



UNIVERSITY OF  
LIVERPOOL

# The Role and Origin of Uterine Natural Killer Cells in Patients with Unexplained Recurrent Miscarriage

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## **ABSTRACT**

The aim of the study was to determine the role of uNK cells by looking at their numbers across three location sites, the epithelial edge, areas of low stromal cell density/oedema and peri-vascular stroma/spiral arteries. The universal NK cell marker, CD56 was used to identify uNK cells in the endometrium. The research determined whether uNK cells were the result of proliferation and differentiation of resident uNK cells or trafficking of pbNK cells. In addition, it was determined whether uNK cells play a part in spiral artery remodelling and trophoblast invasion and also the role they may play in RM.

The hypothesis was tested using Immunohistochemical staining on serial sequential sections of endometrial tissue received from RM patients of extreme phenotype between LH + 5-9 days. Analysis was made on 20 RM patients, 10 patients were grouped as high uNK, defined as an uNK cell density value of  $\geq 5\%$  and 10 were grouped as low uNK, defined as an uNK cell density value of  $\leq 2.5\%$ . The endometrial samples were stained for the antibodies to CD56, KI67, NKp30, L-Selectin and CD16, uNK cell markers of proliferation, differentiation and trafficking.

No association was found between RM and age. The high uNK density group had significantly higher levels for all the antibodies stained ( $P < 0.0001$ ). uNK cells were more proliferative and differentiated in the high uNK cell density group compared to the low. Proliferative and differentiated uNK cells also varied significantly across the location sites ( $P = 0.003$  and  $P = 0.008$  respectively). A significantly increased number of uNK cells staining positive for CD56, KI67 and NKp30 were found clustered around vessels ( $P = 0.021$ ,  $0.001$  and  $P = 0.004$  respectively).

The paucity of L-Selectin<sup>+</sup> and CD16<sup>+</sup> stained cells in both groups, low and high uNK cell density suggests that uNK cells are not trafficking from peripheral blood. Also, the spatial and temporal relationship of proliferative and differentiated uNK cells suggests that uNK cells originate from resident endometrial NK cells and do not arise from peripheral blood. In addition, KI67<sup>+</sup> and NKp30<sup>+</sup> uNK cells may assist with angiogenesis of uterine spiral arteries, which in turn increases uterine artery blood flow and subsequently initiates oxidative stress in the early developing foetus leading to RM.

**LIST OF ABBREVIATIONS**

<b>Ang-2</b>	Angiopoietin 2
<b>APC</b>	Antigen Presenting Cell
<b>APES</b>	3-Aminopropyltriethoxysilane
<b>APS</b>	Antiphospholipid Syndrome
<b>BSA</b>	Bovine Serum Albumin
<b>CMV</b>	Cytomegalovirus
<b>D&amp;C</b>	Dilatation and Curettage
<b>DAB</b>	3,3-Diaminobenzidine
<b>DPX</b>	Distyrene, Plasticiser and Xylene Mountant
<b>FSH</b>	Follicle Stimulating Hormone
<b>GM-CSF</b>	Granulocyte Macrophage - Colony Stimulating Factor
<b>HCG</b>	Human Chorionic Gonadotrophin
<b>HLA</b>	Human Leukocyte Antigen
<b>HRP</b>	Horseradish Peroxidase
<b>IFN</b>	Interferon
<b>IgG</b>	Immunoglobulin G
<b>IL</b>	Interleukin
<b>IUGR</b>	Intrauterine Growth Restriction
<b>KAR</b>	Killer Activation Receptor
<b>KIR</b>	Killer Inhibition Receptor
<b>LH</b>	Luteinising Hormone
<b>MCII Cabinet</b>	Microbiological Safety Class II Cabinet
<b>MHC</b>	Major Histocompatibility Complex
<b>NBF</b>	Neutral Buffered Formalin
<b>NK Cell</b>	Natural Killer Cell
<b>pbNK Cell</b>	Peripheral Blood Natural Killer Cell
<b>RCT</b>	Randomised Controlled Trial
<b>RM</b>	Recurrent Miscarriage
<b>TBS</b>	Tris Buffered Saline
<b>TGF</b>	Transforming Growth Factor
<b>TH1/2</b>	Type 1 or 2 Helper T Cells
<b>TNF</b>	Tumour Necrosis Factor
<b>uNK Cell</b>	Uterine Natural Killer Cell
<b>VEGF</b>	Vascular Endothelial Growth Factor

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## **CHAPTER 1 - INTRODUCTION**

### **1.1 PHYSIOLOGY OF REPRODUCTION**

*The results of sexual reproduction pass through all aspects of mammalian life. At the core of the process lies the creation and fusion of two gametes, the male sperm and the female ovum. These gametes come together at fertilisation. Critical to sexual reproduction is the process of fertilisation.*

#### **1.1.1 FERTILISATION (1)**

During sexual intercourse approximately 300 million sperm are released into the vagina. Only one sperm will fertilise the ovum. The successful sperm is one of many that swims through the cervical mucus, enters the uterus and passes up to the ampulla of the fallopian tube, the site of fertilisation. Both the female and male gametes have a limited period of viability in the female reproductive tract. Sperm are able to retain their fertility for up to 48 hours, whilst the ovum remains viable for only around 12 to 24 hours after ovulation.

Before a sperm fertilises an ovum it must undergo a process called capacitation. This occurs naturally within the female reproductive tract. The process of capacitation takes around 5 hours during which the composition of the plasma membrane of the sperm head is modified. The sperm which meet the ovum in the fallopian tube are confronted by the corona radiata, which makes up the outer layer of the egg. The corona radiata is a highly cellular layer with an intercellular matrix consisting of protein, carbohydrates and hyaluronic acid. After they have penetrated the corona radiata the sperm then undergo what is known as the acrosome reaction as they bind to the zona pellucida. The head of the sperm contains hydrolytic enzymes which are released together with the force generated by the tail of the sperm to help break through this surrounding layer, zona pellucida and penetrate the ovum. The most

important enzyme is acrosin, which is bound to the inner acrosomal membrane. Once the head of the sperm has made its way through the zona and into the perivitelline space, the space between the plasma membrane of the ovum and the zona pellucida, the tail falls off and the zona pellucida thickens further to prevent another sperm from entering. The sperm is now capable of fusing with the egg to complete the first stage of fertilisation. The fertilised egg then undergoes meiotic division which is normally completed within 2 hours of fertilisation. At this point the fertilisation is complete and the fertilised egg is known as a zygote. The process of fertilisation causes metabolic activation of the egg, which is necessary for cleavage and subsequent embryonic development to occur.

### **1.1.2 IMPLANTATION** (1)

The embryo, still encased in its zona pellucida, is transported down the fallopian tube and into the uterus. During this time and immediately after fertilisation the zygote undergoes a pronounced shift in metabolism and begins several days of cleavage. Development proceeds at the rate of one cleavage division per day for the first 2 days. After the two cell stage, one of the cells, also known as blastomere, divides now forming a 3 cells embryo. Continuing cleavage results in the 8-cell stage embryo which undergoes compactation, where the cells bind tightly to each other, forming a compact sphere. The embryo now consists of approximately 16 cells and is called a morula. The morula then becomes filled with fluid secreted by trophoblast cells, the outermost layer. A blastocoele has now been formed.

Approximately six days later, the embryo sheds its zona pellucida allowing the embryo to expand. At this stage it is known as a blastocyst. This consists of an inner cell mass, also called an embryoblast which forms the amnion, yolk sac and allantois. This later gives rise to all the organs. The outermost layer, the trophoblast differentiates into two layers: the inner cytotrophoblast, a single layer of cuboidal cells and the outer syncytiotrophoblast. The blastocyst implants into the uterine wall by forming chorionic villi and lacunae that then fill up with maternal blood. This formation is assisted by hydrolytic enzymes that erode the epithelium.

The syncytiotrophoblast secretes a glycoprotein hormone, human chorionic gonadotrophin (hCG) which initiates changes that can prevent regression of the corpus luteum and shedding of the endometrium. Maintenance of the corpus luteum will allow continuous secretion of progesterone which is required to maintain pregnancy. After 6-8 weeks the placenta is well established to synthesise and secrete sufficient progesterone to continue the remaining gestation period. The implantation process is completed by day 9 or 10.

Complex interactions involving hormones, cytokines and adhesion molecules between the blastocyst and the endometrium mediate implantation. During the mid secretory phase of the menstrual cycle the endometrium expresses factors required for implantation and undergoes cyclical changes. This period is known as the 'implantation window' and it occurs between 5 and 10 days after the LH surge (2). Following this phase the endometrium undergoes decidualisation, which results in the stromal cells changing. Although progesterone appears to be the main factor initiating decidualisation, a number of other factors, including cytokines, appear to facilitate this event. Malfunction at any point may lead to implantation or pregnancy failure. This is why the endometrial sample required for this research is taken at this important time of the menstrual cycle, during the 'implantation window'.

### **1.1.3 PLACENTAL DEVELOPMENT** (1)

The placenta is a unique materno-foetal organ that connects the foetus to the uterine wall. It supplies the foetus with nutrients and oxygen and removes foetal waste via the maternal kidneys. The placenta is made up of two parts, the foetal part (chorion frondosum) and the maternal part (decidua basalis). At the placenta the foetal circulation comes close to the maternal circulation, but there is no actual contact or mixing of blood. The chorionic villi project towards the maternal decidua basalis. Each main villus becomes anchored to the decidua basalis, while secondary villi project into the intervillous space of the placenta. The intervillous space is subdivided by placental septa which cause the foetal surface to form units called the cotyledons. Spiral arteries supply the intervillous space with a pulsatile flow of maternal blood. The blood flows through the intervillous space to be drained from the placenta by endometrial veins. Branches of the umbilical arteries supply the villi with foetal blood. Blood flows through the capillaries of the villi, where gas exchange also occurs, to be collected in the branches of the umbilical vein to be returned to the foetal circulation.

#### **1.1.4 ANATOMY OF PLACENTATION**

Transport occurs between the maternal blood circulation in the intervillous space and the foetal blood circulation. The extravillous trophoblast ensures the blood flow to the maternal intervillous space is sufficient enough to meet the demands of the foetus. This is achieved during early pregnancy when the extravillous trophoblast deeply infiltrates the uterine wall and the placental cells destroy the muscular walls of the uterine arteries. Also, the endothelium is replaced as the endovascular trophoblast cells move down the arteries. This replacement alters the vascular conductance and increased blood flow to the intervillous space results. This is required for normal foetal growth and development (3). It is important for trophoblast invasion to proceed deep enough to prevent many clinical conditions such as, pre-eclampsia, IUGR and still birth, which are a result of incomplete maternal vessel transformation and inadequate blood supply to the foetus later on in the pregnancy (4,5). A compromise between trophoblast invasiveness and maternal resistance is required for a normal pregnancy. There is close contact between mother and foetus as the trophoblast invades and arteries are transformed. Despite this intermingling, there is neither rejection of the foetus or parasitism of the mother, instead a balance is achieved and foetal and maternal cells form a definitive boundary that demarcates their respective territories.

## **1.2 RECURRENT MISCARRIAGE**

### **1.2.1 DEFINITION**

Miscarriage is a loss of a clinically recognised pregnancy at some point prior to the 24th week of gestation. Recurrent miscarriage is defined as the spontaneous loss of three or more consecutive pregnancies, with the same biological father (6). Recurrent miscarriage can be considered a primary or a secondary process. Primary recurrent miscarriage refers to repeated miscarriages, in which a pregnancy has never been carried to viability, whilst in secondary recurrent miscarriage, a live birth has occurred at some time.

Other terminologies used are recurrent pregnancy loss, recurrent spontaneous abortion, recurrent spontaneous miscarriage, idiopathic miscarriage and habitual abortion.



### **1.2.2 EPIDEMIOLOGY**

Miscarriage occurs in 15-20% of all clinically diagnosed pregnancies of reproductive aged women, with the most occurring in the first twelve weeks of gestation. Recurrent miscarriage occurs in 1-2% of these women (7). Prognosis for future successful pregnancy is affected by the previous number of miscarriages. The rate of miscarriage after one increases to approximately 20%; after two to 28%; after 3 to 48%, chances of a future successful pregnancy are around 2 in 3 once miscarriage has occurred (8,9).

Maternal age also plays a significant role in the incidence of recurrent miscarriage. This suggests that pregnancy abnormality is a significant contributory factor to miscarriage given that the incidence of pregnancy abnormality increases with maternal (10). The risk of miscarriage for women younger than the age of 24 is 9.5%. With age this risk rises, it increases to 11% by the age of 30 and reaches 33% in women aged 40. The incidence increases dramatically to 53% in those women over the age of 44 (10,11).

Pregnancy loss rates decrease as pregnancy progresses. Approximately 1 to 5 % are lost at 13 to 19 weeks' gestation, whereas stillbirth occurs in 0.3 % at 20 to 27 weeks' gestation, a rate similar to that of third trimester stillbirth.

### **1.2.3 IMPORTANCE OF RESEARCH**

Miscarriage is not fully understood by clinicians, some miscarriages just happen with no explanation other than nature 'taking a hand'. When this complex process of pregnancy goes wrong it can take a tremendous emotional toll.

Clinical developments in the field of reproduction have expanded opportunities for the alleviation and circumvention of subfertility. The threat posed by miscarriage with no known aetiology continues to place reproduction high on the agenda of medical research.

### **1.2.4 CLASSIFICATION OF MISCARRIAGE** (12-14)

First trimester miscarriage is defined as a miscarriage which occurs within 12 weeks of gestation. A blighted ovum is a very common type of miscarriage that accounts for 60 percent of first trimester miscarriages. It is also known as empty gestation sac. This happens when a fertilised egg successfully attaches to the uterine wall but contains no embryo. All that forms is a placenta and an empty sac (14). A biochemical pregnancy is also a first trimester miscarriage which occurs when the foetus dies immediately after conception. This happens before the embryo has a chance to implant in the uterine wall. It produces a positive pregnancy test but is not seen on ultrasound. An embryonic loss is defined as an early embryo loss before foetal heart activity is visualised on ultrasound, usually before 8 weeks gestation, or the crown rump length is less than 5 mm with no change seen on a rescan 7 days later (15). A foetal loss is said to occur when the foetus has died within 8-12 weeks gestation and no heart activity has been recorded (15).

Second trimester miscarriage is defined as a pregnancy loss between 12 and 24 weeks of gestation (16). However, this is not as common as first trimester miscarriage. Pregnancy loss beyond 24 weeks gestation is known as stillbirth. Stillbirth is common in all countries around the world. In the UK, 5 out of every 1,000 babies will be stillborn. However, in the past thirty years the incidence of stillbirth has dropped considerably (17). Spontaneous second trimester loss is pregnancy loss with spontaneous rupture of the membranes or cervical dilatation. The foetus is between 12 to 24 weeks gestation and has had foetal heart activity (15).

The clinical aspects of miscarriage are considered within six subgroups: threatened, inevitable, incomplete, complete, missed and septic miscarriage. Some treatment is discussed within each category.

Threatened miscarriage is presumed when any vaginal bleeding appears in the first half of pregnancy. This is very common about 25-30% of all pregnancies have some

bleeding during the pregnancy (18). The bleeding is often small, but it may persist for days or weeks. The foetus at this point is still alive, the uterus is of expected size and the cervical os is closed. In 75% of women this will settle and 25% will go on to have a miscarriage. Threatened miscarriage is associated with risk of preterm rupture of membranes and preterm delivery. Women are told to inform their physicians immediately if any vaginal bleeding is observed. The bleeding generally ceases and a normal pregnancy results, therefore no intervention is usually taken place.

Inevitable miscarriage is signalled by gross rupture of the membranes in the presence of cervical dilatation. Under these conditions miscarriage is almost certain. The bleeding is usually heavier, abdominal cramps occur and the cervical os is open. Most often, uterine contractions commence rapidly, resulting in expulsion of the products of conception. The possibility of salvaging the pregnancy is extremely unlikely.

Incomplete miscarriage is where some parts of the foetus have been passed but the cervical os is open. The foetus and placenta are likely to pass together in miscarriages occurring before 10 weeks of gestation, but separately thereafter. Seen on ultrasound with an incomplete miscarriage, the retained products of conception may need to be removed by curettage.

Complete miscarriage is defined by diminished vaginal bleeding. The entire foetus has been expelled, the uterus is no longer distended with pregnancy and the cervical os is closed.

Missed miscarriage is when the undeveloped or deceased foetus is retained within the uterus and is not recognised until bleeding occurs or is seen on an ultrasound scan. The foetus is usually small for dates and the cervical os is closed. Mifepristone and misoprostol may be used to induce evacuation of products from the uterus. However, 50% will require surgical evacuation.

Septic miscarriage usually presents as acute salpingitis. The contents of the uterus are usually infected, causing endometritis. Vaginal loss is frequently offensive and the uterus is tender. A fever may be absent. Abdominal pain and peritonism are suggestive of a pelvic infection. Broad spectrum antibiotics for example co-amoxiclav and metronidazole are usually started one hour prior to uterine curettage.

### **1.2.5 AETIOLOGY**

Isolated non-recurring chromosomal abnormalities account for 60 percent of sporadic miscarriages (19). However with recurrent miscarriage a maternal or paternal cause is more likely. There are many factors which need to be considered.

#### **1.2.5.1 FOETAL FACTORS**

Recurrent miscarriage at an early stage is commonly due to an abnormality in the development of the zygote, embryo, early foetus, or the placenta. Abnormal foetal development in the first trimester is classified into that with an abnormal number of chromosomes, known as aneuploidy, or an abnormal development with a normal chromosome number, known as euploidy.

Pre-implantation genetic diagnosis has shown that women with recurrent miscarriage produce more aneuploid embryos than normal women (20,21). Approximately 50 to 60 percent of recurrent miscarriages are associated with chromosomal anomaly of the conceptus (13). Another study reported one fourth of chromosomal abnormalities were due to maternal gametogenesis errors and 5 percent paternal. Less than 10 percent were due to errors in fertilisation and zygote divisions (13).

In sporadic miscarriage, autosomal trisomy is the most frequently identified chromosomal anomaly associated with first trimester miscarriages, with an incidence of 35 percent (22).

Parental chromosomal anomalies occur in 4 percent of couples with recurrent miscarriage as opposed to 0.2 percent in the normal population (23). The most common is the result of balanced reciprocal or Robertsonian translocation, which produces an unbalanced translocation in the foetus (6,24).

Findings of an additional study were that a 100 percent of the pregnancies that miscarried had lower than expected crown rump length readings for their gestational age and in 61 percent of the women who miscarried, the foetus was significantly smaller than that expected for its gestational age (25).

Research suggests euploid miscarriages are usually lost later in gestation. A study found three fourths of aneuploid miscarriages occurred at or before 8 weeks gestation, while euploid miscarriages peaked at 13 weeks gestation (13).

### **1.2.5.2 MATERNAL FACTORS**

Maternal diseases are usually associated with euploid miscarriages (13). They have been associated with an increased risk for recurrent miscarriages. Maternal diseases include systemic lupus erythematosus, primary antiphospholipid syndrome, uncontrolled diabetes mellitus, thyroid disease, chronic essential hypertension, renal diseases and maternal infections such as *Salmonella typhi*, malaria, cytomegalovirus, *brucella*, toxoplasmosis, *mycoplasma hominis*, *Chlamydia trachomatis* and *ureaplasma urealyticum* (22). Current evidence is that well controlled thyroid disease and diabetes is not associated with recurrent miscarriage (23). Also bacterial vaginosis is associated with second trimester miscarriage, more so than first trimester miscarriage (8).

### **1.2.5.3 ENDOCRINE FACTORS**

Some researchers believe imbalances in certain hormones such as lutenising hormone and progesterone play a role in recurrent miscarriage. As already mentioned systemic maternal disorders, diabetes mellitus and thyroid dysfunction have been associated with miscarriage.

Insufficient progesterone may be produced after ovulation, known as a luteal phase defect. Progesterone is produced by the corpus luteum. Progesterone is needed to sustain the endometrium to support an embryo and developing foetus. A luteal phase

defect is usually the result of suboptimal or poor ovulation. Treatment to improve ovulatory function with clomiphene citrate, or injectable FSH can help correct this problem. It can also be treated by administering supplemental progesterone. However, there has never been an RCT that shows this is an effective treatment.

Luteinising hormone hypersecretion and polycystic ovarian syndrome are considered to be associated with recurrent miscarriage (26,27). However in a comparison study of recurrent miscarriage women with polycystic ovaries and women with normal ovarian morphology, the live birth rate was very similar, 58.5 percent and 60.9 percent respectively (23).

Hyperprolactinaemia has been found to increase the risk of recurrent miscarriage and is also associated with unexplained infertility (28). Tal *et al* (29) reported that prolactin reduced the levels of HCG secreted from the placenta during early pregnancy (29). Hirahara (30) carried out a study looking at pregnancy outcome of recurrent miscarriage women with hyperprolactinaemia who were receiving bromocriptine for their condition. 85.7 percent of women had a successful pregnancy outcome compared to the control group of which was only 52.4 percent (30).

#### **1.2.5.4 UTERINE FACTORS**

The structure of the uterus can also affect the viability and the development of the foetus. Uterine abnormalities are split into congenital or acquired malformations. Congenital malformations include double uterus (uterus didelphys), a singled sided uterus (unicornuate uterus), a heart shaped uterus (bicornuate uterus) and a septate uterus (midline muscle growth in the uterine cavity). Most of these abnormalities decrease the chance of pregnancy, increase the risk of miscarriage and also increase the risk of preterm labour. Research into pregnancy outcomes of women with abnormal uteri found that miscarriage is higher in women with a uterine abnormality than in those with a normal uterus, but these rates varied according to the type of



abnormality. Women with a septate uterus are at a greater risk of miscarriage than women with other abnormalities (31).

Acquired abnormalities include uterine fibroids and intrauterine adhesions. Fibroids (uterine leiomyoma) are benign muscle growths that arise from the wall of the uterus. There are three types of fibroids: 1) submucosal, the fibroid is in or impinging into the uterine cavity, 2) intramural where the fibroid is within the wall of the uterus and 3) subserosal where the fibroid is primarily on the outside (serosal surface) of the uterine wall. Typically, submucosal fibroids are thought to significantly decrease the likelihood of conception by 50-80% and significantly increase the likelihood of miscarriage. Intramural fibroids have less of an impact unless they are greater than 2 cm in size. Submucosal fibroids probably do not have a major impact on miscarriage rates. Fibroids can usually be surgically removed. Uterine adhesions are usually the result of previous uterine surgery. This is primarily a complication of postpartum D&C (dilation and curettage) procedures but can also occur after intrauterine surgery for fibroids or polyps.

Cervical incompetence is a known anatomical cause of recurrent miscarriage. It causes 20 - 25% of miscarriages in the second trimester. Dilation and effacement of the cervix occur without pain or uterine contractions. Cervical incompetence occurs because of weakness of the cervix, which is made to open by the growing pressure in the uterus as pregnancy progresses. The causes of cervical incompetence include D&C and cone biopsy. Treatment is usually cervical cerclage, where a special suture is put into the weakened cervix to keep it closed until term.

#### **1.2.5.5 IMMUNOLOGICAL FACTORS**

The two mechanisms involved in the immunological factors of recurrent miscarriage are said to be either autoimmune or alloimmune. Autoimmune disorders are a result of an individual's immune response to their own cells which results in destruction of their own cells. Examples of autoimmune diseases associated with recurrent

miscarriage include those that express antiphospholipid antibodies, including the lupus anticoagulant and anticardiolipin antibodies. These antibodies are directed against platelets and vascular endothelium, causing blood to clot more easily. It is thought that this results in improper blood flow in the placenta and the growing foetus does not receive the correct amount of oxygen and nutrition needed to survive and as a result, the foetus miscarries. There is evidence for an alternative mechanism by which antiphospholipid antibodies cause miscarriage, by their ability to directly inhibit trophoblast function (32).

The prevalence of antiphospholipid syndrome (APS) in women with recurrent miscarriage is 15 percent (33). The live birth rate in these women with no pharmacological intervention is as low as 10 percent. However APS is a treatable cause of miscarriage. Various treatments include aspirin, heparin, steroids and intravenous immunoglobulin. Although a meta-analysis shows only a combination of aspirin and heparin can significantly improve the live birth rate in women with recurrent miscarriage and antiphospholipid syndrome (34). Pregnancy outcome is enhanced by 54 percent with this treatment combination achieving over a 70 percent live birth rate amongst women with this syndrome (35). Although, there is one study that did not show a benefit of adding heparin to aspirin (36). However, the study used women with low anticardiolipin antibody levels and women were also assigned to the treatment group randomly at a late stage in their pregnancy where they were already likely to have a successful outcome. Moreover the use of pre-conceptual aspirin is controversial, recent publications have suggested that it causes harm.

An alloimmune response is the result of an immune response to the cells of another member of the same species. For example the foetus is an allogenic transplant that is tolerated by the mother for reasons that are incompletely understood. However we know several immunological mechanisms occur to prevent rejection of the foetus. These mechanisms include histocompatibility factors, circulating blocking factors, local suppressor factors and maternal or antipaternal antileukocytotoxic antibodies (13) and many more which are not clearly understood.

All pregnant women have a higher chance of developing a potentially dangerous thrombus because of an increase in coagulation factors in normal pregnancy. The likelihood of having a pregnancy associated thrombus is at least 10 times higher for women with either or both of the two most common inherited blood clotting disorders, factor V Leiden or a prothrombin mutation. Approximately 10-15% of women who have recurrent miscarriages have an inherited thrombophilia. However, factor V Leiden carries a greater risk for miscarriage than a prothrombin mutation. There is up to a 30% chance of miscarriage in those who have factor V Leiden, compared to a 4 to 9% chance for those who have a prothrombin mutation (37).

#### **1.2.5.6 CHEMICAL AGENTS**

Exposure to teratogens increases the chance of miscarriage. Teratogens can be toxic chemicals and radiation, certain viral and bacterial infections, cigarette smoke and alcohol. A 2006 analysis (38) of past research found evidence that occupational exposures to these chemical agents could increase risk of miscarriage: heavy metals (industrial workers, dental assistants), organic solvents (laboratory, industrial, and dry cleaning workers), tetrachloroethylene (dry cleaning workers), glycol ethers (semiconductor employees), 2-Bromopropane (electronics industry), petrochemicals, ethylene oxide (dental assistants), anesthetic gases (surgical staff) and antineoplastic drugs (oncology staff). The first trimester is the period in foetal development that is most vulnerable to teratogens.

Some viral and bacterial infections increase the risk of pregnancy loss. The 'TORCH' infections (Toxoplasmosis, Rubella, Cytomegalovirus and Herpes); endometrial Infection and syphilis may be associated with miscarriage. Infections of the cervix or vagina such as Mycoplasma, Ureaplasma, Bacterial vaginosis, etc. have been associated with pregnancy loss. The incidence of infection in the UK during pregnancy is low. Rubella infection can be prevented by immunisation. Most bacterial infections are relatively easily treated with specific or broad spectrum antibiotics. Viral infections

(CMV, Rubella, Chicken pox, hepatitis, etc) and their association with recurrent miscarriages are less certain.

A recent study showed that in women who have more than 200mg caffeine per day during pregnancy can increase the risk of miscarriage (39).

### **1.2.6 CLINICAL FEATURES**

Most cases of miscarriage present with vaginal bleeding and lower abdominal cramps. Some may have lower back pain and pass products of conception and mistake this for clots. Some patients can present with shock due to the blood loss.

### **1.2.7 TREATMENTS**

Treatment suggested for recurrent miscarriage ranges from bed rest to the most recent advanced medical intervention discovered. However, there is no research on how bed rest may improve pregnancy outcome in recurrent miscarriage. Earlier scanning in subsequent pregnancies has also been suggested. This may reduce anxiety in women with recurrent miscarriage. It has been hypothesised that reducing anxiety can reduce immunological factors that may be seen as harmful to the developing foetus. Studies have suggested giving human chorionic gonadotrophin may be more effective at reducing recurrent miscarriages than placebo. HCG significantly reduced miscarriage compared with placebo, 14% with HCG vs. 40% with placebo (40). However the results may not be reliable since the review included studies of women with two or more consecutive miscarriages. Some studies did not provide information on how their patients were randomised or allocated. Another method of using intravenous immunoglobulin was suggested to improve pregnancy outcome. However, a systemic review of 8 RCT's found no significant difference in live births between intravenous immunoglobulin treatment and controls (41).

