate

- Patient's acceptance of the possibility of randomisation to placebo

The chair of the DMC will report to the Trial Steering Committee (TSC) approximately 2 weeks after each meeting in accordance with recommended trial oversight.

Steering and Management Committees

A trial steering committee (TSC) and trial management group (TMG) have been formed to supervise and manage the trial. The TSC will be responsible for approving the core protocol and any subsequent amendments. The TMG will meet every 4-6 months to discuss:

- the recruitment rate and patient acceptability of the trial

- side effect of steroids

- data collection forms

- potential problems arising from the trial

The TMG will report the above and present any possible solutions to problems or strategies to improve recruitment to the TSC who will meet when deemed necessary by the Chief Investigator.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SQ conceived the study. AT and SQ participated in the design and coordination of the study and drafted the manuscript. AT, SQ, MT and ZA participated in the management of the study. JD managed the laboratory practice. All authors read and approved the final manuscript.

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