Recently low dose aspirin has been given to women with recurrent miscarriage, though it still remains controversial as to whether this offers any benefit. A systematic review found no significant difference in the live birth rate of women taking low dose aspirin and placebo. Low dose aspirin plus unfractionated heparin is more effective at reducing pregnancy compared with low dose aspirin alone. Paternal white cell immunisation and oestrogen supplementation has also been used as treatment for recurrent miscarriage. This has also proved no more effective than previous treatments mentioned at improving live birth rates (41). A systematic review identified 3 RCT's that gave progesterone to women with 3 or more consecutive unexplained miscarriages. The progesterone significantly reduced miscarriage rates compared with control, 25% vs. 47% respectively (42).

### **1.3 - IMMUNE SYSTEM OVERVIEW**

In the absence of a cause for this distressing condition researchers have explored several avenues to a successful treatment. One of these avenues is manipulation of the immune system. Cells making up the immune system develop from pluripotent hematopoietic stem cells at a gestational age of five weeks. As the foetus develops they circulate through various organs in the lymphatic system. The immune system protects from infection with defences of increasing specificity. Bacteria and viruses are prevented from entering via physical barriers. If a pathogen crosses these barriers, the innate immune system provides an immediate, but non-specific response. However, the adaptive immune system is activated if pathogens successfully evade the innate response. The recognition of the pathogen and is then retained in the immunological memory after it has been eliminated. The adaptive immune system can now respond faster and eliminate the pathogen more rapidly if encountered again. The immune system is divided into the innate immune system and the acquired or adaptive immune system (Table 1).

<b>Components of the immune system</b>	
<b>Innate immune system</b>	<b>Adaptive immune system</b>
Antigen non-specific mechanism	Pathogen and antigen specific response
Exposure leads to immediate maximal response	Lag time between exposure and maximal response
Cell-mediated and humoral components	Cell-mediated and humoral components
No immunological memory	Exposure leads to immunological memory
Involves phagocytic cells: leukocytes, macrophages. Mast cells, NK Cells, complement proteins, acute phase proteins, interferons and cytokines.	Involves macrophages, dendritic cells, B, T lymphocytes, cytotoxic T lymphocytes and cytokines.

**Table 1:** Comparing the differences between innate and adaptive immunity.

Humoral immunity is mediated by secreted antibodies produced in B cells. The antibodies bind to antigens on the surfaces of invading microbes which flags them for destruction. It includes Th2 activation, cytokine production, memory cell generation, pathogen and toxin neutralisation, classical complement activation, and opsonin promotion of phagocytosis and pathogen elimination. Opsonins are molecules that coat the microbe and effectively mark them for their destruction. This enhances the efficiency of the phagocytic process.

Cell mediated immunity involves the activation of macrophages, NK cells, antigen-specific cytotoxic T-lymphocytes, and the release of many cytokines in response to an antigen. Cellular immunity provides protection by activating antigen-specific cytotoxic T-lymphocytes which induce apoptosis in cells displaying epitopes of foreign antigen on their surface. These include virus-infected cells, cells with intracellular bacteria, cancer cells displaying tumour antigens, activating macrophages and NK cells. They work by destroying intracellular pathogens. Cellular immunity also protects the body by stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive and innate immune responses.

Both components of the immune system depend on its ability to differentiate between self and non-self molecules. The immune system is able to distinguish *self* molecules from foreign. Conversely, *non-self* molecules are those recognised as foreign molecules.

### **1.3.1 CELLS OF THE IMMUNE SYSTEM** (43,44)

Phagocytes are subdivided into neutrophils and macrophages. They both have the same function, to engulf microbes. Neutrophils have a multilobular nucleus and play a role in the development of acute inflammation. They are also categorised as granulocytes since they contain granules which include acidic and alkaline phosphatases, defensins and peroxidase, all which are required to eliminate the unwanted microbe. Macrophages (also known as monocytes if found in the blood stream) are large cells with a horse shoe shaped nucleus. Its properties include phagocytosis and antigen presentation to T cells. Unlike neutrophils which are short lived, these cells are found in chronic inflammation since they are long lived cells.

Mast cells and basophils are very similar in nature. In the cytoplasm they both contain electron dense granules. Unlike mast cells which reside in close proximity to blood vessels in connective tissue, basophils are present in the circulation. Both cell types are involved in the initiation of an acute inflammatory response. Release of pro-inflammatory mediators including histamine and various cytokines is achieved by degranulation. This is achieved by binding to components of the complement system or by cross linkage of IgE antibodies. Histamine release induces vasodilation and enhances vascular permeability, whilst cytokines attract neutrophils and eosinophils. Eosinophils are granulocytes with a multilobed nucleus that also possess phagocytic properties. Even though they make up 2-5% of the total leukocyte population, they are involved in the eliminating parasites that are too big to be phagocytosed.

Dendritic cells consist of Langerhans' and interdigitating cells. They form an important bridge between innate and adaptive immunity. They are also known as antigen presenting cells as they present the antigenic peptide to the T helper cell.

There are 3 major molecules of the immune system. They are the complement, acute phase proteins and interferons. The complement system is made up of a large group of independent proteins secreted by hepatocytes and monocytes. The complement system consists of two pathways, an alternative and a classical pathway. Alternative



pathway is activated by the microbe itself and the classical pathway requires interaction of the antibody with a specific antigen. The complement system has the following functions, opsonisation, lysis (destruction of cells by damage or rupture of the plasma membrane), chemotaxis (migration of immune cells) and initiation of inflammation via direct activation of mast cells. Complement regulation is important to protect host cells from damage or total destruction. Acute phase proteins are produced in high numbers by the hepatocytes in response to cytokines released by macrophages. Interferons are molecules which prevent the spread of viral infections. They are split into two categories, type I and type II. Type I are further divided into IFN- $\alpha$  and  $\beta$ . IFN- $\gamma$  is the only type II interferon. Type I is induced by viruses, pro-inflammatory cytokines and endotoxins from gram negative bacterial cell walls. Type II is produced by T helper cells and NK cells and includes both antigen presenting properties and phagocytic properties.

Lymphocytes include T, B and NK cells. However, only T and B exhibit memory and specificity. B lymphocytes react with the free antigen directly when it binds to their cell surface immunoglobins which act as receptors, whereas T lymphocytes make use of APC's to phagocytose the antigen and then express its component protein on the cell surface. This needs to occur in order for the T cell to recognise the antigen.

T cells are divided into helper T ( $T_H$ ) and cytotoxic ( $T_C$ ) T cells.  $T_H$  is further subdivided into  $T_{H1}$  and  $T_{H2}$ . The former stimulate macrophages and are pro-inflammatory T cells, whilst the latter manage B cell differentiation and maturation and hence are involved in the production of humoral immunity. The third T-helper cell population is that of the  $T_{H0}$  cells; these are precursor cells which can be converted to either  $T_{H1}$  or  $T_{H2}$  type cells and can produce  $T_{H1}$  and  $T_{H2}$  cytokines as well as TNF- $\alpha$  and granulocyte-macrophage colony stimulating factor (GM-CSF) (45).

T cells express cell surface proteins,  $T_H$  cells express CD4 molecules on their cell surface which allows lymphocytes to bind to MHC class II molecule. The T cell receptor can only identify antigen when it is associated with MHC class molecules on the surface of

the cell. Cytotoxic T cells are involved in the destruction of infected cells, i.e. viruses. Unlike helper T cells, cytotoxic T cells possess CD8 surface molecules which bind to antigenic peptides expressed on MHC class I molecules. Considerable interest into the role of cytokines in pregnancy has developed over recent years.  $T_{H2}$  cytokines are found to favour successful pregnancy, whilst  $T_{H1}$  cytokines are considered to be detrimental (46). Although the original hypothesis was based on T helper cell cytokine production.

B cells are lymphocytes that produce antibodies (immunoglobulins) and can recognise free antigens directly. They originate from the bone marrow and migrate to secondary lymphoid organs. B cells are responsible for humoral immunity. B cells mature in antibody secreting plasma cells once they are activated by a foreign antigen. Not all B cells proliferate and develop into plasma cells. Clonal selection allows a significant proportion to remain as memory B cells. This vital process eliminates the antigen if in the future the body became re-exposed to it. T cells are also clonally selected to form memory T cells. Both T and B cells are able to re-circulate and migrate around the body, which increases the efficiency with which cells of the immune system can home onto the invading antigen. Antibodies play two roles, firstly to bind with the antigen and the second is to interact with host tissues and effector systems to ensure removal of the antigen (44).

Major histocompatibility complex (MHC) are cell surface proteins classified as class I (also known as human leucocytic antigen [HLA] A, B and C) which are found on all nucleated cells and class II (also known as HLA, DP, DQ and DR) which are found on all antigen presenting cells (APCs). MHC molecules are the outcome of T cell induced immunity.

Natural Killer cells are also known as large granuloocytes which are mainly found in the circulation. They make up 5-15% of the total lymphocyte population (44). They have receptors to IgG and also contain two unique cell surface receptors known as killer activation receptor (KAR) and killer inhibition receptor (KIR). Activation of KAR initiates

cytokine ‘communication’ molecules from the cell and activation of KIR inhibits this action. NK cells play an important role in destroying virally infected cells in addition to certain tumour cells. The granules within the NK cell release perforins and granzymes which induce apoptosis of the infected cell. NK cells also secrete IFN- $\gamma$  which serves two purposes, first, to prevent healthy host cells from being infected by a virus and second, to enhance the T cell response to other virally infected cells.

Cytokines, also known as interleukins (meaning ‘between white blood cells’) are small molecules which act as a signal between cells (Table 2). Cytokines play a role in chemotaxis, cellular growth and cytotoxicity. They have been described as ‘hormones’ of the immune system due to their ability to control immune activity.

Cytokine	Source	Function
IL-1	Macrophages	Inflammatory response, Increase adhesion factor expression on endothelial cells
IL-2	T <sub>H1</sub> cells	Stimulates cytotoxic T Cells
IL-4	T <sub>H2</sub> cells Mast cells	B cell activation
IL-5	T <sub>H2</sub> cells	B cell activation
IL-6	T <sub>H2</sub> cells	B cell activation
IL-8	Macrophages T cells Fibroblasts Keratinocytes	Chemotaxis of PMNs
IL-10	T <sub>H2</sub> cells Macrophages	B cell activation Suppress macrophages
IL-12	B cells	Stimulate T <sub>H1</sub> Inhibit T <sub>H2</sub>
TGF $\beta$ (transforming growth factor)	T cells	Inhibits other cytokines
TNF $\alpha$ (tumour necrosis factor)	Macrophages	Inflammation

**TABLE 2:** Highlighting the various types of cytokines, where they come from and the role they play in the immune system.

### **1.3.2 PREGNANCY AND THE IMMUNE SYSTEM**

Pregnancy is a biological paradox. Genetically foreign material will be rejected by the mother's immune system. However, the immune system yet does not reject a genetically foreign foetus, which implants into the uterine wall and establishes an intimate contact with the maternal blood supply. For years now it has been speculated that a defect in the mother's immune system towards the allogenic foetus may be involved in the mechanism of recurrent miscarriage. Maternal Cytotoxic T cells are said to be inhibited during pregnancy, this allows the foetus to avoid rejection by its mother. However this finding was discovered in a previous study involving mice. Recent work in humans has suggested that immunity of pregnancy is likely to be much more complicated. Although there are changes in the levels of T-cell during pregnancy, the first phase is localised in the uterus and is mediated by the mother's NK cells (44).

### **1.3.3 IMMUNOLOGY OF THE FETO-MATERNAL INTERFACE**

The only site of direct contact between foetus and the mother is at the foeto–maternal interface in the uterus. The trophoblast for many years has been seen to act as a barrier between the transport of maternal and foetal cells. The interface is made up of trophoblast cells on the foetal side and immune-competent cells and decidual tissue on the maternal side. All important immune reactions occur between these cells at the interface. Consequently, the antigens and other immunological markers expressed by them will be of considerable interest (47).

The maternal interface protects the pregnancy from the maternal immune system. The placenta also uses many mechanisms to avoid being rejected by the mother's immune system. Neurokinin B containing phosphocholine molecules are secreted as well as small lymphocytic suppressor cells which inhibit maternal Cytotoxic T cells by inhibiting the response to interleukin 2. The foetus also secretes immunosuppressive agents such as platelet-activating factor, hCG, early pregnancy factor, immunosuppressive factor, prostaglandin E2, interleukin 1- $\alpha$ , interleukin 6, interferon- $\alpha$ , leukaemia inhibitory factor and colony stimulating factor, which all help avoid rejection by the immune system of the mother.

## **1.4 - NATURAL KILLER (NK) CELLS**

NK cells are specific lymphocytes which originate from stem cell precursors in the bone marrow and are a part of the innate immune system. They acquire cytotoxic qualities. NK cells secrete several cytokines (e.g. IFN- $\gamma$ , IL-1, tumour-necrosis factor  $\alpha$  [TNF- $\alpha$ ]), and are a major source of IFN- $\gamma$ . By secreting IFN- $\gamma$ , NK cells can promote differentiation of type 1 helper T (T<sub>H1</sub>) cells and inhibit that of type 2 (T<sub>H2</sub>) cells. NK cells also recognise target cells by the decreased or absent expression of MHC class I molecules. NK cells are able to differentiate between self and foreign by the presence or absence of MHC class I molecules. Non-infected cells express MHC class I molecules on their surface, but virus-infected and malignant cells have a reduced expression, so NK cells will eliminate the cells that are infected. Many viruses and tumour cells use this tool to evade detection. NK cells are an active subject of investigation because they are able to differentiate between self and non-self molecules, a process that goes awry in diseases associated with the immune system.

A network of specific activating and inhibitory receptors tightly regulate NK cell function. In particular, CD69 is one of the earliest cell surface activation markers expressed by NK cells. NK cells also express a variety of killer inhibitory receptors (KIRs) and killing activating receptors (KARs), which recognize HLA-G expressed on extra villous trophoblast (48).

Peripheral blood and the uterine mucosa each contain NK cells, but the cells in these two areas have important phenotype and functional differences and therefore should be considered as separate lymphoid subsets.

### **1.4.1 PERIPHERAL BLOOD NK (pbNK) CELLS**

Typical pbNK cells make up 5 to 15% of mononuclear cells. They induce apoptosis of infected or abnormal cells by a number of pathways. NK cells originate from the bone marrow. After differentiation, they enter into the peripheral circulation at a very early stage of maturation (49). They are cells of the innate immune system and therefore they lack antigen-specific receptors and immunological memory. pbNK cells are best characterised by CD2<sup>+</sup>, CD3<sup>-</sup>, CD4<sup>-</sup>, CD8<sup>+</sup>, CD16<sup>+</sup> and CD56<sup>+</sup> surface markers. The cell surface antigen CD16 is expressed on the majority of cells and is a low affinity receptor for IgG complexes. It is responsible for NK-mediated, antibody-dependent cellular cytotoxicity.

Based on the intensity of the CD56 expression, pbNK cells are divided into two populations: CD56<sup>dim</sup> and CD56<sup>bright</sup>.

### **1.4.2 UTERINE NK (uNK) CELLS**

The presence of granulated cells within the human endometrium has been recognised since the 1920s and several later reports described their distribution and staining characteristics (50). It should be remembered, however, that at least in humans uNK cells are not really 'uterine' but 'endometrial', being absent in myometrium. NK cells are the most abundant immune cells infiltrating the endometrium. 70% of infiltrating leukocytes are CD56<sup>bright</sup> NK cells, together with some macrophages and a small number of T cells. B cells are practically absent (48). uNK cells are best characterised by CD56<sup>bright</sup>, CD16<sup>-</sup>, CD3<sup>-</sup> and CD4<sup>-</sup> antigens (50-52).

After ovulation, uNK cells proliferate vigorously so that by the late secretory phase they account for at least 30% of the endometrial stroma (53). Endometrial derived IL-15 and prolactin have been implicated in the proliferation and differentiation of these cells. They are both produced by the mucosal stromal cells and their expression is upregulated by progesterone (54,55). Mice lacking genes for the common  $\beta$  chain, common  $\gamma$  chain or IL-15 display severe deficiency of uNK cells, suggesting that IL-15 receptor signalling is essential for uNK cell differentiation (56).

uNK cells contribute to the cytokine response at the materno-foetal interface either as a T<sub>H1</sub> or a T<sub>H2</sub> type cytokine response. Normal pregnancy is said to be the result of a T<sub>H2</sub> type cytokine response, in which blocking antibodies disguise foetal trophoblast antigens from immunological recognition by a maternal T<sub>H1</sub> cell-mediated cytotoxic response (46). By contrast, women with RM tend to produce a predominantly T<sub>H1</sub> type response both in the period of embryonic implantation and during pregnancy (57-59). These findings support the view that disturbances of the immune tolerance of the foetus could contribute to RM.

The pattern of expression of inhibitory receptor CD94/NKG2A found on uNK cells for which the ligand is HLA-E has shown that it is different from the expression pattern of pbNK cells taken from the same woman at the same time. Maternal cells and trophoblast cells both express HLA-E, this interaction might prevent the lysis of any



tissue cells (either maternal or foetal) in the vicinity (60). Receptors for HLA-C have also been found to be expressed by uNK cells (61-65). These are members of the KIR multigene family. They regulate the killing function of NK cells by interacting with MHC class I molecules, which are expressed on all cell types. This interaction allows them to detect virally infected cells or tumour cells that have a characteristic low level of Class I MHC on their surface. Most KIRs are inhibitory, meaning that their recognition of MHC suppresses the cytotoxic activity of their NK cell. KIR's that are specific for HLA-C are expressed by a greater proportion of uNK cells of pregnant women compared to pbNK cells, which indicates that NK cell receptor repertoire is skewed towards recognition of HLA-C in the uterus (66,67). Interaction should occur between foetal HLA-C and maternal KIR's during pregnancy (67). KIR molecules are highly polymorphic meaning their gene sequences differ greatly between individuals, therefore each pregnancy will involve different combinations of foetal HLA-C and maternal KIR's, it is possible that some combinations may be less optimal for implantation and therefore these pregnancies fail.

Moreover, polymorphisms in KIR molecules on uNK cells in combination with specific polymorphisms in their ligand on fetal trophoblast have been associated with pre-eclampsia, a poor trophoblast invasion (68,69).

The presence of this unique type of lymphocyte in the uterus at implantation and during early placentation is intriguing. Despite much speculation, the function of uterine NK cells is completely unknown. However, it has been suggested that uNK cells may be relevant to that pathogenesis of recurrent miscarriage. For example, Women with RM have a higher number of uNK cells compared to controls, and those with higher numbers have a correspondingly higher rate of miscarriage in subsequent pregnancies without treatment (69-71). These findings have prompted us, and others, to examine uNK cells in detail. An overview of uNK cells is now given to set the scene for the experimental work in this thesis.

### **1.4.2.1 RECEPTOR EXPRESSION OF UNK CELLS**

A series of studies in mice, humans and *in vivo* suggest that uNK cells are hormonally regulated (72). As uNK cells increase in number in the mid-luteal phase of the menstrual cycle, they were at first thought to be controlled by progesterone. The menstrual cycle dependent variation of uNK cells bears a resemblance to that of serum progesterone concentration, suggesting the involvement of progesterone in the proliferation of these uNK cells.

However, uNK cells do not express progesterone receptors but do express the prolactin receptor, oestrogen receptor  $\beta$  and glucocorticoid receptors (54,73). It has been thought that progesterone shows a low avidity to glucocorticoid receptors (74). For this reason, ovarian steroids have been considered to have the potential to stimulate *in situ* proliferation of uNK cells directly. uNK cells may be regulated by the action of oestrogen via oestrogen receptor  $\beta$  or by progesterone via a receptor that is yet to be discovered. Although, a study which used isolated uNK cells found that neither progesterone nor  $\beta$ -estradiol affected the proliferation activity, cytolytic activity or cytokine secretion of these uNK cells *in vitro* (75). It is therefore unlikely that ovarian steroids directly affect *in situ* proliferation of uNK cells at a physiological concentration.

Prolactin is produced by endometrial stromal cells between the mid and late-secretory phase. In the event of pregnancy prolactin synthesis increases significantly and peaks at around 20–25 weeks gestation (76). It is believed that the prolactin plays an important role in implantation and the maintenance of pregnancy. The pattern of expression of prolactin corresponds with the temporal rise in uNK cell number during the luteal phase and in pregnancy, suggesting a possible role of prolactin in this process. The precise function of prolactin on uNK cells remains to be uncertain, though it may regulate uNK cell proliferation or maturation (54). The prolactin receptor was identified on uNK cells by *in situ* immunofluorescence and in purified decidual NK cells by real-time PCR and Western blot analysis. Therefore, through inducing extracellular

signal-regulated kinases (ERK) phosphorylation directly on uNK cells, prolactin may regulate their differentiation and proliferation (54).

Alternatively, uNK cells could be regulated by an indirect mechanism within which progesterone acts on the endometrial T cells and stromal cells, affecting VEGF and macrophage inflammatory protein-1 $\beta$  to enhance recruitment of uNK cells to the uterus, as well as acting through prolactin, via IL-15, to increase proliferation and differentiation of uNK cells (72). Several cytokines have been shown to stimulate the proliferation of uNK cells *in vitro*. One of these is cytokines is IL-2 (77), but IL-2 is not evident in the non-pathologic human endometrium and decidua (78,79). IL-12 also has the potential to stimulate proliferation of uNK cells, but in parallel, IL-12 stimulates their cytolytic activity (80). Stem cell factor may be a candidate cytokine expressed in the human endometrium (81) but alone, in the absence of IL-2 or IL-15, stem cell factor cannot stimulate proliferation of uNK cells (82). Lymphotoxin- $\beta$  receptor, a TNF- $\alpha$  receptor family, considered essential for NK cell differentiation in bone marrow of murine models (83), was found expressed in all cell types in the human endometrium. However, its expression in the human endometrium did not vary with menstrual cycle-dependent fluctuation. From these findings, these cytokines are less likely to be the involved in *in situ* proliferation of uNK cells.

IL-15 is considered a T cell growth factor which shares several properties with IL-2 (84). IL-15 is believed to selectively stimulate proliferation of pbNK cells compared with other peripheral blood lymphocytes (85) IL-15 also stimulates uNK cell proliferation without increasing its cytolytic activity against trophoblasts (67,86). IL-15 protein is rarely detected even though it is expressed largely in various organs (84). IL-15 is expressed in the human endometrium at the protein level. There is a higher expression of IL-15 in the secretory phase of the endometrium compared to the proliferative phase. It originates from endometrial epithelial cells, macrophages and peri-vascular stromal fibroblasts. The production of IL-15 by the endometrial stromal fibroblasts *in vitro* is enhanced by progesterone (75). These findings suggest that IL-15 is a strong possible stimulator for *in situ* proliferation of uNK cells.

Stem cell factor or IL-18 may assist with IL-15, but no evidence exists that IL-18 single-handedly stimulates the proliferation of uNK cells (87). It is possible that IL-15 is produced by progesterone stimulation from endometrial stromal fibroblasts which, in turn, stimulates the proliferation of uNK cells (88).

In favour of *in situ* proliferation of uNK cells is their expression of a cell proliferation associated nuclear marker, Ki67. Immunohistochemical studies carried out on frozen endometrial sections and purified cells have established that up to 40% of CD56<sup>+</sup> uNK cells that are isolated from late secretory phase of the endometrium express the antigen for Ki67 (89).

The expression of three natural cytotoxicity receptors (NCRs) NKp30, NKp44, NKp46 and NKG2D, also known as activating receptors on uNK cells in the endometrium have not been looked at in a great deal. Although, it has already been found that all the activating receptors mentioned are expressed in the pregnant decidua and instead of triggering cytotoxicity, these receptors act to stimulate secretion of cytokines and angiogenic factors essential for trophoblast invasion and vascular remodelling (90).

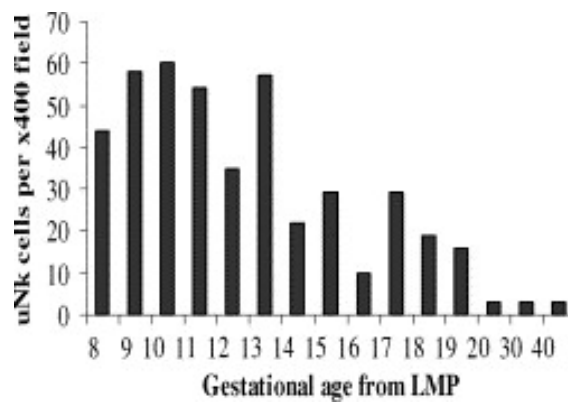
A study found an increase in the expression of NKp30 and NKp44 on uNK cells in the presence of IL-15. However, these results suggest that uNK cells may exist as immature subsets which differentiate once activated. uNK cells are said to be inert lymphocytes in the endometrium that are incapable of killing target cells or secreting NK known cytokines and growth factors, before IL-15 activation (91). However these findings exist *in vitro* and may not necessarily reflect what is happening *in vivo*, this has yet to be investigated.

Moreover, another study did find NKp30 to be selectively expressed in resting and activated NK cells. NKp30 protein was localised to endometrial glands and luminal epithelium during early-, mid- and late secretory stages of the menstrual cycle and replacing the NKp30 antibody with an equivalent amount of control goat IgG resulted in the complete absence of immunoreactivity. The NKp30 protein was found to be expressed in high levels at the time of implantation, while hormonal regulation studies

showed that the expression NKp30 can be induced by progesterone. These observations suggest a possible role for NKp30 in the process of implantation (92).

### **1.4.3 uNK CELLS AND THE MENSTRUAL CYCLE**

The endometrium supports a variety of leukocyte populations whose numbers and proportions fluctuate cyclically with menstrual cycle stage. During the proliferative phase, leukocytes are less frequent and scattered. Ovulation, induced by a mid-cycle LH surge, terminates this phase, and leads to progesterone secretion from the corpus luteum and to leukocyte recruitment (93). As seen in Figure 1, uNK cells are maximal in number in the mid and late secretory phase when implantation occurs and continue into early pregnancy (50).



*Fig 1: The graph above highlights the relationship between uNK cell numbers in the decidua and gestational age. Placental bed cells were counted from pregnant hysterectomy samples (n = 35) (taken with permission from Bulmer and Lash 2005 (50)).*

uNK cells are small and agranular in the proliferative pre-ovulatory phase, then they proliferate and enlarge and become increasingly granulated in the post-ovulatory secretory phase. Two days before menstruation, when progesterone levels begin to decline uNK cells undergo nuclear changes of which cell death is indicated although the classical apoptosis features are absent. This morphological feature indicates that menstrual breakdown of the uterine lining will occur. During early pregnancy the uNK cells densely infiltrate around the trophoblast cells, but mid-gestation onwards they progressively decrease and by term they have significantly decreased (94). This presence of uNK cells coincides with trophoblast invasion and placentation, which is completed by 20 weeks gestation after which the uNK cells progressively disappear (48,95-97).

The fall of uNK cell numbers towards the end of pregnancy and also at the end of the menstrual cycle remains unexplained. However, cellular death by apoptosis and necrosis has been proposed for the loss of uNK cells in the later stages of mouse pregnancy (98) but no information is yet available concerning human pregnancy. Although a study has reported apoptosis of decidual leucocytes (99) another study failed to detect this in early human pregnancy (100). The possibility that uNK cells degranulate in late pregnancy has also been raised (101) to possibly account for the discrepancies in the number of CD56<sup>+</sup> cells noted in immunohistochemical studies and the number of cells recognised in older studies by their cytoplasmic granules. However, it has been suggested that this could be addressed by analysis of perforin, a cytolytic protein found in the granules of NK cells, at different stages of pregnancy.

Also the rounded hyperchromatic nuclei observed in uNK cells in the pre-menstrual endometrium has led to the proposal that these cells undergo apoptosis pre-menstrually. However, a study found no evidence for this (102). Furthermore, it was found that the CD56<sup>+</sup> cells once separated from the late secretory phase and premenstrual endometrium were still capable of proliferative activity and cytotoxicity. Up to 40% still expressed Ki67 indicating proliferation rather than apoptosis (89,103,104).

On the other hand, uNK cells in the non-pregnant murine endometrium exist in a small number. They appear within 24 hours after embryo implantation (on gestational day 5) in the decida basalis, mesometrial triangle and mesometrial lymphoid aggregates of pregnancy (these are lymphoid structures that develop in the myometrium at implantation sites surrounding uterine arterial branches). Murine uNK cells increase until gestational day 12, and then they start to degenerate between gestational days 12–15 (94). The increase of murine uNK cells is seen also in the pseudopregnant uterus, i.e. the artificially induced deciduomata (105).

#### **1.4.4 ROLE OF uNK CELLS**

The function of uNK cells is unknown. The temporal and spatial distribution of uNK cells suggests they contribute to the control of trophoblast invasion. Attention has also been directed at their possible role in structural adaptation of the uterine spiral arteries (53). Although, there is little information regarding the distribution of uNK cells in the second trimester, since tissues from this stage of normal pregnancy is unavailable.

##### **1.4.4.1 CONTROL OF TROPHOBLAST INVASION**

Invasion of uterine tissues and spiral arteries is an essential feature of successful pregnancy in humans (106). This invasion is tightly controlled; too much or too little invasion can cause pre-eclampsia, foetal growth restriction, preterm labour and miscarriage (106). Invasion of the trophoblast into the uterine wall occurs from implantation onwards. It extends by the end of the first half of pregnancy into the inner third of the myometrium. The uterine spiral arteries are invaded by the trophoblast cells, which then migrate within the lumen to as far as the inner third of the myometrium. The end result of the trophoblast invasion is the loss of the arterial media smooth muscle and elastin. The spiral arteries are now dilated vascular channels which are able to supply the low resistance and increased maternal blood supply required to the fetoplacental unit and do not respond to any vasomotor influences (50).

It has been proposed, due to their interaction with trophoblast cells in early pregnancy that uNK cells play an active role in the regulation of trophoblast invasion. Several mechanisms have been suggested for this association, they include cytotoxicity, local cytokine production and trophoblast apoptosis. Although many studies have found unlike pbNK cells, uNK cells possess very little cytotoxic activity against trophoblast cells without prior activation with IL-2 (107-110).



As already noted uNK cells are a rich source of many different cytokines and growth factors (111-114). Altered production of IFN- $\gamma$  has been reported in decidua basalis compared with decidua parietalis (115,116) has reported an increase in the production of IFN- $\gamma$  and VEGF by uNK cells in response to HLA-G. However, Ang-2 (an angiopoietin, a protein growth factor that promotes angiogenesis from pre-existing blood vessels) (117), TNF- $\alpha$  (118), IFN- $\gamma$  and TGF- $\beta$ 1 (119) have all been shown to inhibit trophoblast invasion. Although the cytokines and growth factors produced by uNK cells have been studied using in vitro invasion assays. However, the overall effect of uNK cells on trophoblast invasion has not yet been investigated in detail.

A study found 30% of trophoblast cells determined by immunostaining are undergoing apoptosis in the early gestational placental beds of women with uncomplicated pregnancies (50). Double immunohistochemical labelling has revealed that many apoptosing trophoblast cells are surrounded by uNK cells. Though, the stimulus for the initiation of apoptosis in the trophoblast cells still remains uncertain. The ability of uNK cells to initiate cellular apoptosis is poorly studied. Another probable explanation for the association between apoptosing trophoblast cells and uNK cells may be that the trophoblast cells which undergo apoptosis recruit uNK cells after apoptosis has been initiated. If uNK cells are believed to regulate trophoblast invasion then the mechanism by which this occurs is most likely mediated by uNK cell cytokine secretion (50).

In about two-thirds of first trimester miscarriages there is histological evidence of premature onset of maternal blood flow into the intervillous space throughout the placenta (120,121). This blood flow is found to lead to a state of oxidative stress which is said to be associated with trophoblast apoptosis. This finding is independent of the foetal karyotype (122-124).

There is no convincing evidence that uNK cells kill placental trophoblast cells. Instead, they are said to have an essential, beneficial effect on the trophoblast by secreting cytokines that alter the depth of placental invasion. Unlike T lymphocytes, NK cells are able to spontaneously kill cells in a non-MHC restricted manner. NK cells have a

misleading name in reproduction, and the powerful illustration of maternal cells attacking the foetus is controversial and easily exploited (53).

#### **1.4.4.2 CONTROL OF VASCULAR REMODELLING**

uNK cells have also been proposed to play a role in spiral artery transformation (94). Although most of the evidence for uNK cell involvement in spiral artery transformation has come from mouse models. For example, NK, T and B cell deficient female mice that do not have fertility problems but experience >50% foetal loss between 10 and 14 days post conception and of those that survive have a very small placenta and they remain small throughout their adult lives compared to controls. Also, there is a lack of remodelling of the uterine spiral arteries in these mice. However, transplantation of bone marrow from donors results in a reconstitution of uNK cells without a reconstitution of T cells. This reverses the reproductive deficiencies of the mice (125). It has also been demonstrated that the key uNK cell product accountable for the spiral artery remodelling defects is IFN- $\gamma$ . Mice deficient in uNK cells or IFN- $\gamma$  signalling have implantation site abnormalities and failure of artery remodelling. These defects are not overturned when the deficient mice are given bone marrow from IFN- $\gamma$  deficient controls. This strongly suggests the function of uNK cells in mouse are mediated by IFN- $\gamma$  (126).

Much less is known concerning the mechanisms involved in spiral artery remodelling in humans. Early structural changes occur before cellular interaction with trophoblast such as dilatation in the spiral arteries (127) but at a time when uNK cells are present (95) uNK cells are seen to reduce in number after 20 weeks' gestation when vascular changes are normally complete. In the placental bed uNK cells are seen to be very closely associated with non-transformed, transformed and transforming spiral arteries (50).

In addition to IFN- $\gamma$ , Ang-2 may be an important uNK cell derived mediator of spiral artery transformation. On binding to Tie-2 (the cell-surface receptor which binds and is activated by the angiopoietins) Ang-2 acts to counteract the effects of VEGF-A and

Ang-1 to destabilise vessel structure. It is only found to be expressed in tissues where vascular remodelling is taking place. uNK cells are a rich source of Ang-2 (114) and are found mostly in association with vessels undergoing remodelling in the placental bed. The mechanisms for spiral artery transformation are poorly understood due to a lack of appropriate human models for the study. Additionally, the changes that the remodelled vessels undergo are inadequately defined.

uNK cells may affect the endometrium by controlling vascular function through secretion of angiogenic growth factors. In this way they may be crucial in the decision to switch from endometrial breakdown (menstruation) to decidualisation in pregnancy (128). Investigation of uNK cells in human pregnancy is held back by the lack of availability of tissues from the first half of the second trimester of pregnancy when vascular remodelling occurs and also by possible differences between cells from different regions of decidua (50).

The following need to be considered before the role of uNK cells is established. uNK cells are confined to the endometrium, whereas trophoblast invasion and spiral artery transformation extends into the inner third of the myometrium where there are no uNK cells. A possible theory may be that uNK cells initiate the trophoblast invasion and spiral artery transformation. Also, Invasion by trophoblast occurs in ectopic pregnancies in the absence of uNK cells (129,130). It is difficult to assess whether this invasion is extreme given that the wall of the fallopian tube is not equivalent to that of the uterus and that the rupture of the fallopian tube may be due to mechanical reasons rather than excessive trophoblast invasion. uNK cells are also present in numerous species which do not go through invasive placentation and so an alternative role would need to be proposed for these.

### **1.4.5 UNK CELLS AND RM**

Many clinicians believe that some women with RM have an endometrial factor contributing to the pregnancy losses (23). Studies of the preimplantation endometrium have found differences in the uNK cell populations in women suffering idiopathic RM. Using flow cytometry, CD16<sup>-</sup>CD56<sup>bright</sup> NK cells were decreased and CD16<sup>+</sup>CD56<sup>dim</sup> NK cells were increased in the luteal phase endometrium from women who had suffered RM (69). With immunohistochemistry increased numbers of CD56<sup>+</sup> cells were detected in the mid-luteal phase endometrium compared with fertile controls (70,71,131). Moreover, high numbers of uNK cells in women with RM were predictive of miscarriage in subsequent pregnancy (71). However there are two studies that have failed to confirm an association between uNK cells in RM (132,133) although they did include women with only two miscarriages. Though, others have found that women with just two miscarriages cannot be considered to be suffering from any pathology since they have such a high live birth rate (85%) in subsequent pregnancies (134-136).

An association between high numbers of uNK cells and women with idiopathic RM of severe phenotype has been confirmed (131). Research has identified those women who have had a previous birth, have a significantly lower number of uNK cells compared to women who have not previously given birth. This is said to be the result of the complex process of pregnancy and birth which involves extensive changes in the size and vascularisation of the uterus. However, in a study of five women who all had a previous live birth had uNK cell numbers of >5%. This excludes the possibility that a live birth reduces the uNK cell numbers to <5% in all women regardless of whether they have given birth in the past (131). Much previous work on the endometrium of women with RM has suggested that a luteal phase defect may be involved. Luteal phase defect is believed to interfere with the implantation of embryos (23). The results of another study suggest that the increased numbers of uNK cells seen in the endometrium of women with RM are independent of luteal phase defects (137).

Two competing hypotheses have been suggested for how uNK cells may lead to RM. Firstly, uNK cells may be hostile to the invading trophoblast or secondly, uNK cells facilitate the implantation of abnormal blastocysts, which leads to the clinical presentation of RM (138). A problem with the first hypothesis is that uNK cells are needed for normal pregnancy to occur, at least in mice (139). There is uncertainty as to whether high numbers of uNK cells may be harmful and whether they should be decreased. The second hypothesis is supported by data which shows that uNK cells are more numerous in the decidua from chromosomally abnormal miscarriages than in those from chromosomally normal miscarriages (140). Differences were found in the decidual leucocytes from the miscarried women with unexplained RM and a normal foetal karyotype compared to women with unexplained RM and abnormal foetal karyotype (141).

### **1.4.6 pbNK CELLS AND RM**

Some studies have found an association between pbNK cells and RM. Studies have shown that women with unexplained RM have increased numbers and activity of pbNK cells and that increased levels of these cells predict subsequent miscarriages in women with RM (142,143). However, such increases may not reflect a condition of overactive immunity but may result from a transient increase in the number of NK cells because of the stress associated with blood withdrawal (144).

However, one recent study found no significant difference in the number of pbNK cells between women with RM and control subjects. Only 5% of women with RM had high pbNK cells. The number of previous miscarriages did not appear to have an impact on the number of NK cells. Therefore it concludes that there is limited value in the routine measurement of pbNK cells in women with RM (145).

Based on the alleged similarities between pbNK and uNK cells, it has become increasingly common to examine pbNK cells in women with RM and infertility. These tests are based on the theory that these women have abnormalities in uNK cell function, and it has been implied that these are apparent from analysis of pbNK cells (146,147). This approach has several problems. Firstly, as mentioned earlier, uNK cells are different from those in peripheral blood. Examination of pbNK cells will not tell us what is happening in the uterus.

Secondly, it is unclear what an abnormal NK cell number is. The percentage of CD56<sup>+</sup> pbNK cells in normal healthy individuals varies from 5% to 29% (148). Despite this, a finding of more than 12% pbNK cells in women with infertility or RM has been arbitrarily defined as the cut-off point for abnormally raised NK cells. Hence, individuals with entirely normal results are being labelled as having raised NK cell numbers and is used as an indication for treatment (149). Many factors are said to affect the percentage of pbNK cells, these include sex, ethnicity, stress, and age, but there is no indication that concentrations in the upper end of the normal range are

ever harmful. Furthermore, CD56 can also be expressed by activated T cells and NK T cells and the inclusion of these may alter results.

Thirdly, NK cell activity is measured by a range of assays and the results vary in different laboratories. The most commonly used in vitro assay is cytotoxicity, which may not have much relevance to NK cell function in vivo. Certainly, in viral infection, NK cells function mainly by producing cytokines. Furthermore, uNK cells have a much lower cytolytic activity than pbNK cells. Thus, no clinically relevant information is gained from studying either the percentage or cytotoxicity of pbNK cells in women with pregnancy failure. Therefore it is important to look at uNK cells as a separate lymphoid subset to identify their origin, location and function to help achieve a better understanding of their role in women with RM and infertility.

Patient's expectations for an answers or even cures for their reproductive failures are ever increasing leaving them vulnerable to exploitation by those implementing new investigations and treatments that have little scientific validity. Some units have charged couples for pbNK cell estimations during the work up for recurrent miscarriage. No association between the levels of pbNK cells and uNK cells has been recorded, and levels of pbNK cells are not predictive of pregnancy outcome in women with unexplained RM. Therefore, the value of testing women with RM for levels of pbNK cells is questionable (53).

### **1.4.7 ORIGIN OF UNK CELLS**

The origin of uNK cells is unknown, but they are said to closely resemble the minor CD56<sup>bright</sup> pbNK cell population. One possible theory suggested for this is that the pbNK cells may move into the uterus, this is known as trafficking of pbNK cells. They are then said to undergo proliferation, differentiation and enlargement and acquire cytoplasmic granules (48). Another possible theory suggested is that haematopoietic progenitor cells that reside in the endometrium proliferate and differentiate into uNK cells (150).

#### **1.4.7.1 PROLIFERATION & DIFFERENTIATION**

In favour of *in situ* proliferation of uNK cells is their expression of a cell proliferation associated nuclear marker, Ki67. Immunohistochemical studies carried out on frozen endometrial sections and purified cells have established that up to 40% of CD56<sup>+</sup> uNK cells that are isolated from late secretory phase of the endometrium express the antigen for Ki67 (89) and, stromal mitoses is a recognised feature of the pre-menstrual endometrium. Also, a dual immunohistochemical study demonstrated that the positive rate of Ki67 in uNK cells was higher in the secretory phase than in the proliferative phase (151). This was confirmed using three different methods (152). It is therefore possible that local proliferation of uNK cells could account for their dramatic increase in number. The increase in uNK cell number by local proliferation would have to be from stem cell precursors within the endometrium remaining after menstruation or from residual CD56<sup>+</sup> cells in the stratum basalis which is not shed at menstruation, since at no point during the menstrual cycle is there any substantial number of CD16<sup>+</sup> NK cells (50). Up-regulation of genes controlling proliferation has been found to occur in the luteal phase endometrium (153).

In murine models uNK precursor cells have limited self-renewal capacity in the uterus but are capable of differentiating into mature uNK cells. Mature uterine NK cells show *in situ* proliferative capacity in mid-pregnancy (154,155).



### **1.4.7.2 TRAFFICKING**

Other researchers explain the increased number of uNK cells in the endometrium as an influx of pbNK cells from the circulation with modification to the specialised uNK cell type within the uterine microenvironment (50). Several studies believe expression of adhesion molecules by uNK cells in the endometrium could explain homing of uNK cells to the endometrium from peripheral blood and also may account for their distribution in a peri-vascular position (156-158). In both humans and mice uNK cells are seen in a collection around blood vessels. Since uNK cells are CD16<sup>-</sup>, recruitment from the blood would suggest recruitment of CD56<sup>bright</sup> NK cells or their precursors within the circulation which then undergo modification within the uterus.

In the late secretory phase endometrium and early pregnancy uNK cells are detected in the stratum functionalis where they have been found often forming aggregates around spiral arteries and glands (95). This peri-vascular distribution was initially said to represent the effect of diffusion of progesterone from blood into peri-vascular tissues but more recently has been considered to reflect trafficking of uNK cells or their precursors from the peripheral circulation (97,159). An alternative explanation is that the peri-vascular distribution may reflect a role in the remodelling of spiral arteries which is an essential feature of pregnancy.

Another interpretation of the peri-vascular location of uNK cells is that they may have a role in decidualisation of stroma (50).

Evidence from mouse pregnancy supports trafficking of precursor cells to the uterus in early pregnancy in mouse (155). It is suggested that an increase in uNK cell number seen in decidualisation and implantation is a result of endocrine signals that coordinate and recruit uNK stem cell precursors from the spleen into the uterus. Other studies have shown that steroids produced by the ovaries and the LH surge enhance the adhesive property of NK cells to the murine uterus using the L-Selectin dependent mechanism, although it remains unclear whether their effect is direct or indirect (93,159,160). These findings point towards that uNK cells in murine models are

established from NK-precursor cells recruited from the peripheral circulation into the implantation site, rather than exclusively from *in situ* proliferation.

Overall these studies of murine uNK cells are based on *in vivo* assays. They give us many clues to the understanding of the underlying mechanisms in the post-ovulatory rise of human CD16<sup>+</sup> uNK cells. At the same time, uNK cells found in mice are different from those human endometrial CD16<sup>+</sup> uNK cells in several different ways. For example, murine uNK cells do not increase in number after ovulation, but they do after implantation. In addition, uNK cells also accumulate in the murine-specific placental structure (the mesometrial triangle and mesometrial lymphoid aggregates of pregnancy) and not only in the decidua basalis. Consequently, direct relevance of these findings to humans should be made cautiously (88).

In humans implantation is a complex process composed of three distinct and consecutive phases: apposition, attachment, and invasion (161). Selectins are a group of cell adhesion molecules that include P-selectin (CD62P), L-selectin (CD62L), and E-selectin (CD62E). Evidence indicates that in the uterus the initial attachment of an embryo to the endometrium depends on binding of L-selectin. This is expressed by the trophoblast and oligosaccharide-based ligands which are expressed by the endometrium (162). Interactions between L-Selectin and its respective ligand in the endometrium may behave as a bridge for the initial attachment during implantation (163). This supports the notion that the expression of L-Selectin ligands might reflect the receptivity of the endometrium. However the expression pattern of L-Selectin ligands in the human endometrial cycle remains indefinite.

A study (164) looked into the L-Selectin ligand in the luminal and glandular epithelium of the endometrium at various phases of the menstrual cycle. L-Selectin ligand was expressed differentially at various phases in the endometrium. In the luminal epithelium, the expression of L-Selectin ligand was greatest from the peri-ovulatory phase through to the mid-secretory phase which coincides with the window of implantation.

However, in the glandular epithelium, L-Selectin ligand expression increased steadily from the proliferative phase to the secretory phase, the expression of L-Selectin ligand was greatest in the mid-secretory phase. The level of L-Selectin ligand was considerably elevated at the beginning of the secretory phase until 5–7 days post ovulation. 9-11 days after ovulation the level of L-Selectin ligand fell in both the luminal and glandular epithelium. This pattern is consistent with the concept that the luminal epithelium serves as an initial contact between the blastocyst and the endometrium, whereas the glandular epithelium participates at a later stage. It is possible that defects in the Selectin adhesion system may account partly for certain instances of implantation failure and early pregnancy loss (162).

L-Selectin and their ligands that play a part in cell-to-cell interactions are essential in the rolling, tethering, and attachment processes which allow leucocytes to pass through the endothelial barrier to reach lymphatic organs and inflammation sites (165-167). The binding of L-Selectin to its ligand may activate a signalling cascade (168) which in turn triggers signalling pathways that modify the uterine environment to allow a more stable adhesion of the embryo to the endometrium. L-Selectin may serve as a possible biochemical marker of endometrial receptivity and may help clarify the implantation process. Further evaluation of the L-Selectin is important to determine if it can help identify patients with defects in endometrial receptivity, unexplained infertility, and RM.

### **1.4.8 MARKERS USED IN THIS RESEARCH**

NK cells express receptors for many antigens, the following listed in table 3 are the ones that were stained for in this particular study. The following markers will help us understand the origin and the function of uNK cells in the endometrium. This may in future help us explain why women experience RM and implantation failure.

NK cell Markers	Evidence	Implication in this research
<b>CD56</b>	Universal NK Cell marker	All NK cells will stain positive for this marker.
<b>CD16</b>	90% of pbNK cells are CD16+, whereas only approximately 10% of uNK cells are CD16+ (169)	If increase of CD16+ cells found in research samples, then uNK cells could possibly be the result of trafficking from peripheral blood
<b>CD62-L</b>	Is an adhesion molecule. May also be a marker of endometrial receptivity (164)	If increased CD62-L+ cells then possibility that the endometrium displays adhesive properties that allow NK cells to home into the uterus from peripheral blood. i.e. support trafficking theory
<b>KI67</b>	40% of CD56+ NK cells express the antigen for KI67 in the secretory phase endometrium (89)	If increased numbers of KI67+ Cells found in research samples stained then we can say that NK cells are proliferating and may result from resident uNK cells.
<b>NKp30</b>	It is an activating/differentiating marker for all NK cells, belongs to the NCR family. Activated by IL-15 (91,92)	We know it is expressed in decidual NK cells, but we want to know if uNK cells in the secretory phase endometrium express the marker NKp30.

**TABLE 3:** NK Cell markers stained for in this particular study are highlighted above. Also highlighted is the evidence of how these cells were discovered and what implication the markers have in this study.

## **1.5 - CONCLUSION**

The latest research has given us an in-depth understanding of the complexities of an evolving pregnancy and of the potential hazards that it may face at different developmental stages. For many years it has been thought that RM is caused by maternal rejection of normal fetuses. However, recent evidence has challenged this assumption. The endometrial leukocyte profile in women with RM has recently been a focus of interest. Recent research has shown that high levels of uNK cells within the endometrium may be associated with idiopathic RM. uNK cells are seen to be the most abundant leukocytes in the endometrium. In order to improve both the management and research into RM, it is important to determine the role uNK cells play in the endometrium at implantation in patients with RM.

The function of uNK cells is unknown and their role played in implantation remains unclear. Maternal uNK cells are adjacent to, and have the ability to interact directly with, foetal trophoblast cells. Their presence in the pre-implantation endometrium indicates that they are not simply part of an immune response to embryonic demise. Extensive trophoblast invasion of the uterine tissue is necessary for successful implantation and placentation. This process must be finely regulated.

There is considerable controversy concerning the origin and the function of uNK cells. There are many competing hypotheses. uNK cells could be the result of pbNK cells trafficking into the uterus or the result of proliferation and differentiation of uNK cells already resident in the endometrium. uNK cells may cause the endometrium of RM patients to become hostile to the invading trophoblast. Or uNK cells could make the endometrium of RM patients more receptive which may lead to the implantation of abnormal fertilised fetuses which subsequently miscarry.

It is already well-known that endometrial uNK cells are important for the establishment of pregnancy and alterations in the uNK cell population are associated with early pregnancy failure. However a causal link remains to be established and

further studies are needed to determine the precise role of uNK cells in the endometrium of RM patients.

### **1.5.1 IMPORTANCE OF CONDUCTING THIS RESEARCH**

Much of the present research surrounds how uNK cells can act in a way that is harmful to the foetus resulting in miscarriage. Therefore, prediction of uNK cell function and its therapeutic development are desirable and a potential revenue generating goals. This desire to know whether uNK manipulation could elevate success rates for RM patients is mesmerising. Interest in uNK cells does not end with successful pregnancy outcome since intrauterine programming has continuing consequences on the offspring. The entire next generation will benefit from a greater understanding of uNK cell function.

Not only is this research beneficial to humans, therapeutic manipulation of uNK cells also interests livestock producers. They desire a more economic production by increasing offspring numbers and gestational success of unique, high cost or genetically modified embryos.

## **1.6 - HYPOTHESIS**

The hypothesis for the following research is that the uNK cells in patients with RM will show evidence of proliferation and differentiation of resident uNK cells but not trafficking of pbNK cells. This will be determined using various uNK cell markers for proliferation; KI67, differentiation; NKp30 and trafficking; CD16 and L-Selectin.

### **1.6.1 AIM**

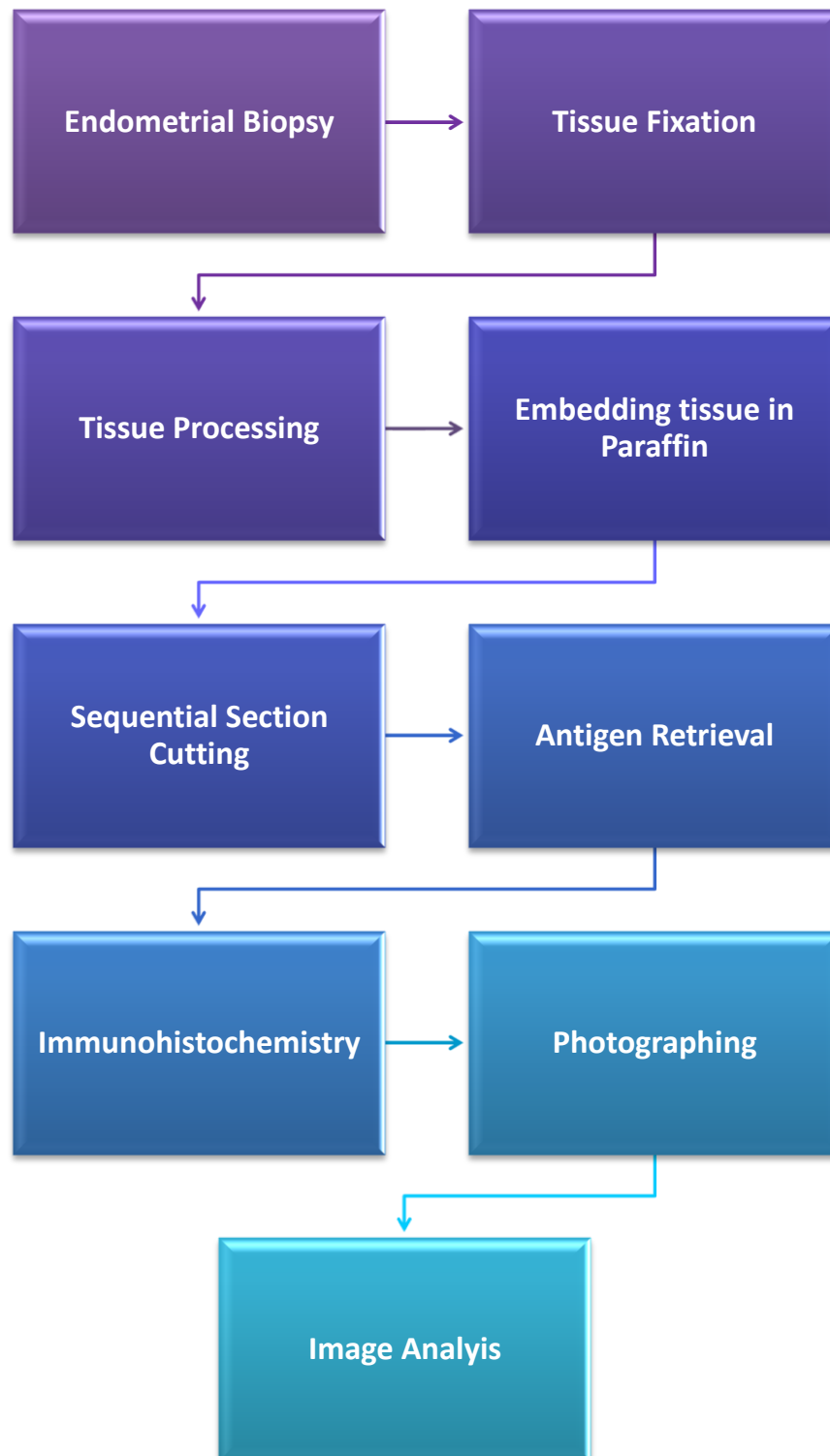
This hypothesis will be tested using Immunohistochemical staining of endometrial tissue received from RM patients of extreme phenotype between LH + 7-9 days will allow us to determine the role of uNK cells by looking at their numbers in different locations, the epithelial edge, areas of low stromal cell density and peri-vascular stroma. The universal NK cell marker, CD56 will be used to identify uNK cells in the endometrium. The research will determine whether uNK cells may play a part in spiral artery remodelling and trophoblast invasion.

In addition, the results will be compared and contrasted for the occurrence of these marker in the endometrial tissue of RM patients already identified as having a 'high' or 'low' density of uNK cells.

The hypothesis will be acknowledged as correct if the majority of uNK cells stain positive for ki67 and NKp30 and very few stain positive for L-Selectin or CD16. Furthermore in cases with high density of uNK cells there will be more positively stained cells for KI67. If uNK cells that surround the blood vessel in the endometrium are highly positive for L- Selectin and CD16 then we can say that the uNK cells may result from trafficking of pbNK cells. However, if uNK cells stain highly positive for NKp30 and KI67 and not so much for L-Selectin and CD16 then we can say that uNK cells may proliferate and differentiate from resident uNK cells.



## CHAPTER 2 – METHODS



*Fig 2: Schematic diagram representing an overview of the method from the tissue sampling to image analysis*

## **2.1 PATIENT SELECTION**

From a database of an ongoing study of a larger population of female patients (N = 500), 20 recurrent miscarriage patients ( $\geq 3$  consecutive miscarriages) of extreme phenotype were selected. They were recruited from throughout the UK via referrals from general practitioners or local hospital consultants. 10 patients were grouped as high uNK, defined as an uNK cell density value of  $\geq 5\%$  (131) and 10 were grouped as low uNK, defined as an uNK cell density value of  $\leq 2.5\%$ . The low cut off point was chosen at this level because it will allow comparisons to be made with those in the high group. It is a pragmatic way of having a demarcated difference between the two groups. Anything lower would make it difficult to count the cells reliably. Only those women who had a regular 28 day menstrual cycle were included. This then excluded all peri-menopausal women. Women were also excluded if they tested positive for any of the following possible contributing factors for pregnancy loss; antiphospholipid syndrome, thrombophilia, uterine anomaly, polycystic ovarian syndrome, diabetes, abnormal thyroid function tests or parental balanced translocations.

## **2.2 ENDOMETRIAL SAMPLING**

All women used a commercially available urine LH kit to confirm the mid-cycle urinary LH surge. They then rang the hospital to arrange for an endometrial biopsy 7-9 days after the LH surge. At sample collection all women had a transvaginal scan to ensure normal endometrial thickness. The samples were obtained using a pipelle sampler to attain an endometrial biopsy. All women had given written informed consent for an endometrial biopsy and a chaperone was present at all times.

### **2.3 ENDOMETRIAL BIOPSY RECEPTION**

The fixation of endometrial biopsy samples in 10% neutral buffered formalin (NBF) was carried out as described below. Laboratory coats and nitrile gloves were worn at all times when handling the unfixed biopsies. All manipulations on the fresh samples were performed in the class II microbiological safety cabinet.

Equipment Information:

- Dry ice, Virkon and laboratory plastics (e.g. universals etc) are purchased from Liverpool Women's Hospital (NHS) purchasing department.
- Nunc cryotubes are purchased from Corning. Distributor address: PO Box 75089, 1117 ZP Schiphol, Koolhavenlaan, The Netherlands.
- Forceps and scissors are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.
- TriGene Advance is obtained from Medichem International. Supplier address: PO Box 237, Seven Oaks, Kent, TN15 0ZJ. Telephone: 01732 763555.
- Neutral Buffered Formalin solution is purchased from Sigma Aldrich. Supplier address: Fancy road, Poole, Dorset, BH12 4QH. Telephone 01202 712300.
- Biomat<sup>2</sup> Recirculating microbiological safety cabinet was purchased from Bioquell. Supplier information: Walworth road, Andover, Hampshire, SP10 5AA.

Firstly the lab was prepared to receive the endometrial biopsy. This was done by cleaning the surfaces of the microbiological safety class II (MCII) cabinet with 1% TriGene Advance solution and dried with absorbent towels. The following items were collected and placed on a clean plastic tray in the MCII cabinet: Universal tube containing 4% neutral buffered formalin (NBF), which was labelled with the date and the patient number, small weighing boats, forceps and scissors, discard pot containing 1% Virkon solution, equipment decontamination container (small plastic container filled to approximately 50% capacity with 2% TriGene Advance Solution, small bottle of sterile PBS, a box of dry ice and liquid nitrogen. A Nunc cryotube was then labelled

with the patient number and the date. The labelled cryotube and a clean pair of forceps were then placed in the box of dry ice to cool.

The biopsy specimen was collected in a Wallach endometrial sampler and delivered to the class II laboratory in a transport box. The sample was then transferred to the MCII cabinet. The end of the endometrial sampler was cut and the biopsy pushed out onto a weighing boat. The endometrial sampler was then filled with 1% Virkon and left for approximately 10 minutes to decontaminate, then discarded in the clinical waste sharps bin. In cases where a large amount of blood was present the tissue was then immersed in sterile PBS. Mucous and blood clots were removed from the tissue and placed in the 1% Virkon discard pot. The tissue was then placed into a labelled Universal tube (containing 4% NBF) and stored overnight in a fume cabinet. If the sample was collected on a Friday then the NBF pot was stored in the fridge to minimise evaporation.

If a large sample was collected then frozen blocks were also prepared. The tissue was cut into appropriate lengths and half was placed into the labelled NBF pot, whilst the remaining was transferred onto a fresh weighing boat. The sample was then manipulated so that it coiled into a 'Danish pastry' shape. The weighing boat then was carefully placed to float on the surface of the liquid nitrogen using long forceps. Freezing occurred within a couple of seconds, the weighing boat was then removed from the liquid nitrogen using long forceps and placed immediately onto dry ice. It is then transferred into the labelled cryotube using the pre-cooled forceps.

The cryotube was then placed in the storage boxes in the freezer. This information was all logged in a patient specimen freezer file. The patient and sample information was then also entered into the lab samples database and the patient progress log updated with the specimen code and the collection date.

The plastic weighing boats and any waste tissue was placed into a discard jar containing 1% Virkon. Items placed in discard jars were completely submerged and

incubated for at least 1 hour to ensure effective decontamination. The contents of the discard jar were then washed down a sink through a sieve, colander or filter paper. The disinfected solids then were placed into the hospital clinical waste bags for disposal.

## **2.4 TISSUE PROCESSING**

The formalin fixed tissue was firstly dehydrated, cleared then impregnated with paraffin wax using the automated Shandon Citadel 1000 processing machine. The total processing time was 18  $\frac{3}{4}$  hours. The processing schedule was made up of: 45 minutes in 4% formalin in neutral buffer, 1 hour in 60% Ethanol, 1 hour in 70% Ethanol, 1 hour in 90% Ethanol, 1 hour in 100% Ethanol 1, 1  $\frac{1}{2}$  hours in 100% Ethanol 2, 2 hours in 100% Ethanol 3, 1 hour in Xylene 1, 1  $\frac{1}{2}$  hours in Xylene 2, 2 hours in Xylene 3, 2  $\frac{1}{2}$  hours in Wax 1 and 3  $\frac{1}{2}$  hours in Wax 2.

### **Equipment information:**

- Citadel processor manufacturer: Thermo electron Corporation. Manufacturer's address: Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England.
- Ethanol and xylene are purchased from Chemistry solvent stores (University of Liverpool).
- The wax (Paramat Paraplast Gurr) and the neutral buffered formalin are purchased from VWR International Ltd (UK). VWR address: Hunter Boulevard, Magna Park, Lutterworth, Leicester, England, LE17 4XN

Between processing runs, the ethanol solutions were stored in labelled bottles to prevent evaporation. Therefore the solvent containers were re-filled before tissue processing. Firstly, plastic cassettes were labelled with the sample ID number using pencil. The tissue was then removed from the formalin pot using forceps and placed into small wire inserts, which were secured into the plastic cassettes and the sample ID was re-checked once more. This was all done in the fume hood. The used formalin was then disposed of in a solvent waste can. The cassettes were loosely spaced in the processing baskets which were then lowered into formalin. The following method was used as shown in table 4.

Date of Processing	Processing Time	Hand Held Controller Action
Monday-Thursday	Before 2pm	Press <b>delay on</b> button
Monday-Thursday	After 2pm	Press <b>autostart</b> button
Friday	Before 2pm	Press <b>days delay</b> , scroll to 2 days using the + button then press <b>delay on</b> .
Friday	After 2pm	press <b>days delay</b> , scroll to 1 day using the + button, then press <b>delay on</b>

**Table 4:** Highlights the processing schedule dependent on which day and time the samples were put in the processor.

Once the processing cycles were completed, the samples were embedded using the embedding machine.

The solvents were changed on a regular basis to ensure efficient processing of the tissue samples. Waste solvents were placed in red solvent waste containers and identified clearly with the appropriate labels. Contaminated waste wax was discarded by pouring the molten solution into a container and cooling until the wax was set. The wax container was then disposed of in clinical waste.



## **2.5 TISSUE EMBEDDING**

The Shandon Histocentre 3 embedding machine was used to embed endometrial tissue samples after they had been processed as mentioned previously. This was done in the following way: the cassettes removed from the holder in the processor ensuring that wax does not drip on the floor. The block then placed into the heated reservoir in the embedding machine. Absorbent towels used to remove surplus wax from the cassette and placed into the oven (heated to  $\sim 100^{\circ}\text{C}$ ) for several minutes to remove any residue. A metal mould from the heated unit was then placed underneath the wax tap and half-filled with wax. The cassette was then placed on the heated area near the wax tap to prevent solidification of the wax. The cassette was then opened and the sample transferred to the wax-filled mould using hot forceps. The mould was then placed on the "cold spot" at the front of the dispensing unit and hot forceps used to position the tissue in the mould at the bottom. The sample was then covered with a plastic cassette which was numbered accordingly. The wax was topped up and placed on a pre-cooled cooling block to set. After  $\sim 30$  minutes the cassette came cleanly away from the metal mould, if necessary forceps or a scalpel was used to loosen the edges. The block was then stored in metal storage cabinets in numerical order ready to be cut. The metal biopsy inserts were placed in Xylene to clean off any remaining wax ready for re-use.

## **2.6 CUTTING**

### Equipment Information:

- Micron HM335 rotary microtome is supplied by MICROM Company. Manufacturer's address: Microm UK Ltd, 8 Thame Park Business Centre, Leinman Road, Thame, OX9 3XA. <http://www.micron-online.com>
- Microscope slides (super premium twin frost, catalogue number 631-0111) are purchased from VWR International. UK based distributor is located at: Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN.
- Microtome blades (feather, stainless steel S35) are purchased from Liverpool Women's Hospital (NHS) purchasing department.
- Forceps, paintbrush, section dryer and water bath are supplied by Raymond A Lamb Ltd. Manufacturer's address; Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.

The water bath was first filled with distilled water and set to approximately 40°C. The sample block to be cut was placed in the fridge 30 minutes prior to use. The blade angle was pre-set to 10-12°. This setting ensured minimal amount of tissue was wasted when many sections were prepared from the same block. A new block was set to trim at 10-20µm until a representative amount of tissue was exposed in the tissue block. The section thickness was then set between 3-5µm. All the sections used in this study were cut at 4µm. After the first section was cut, using forceps the end was gently held onto whilst cutting continued to produce a ribbon of 8 or more sequential sections. The sections were then carefully placed to float onto the water in the pre-warmed water bath. The sections were separated gently using the pressure of forceps. They then were floated onto APES coated slides (3-aminopropyltriethoxysilane, Sigma Chemical Co, Poole, UK) in order of when they were cut. Each section was placed onto the slide in the same orientation as the previous one. The frosted part of the slide was labelled with the specimen ID and thickness (using a pencil). The slides were then placed in a rack to dry at room temperature overnight. They were then ready to be baked as required for the immunohistochemical staining procedure.

## **2.7 STAINING**

The endometrial tissue was stained using immunohistochemistry, with antibodies to CD56, KI67, NKp30, L-Selectin and CD16. They are uNK cell markers of proliferation, differentiation and migration/trafficking respectively.

Immunohistochemistry is the process of localising antigens (e.g. proteins) in cells of a tissue section. This is done by the use of labelled antibodies as specific reagents through antigen-antibody interactions that are then visualised by a marker such as fluorescent dye, enzyme, or colloidal gold (170).

Tissue preparation is the foundation of immunohistochemistry. For this reason the endometrial tissue sample is prepared initially to ensure preservation of architecture and cell morphology. This is achieved by prompt and adequate fixation of the sample in 10% NBF. Inadequate or prolonged fixation can reduce the antibody binding capability to a great extent. Formalin-fixation and paraffin-embedding is widely used in routine immunohistochemistry. However, certain antigens are not capable of surviving this technique and the tissue has to be frozen. The disadvantages of frozen sections are poor morphology, poor resolution at higher magnifications and cutting difficulty over paraffin sections (170).

The expression of the antigen can be also be improved by the pre-treatment of the tissue sample with an antigen retrieval reagent such as citrate buffer solution. Antigen retrieval is carried out in a pressure cooker to allow breakage of the protein cross-links formed by the earlier fixation in formalin and thus uncover the hidden antigen sites (170).

The antibodies used for specific detection are either polyclonal or monoclonal. Monoclonal antibodies are generally considered to display greater specificity. Antibodies can also be classified as primary or secondary reagents. Primary antibodies are raised against an antigen of interest and are unconjugated (unlabelled), whilst the secondary antibodies are raised against the primary antibodies. Also, secondary

antibodies recognise immunoglobulins of a particular species and are conjugated to a reporter enzyme such as horseradish peroxidase (HRP).

There are two methods used for the detection of antigens in tissues, the *direct* method and the *indirect* method. We used the indirect method within which an unlabelled primary antibody (first layer) reacts with the tissue antigen and a labelled secondary antibody (second layer) then reacts with the primary antibody. The second layer antibody is then labelled with an enzyme and reacted with DAB to produce a brown staining wherever primary and secondary antibodies are attached (170).

The staining protocol overall is:

- i) Application of a primary antibody
- ii) Application of enzyme labelled polymer, a secondary antibody
- iii) Application of the substrate chromagen (DAB).

The antibodies were validated using various different concentrations to find the most optimal staining for the endometrial tissue. This is highlighted in table 5 on the next page. For each validated staining run tonsil was used as a positive control, since it has already been validated to stain extremely well for CD56<sup>+</sup> NK cells.

**Validation of antibody concentration:**

Antigen	Antibody /cat#	Clone	Polymer	Company	Dilution	Duration
<b>Ki67</b>	NCL-Ki67-MM1	MM1	Mouse	Novocastra	1:100, 1:150, 1:200, 1:400	60 mins
<b>CD56</b>	NCL-L-CD56-1B6	1B6	Mouse	Novocastra	1:50	60 mins
<b>CD16</b>	DJ13OC	VIFcRIII	Mouse	Dako	1:50, 1:100, 1:200	60 mins
<b>CD62-L</b>	AB49508-500	9H6	Mouse	Abcam	1:25, 1:50, 1:100	60 mins
<b>NKp30</b>	SC-20477	SC-20477	Goat	Santa Cruz	1:100, 1:200, 1:400	60 mins
<b>Mouse IgG</b>	MCA928	IgG1	Mouse	Serotech	1:100	60 mins
<b>Goat IgG</b>	Sc-2028	Sc-2028	Goat	Santa Cruz	1:200	60 mins

**Working Dilutions used:**

Antigen	Antibody /cat#	Clone	Polymer	Company	Dilution	Duration
<b>Ki67</b>	NCL-Ki67-MM1	MM1	Mouse	Novocastra	1:150	60 mins
<b>CD56</b>	NCL-L-CD56-1B6	1B6	Mouse	Novocastra	1:50	60 mins
<b>CD16</b>	DJ13OC	VIFcRIII	Mouse	Dako	1:100	60 mins
<b>CD62-L</b>	AB49508-500	9H6	Mouse	Abcam	1:25	60 mins
<b>NKp30</b>	SC-20477	Polyclonal	Goat	Santa Cruz	1:100	60 mins
<b>Mouse IgG</b>	MCA928	IgG1	Mouse	Serotech	1:100	60 mins
<b>Goat IgG</b>	Sc-2028	sc-2028	Goat	Santa Cruz	1:200	60 mins

**Detection Information:**

Detection Kit	Kit /cat#	Company	Dilution	Duration
<b>DAB EnVision</b>	K4006	Dako	1:50	10 mins
<b>Dako (anti-mouse)</b>	K4003	Dako	1 Drop	30 mins
<b>Vector ImmPress Kit (anti-goat)</b>	MP-7405	Vector Labs	1 Drop	30 mins

**Table 5:** Details of all solutions used in the study

Prior to carrying out the staining, the slides with the samples to be stained were baked in an oven at 37°C overnight. The following day they were put through a set of solutions to remove as much wax off the sample as possible. This was done by incubating the samples in 2 changes of xylene for 10 minutes, 2 changes of absolute ethanol for 3 minutes each, 90% ethanol for 1 minute and 70% ethanol for 1 minute prior to transfer into water. The slides then underwent antigen retrieval as they are placed in a pressure cooker containing citrate buffer solution made up of 3.15g of citric acid, 1.5L dH<sub>2</sub>O at pH 6 for 1 minute.

The slides were then placed in a staining dish containing Tris Buffered Saline solution (TBS) and incubated for 5 minutes at room temperature. Endogenous peroxidase activity was blocked by incubating the slides in 0.3% H<sub>2</sub>O<sub>2</sub> for 10 minutes at room temperature. Humidifying chambers were prepared by placing folded paper towels in the centre gulleys and soaking them with distilled water. The slides were washed twice more with TBS for 5 minutes at room temperature each time. Area to be stained was marked with a DAKO hydrophobic marker pen ensuring that the tissue was surrounded with sufficient space to allow spreading of the antibodies. Antibody diluent made up of TBS and 0.5 % BSA was prepared in the following ratio (e.g. 250 µl 10% BSA + 4750 µl TBS). Working dilutions of each antibody were also prepared allowing 50 µl per section. The slides were then placed in the humidifying chamber and 50µl of the appropriate antibody to each section was applied, which was spread using parafilm to ensure the entire section was covered. The slides were then left to incubate for 60 minutes at room temperature.

The slides were then tapped off and placed in a staining dish filled with TBS for 5 minutes at room temperature. The TBS was decanted and refilled and incubated for another 5 minutes. The slides were then returned to the humidifying chamber and a drop of mouse or goat polymer-HRP applied accordingly, ensuring the entire section was covered using parafilm. The slides were then incubated further for 30 minutes at room temperature. The slides again were tapped off and incubated in TBS twice for 5 minutes each time.

DAB (3, 3-diaminobenzidine), a substrate and chromagen solution, was prepared using the following ratio, also allowing for 50 µl per section: 20µl/1000µl substrate. The slides were returned to the humidifying chamber and the substrate and chromagen solution was applied and spread using parafilm on each section. They were left to incubate for 10 minutes. The slides were then immersed immediately in tap water to stop the reaction.

The endometrial samples were lastly counterstained using filtered Gill 2 haematoxylin for 2 minutes, and were then immersed in tap water and rinsed until the water ran clear. First, they were briefly dipped in acid alcohol to remove the background haematoxylin, and then immediately back into tap water for 5 minutes, The slides then underwent the following set of solutions to dehydrate the sample: 1 minute in 70% ethanol, incubated for 1 minute in 90% ethanol, incubated for 3 minutes in 100% ethanol<sup>1</sup> and then 2, incubated for 5 minutes in Xylene 1 and incubated for 10 min in Xylene 2. Sufficient DPX was applied to cover all the section and a coverslip was applied over each. Air bubbles were removed and the slides left to dry overnight in the fume hood.

Appropriate positive controls were also stained with each staining run to confirm staining, and negative controls were stained for each sample by replacing the primary antibody with mouse or goat IgG.

## **2.8 PHOTOGRAPHING**

All 20 RM women had their endometrial samples photographed for epithelial edge, areas of low stromal cell density, also known as oedema and peri-vascular areas. First for each RM patient, the whole tissue was glanced at under the microscope, to appreciate what the remaining tissue was like, whether it remained on the slide, whether it was inconsistent and therefore cannot be photographed. All the slides for the RM patient were first looked at in this way, before sequential photographs were taken.

A photographing log was kept of all the slides, staining and photographing. When the tissue was first looked at under the microscope, a drawing by hand was made of the outline of the tissue. Areas of the tissue that were inconsistent were noted down for future reference. Other comments were also noted to help identify the tissue, for example if the staining was weak or there was a particular pattern of staining for a certain antibody; e.g. more staining around vessels, was all noted.

Sequential micrographs were then produced for each RM. Three areas of epithelium, three areas of low stromal cell density and three areas of per-vascular areas, all images had glands in them, they were used as reference points when trying to find the same location on the next sequential section. As seen in table 6, the order of the antibodies listed are the same as how the sequential sections were stained. For every antibody either side, a CD56 in the middle was stained. This was so a comparison could be made with the CD56 stained sections.



RM Number...			
	Location		
Antibody	Epithelial Edge x3	Low stromal cell density x3	Spiral arteries x3
IgG Goat			
NKp30			
CD56			
KI67			
CD16			
CD56			
CD62-L			
IgG Mouse			

**Table 6:** An example of the photographing log used to record the information about the sections once looked at under the microscope.

## **2.9 IMAGE ANALYSIS**

uNK cell density was assessed using an Image analysis software. This was chosen to analyse micrographs because it offers the benefit of reducing inter-observer variability. This method was previously validated by the department. There were two parts to the image analysis, firstly, it was to count number of stromal cells and secondly to count positively stained cells, with the aim to produce a percentage for each stain per stromal count for each micrograph, for each area to be looked at. The advantage of producing three micrographs per stain per location enhanced accuracy by enabling use of an average in the analysis of results.

Equipment information:

### Setup 1

- Nikon Biophot Microscope, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon DS-5M camera head 5M pixel, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon Digital Sight DS-U1 digital control unit, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon C-Mount TV adaptor 0.6x, Nikon Corporation, Tokyo 100-8331, Japan
- Eclipsenet software, developed by Laboratory Imaging s.r.o. for Nikon Instruments Europe B.V., 1170AE Badhoevedorp, The Netherlands
- Adobe Photoshop CS2 software, Education Version. Adobe Systems Incorporated. 345 Park Avenue, San Jose, CA 95110-2704 USA [www.adobe.com](http://www.adobe.com)

### Setup 2 (supplied by Jencons-PLS)

- Nikon Eclipse 50i Microscope, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon DS-Fi1 digital camera Head 5M pixel, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon Digital control unit DS-U2 USB, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon C-Mount TV adaptor, 0.63x, Nikon Corporation, Tokyo 100-8331, Japan

- NIS-Elements-F software, developed for Nikon Instruments
- Adobe Photoshop CS3 software, Education Version. Adobe Systems Incorporated.  
345 Park Avenue, San Jose, CA 95110-2704 USA [www.adobe.com](http://www.adobe.com)

Both systems

- Personal computer (minimum specification 1GB RAM, 2.8GHz processor)
- WCIF Image J <http://www.uhnresearch.ca/facilities/wcif/fdownload.html>

Nine digital images; 3 of epithelial edge, 3 of low stromal density and 3 of endometrial peri-vascular areas at high power (x400) fields were captured on either setup 1 or setup 2 for each patient per antibody.

### **2.9.1 STROMAL COUNTING**

Adobe Photoshop was used to prepare the micrographs for image analysis. Each file to be analysed had every gland and vessel manually removed by using the Magic Lasso tool, an outline of each structure was produced then deleted. Following this, it was important to remove the DAB staining to just leave the blue stained stromal cells. This was achieved by using the Magic wand tool, to select and delete areas of 'brown' positive staining. At each step images were saved in TIFF format, to prevent detail being lost in other compression formats.

A stromal cell count was obtained using Image J software. Each file with DAB removed (stroma images) were opened with Image J and subsequently saved as 8-bit grey scale images (Image|Type|8 bit). A threshold could now be applied to the image (Image|Adjust|Threshold) set at a level which accurately represents the majority of cells visible in 'red' without background being selected also. The threshold number was noted to enable adjustment at a later stage if necessary. If needed 'smooth' function was applied to smoothen the cellular edges and have more defined outlines. The threshold was then applied. If a significant proportion of the cells were patchy they were filled using Process|Binary|Fill. Cells were separated using the watershed function (Process|Binary|Watershed) to enable distinct cells to be counted, instead of a cluster. To count the number of nuclei, the analyse particles function was used (Analyse|Analyse Particles) with the following settings (Setup 1 Size: 0.0015-0.1, Setup 2 Size: 36-2250, with both setups using the following: Circularity: 0.3-1, Show outline, Clear results, Summarise). An outline of the nuclei was then displayed. This image was then inverted and merged with the original. The cells were visually checked to see whether the outlines match with the cell nuclei of the original picture and that the clusters of cells are split and identified appropriately. A summary box of the total cell count was produced. This number was noted down.

### **2.9.2 POSITIVELY STAINED CELL COUNTING**

Image J software was used for the identification of positively stained cells. Each original file was opened and the positively stained cells counted using the 'point picker' tool (Plugins|Particle Analysis|Pointpicker), after applying a grid to make counting easier and more accurate (Plugins|Particle Analysis|Grid Average per point = 15170, pixel width = 2). For the nucleus markers, NKp30 and Ki67, the positively stained cells were identified as those in which there was a brown stained nucleus instead of the haematoxylin stained blue stromal nuclei. As for the cytoplasmic markers CD56, CD16 and CD62-L/L-Selectin positively stained cells were those which contain a blue stained nuclei surrounded by brown DAB staining to these antibodies. Positively identified cells were marked with a cross. Markers could be removed and moved by selecting corresponding buttons on the menu bar. When all positively stained cells had been identified, a results box was obtained by selecting 'show' on the menu bar. The total number of cells identified was noted.

To obtain a percentage of stromal cells stained the stained cell count was divided by the total stromal count and multiplied by 100.

## **2.10 UNK CELL COUNTING VALIDATION**

A selection of micrographs were counted and identified as the ones used for validation. A number of micrographs of patients were counted by me, uNK and stromal counts. These patients had already been counted by those who have already been validated to count in the department. The counts were then compared against their counts and a Bland-Altman graph was produced. The counting process was repeated until our differences were very small.

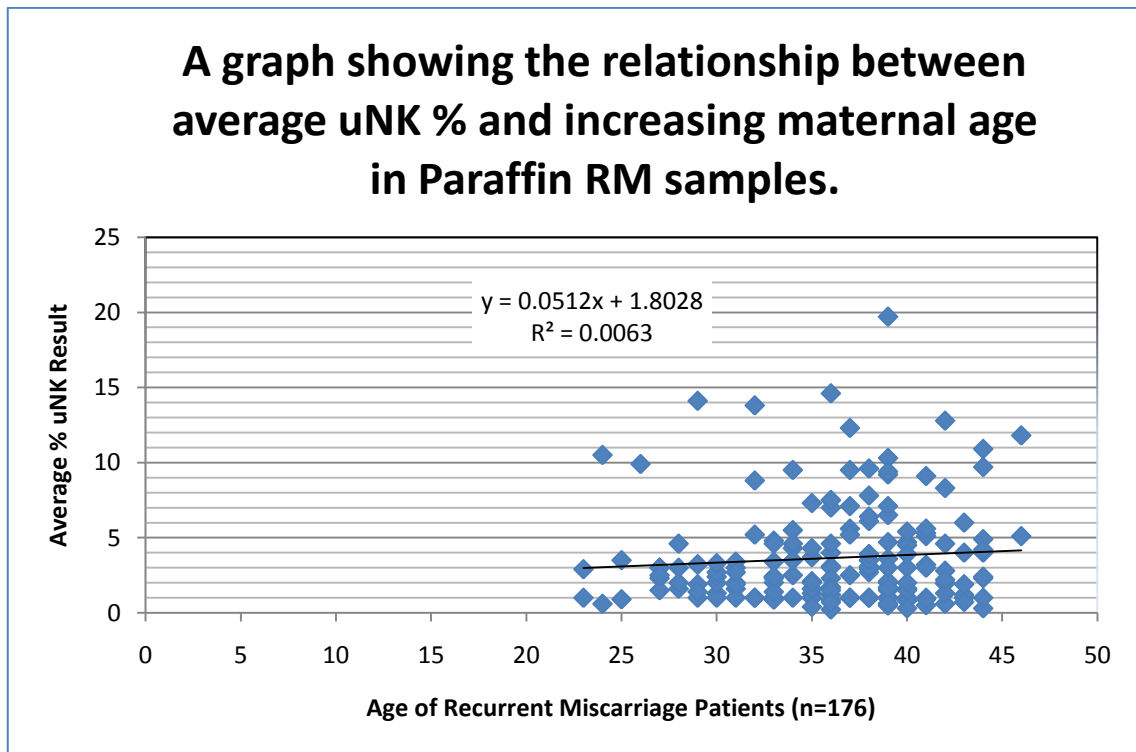
## **2.11 STATISTICAL METHODS USED FOR RESULTS ANALYSIS**

SPSS version 16 was used for all statistical analyses. Each condition was measured more than once for each individual, varying from 2 replicates per condition to 9 replicates per condition. The mean value for each condition for each individual was used in the analysis. The first step in analysis was to summarise the demographic characteristics of the participants. Then, the possibility of an association between age and uNK cell density was explored using correlation analysis. The measurements of uNK cell density made by me were compared to measurements made by other workers in the lab. Bland-Altman plots were used to check whether there were any systematic differences between experienced cell counters, myself and a colleague. Typical examples of immunohistochemical staining were prepared.

The next step in analysis was to express the expression for each antibody at each location in graphical format, grouping by uNK cell density status. The interpretation of the graphical analysis was supplemented using Analysis of Variance. Each woman contributed data for each antibody in three conditions. This meant that there was an element of repeated measurement. As a pragmatic measure to overcome any effects of repeated measurements, study patient number was used as a covariate. Post hoc tests are not possible with a covariate. Accordingly, a simple contrast was used to compare the locations, with epithelium as a reference group. An interaction term to examine whether differences in location differed according to uNK cell density status was also included. This interaction term was not significant in any of the analyses and so is not presented in the results.

## **CHAPTER 3 - RESULTS**

Maternal age at conception and previous reproductive history are strong and independent risk factors for miscarriage. The chance of a successful pregnancy in a woman aged 40 years or more is poor.



*Fig 3: A graph demonstrating the relationship between age of women with RM and their uNK results*

Figure 3 demonstrates the relationship between maternal age of RM patients and uNK cell level. The correlation coefficient ( $R^2$ ) of 0.0063 indicates the strength and direction of the linear relationship between maternal age and uNK cell level. The degree of correlation is so diminutive that the relationship is nonexistent. Therefore this does not interfere as a confounding factor with the analysis of the results.



### **3.1 DEMOGRAPHIC DETAILS OF WOMEN INCLUDED IN STUDY**

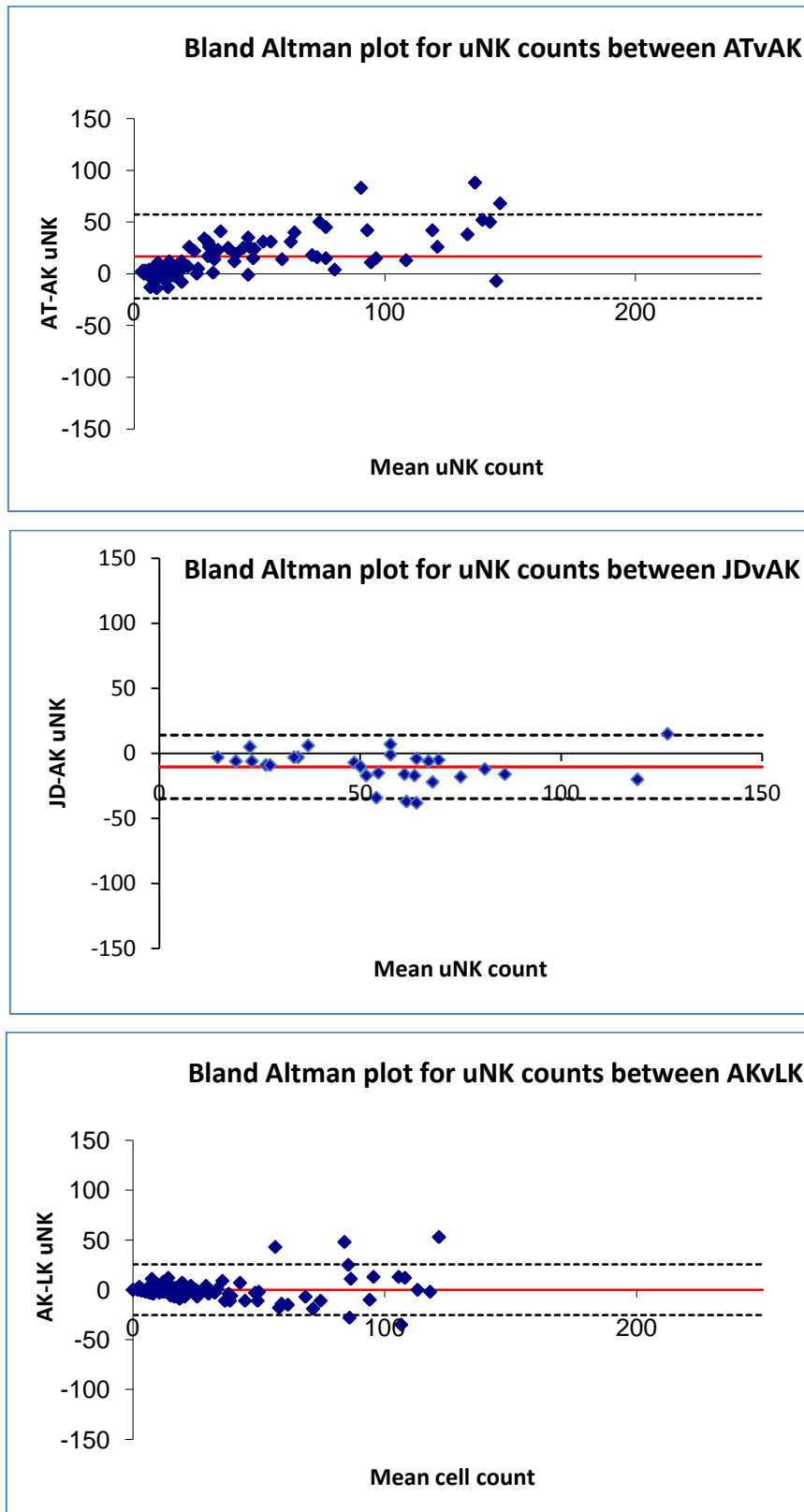
	<b>HIGH uNK CELL DENSITY (N=10) MEDIAN (RANGE)</b>	<b>LOW uNK CELL DENSITY (N=10) MEDIAN (RANGE)</b>
<b>uNK (%)</b>	8.1 (5.2-13.8)	1.95 (1-2.4)
<b>AGE (YEARS)</b>	37 (32-44)	36 (31-44)
<b>FIRST TRIMESTER</b>	6 (5-7)	7 (5-17)
<b>SECOND TRIMESTER</b>	0 (0-3)	0
<b>STILL BIRTH</b>	0 (0-1)	0
<b>LIVE BIRTH</b>	0 (0-5)	0 (0-1)
<b>ECTOPIC PREGNANCY</b>	0	0

**Table 7:** *The demographics of the patients included in the study*

As seen in table 7, the median age is very similar for both high and low uNK cell density groups. This is also similar for the number of miscarriages for all women. The patients in the two groups were matched as closely as possible, in terms of age, number of miscarriages, as shown above, to try and minimise any confounding variables.

### **3.2 uNK CELL COUNTING VALIDATION**

A Bland-Altman plot is a statistical method used to compare two measurement techniques, in this case the number of uNK cells counted. The differences between the two counts are plotted against the averages of the two counts. Horizontal lines are drawn at the mean difference and at the limits of agreement, which are defined as the mean difference plus and minus 1.96 times the standard deviation of the differences. This was produced to analyse the similarity between me and others in the department who have already been validated previously. The same parameter is measured and a correlation is produced. This is demonstrated in Figure 4 on the following page.

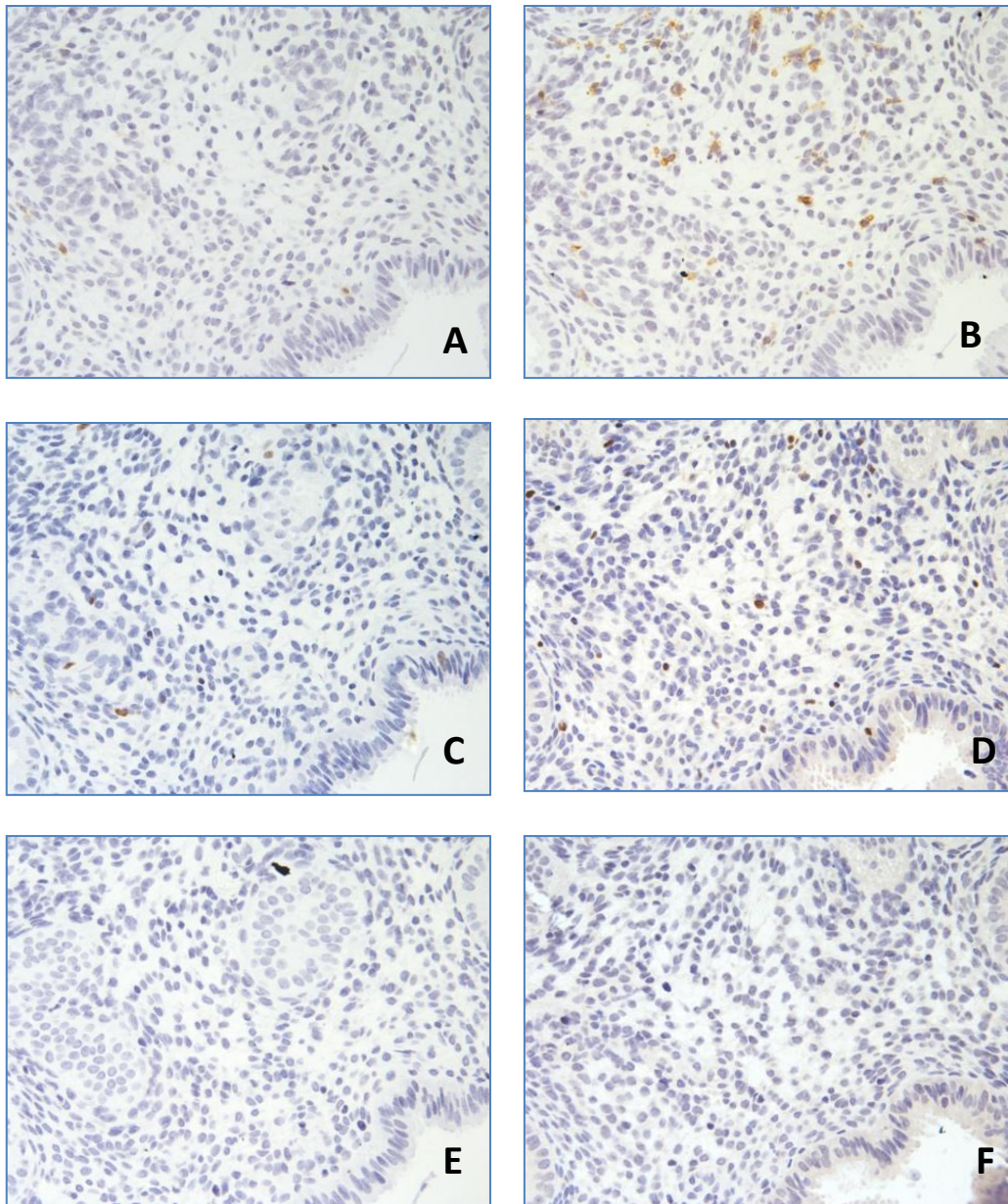


**Fig 4:** The graphs display a scatter diagram of the differences plotted against the averages of the two measurements, AT vs. AK, JD vs. AK, AK vs. LK. Horizontal lines are drawn at the mean difference, and at the limits of

agreement, which are defined as the mean difference plus and minus 1.96 times the standard deviation of the differences.

### **3.3 MICROGRAPHS OF LOW uNK CELL DENSITY RM PATIENTS**

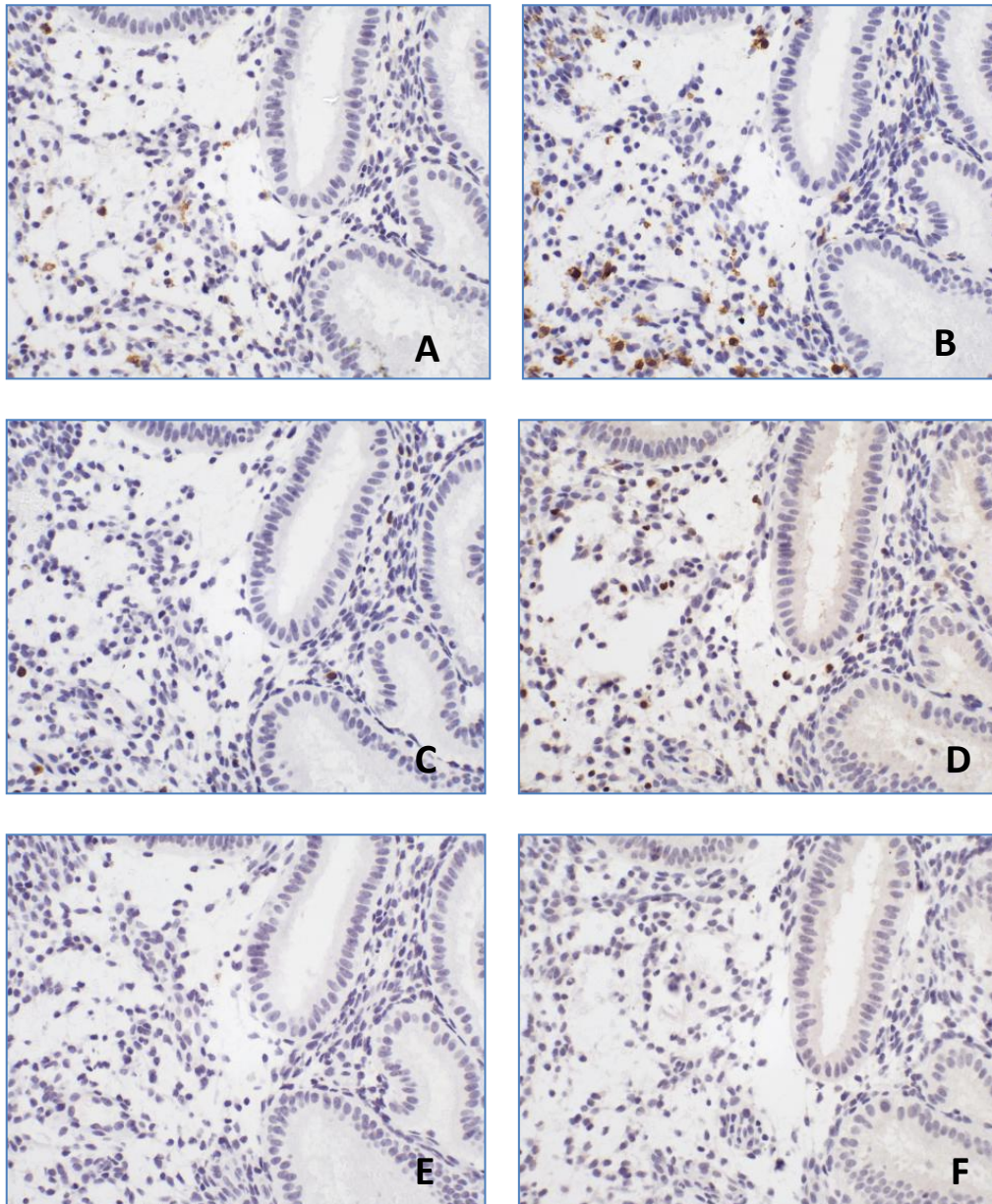
#### **3.3.1 EPITHELIAL EDGE**



**Fig 5:** Example of Epithelial Edge staining for a low uNK Cell density patient, A-CD16, B-CD56, C-KI67, D-NKp30, E-CD62-L, F-IgG Goat

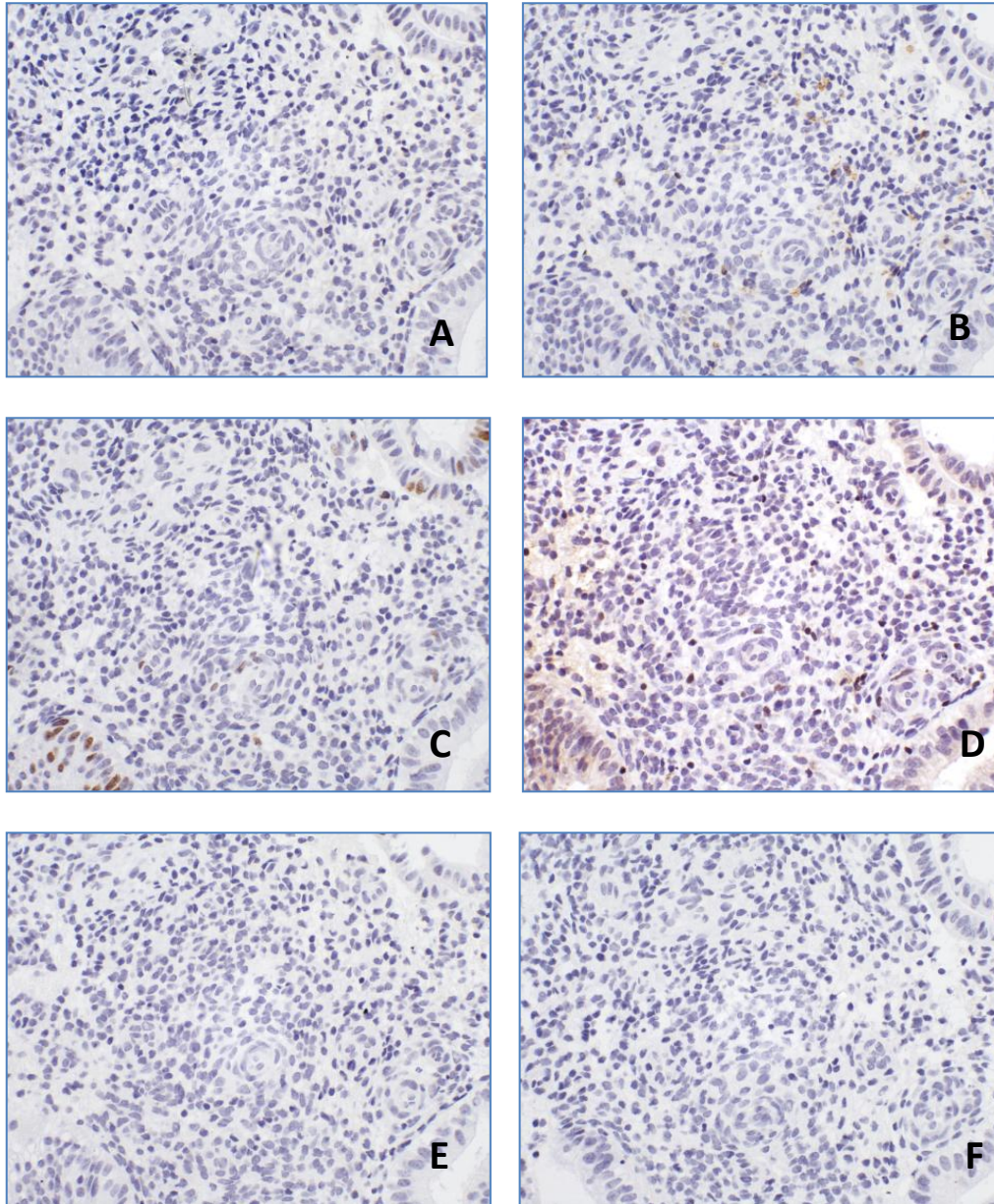


### **3.3.2 LOW STROMAL CELL DENSITY/OEDEMA**



**Fig 6:** Example of Low stromal cell density/oedema staining for a low uNK Cell density patient, A-CD16, B-CD56, C-KI67, D-NKp30, E-CD62-L, F-IgG Goat

### **3.3.3 SPIRAL ARTERIES**

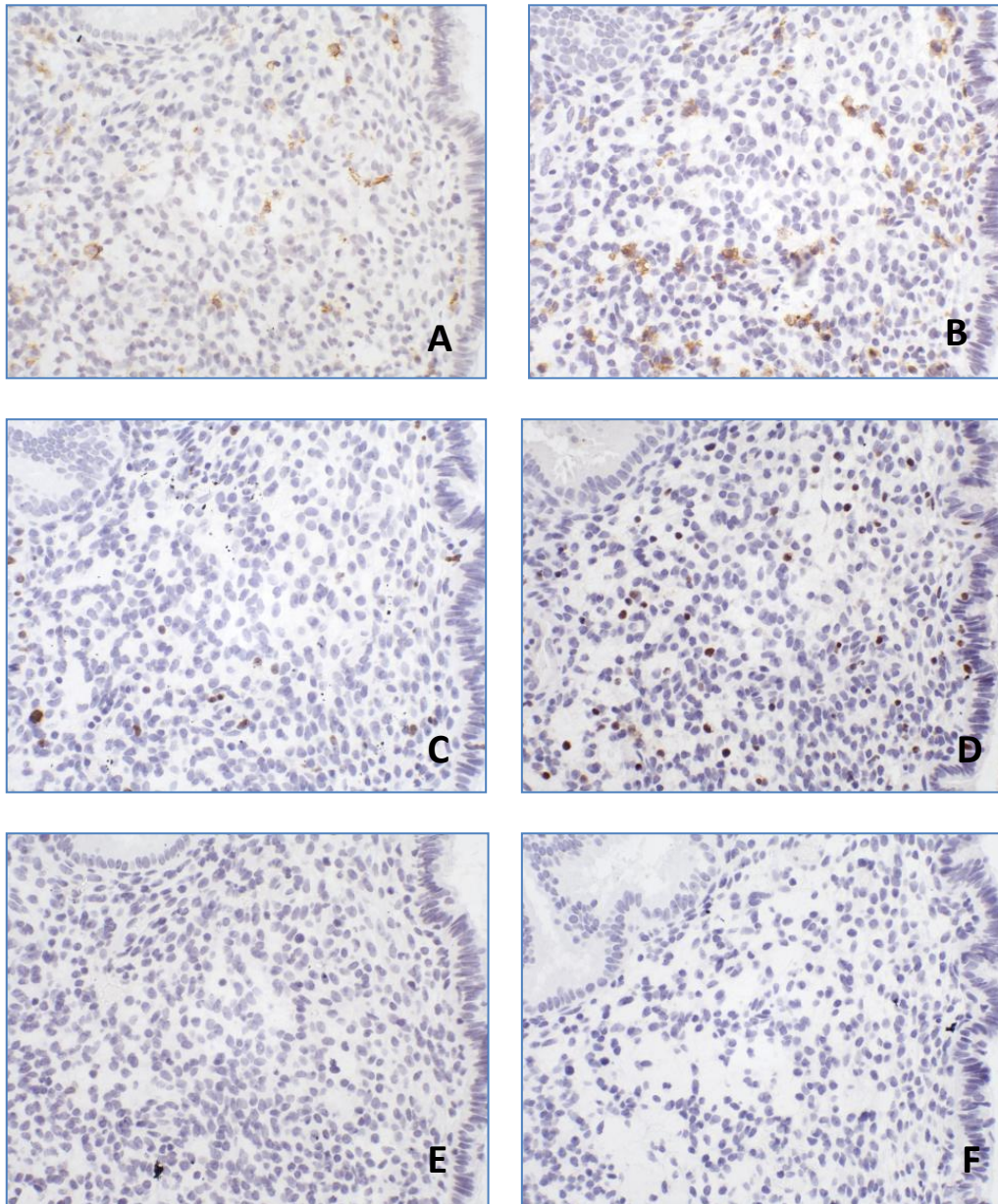


**Fig 7:** Example of stromal staining around spiral arteries for a low uNK Cell density patient, A-CD16, B-CD56, C-Ki67, D-NKp30, E-CD62-L, F-IgG Mouse



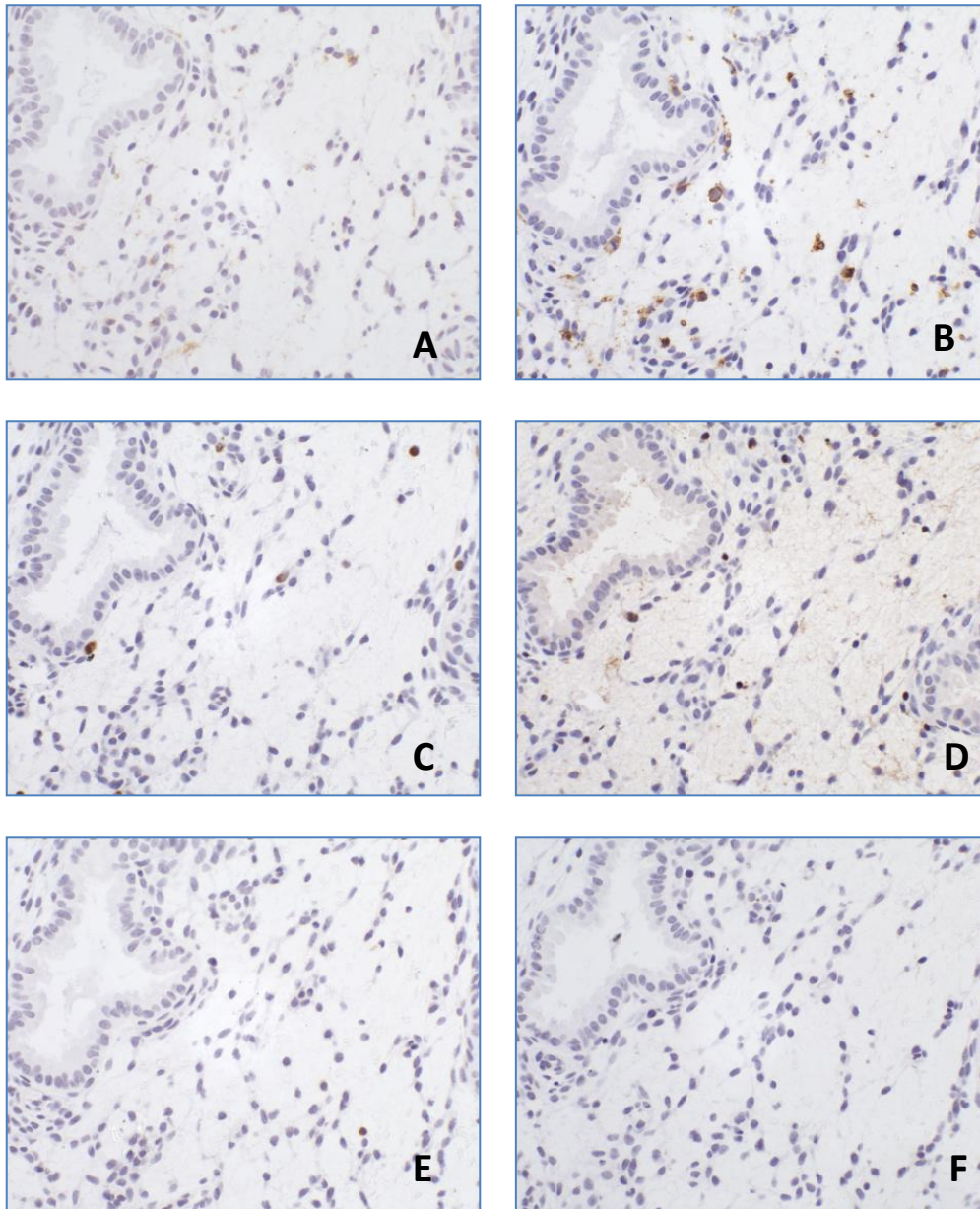
### **3.4 MICROGRAPHS OF HIGH UNK CELL DENSITY RM PATIENTS**

#### **3.4.1 EPITHELIAL EDGE**



**Fig 8:** Example of stromal staining near epithelial edge for a high uNK Cell density patient, A-CD16, B-CD56, C-KI67, D-NKp30, E-CD62-L, F-IgG Mouse

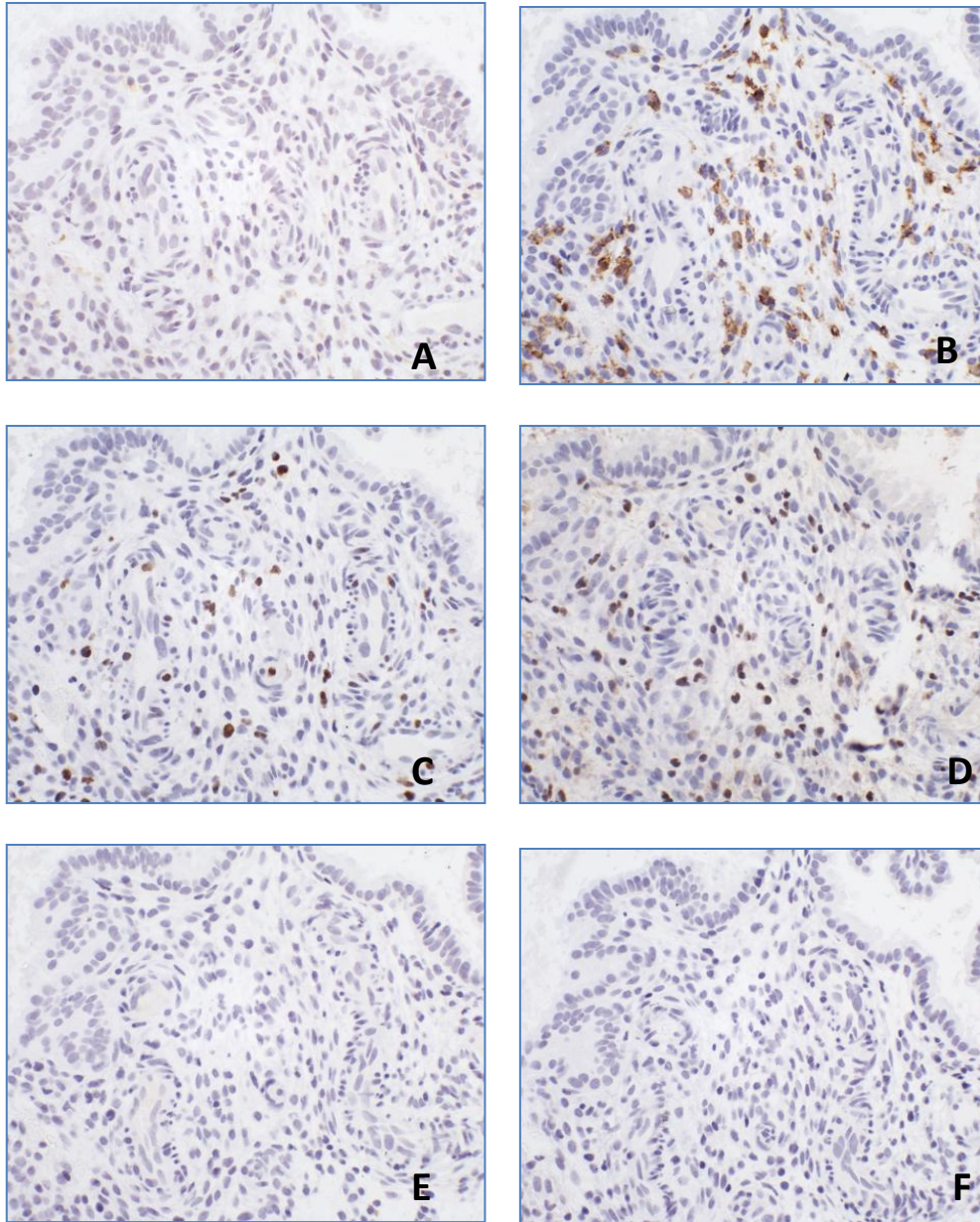
### **3.4.2 LOW STROMAL CELL DENSITY/OEDEMA**



**Fig 9:** Example of stromal staining in low stromal cell density/oedema areas for a high uNK Cell density patient, A-CD16, B-CD56, C-KI67, D-NKp30, E-CD62-L, F-IgG Mouse



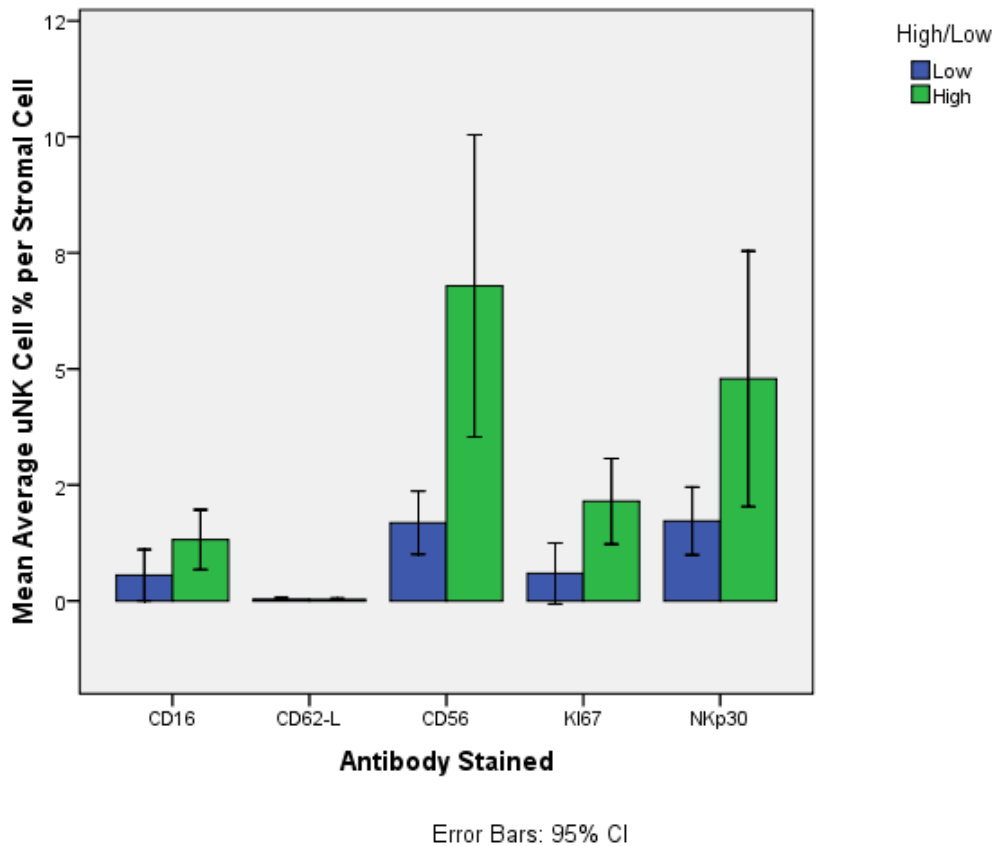
### **3.4.3 SPIRAL ARTERIES**



**Fig 10:** Example of stromal in a peri-vascular area for a high uNK Cell density patient, A-CD16, B-CD56, C-KI67, D-NKp30, E-CD62-L, F-IgG Mouse

### 3.5 GRAPHS

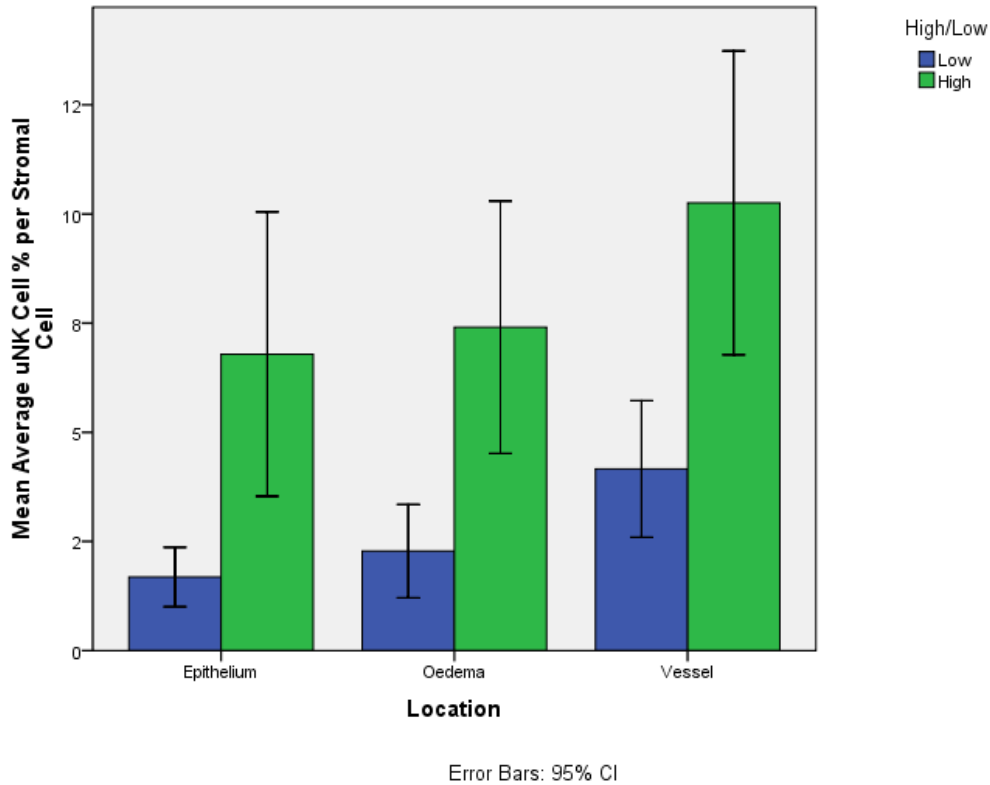
The Difference in Number of Antibody Stained Cells Between the High and Low uNK Cell Density Groups at the Epithelial Edge



**Figure 11:** Graph illustrating the number of antibody Stained cells in the two uNK Cell Density groups

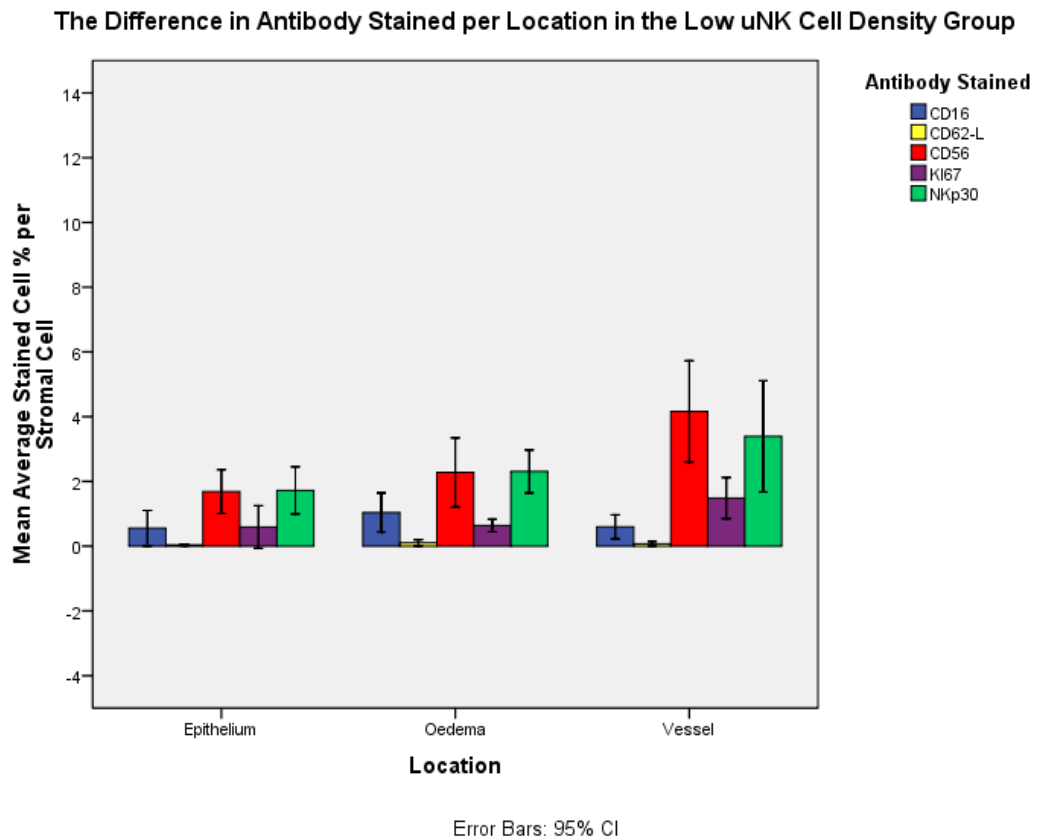
Figure 11 highlights the difference between the high and low uNK cell density groups and how they differ for all the stained antibodies. The high uNK cell density group has much higher numbers of stained uNK cells compared to low for all antibodies stained.

The Difference in Location of CD56+ uNK Cells Between the High and Low uNK Cell Density Groups



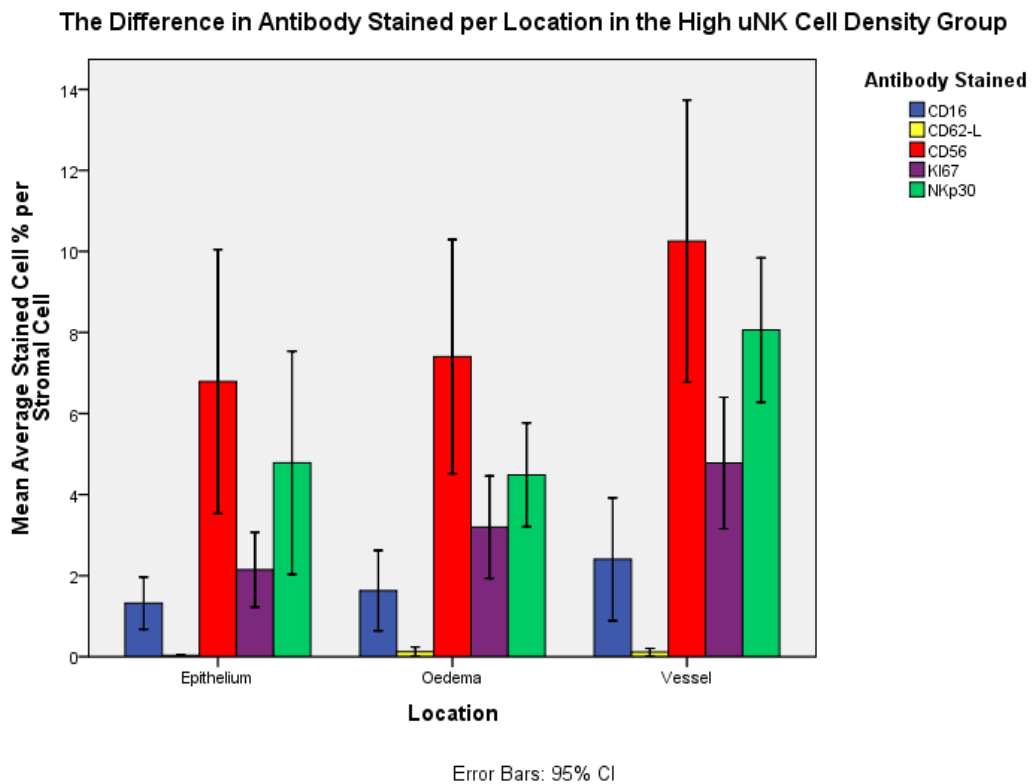
**Figure 12:** Graph illustrating the difference in location of CD56+ uNK cells in the two uNK cell density groups

Figure 12 above highlights the difference between the high and low uNK cell density groups and how they differ for all three locations. The high uNK cell density group has much higher numbers of uNK cells compared to low, this is as expected and can be seen in all three areas. The difference between the two groups at each location is statistically significant because the standard error bars show no overlap between the two groups for each location.



**Figure 13:** Graph illustrating the difference in antibody stained per location in the low uNK cell density group

Figure 13 shows how the Unk cell numbers vary for each antibody stained in each of the three locations for the low Unk cell density group. As seen from the graph there is great variation between all the antibodies at each location. Generally, the number of positive cells stained for the antibodies are seen highest around vessels.



**Figure 14:** Graph illustrating the difference in antibody stained per location in the high uNK cell density group

Figure 14 shows how the uNK cell numbers differ for each antibody stained in each of the three locations for the high uNK cell density group. As seen from the graph there is large variation between all the antibodies for each location. However not all of these are significantly different since the standard error bars overlap to a great extent. This is explored in more detail later. Although, for the high uNK cell density group the number of cells stained for each antibody also seems to be higher around vessels, similar to that of the low uNK cell density group.

### 3.6 OVERALL RESULTS

Antibody Stained	High/Low		Location			
	Overall % mean difference (95% CI)	% significant difference	Overall significant difference	Location comparison	Mean difference (95% CI)	Significance of difference
<b>CD16</b>	1.556 (0.933-2.179)	P<0.0001	NS	Oedema Vs. Epithelium	0.531 (-0.212-1.275)	NS
				Vessel Vs. Epithelium	0.817 (0.073-1.561)	P=0.032
<b>L-Selectin (CD62-L)</b>	0.035 (-0.034-0.104)	NS	NS	Oedema Vs. Epithelium	0.085 (0.002-0.167)	P=0.044
				Vessel Vs. Epithelium	0.061 (-0.021-0.144)	NS
<b>CD56</b>	4.591 (2.470-6.712)	P<0.0001	P=0.055	Oedema Vs. Epithelium	0.77 (-1.761-3.302)	NS
				Vessel Vs. Epithelium	3.011 (0.480-5.543)	P=0.021
<b>K167</b>	2.092 (1.358-2.826)	P<0.0001	P=0.003	Oedema Vs. Epithelium	0.643 (-0.233-1.519)	NS
				Vessel Vs. Epithelium	1.567 (0.691-2.443)	P=0.001
<b>NKp30</b>	2.819 (1.369-4.269)	P<0.0001	P=0.008	Oedema Vs. Epithelium	0.335 (-1.397-3.066)	NS
				Vessel Vs. Epithelium	2.586 (0.855-4.318)	P=0.004

**Table 8:** The following table demonstrates the overall results of the study. It shows how the staining varied between the high and low uNK cell density groups and also location. For each antibody an analysis of variance was performed. The patient number was included as a covariate and locations were compared using a simple contrast with epithelium as a reference category.

### **3.6.1 HIGH VS. LOW UNK CELL DENSITY GROUP**

All the antibodies showed a significant difference between the high and low uNK cell density groups except CD62-L (Table 8). The difference in CD56<sup>+</sup> between the two groups was as expected because of the selection criteria used in the study.

### **3.6.2 LOCATION**

Table 8 shows that the staining varies significantly overall for each antibody with regards to location, except for CD16 and L-Selectin. However, when these results are looked at in further detail with regards to specific location sites the results do show some significance.

#### **3.6.2.1 CD16**

Although no significant difference is seen overall in between location sites for CD16<sup>+</sup> cells, when explored in further detail, it can be seen that there is an increase in the number of cells stained in the peri-vascular areas when compared to areas of epithelial edge. This difference is statistically significant (P=0.032).

#### **3.6.2.2 L-Selectin**

L-Selectin also produced no statistical difference between location sites overall. Although, the results did show that a larger number of cells were stained positive in areas of low stromal density, or oedema when compared to epithelial edge. This difference was statistically significant (P=0.044).

#### **3.6.2.3 CD56**

The pattern of staining for CD56<sup>+</sup> cells with regards to location on the whole, almost attains nominal significance (P= 0.055). However when looked at further, the number

of CD56<sup>+</sup> cells is significantly higher in the peri-vascular areas compared to the epithelial edge (P=0.021).

#### **3.6.2.4 KI67**

KI67 was found to statistically differ across all location sites overall (P=0.003). When the location sites were looked at in more detail, a significant increase in the numbers of positively stained cells was found at the peri-vascular areas compared to epithelial edge (P=0.001).

#### **3.6.2.5 NKp30**

Positively stained cells for NKp30 also demonstrated a significant difference across the location sites (P=0.008). As with the previous two antibodies, this antibody also demonstrated a higher number of positively stained cells in the peri-vascular area compared to epithelial edge.



## **CHAPTER 4: DISCUSSION**

Endometrial expression of markers of cell differentiation and proliferation in patients with unexplained RM and high and low uNK density are described in the thesis. The distribution of each of these cell markers was examined. This allows us to comment on the spatial differentiation of NK cells and the implications for function of that distribution. Comments are also made on whether patients with high and low uNK cell density are associated with markers of uNK cell trafficking. This allows us to address a controversial area of clinical relevance in endometrial biology.

### **4.1 TRAFFICKING OF UNK CELLS**

As seen from the result graphs, the paucity of L-Selectin<sup>+</sup> and CD16<sup>+</sup> stained cells in both groups, low and high uNK cell density suggests that uNK cells are not trafficking from peripheral blood. These two markers are present on the majority of pbNK cells. This result correlates with the findings of many other studies who (171-173) found that the majority of uNK cell population to be CD56<sup>+</sup> CD16<sup>-</sup>. However, there are few other studies that investigated L-Selectin. Had trafficking been the reason that some women have a much higher uNK cell density than others then an increased density of L-Selectin would have been expected in the peri-vascular areas. Furthermore there would have been more L-Selectin positive cells in the high uNK cells density group compared to the low uNK cell density group. Hence this lack of L-Selectin positive is an important negative finding that I will explain further later in this section.

A significant difference was found between the high and low uNK cell density groups for CD16. This could be explained by the fact that there are more CD56<sup>+</sup> cells in the high uNK cell density group compared to the low, and a consistent proportion of CD56 cells were CD16<sup>+</sup>. However, other factors could explain the increase in CD16 in the high density CD56 group. The CD16 antigen is not specific to NK cells; it is also found on the surface of neutrophils, macrophages and mast cells. These cells are found in many conditions associated with the endometrium, for example, endometritis which is

inflammation of the endometrium. However, none of the patients sampled had any clinical symptoms or signs of infection. CD16 was used in this project as a marker for pbNK cells in the endometrium given that 90% of pbNK cells are CD56<sup>+</sup> and CD16<sup>+</sup> (151,174). Seeing as a few CD16<sup>+</sup> cells were found in the endometrial samples, it is therefore reasonable to assume that the majority of CD56<sup>+</sup> uNK cells are not those found in the peripheral blood.

Although some argue that the uNK cells in the endometrium may result from the minority 10% of pbNK cell population that are CD56<sup>bright+</sup> and CD16<sup>-</sup>. Moreover, this tiny pbNK cell population also expresses the receptor for the adhesion molecule, L-Selectin (150) and given that the result found is so small concerning the number of L-Selectin<sup>+</sup> cells in the endometrium, this implication can be ruled out.

As seen from the bar graphs, L-Selectin<sup>+</sup> cells are practically absent in the endometrium of patients in both the high and the low uNK cell density groups. The difference between the two groups although not statistically significant causes uncertainty to arise as to whether the staining actually worked. However, this little L-Selectin<sup>+</sup> staining found in the endometrium is consistent with the findings of another study (104).

Nevertheless, my colleague, with whom I carried out immunohistochemistry, also undertook a similar project. She looked at IVF patients with implantation failure and found an exceptional patient who had L-Selectin<sup>+</sup> staining. This area was identified as lymphoid aggregate by an experienced pathologist. The positively stained cells for L-Selectin did not represent the typical stromal appearance of the endometrium at this stage. This shows that it was not the staining that did not work but that the number of positively stained cells for L-Selectin is scarce in the endometrium at the time of implantation.

Lymphocytes are known to migrate to areas of inflammation by using a number of adhesive mechanisms via a family of adhesion molecules called homing receptors. A

well characterised homing receptor is L-Selectin. Studies have found that L-Selectin is required for homing of naive T cells to lymphoid organs such as peripheral lymph nodes (175). L-Selectin is also known to be expressed on many other leukocytes, and when blocked with the L-Selectin antibody, nonspecific triggers inhibit accumulation of neutrophils at the site of inflammation (176) indicating that L-Selectin has more diverse functions than just mediating entry of T cells.

Studies have proposed a three step model for lymphocyte endothelial interactions. The initial interaction, also known as rolling of circulating leukocytes with endothelia is induced by L-Selectin. The second step is that the initial binding of leukocytes brings the leukocytes closer to chemoattractants released from the endothelium. Upon binding of the chemoattractants to the appropriate receptors on the leukocytes certain integrins are activated. In the final step, integrins bind to their counter receptors on the endothelium, thus increasing the adhesion and resulting in arrest of the rolling of leukocytes. Leukocytes then follow cues from chemoattractants and, using integrins for traction control; they cross the endothelium and enter the appropriate tissues (177).

Several studies believe expression of adhesion molecules by uNK cells in the endometrium could explain homing of uNK cells to the endometrium from peripheral blood (156-158). However the results from this study suggest that uNK cells do not express L-Selectin and therefore cannot support the theory that they arrive from trafficking of pbNK cells. Also studies by Croy *et al* and Van Den Heuvel *et al* suggest ovarian steroids and the LH surge enhance the adhesiveness of NK cells and therefore aid trafficking of the cells into the uterus via the L-Selectin mechanism (93,159). Although these findings were from murine studies and therefore a direct comparison to the human uterus cannot be made, the findings of this study do not support this proposition. Since endometrial samples were taken at only LH + 7-9 you would expect the effect of the LH surge as suggested to result in an increase in the number of L-Selectin<sup>+</sup> cells. However, this is not the case in the studied RM patients.

If the uNK cells were to enter the endometrium from peripheral blood then a perivascular distribution of the number of L-Selectin<sup>+</sup> cells stained no matter how small should be expected. However, the results of this study show that most of the L-Selectin<sup>+</sup> cells are significantly located in the oedema compared to vessels and epithelium.

In addition, Tedder *et al* carried out a study to determine the role of L-Selectin adhesion pathway in the recruitment of leukocytes during inflammation. He found significant inhibition of neutrophil, lymphocyte and monocyte migration into an inflammatory site in L-Selectin deficient mice (178). This finding is consistent with previous studies (179-182). The results indicate that the neutrophils, lymphocytes and monocytes lacking L-Selectin are unable to exit the bloodstream effectively. This may imply that uNK cells lacking L-Selectin are not a result of NK cells trafficking from peripheral blood as maybe they are unable to exit the blood and enter the endometrium.

On the other hand, a few studies have found that once mature naive T cells are exposed to antigens and are subsequently activated, they then shed their surface L-Selectin (183-185). This may represent what is happening in the endometrium. uNK cells may express L-Selectin initially but then lose it once they are activated. The study here demonstrates that many uNK cells at this point in time are activated since the levels of NKp30<sup>+</sup> cells is seen to be quite high in both the high and low uNK cell density groups especially when compared to CD56<sup>+</sup> cell numbers. This may possibly suggest that the uNK cells may have already shed their L-Selectin. However for this theory to be applied it would mean uNK cells are trafficking from peripheral blood. For this reason other trafficking markers need to be looked at in further detail to confirm whether uNK cells are trafficking from the blood or arise from resident NK cells within the endometrium. Also the endometrium needs to be looked at during all the phases of the menstrual cycle and not at just one point in time to conclude further whether L-Selectin numbers vary.

A study by Smolen *et al.* (168) suggests that the initiation of the L-Selectin cascade is perhaps essential to modify the uterine environment to allow for a more stable adhesion of the embryo to the endometrium. Therefore L-Selectin may serve as a possible biochemical marker of endometrial receptivity (168). This implies that L-Selectin is a vital feature of a positive pregnancy outcome and maybe the minute numbers seen in this study may help explain why these women have recurrent miscarriages.

## **4.2 PROLIFERATION OF UNK CELLS**

The results of this study are consistent with the findings of previous studies in relation to KI67. The number of KI67<sup>+</sup> stained cells is significantly higher in the high uNK cell density group compared to the low (P<0.0001). The difference also varies significantly across location sites (P=0.003). A significantly higher number of KI67<sup>+</sup> cells are found at the peri-vascular location compared to the epithelial edge (P=0.001). This implies that women with an increased uNK cell density have more proliferative uNK cells that are more frequently found surrounding endometrial vessels. This finding together with the few numbers of CD16<sup>+</sup> and L-Selectin<sup>+</sup> cells suggests that uNK cells originate from resident endometrial NK cells and do not arise from peripheral blood.

KI67<sup>+</sup> uNK cells may assist with angiogenesis of uterine spiral arteries. Embryo implantation and early placental development occur in a relatively hypoxic environment of 2-3% oxygen (186,187). The placental syncytiotrophoblast undergoes a minor degree of oxidative stress damage when maternal blood flow initially enters the intervillous space, however this soon adapts to the higher oxygen levels (188). However inappropriate maternal blood flow to the intervillous space may be a cause of RM. It has already been established that uNK cells are essential for spiral artery remodelling (159,189). A high number of proliferating uNK cells may increase uterine vessel formation too early and too quickly which in turn increases uterine artery blood flow and subsequently initiates oxidative stress in the early developing foetus. This demonstrates that a balance needs to be achieved. Patients with RM may have an abnormally large number of proliferating uNK cells compared to those who have no fertility problems. However this does not provide an explanation for the low uNK cell density group. The endometrial pathologies of these two groups may be different and therefore needs to be explored further, normal KI67 uNK levels need to be defined.

### **4.3 DIFFERENTIATION OF UNK CELLS**

According to the results of this study, a larger number of uNK cells are active and differentiated in the high uNK cell density group compared to the low ( $P < 0.0001$ ). This is as expected since the numbers of CD56+ cells are higher in this group also. The pattern of NKp30<sup>+</sup> uNK cells also varies across the location sites, with the highest numbers around vessels ( $P = 0.004$ ). This pattern of staining again suggests that the uNK cells may contribute to spiral artery transformation therefore causing the arteries to develop earlier than usual. This may contribute to the early increased blood flow to the endometrium which means there is an increased level of oxygen which results in oxidative stress and causes early pregnancy loss.

An alternative explanation for the peri-vascular distribution of uNK cells is that the cells are closely associated with endometrial stromal cell decidualisation and it is in the peri-vascular areas that the endometrial stromal cells first undergo pre-decidual change in late secretory phase endometrium (190,191).

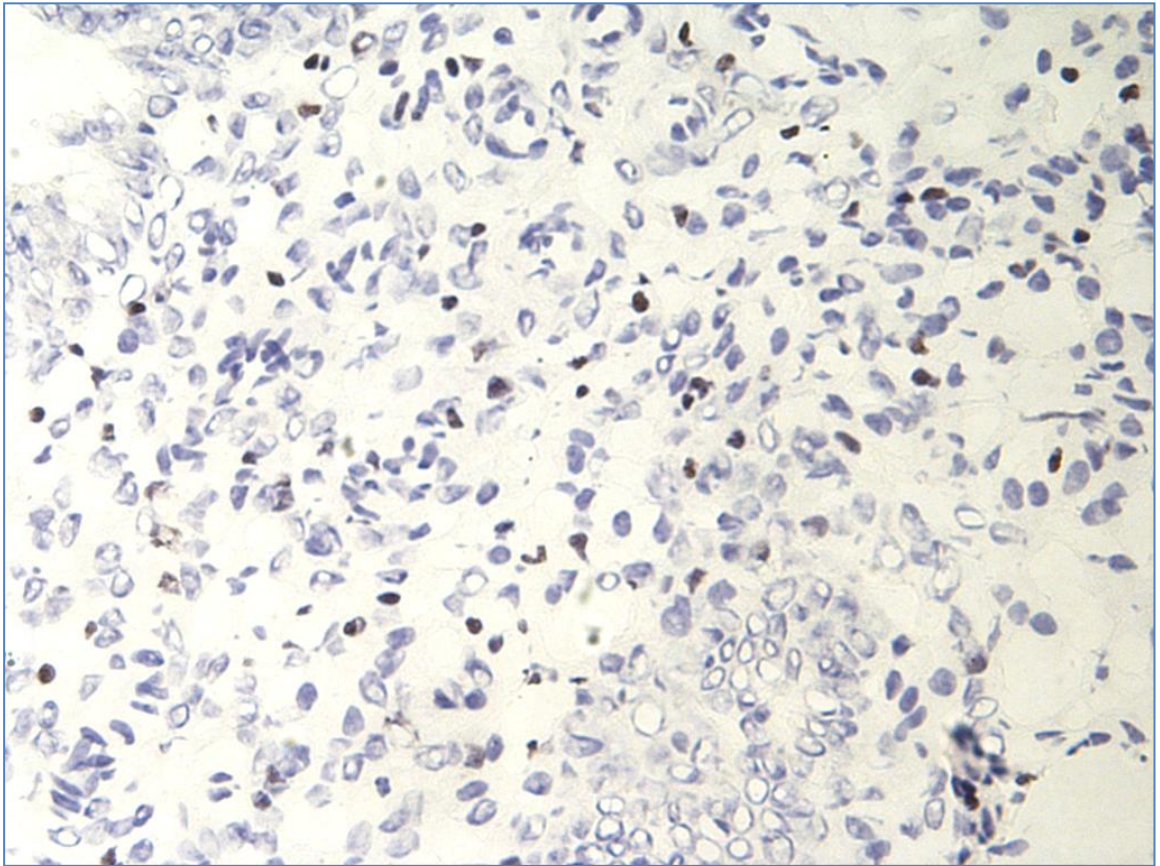
However, a study by Manaster *et al* (91) found conflicting results. He believes uNK cells are negative for NKp30 receptor expression before IL-15 stimulation and therefore are inactive in the endometrium before conception. However there are many problems with this study. Firstly, flow cytometry analysis was used to determine the results. This method is not comparable to immunohistochemistry, which is what was used to conclude the results of this study. Secondly, the patients sampled in his study may not have been timed correctly for the phases of the menstrual cycle, since the gold standard criteria developed by Noyes *et al* (192) or the LH surge was not used to detect the phases. The patients were selected by ultrasound confirmation and serum progesterone levels. Using progesterone levels to date the endometrium is not as accurate as the LH surge since it covers a broader time frame which allows for more variability. Thirdly, an explanation for the differences in the results may be explained by the fact that his patient samples were given IL-15 *in vitro*. This may not reflect what

goes on *in vivo*; many other factors are always involved. Lastly, no statistical method has been used to say whether the results of the study were significant.

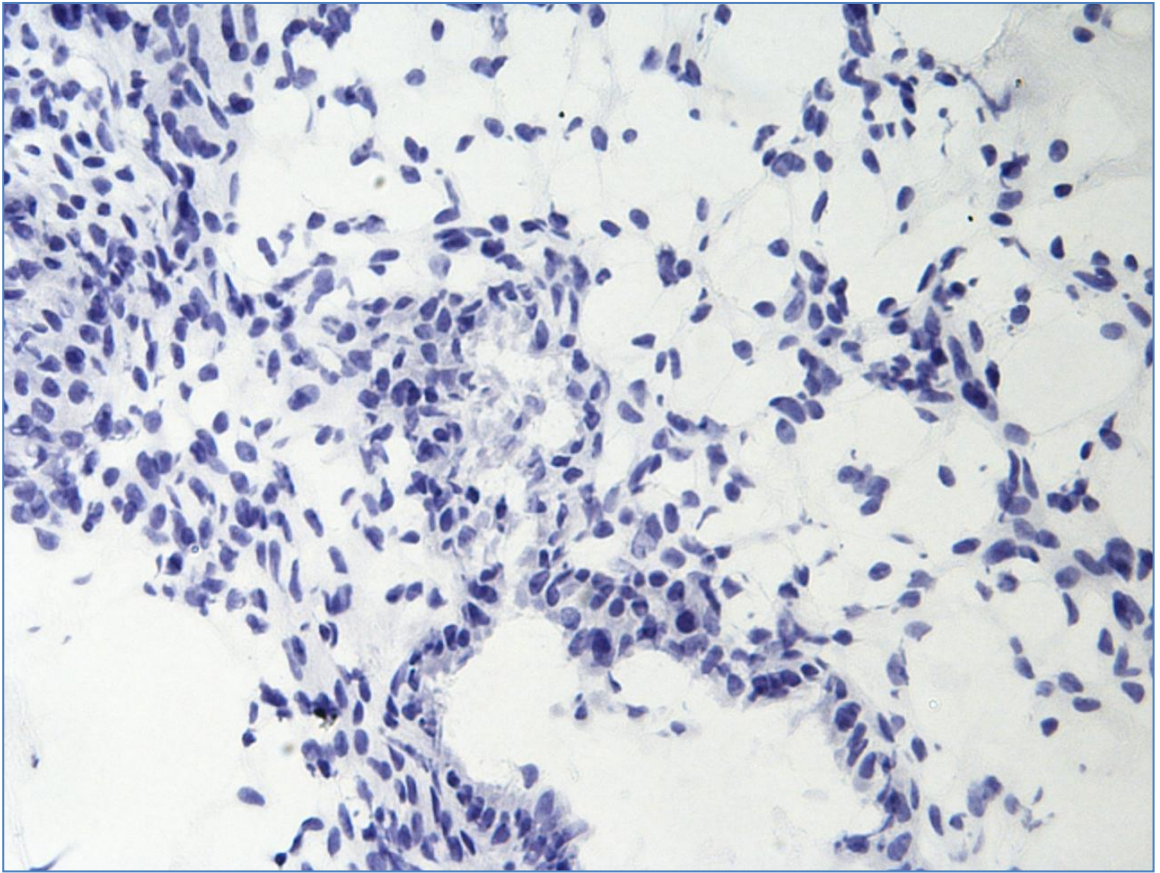
A study by Ponnampalam *et al* (92) investigated the cyclical changes of NKp30 in the human endometrium during the menstrual cycle. Little or no immunostaining for NKp30<sup>+</sup> cells were found in the proliferative phase endometrium. Although, the results showed NKp30<sup>+</sup> staining in the endometrial glands and the luminal epithelium in the early, mid and late secretory phase endometrium. This study was the only one to demonstrate the presence of NKp30 staining in the endometrium epithelium. Conversely, the results of our study found NKp30<sup>+</sup> cells in the stroma near the epithelial edge, in low stromal density areas and surrounding vessels but not in glands or in the epithelium. The NKp30 staining identified by Ponnampalam *et al* is debatable. The staining in the epithelium and glands may be considered as background staining, since the goat polymer to this antibody is known to stain background more readily compared to the mouse polymer.

Although the findings of this study are similar concerning the levels of NKp30 found at the time of implantation it cannot be compared directly. The results revealed in Ponnampalam *et al* study were carried out on frozen endometrial sections. To see whether we could detect a similar pattern of NKp30 expression, frozen sections of a few of our samples were also stained for NKp30 in our study. However, we failed to replicate the Ponnampalam results as shown in figure 15. In our laboratory NKp30 continued to stain frozen sections in a similar manner to that seen in the micrographs of paraffin sections in the previous chapter.





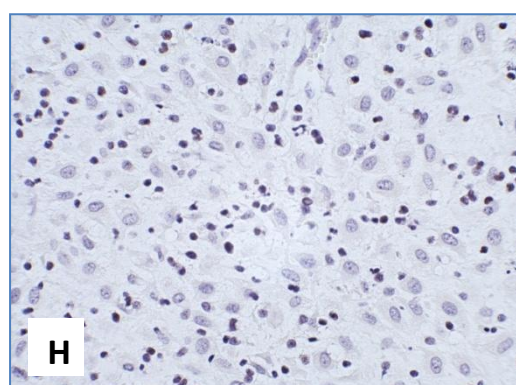
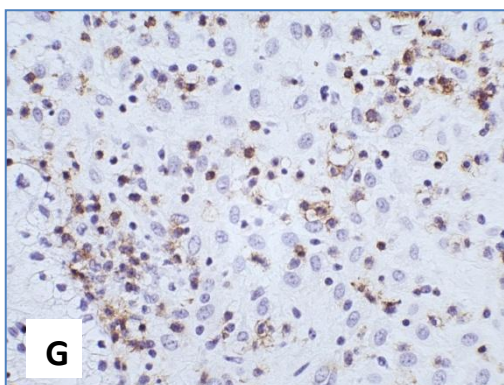
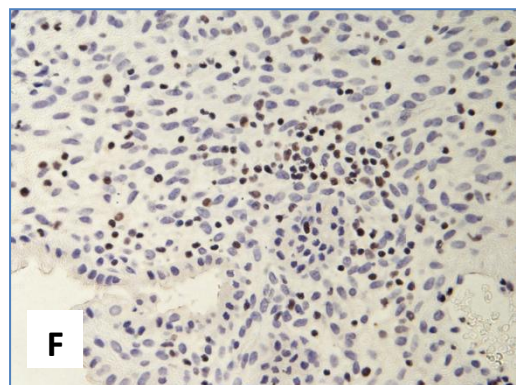
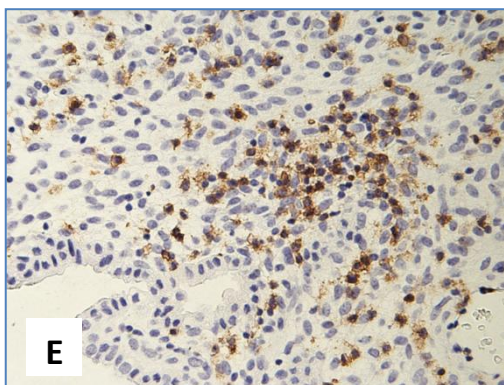
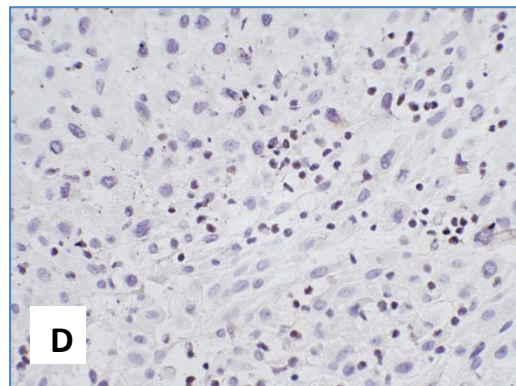
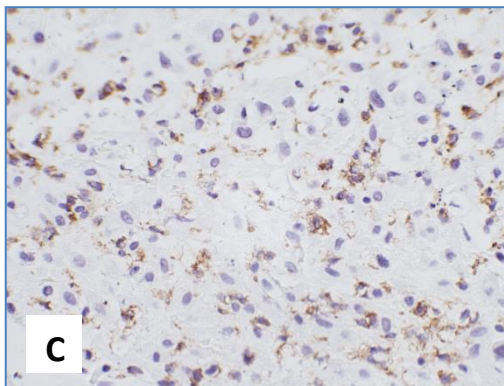
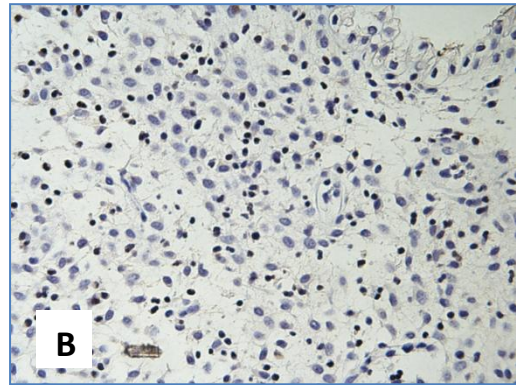
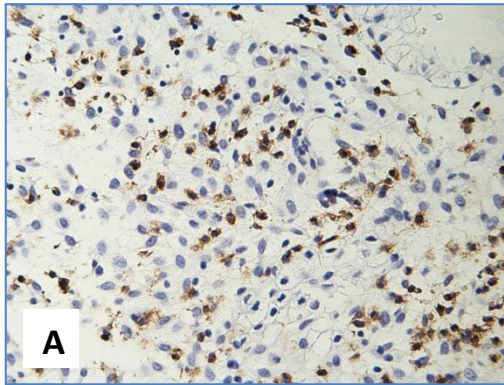
**Fig 15:** The micrograph above is an example of NKp30<sup>+</sup> staining on a frozen endometrial section of a patient from the high uNK cell density group. As seen above the positively stained cells are still staining in a nuclear fashion. This was cut and stained by a member of our department.

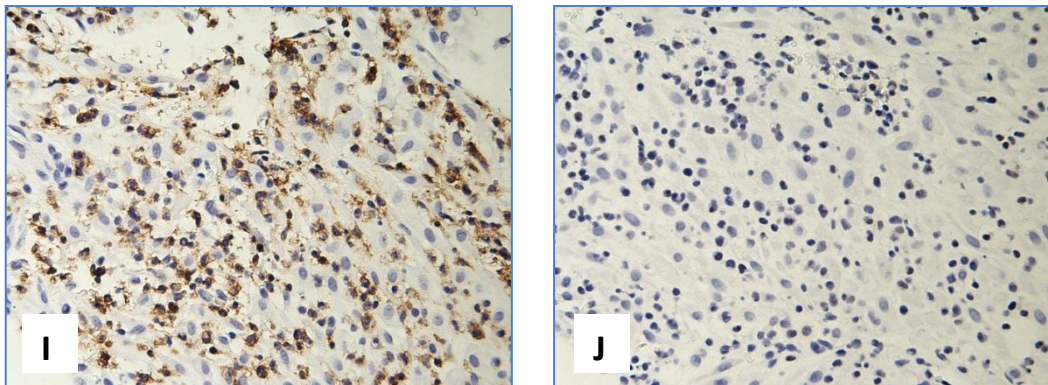


**Fig 16:** *The micrograph above is an example of IgG Goat staining on a frozen endometrial section from the same patient in the high uNK cell density group as shown on the previous page. No positive staining is seen. This was cut and stained by a member of our department*

Another difference highlighted between the findings of our study and that of Ponnampalam *et al* and Manaster *et al*'s study is that the NKp30 staining was noticed to be nuclear in our data. To address this difference observed we looked at the staining pattern of NKp30 in termination of pregnancy samples from the first trimester decidua of varying gestational age (Figure 17). If in the following studies, the NKp30 positive peaks seen on flow cytometry were only observed once stimulated with IL-15, *in vitro*, it could be that changes from nuclear staining to cytoplasmic staining could occur as part of differentiation of cells. However, the decidual staining produced was not any different to that found in the late secretory phase endometrium in that the NKp30 staining remained nuclear. This is illustrated by the micrographs in figure 17. From this we must conclude that our staining of the novel NKp30 is the first to truly be observed in frozen and paraffin late-secretory phase endometrial sections and first trimester decidua.







**Fig 17:** The micrographs above show staining on A- CD56<sup>+</sup>, B- NKp30<sup>+</sup> a 6 week decidua. As seen the NKp30 is still producing nuclear staining. C- CD56<sup>+</sup> staining, D- NKp30<sup>+</sup> staining a 7 week decidua, again nuclear stained cells are seen. E- CD56<sup>+</sup> staining, F- NKp30<sup>+</sup> staining an 8 week decidua, again nuclear stained cells are seen. G- CD56<sup>+</sup> staining, H- NKp30<sup>+</sup> staining a 10 week decidua, again nuclear stained cells are seen. I- CD56<sup>+</sup> staining, J- NKp30<sup>+</sup> staining a 12 week decidua, again nuclear stained cells are seen. All the sections were cut and stained by a member of the department.

## **5 – CONCLUSIONS**

### **5.1 METHODOLOGICAL LIMITATIONS**

There are fundamental limitations in the methodologies used in previous studies. The levels and activation of NK cells is dependent on many variables, these include, whether whole blood or fractionated mononuclear cells are used, the time of day the sample is taken, whether any physical exercise has been performed prior to the biopsy, the parity of the patient and whether the samples have been previously frozen (193-197).

Another problem is that the number of uNK cells during a menstrual cycle change rapidly and the density of endometrium is not constant, therefore the results produced by the endometrial samples in different studies that are not analysed at the same depth beneath the surface epithelium cannot be compared directly. Also, comparison of data from the different studies is complicated due to the varying approaches to uNK cell separation and purification, which is likely to lead to the inclusion of different cell types. Therefore comparisons cannot be made directly between different methodologies, for example flow cytometry and immunohistochemistry.

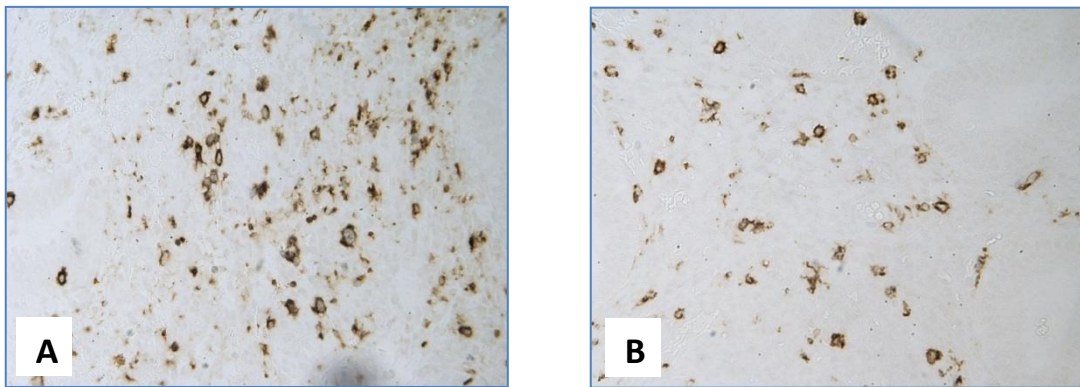
Furthermore, the possibility that tissue disaggregation and cell purification may lead to cell activation should also be considered (50). This may explain the inconsistency between the results of NKp30 numbers and staining by the different studies.

The advantages of using immunohistochemistry over other methods such as flow cytometry allowed location of uNK cells to be studied. However, it cannot be said with certainty that for example a cell stained positive for Ki67 is definitely a CD56<sup>+</sup> uNK cell. For this reason serial sequential sections were used to allow for comparisons to be made. However, this method is not very accurate since a cell's diameter ranges



approximately from 5-15 $\mu$ m (198) and therefore the same cell may not be identifiable on the next serial section micrograph if it is only 5 $\mu$ m in diameter as each section used in this study was 4 $\mu$ m.

To overcome this problem double staining was also carried out on a small selection of the endometrial samples to determine whether a CD56<sup>+</sup> cell was also a KI67<sup>+</sup> cell. This is illustrated by the micrographs in figure 18. As demonstrated by the micrographs, it is difficult to determine the structures, such as vessels and glands of the endometrium. Again, this makes it difficult to determine the distribution of uNK cells across different locations.



**Fig 18:** The micrographs above show double staining from, A- A high uNK cell density patient and B- A low uNK cell density patient. The brown staining represents CD56 and the grey staining represents KI67. As seen on the micrographs it is difficult to appreciate any structures within the endometrium. Also double staining cannot be seen clearly.

## **5.2 CLINICAL IMPLICATIONS OF RESULTS**

The results of the current study have provided more data to that already published to show us that uNK cells are a unique subset of natural killer cells that are solely confined to the endometrium. From the following results and that of previous studies, it can be said that there is little scientific justification for testing peripheral blood for pbNK cells to identify the cause of RM. As this investigation does not reflect what pathology is going on in the endometrium. The biologically important tissue is the endometrium, the site of implantation and the materno-foetal interface.

Studying uNK cells at the different location sites has helped us determine the role of uNK cells to some extent. Previous work found that uNK cell density correlated to endometrial vessel density (169) but this work raised the following question. Are the uNK cell angiogenic cytokines causing vessel differentiation in the endometrial tissue? Alternatively, are the uNK cells in increased density because of increased vessel density and thus increased trafficking from the blood. My study was designed to answer this question and the evidence all points to the former possibility, that uNK cells in the pre-implantation endometrium are proliferative, differentiated and thus influencing endometrial vessel development, as they were found to be differentiated and more proliferative around vessels. The CD56<sup>+</sup> uNK cells were seen clustered around vessels at higher numbers compared to those in the epithelial edge and low stromal density areas.



### **5.3 FUTURE WORK**

Reproductive immunology advances has led to a more multidisciplinary approach to studying and treating couples with RM. The chance of a successful pregnancy in couples with unexplained RM together with the small number of data from randomised control trials means that clinicians should not use empirical treatments which may be of no benefit or may even cause harm. Instead, patients with RM should be recruited to an adequately powered placebo-controlled study. A trial is currently ongoing in the Liverpool Women's Hospital where RM patients are receiving prednisolone or placebo. This trial will determine whether steroids increase the chance of a successful pregnancy outcome.

The relationship between uNK cells and endometrial cytokine production also requires further investigation and how these processes may be defective in RM.

The study has produced many interesting findings that could be investigated further. The uNK cell activating markers need to be looked at in more detail since we are the first to find the nuclear staining pattern of NKp30<sup>+</sup> uNK cells. NKp30 should be looked at across the phases of the endometrium to determine whether the cells are differentiated differently at different stages and also whether they change from nuclear to cytoplasmic or vice versa. This may help determine the role of uNK cells within the endometrium and their effect on pregnancy outcome.

Other markers of trafficking could be examined to completely rule out the theory of NK cell migration from the blood.

## **REFERENCES**

- (1) Johnson M, Everitt BJ. Essential reproduction. 6 Rev ed.: Blackwell Pub; 2007.
- (2) Psychoyos A. Hormonal control of ovoimplantation. *Vitamins and Hormones* 1973;31:201-256.
- (3) Pijnenborg R. Trophoblast Invasion. *Reprod Med Rev.* 1994;3:53-73.
- (4) Brosen IA. Morphological changes in the utero-placental bed in pregnancy hypertension. *Clin.Obstet.Gynecol.* 1977;4:573-593.
- (5) Khong TY, De Wolf F, Robertson WB, Brosens I. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. *Br.J.Obstet.Gynaecol.* 1986;93:1049-1059.
- (6) Horne AW, Alexander CI. Recurrent miscarriage. *J.Fam.Plann.Reprod.Health.Care.* 2005 Apr;31(2):103-107.
- (7) Kutteh WH. Recurrent pregnancy loss. *Infertility* 2005:467.
- (8) Collier J, Longmore M, Brinsden M. *Oxford Handbook of Clinical Specialties . 7th ed.:* Oxford University Press; 2006.
- (9) Tucker D. Pregnancy Loss: *Miscarriage*. Available at: <http://www.womens-health.co.uk/miscarr.asp>, 2009.
- (10) Quenby SM, Farquharson RG. Predicting recurring miscarriage: what is important? *Obstetrics And Gynecology-New York-* 1993;82:132-132.
- (11) Reproductive Genetics Institute. infertility: Repeated Pregnancy Loss. 2005; Available at: [http://www.reproductivegenetics.com/repeated\\_loss.html](http://www.reproductivegenetics.com/repeated_loss.html). Accessed February, 2009.
- (12) Longmore M, Wilkinson IB, Turmezei T, Cheung CK. *Oxford handbook of clinical medicine.* 7th ed. Oxford: Oxford University Press; 2007.
- (13) Cunningham FG, Mac-Donald PC, Gant NF, Leveno KJ, Gilstrap LC, Hankins MD, et al. *Williams obstetrics.* 22nd ed.: Appleton & Lange; 2001.

- (14) Tucker D. Pregnancy Loss, *Blighted Ovum*. 1997; Available at: <http://www.womens-health.co.uk/blighted.html>, 2009.
- (15) Quenby S. Recurrent miscarriage. *Obstetrics, Gynaecology & Reproductive Medicine* 2007;17(10):296-300.
- (16) Baker PN, Monga A. *Obstetrics by Ten Teachers*. 18th ed. London: Hodder Arnold; 2006.
- (17) Tucker D. Pregnancy Loss: Stillbirth. Available at: <http://www.womens-health.co.uk/stillbirth.html>, 2009.
- (18) Puscheck EE, Pradhan A. First-Trimester Pregnancy Loss. 2006; Available at: <http://emedicine.medscape.com/article/266317-overview>.
- (19) Impey L. *Obstetrics and gynaecology*. 2nd ed.: Blackwell Pub; 2004.
- (20) Simon C, Rubio C, Vidal F, Gimenez C, Moreno C, Parrilla JJ. Increased chromosome abnormalities in human preimplantation embryos after in-vitro fertilization in patients with recurrent miscarriage. *Reprod.Fertil.Dev.* 1998;10:87–92.
- (21) Gianaroli L, Magli MC, Ferraretti AP. The in vivo and in vitro efficiency and efficacy of PGD for aneuploidy. *Mol Cell Endocrinol* 2001;183(Suppl. 1):13–18.
- (22) Monga AK, Baker P. *Gynaecology by ten teachers*. 18th ed. London: Arnold; 2006.
- (23) Li T, Makris M, Tomsu M, Tuckerman E, Laird S. Recurrent miscarriage: aetiology, management and prognosis. *Hum.Reprod.Update* 2002;8(5):463-481.
- (24) Longmore M, Wilkinson IB, Turmezei T, Cheung CK. *Oxford Handbook of Clinical Medicine*. 7th ed.: Oxford University Press; 2007.
- (25) OnMedica Staff. Researchers identify marker for early miscarriage. 2008; Available at: <http://www.onmedica.com/news/37993cac-9012-4d91-b1ad-7faa265c873e/researchers-identify-marker-for-early-miscarriage>, 2009.
- (26) Homburg R, Armar NA, Eshel A, Adams J, Jacobs HS. Influence of serum luteinising hormone concentrations on ovulation, conception and early pregnancy loss in polycystic ovary syndrome. *Br.Med.J* 1988;297(6655):1024-1026.
- (27) Regan L, Owen EJ, Jacobs HS. Hypersecretion of luteinising hormone, infertility and miscarriage. *Lancet* 1990;336:1141–1144.

- (28) Ben DM. Transient Iso-B Hyperprolactinemia in infertile women with normal cycle and its involvement with unexplained infertility, HMG failure, and repeated miscarriages: detection and treatment. In: Blackwell RE, Chang RL, editors. *Prolactin-Related Disorders*. 1st ed. New Jersey: Macmillan; 1987. p. 75-84.
- (29) Tal J, Kaplan M, Sharf M, Barnea ER. Stress-related hormones affect human chorionic gonadotrophin secretion from the early human placenta. *in vitro*. *Hum. Reprod* 1991;6:766-769.
- (30) Hirahara F, Andoh N, Sawai K, Hirabuki T, Uemura T, Minaguchi H. Hyperprolactinemic recurrent miscarriage and results of randomised bromocriptine treatment trials. *Fertil. Steril* 1998;70:246-252.
- (31) Lin PC. Reproductive Outcomes in Women with Uterine Anomalies. *Journal of Women's Health* 2004;13:33-39.
- (32) Quenby S, Mountfield S, Cartwright JE, Whitley GSJ, Chamley L, Vince G. Antiphospholipid antibodies prevent extravillous trophoblast differentiation. *Fertil.Steril*. 2005;83(3):691-698.
- (33) Rai R, Regan L. Recurrent miscarriage. *Lancet* 2006 Aug 12;368(9535):601-611.
- (34) Empson M, Lassere M, Craig JC, Scott JR. Recurrent pregnancy loss with antiphospholipid antibody: A systematic review of therapeutic trials. *Obstet.Gynecol*. 2002;99:135-144.
- (35) Rai R, Cohen H, Dave M, Regan L. Randomised controlled trial of aspirin and aspirin plus heparin in pregnant women with recurrent miscarriage associated with phospholipid antibodies (or antiphospholipid antibodies). *Br.Med.J*. 1997;314(7076):253.
- (36) Farquharson RG, Quenby S, Greaves M. Antiphospholipid syndrome in pregnancy: a randomized, controlled trial of treatment. *Obstetrics & Gynecology* 2002;100(3):408.
- (37) Varga E. Recurrent Pregnancy Loss: Women and Thrombophilia. 2008; Available at: <http://www.dnadirect.com/web/article/testing-for-genetic-disorders/recurrent-pregnancy-loss/79/women>, 2009.
- (38) Figa-Talamanca I. Occupational risk factors and reproductive health of women. *Occup Med (Lond)* 2006 December 1;56(8):521-531.

- (39) Dlugosz L, Belanger K, Hellenbrand K, Holford TR, Leaderer B, Bracken MB. Maternal Caffeine Consumption and Spontaneous Abortion: A Prospective Cohort Study. *Epidemiology* 1996;7(3):250.
- (40) Scott JR, Pattison N. Human chorionic gonadotrophin for recurrent miscarriage. *Cochrane database of systematic reviews* [Withdrawn].
- (41) Scott JR. Immunotherapy for recurrent miscarriage. *Cochrane Database Syst.Rev.* 2000;(2)(2).
- (42) Oates-Whitehead R, Haas D, Carrier J. Progestogen for preventing miscarriage (Review). *Cochrane database of systematic reviews* 2003;4:CD003511.
- (43) Abbas AK, Lichtman AH, Pober JS, Wonsiewicz MJ. Cellular and molecular immunology. : Saunders Philadelphia; 1991.
- (44) Chong PJ, Matzner WL, Ching WTW, Smith W. Miscarriages Can Be Prevented.
- (45) Laird S, Tuckerman E, Cork B, Linjawi S, Blakemore A, Li T. A review of immune cells and molecules in women with recurrent miscarriage. *Hum.Reprod.Update* 2003;9(2):163-174.
- (46) Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: Is successful pregnancy a TH2 phenomenon? *Immunology Today* 1993;14(7):353-356.
- (47) Christiansen OB. A fresh look at the causes and treatments of recurrent miscarriage, especially its immunological aspects. *Hum.Reprod.Update* 1996;2(4):271-293.
- (48) Moffett-King A. Natural killer cells and pregnancy. *Nature Reviews Immunology* 2002;2(9):656-663.
- (49) Ntrivalas EI, Kwak-Kim JYH, Gilman-Sachs A, Chung-Bang H, Ng SC, Beaman KD, et al. Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. *Human Reproduction* 2001;16(5):855-861.
- (50) Bulmer JN, Lash GE. Human uterine natural killer cells: a reappraisal. *Mol.Immunol.* 2005 2;42(4):511-521.

- (51) Koopman LA, Kopcow HD, Rybalov B, Boyson JE, Orange JS, Schatz F, et al. Human decidua natural killer cells are a unique NK cell subset with immunomodulatory potential. *J.Exp.Med.* 2003;198(8):1201.
- (52) Starkey P, Sargent I, Redman C. Cell populations in human early pregnancy decidua: characterization and isolation of large granular lymphocytes by flow cytometry. *Immunology* 1988;65(1):129.
- (53) Moffett A, Regan L, Braude P. Natural killer cells, miscarriage, and infertility. *Br.Med.J.* 2004;329(7477):1283-1285.
- (54) Gubbay O, Critchley HO, Bowen JM, King A, Jabbour HN. Prolactin induces ERK phosphorylation in epithelial and CD56(+) natural killer cells of the human endometrium. *J Clin Endocrinol Metab.* 2002;87(5):2329-35.
- (55) Dunn CL, Critchley HO, Kelly RW. IL-15 regulation in human endometrial stromal cells. *J Clin Endocrinol Metab* 2002;87(4):1898-901.
- (56) Ashkar AA, Croy BA. Functions of uterine natural killer cells are mediated by interferon gamma production during murine pregnancy. *Semin Immunol* 2001;13(4):235-41.
- (57) Reinhard G, Noll A, Schlebusch H, Mallmann P, Ruecker AV. Shifts in the TH1/TH2 balance during human pregnancy correlate with apoptotic changes. *Biochem.Biophys.Res.Commun.* 1998;245(3):933-938.
- (58) Wolff M, Thaler C, Strowitzki T, Broome J, Stolz W, Tabibzadeh S. Regulated expression of cytokines in human endometrium throughout the menstrual cycle: dysregulation in habitual abortion. *Mol.Hum.Reprod.* 2000;6(7):627-634.
- (59) Raghupathy R, Makhseed M, Azizieh F, Omu A, Gupta M, Farhat R. Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion. *Human Reproduction* 2000;15(3):713-718.
- (60) King A, Allan D S, Bowen M, Powis S J, Joseph S, Verma S, et al. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. *European journal of immunology* 2000;30(6):1623-31.
- (61) Braud VM, Allan DSJ, O'Callaghan CA, Söderström K, D'Andrea A, Ogg GS, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 1998;391(6669):795-799.

- (62) Lee N, Llano M, Carretero M, Ishitani A, Navarro F, López-Botet M, et al. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc.Natl.Acad.Sci.U.S.A.* 1998;95(9):5199.
- (63) Boyington JC, Brooks AG, Sun PD. Structure of killer cell immunoglobulin-like receptors and their recognition of the class I MHC molecules. *Immunol.Rev.* 2001;181:66.
- (64) Long EO, Barber DF, Burshtyn DN, Faure M, Peterson M, Rajagopalan S, et al. Inhibition of natural killer cell activation signals by killer cell immunoglobulin-like receptors (CD158). *Immunol.Rev.* 2001;181:223.
- (65) Vilches C, Parham P. K IR: Diverse, Rapidly Evolving Receptors of Innate and Adaptive Immunity. *Annu.Rev.Immunol.* 2002;20(1):217-251.
- (66) Hiby SE, King A, Sharkey AM, Loke YW. Human uterine NK cells have a similar repertoire of killer inhibitory and activatory receptors to those found in blood, as demonstrated by RT-PCR and sequencing. *Mol.Immunol.* 1997;34(5):419-430.
- (67) Verma S, King A, Loke YW. Expression of killer cell inhibitory receptors on human uterine natural killer cells. *Eur.J.Immunol.* 1997;27(4).
- (68) Hiby SE, Walker JJ, O'Shaughnessy KM, Redman CWG, Carrington M, Trowsdale J, et al. Combinations of Maternal KIR and Fetal HLA-C Genes Influence the Risk of Preeclampsia and Reproductive Success Abbreviations used in this paper: EVT, extravillous trophoblast; KIR, killer immunoglobulin receptors; uNK, uterine NK. *J.Exp.Med.* 2004;200(8):957-965.
- (69) Lachapelle M, Miron P, Hemmings R, Roy D. Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome. *The Journal of Immunology* 1996;156(10):4027-4034.
- (70) Clifford K, Flanagan A, Regan L. Endometrial CD56 natural killer cells in women with recurrent miscarriage: a histomorphometric study. *Human Reproduction* 1999;14(11):2727-2730.
- (71) Quenby S, Bates M, Doig T, Brewster J, Lewis-Jones D, Johnson P, et al. Pre-implantation endometrial leukocytes in women with recurrent miscarriage. *Human Reproduction* 1999;14(9):2386-2391.
- (72) Dosiou C, Giudice LC. Natural killer cells in pregnancy and recurrent pregnancy loss: endocrine and immunologic perspectives. *Endocr.Rev.* 2005;26(1):44-62.

(73) Henderson TA, Saunders PTK, Moffett-King A, Groome NP, Critchley HOD. Steroid receptor expression in uterine natural killer cells. *Journal of Clinical Endocrinology & Metabolism* 2003;88(1):440-449.

(74) Kontula K, Paavonen T, Luukkainen T, Andersson LC. Binding of progestins to the glucocorticoid receptor. Correlation to their glucocorticoid-like effects on in vitro functions of human mononuclear leukocytes. *Biochem.Pharmacol.* 1983 May 1;32(9):1511-1518.

(75) Kitaya K, Yasuda J, Yagi I, Tada Y, Fushiki S, Honjo H. IL-15 expression at human endometrium and decidua. *Biol.Reprod.* 2000;63(3):683-687.

(76) Wu W, Brooks J, Glasier A, McNeilly A. The relationship between decidualization and prolactin mRNA and production at different stages of human pregnancy. *J.Mol.Endocrinol.* 1995;14(2):255-261.

(77) Ferry B, Starkey P, Sargent I, Watt G, Jackson M, Redman C. Cell populations in the human early pregnancy decidua: natural killer activity and response to interleukin-2 of CD56-positive large granular lymphocytes. *Immunology* 1990;70(4):446.

(78) Hamai Y, Fujii T, Yamashita T, Kozuma S, Okai T, Taketani Y. Pathogenetic implication of interleukin-2 expressed in preeclamptic decidual tissues: A possible mechanism of deranged vasculature of the placenta associated with preeclampsia. *American journal of reproductive immunology(1989)* 1997;38(2):83-88.

(79) Lim K, Odukoya O, Ajjan R, Li T, Weetman A, Cooke I. Profile of cytokine mRNA expression in peri-implantation human endometrium. *Mol.Hum.Reprod.* 1998;4(1):77-81.

(80) Hayakawa S, Nagai N, Kanaeda T, Karasaki-Suzuki M, Ishii M, Chishima F, et al. Interleukin-12 augments cytolytic activity of peripheral and decidual lymphocytes against choriocarcinoma cell lines and primary culture human placental trophoblasts. *Am.J.Reprod.Immunol.* 1999 May;41(5):320-329.

(81) Kauma S, Huff T, Krystal G, Ryan J, Takacs P, Turner T. The expression of stem cell factor and its receptor, c-kit in human endometrium and placental tissues during pregnancy. *Journal of Clinical Endocrinology & Metabolism* 1996;81(3):1261-1266.

(82) Umekage H, Saito S, Morikawa H. Enhancement by stem cell factor of interleukin-2 (IL-2)-induced DNA synthesis in human decidual CD16<sup>+</sup> CD56<sup>bright</sup> natural killer cells mediated by increased expression of the IL-2 receptor  $\alpha$  chain. *J.Reprod.Immunol.* 1998;40(1):1-24.



- (83) Wu Q, Sun Y, Wang J, Lin X, Wang Y, Pegg LE, et al. Signal Via Lymphotoxin- $\beta$ R on Bone Marrow Stromal Cells Is Required for an Early Checkpoint of NK Cell Development 1. *The Journal of Immunology* 2001;166(3):1684-1689.
- (84) Fehniger TA, Caligiuri MA. Interleukin 15: biology and relevance to human disease. *Blood* 2001;97(1):14.
- (85) Carson W, Giri J, Lindemann M, Linett M, Ahdieh M, Paxton R, et al. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J.Exp.Med.* 1994;180(4):1395-1403.
- (86) Kitaya K, Nakayama T, Okubo T, Kuroboshi H, Fushiki S, Honjo H. Expression of macrophage inflammatory protein-1 $\beta$  in human endometrium: its role in endometrial recruitment of natural killer cells. *Journal of Clinical Endocrinology & Metabolism* 2003;88(4):1809-1814.
- (87) Verma S, Hiby SE, Loke YW, King A. Human decidual natural killer cells express the receptor for and respond to the cytokine interleukin 15. *Biol.Reprod.* 2000;62(4):959.
- (88) Kitaya K, Yamaguchi T, Yasuo T, Okubo T, Honjo H. Post-ovulatory rise of endometrial CD16(-) natural killer cells: in situ proliferation of residual cells or selective recruitment from circulating peripheral blood? *J.Reprod.Immunol.* 2007 12;76(1-2):45-53.
- (89) Jones RK, Searle RF, Stewart JA, Turner S, Bulmer JN. Apoptosis, bcl-2 expression, and proliferative activity in human endometrial stroma and endometrial granulated lymphocytes. *Biology of Reproduction* 1998 April;58(4):995-1002.
- (90) Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat.Med.* 2006;12(9):1065-1074.
- (91) Manaster I, Mizrahi S, Goldman-Wohl D, Sela HY, Stern-Ginossar N, Lankry D, et al. Endometrial NK Cells Are Special Immature Cells That Await Pregnancy. *J.Immunol.* 2008 August 1;181(3):1869-1876.
- (92) Ponnampalam AP, Gargett CE, Rogers PAW. Identification and hormonal regulation of a novel form of NKp30 in human endometrial epithelium. *Eur.J.Immunol.* 2008;38(1).

- (93) Croy A, van den Heuvel MJ, Borzychowski AM, Tayade C. Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. *Immunol.Rev.* 2006;214(1):161.
- (94) Croy BA, Esadeg S, Chantakru S, van den Heuvel M, Paffaro VA, He H, et al. Update on pathways regulating the activation of uterine natural killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. *J.Reprod.Immunol.* 2003;59(2):175-191.
- (95) Bulmer JN, Morrison L, Longfellow M, Ritson A, Pace D. Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies. *Human Reproduction* 1991;6(6):791-798.
- (96) Bulmer JN, Sunderland C. Immunohistological characterization of lymphoid cell populations in the early human placental bed. *Immunology* 1984;52(2):349-357.
- (97) Trundley A, Moffett A. Human uterine leukocytes and pregnancy. *Tissue Antigens* 2004;63(1):1.
- (98) Kusakabe K, Okada T, Sasaki F, Kiso Y. Cell death of uterine natural killer cells in murine placenta during placentation and preterm periods. *Journal of Veterinary Medical Science (Japan)* 1999.
- (99) Hammer A, Dohr G. Apoptotic nuclei within the uterine decidua of first trimester pregnancy arise from CD45 positive leukocytes. *Am.J.Reprod.Immunol.* 1999 Aug;42(2):88-94.
- (100) Pongcharoen S, Bulmer J, Searle R. No evidence for apoptosis of decidual leucocytes in normal and molar pregnancy: implications for immune privilege. *Clin.Exp.Immunol.* 2004;138:330-336.
- (101) Spornitz UM. The functional morphology of the human endometrium and decidua. *Adv.Anat.Embryol.Cell Biol.* 1992;124:1-99.
- (102) Jones R, Searle R, Bulmer J. Apoptosis and bcl-2 expression in normal human endometrium, endometriosis and adenomyosis. *Hum.Reprod.* 1998 December 1;13(12):3496-3502.
- (103) Pace D, Morrison L, Bulmer J. Proliferative activity in endometrial stromal granulocytes throughout menstrual cycle and early pregnancy. *Br.Med.J.* 1989;42(1):35-39.

- (104) Searle RF, Jones RK, Bulmer JN. Phenotypic Analysis and Proliferative Responses of Human Endometrial Granulated Lymphocytes during the Menstrual Cycle. *Biology of Reproduction* 1999 April;60(4):871-878.
- (105) Peel S, Stewart I, Bulmer D. Metrial gland cells in deciduomata of pseudopregnancy. *J.Anat.* 1979;129(Pt 1):21.
- (106) Pijnenborg R, Bland J, Robertson W, Brosens I. Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. *Placenta(Eastbourne)* 1983;4(4):397-413.
- (107) King A, Birkby C, Loke YW. Early human decidual cells exhibit NK activity against the K562 cell line but not against first trimester trophoblast. *Cell.Immunol.* 1989 2;118(2):337-344.
- (108) Christmas S, Bulmer J, Meager A, Johnson P. Phenotypic and functional analysis of human CD3-decidual leucocyte clones. *Immunology* 1990;71(2):182.
- (109) Ferry BL, Sargent IL, Starkey PM, Redman CWG. Cytotoxic activity against trophoblast and choriocarcinoma cells of large granular lymphocytes from human early pregnancy decidua. *Cell.Immunol.* 1991 1;132(1):140-149.
- (110) Rouas-Freiss N, Gonçalves RM, Menier C, Dausset J, Carosella ED. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytotoxicity. *Proceedings of the National Academy of Sciences of the United States of America* 1997 October 14;94(21):11520-11525.
- (111) Jokhi P, King A, Sharkey A, Smith S, Loke Y. Screening for cytokine messenger ribonucleic acids in purified human decidual lymphocyte populations by the reverse-transcriptase polymerase chain reaction. *The Journal of Immunology* 1994;153(10):4427-4435.
- (112) Jokhi PP, King A, Loke YW. Cytokine Production And Cytokine Receptor Expression By Cells Of The Human First Trimester Placental–Uterine Interface. *Cytokine* 1997 2;9(2):126-137.
- (113) Rieger L, Kammerer U, Hofmann J, Sutterlin M, Dietl J. Choriocarcinoma cells modulate the cytokine production of decidual large granular lymphocytes in coculture. *Am.J.Reprod.Immunol.* 2001;46(2):137-143.

- (114) Li XF, Charnock-Jones DS, Zhang E, Hiby S, Malik S, Day K, et al. Angiogenic Growth Factor Messenger Ribonucleic Acids in Uterine Natural Killer Cells. *J.Clin.Endocrinol.Metab.* 2001 April 1;86(4):1823-1834.
- (115) von Rango U, Classen-Linke I, Raven G, Bocken F, Beier HM. Cytokine microenvironments in human first trimester decidua are dependent on trophoblast cells. *Fertil.Steril.* 2003 5;79(5):1176-1186.
- (116) van der Meer A, Lukassen HGM, van Lierop MJC, Wijnands F, Mosselman S, Braat DDM, et al. Membrane-bound HLA-G activates proliferation and interferon- $\gamma$  production by uterine natural killer cells. *Mol.Hum.Reprod.* 2004 March 1;10(3):189-195.
- (117) Dunk C, Shams M, Nijjar S, Rhaman M, Qiu Y, Bussolati B, et al. Angiopoietin-1 and angiopoietin-2 activate trophoblast Tie-2 to promote growth and migration during placental development. *Am.J.Pathol.* 2000;156(6):2185-2199.
- (118) Bauer S, Pollheimer J, Hartmann J, Husslein P, Aplin JD, Knofler M. Tumor Necrosis Factor- $\alpha$  Inhibits Trophoblast Migration through Elevation of Plasminogen Activator Inhibitor-1 in First-Trimester Villous Explant Cultures. *J.Clin.Endocrinol.Metab.* 2004 February 1;89(2):812-822.
- (119) Graham CH, Connelly I, MacDougall JR, Kerbel RS, Stetler-Stevenson WG, Lala PK. Resistance of Malignant Trophoblast Cells to both the Anti-proliferative and Anti-invasive Effects of Transforming Growth Factor- $\beta$ . *Exp.Cell Res.* 1994 9;214(1):93-99.
- (120) Hempstock J, Jauniaux E, Greenwold N, Burton GJ. The contribution of placental oxidative stress to early pregnancy failure. *Hum.Pathol.* 2003 12;34(12):1265-1275.
- (121) Jauniaux E, Greenwold N, Hempstock J, Burton GJ. Comparison of ultrasonographic and Doppler mapping of the intervillous circulation in normal and abnormal early pregnancies. *Fertil.Steril.* 2003 1;79(1):100-106.
- (122) Hustin J, Jauniaux E, Schaaps JP. Histological study of the materno-embryonic interface in spontaneous abortion. *Placenta* 1990 12;11(6):477-486.
- (123) Sebire NJ, Fox H, Backos M, Rai R, Paterson C, Regan L. Defective endovascular trophoblast invasion in primary antiphospholipid antibody syndrome-associated early pregnancy failure. *Hum.Reprod.* 2002 April 1;17(4):1067-1071.

- (124) Greenwold N, Jauniaux E, Gulbis B, Hempstock J, Gervy C, Burton GJ. Relationship among maternal serum endocrinology, placental karyotype, and intervillous circulation in early pregnancy failure. *Fertil.Steril.* 2003 6;79(6):1373-1379.
- (125) Guimond M, Wang B, Croy BA. Engraftment of Bone Marrow from Severe Combined Immunodeficient (SCID) Mice Reverses the Reproductive Deficits in Natural Killer Cell-deficient tgepsilon 26 Mice. *J.Exp.Med.* 1998 January 19;187(2):217-223.
- (126) Ashkar AA, Di Santo JP, Croy BA. Interferon {gamma} Contributes to Initiation of Uterine Vascular Modification, Decidual Integrity, and Uterine Natural Killer Cell Maturation during Normal Murine Pregnancy. *J.Exp.Med.* 2000 July 17;192(2):259-270.
- (127) Craven CM, Morgan T, Ward K. Decidual spiral artery remodelling begins before cellular interaction with cytotrophoblasts. *Placenta* 1998 5;19(4):241-252.
- (128) King A. Uterine leukocytes and decidualization. *Hum.Reprod.Update* 2000 January 1;6(1):28-36.
- (129) Vassiliadou N, Bulmer JN. Characterization of Tubal and Decidual Leukocyte Populations in Ectopic Pregnancy: Evidence That Endometrial Granulated Lymphocytes are Absent from the Tubal Implantation Site. *Fertil.Steril.* 1998 4;69(4):760-767.
- (130) von Rango U, Classen-Linke I, Kertschanska S, Kemp B, Beier HM. Effects of trophoblast invasion on the distribution of leukocytes in uterine and tubal implantation sites. *Fertil.Steril.* 2001 7;76(1):116-124.
- (131) Quenby S, Kalumbi C, Bates M, Farquharson R, Vince G. Prednisolone reduces preconceptual endometrial natural killer cells in women with recurrent miscarriage. *Fertil.Steril.* 2005;84(4):980-984.
- (132) Michimata T, Ogasawara Ms, Tsuda H, Suzumori K, Aoki K, Sakai M, et al. Distributions of endometrial NK cells, B cells, T cells, and Th2/Tc2 cells fail to predict pregnancy outcome following recurrent abortion. *American Journal Of Reproductive Immunology* 2002;47(4):196-202.
- (133) Shimada S, Kato EH, Morikawa M, Iwabuchi K, Nishida R, Kishi R, et al. No difference in natural killer or natural killer T-cell population, but aberrant T-helper cell population in the endometrium of women with repeated miscarriage. *Hum.Reprod.* 2004 April 1;19(4):1018-1024.

- (134) Liddell H, Pattison N, Zanderigo A. Recurrent miscarriage-outcome after supportive care in early pregnancy. *Australian and New Zealand Journal of Obstetrics and Gynaecology* 1991;31(4):320-322.
- (135) Brigham S, Conlon C, Farquharson R. A longitudinal study of pregnancy outcome following idiopathic recurrent miscarriage. *Human Reproduction* 1999;14(11):2868-2871.
- (136) Ogasawara M, Aoki K, Okada S, Suzumori K. Embryonic karyotype of abortuses in relation to the number of previous miscarriages. *Fertil.Steril.* 2000 Feb;73(2):300-304.
- (137) Tuckerman E, Laird S, Stewart R, Wells M, Li T. Markers of endometrial function in women with unexplained recurrent pregnancy loss: a comparison between morphologically normal and retarded endometrium. *Human Reproduction* 2004;19(1):196-205.
- (138) Quenby S, Vince G, Farquharson R, Aplin J. Recurrent miscarriage: a defect in nature's quality control? *Human Reproduction* 2002;17(8):1959-1963.
- (139) Guimond M, Wang B, Croy BA. Engraftment of Bone Marrow from Severe Combined Immunodeficient (SCID) Mice Reverses the Reproductive Deficits in Natural Killer Cell-deficient tgepsilon 26 Mice. *J.Exp.Med.* 1998 January 19;187(2):217-223.
- (140) Yamamoto T, Takahashi Y, Kase N, Mori H. Role of decidual natural killer (NK) cells in patients with missed abortion: differences between cases with normal and abnormal chromosome. *Clin.Exp.Immunol.* 1999;116:449-452.
- (141) Quack KC, Vassiliadou N, Pudney J, Anderson DJ, Hill JA. Leukocyte activation in the decidua of chromosomally normal and abnormal fetuses from women with recurrent abortion. *Hum.Reprod.* 2001 May 1;16(5):949-955.
- (142) Kwak J, Beaman K, Gilman-Sachs A, Ruiz J, Schewitz D, Beer A. Up-regulated expression of CD 56 , CD 56 /CD 16 , and CD 19 cells in peripheral blood lymphocytes in pregnant women with recurrent pregnancy losses. *American journal of reproductive immunology(1989)* 1995;34(2):93-99.
- (143) Beer A, Kwak J, Ruiz J. Immunophenotypic profiles of peripheral blood lymphocytes in women with recurrent pregnancy losses and in infertile women with multiple failed in vitro fertilization cycles. *American journal of reproductive immunology(1989)* 1996;35(4):376-382.

- (144) Shakhar K, Rosenne E, Loewenthal R, Shakhar G, Carp H, Shamgar BE. High NK cell activity in recurrent miscarriage: what are we really measuring? *Human Reproduction* 2006;21(9):2421.
- (145) Wang Q, Li TC, Wu YP, Cocksedge KA, Fu YS, Kong QY, Yao SZ. Reappraisal of peripheral NK cells in women with recurrent miscarriage. *Reprod Biomed Online* 2008;17(6):814-819.
- (146) Aoki K, Kajiura S, Matsumoto Y, Ogasawara M, Okada S, Yagami Y, et al. Preconceptional natural-killer-cell activity as a predictor of miscarriage. *The Lancet* 1995 5/27;345(8961):1340-1342.
- (147) Ntrivalas EI, Kwak-Kim JYH, Gilman-Sachs A, Chung-Bang H, Ng SC, Beaman KD, et al. Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. *Hum.Reprod.* 2001 May 1;16(5):855-861.
- (148) Eidukaite A, Siaurys A and Tamosiunas V. Differential expression of KIR/NKAT2 and CD94 molecules on decidual and peripheral blood CD56bright and CD56dim natural killer cell subsets. *Fertil Steril* 2004;81(Suppl 1):863–868.
- (149) Emmer PM, Nelen WLDM, Steegers EAP, Hendriks JCM, Veerhoek M, Joosten I. Peripheral natural killer cytotoxicity and CD56posCD16pos cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. *Hum.Reprod.* 2000 May 1;15(5):1163-1169.
- (150) Manaster I, Mandelboim O. The unique properties of human NK cells in the uterine mucosa. *Placenta* 2008;29:60-66.
- (151) King A, Balendran N, Wooding P, Carter N, Loke Y. CD3-leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterization of the CD56 population. *Dev.Immunol.* 1991;1(3):169.
- (152) Kämmerer U, Marzusch K, Kröber S, Ruck P, Handgretinger R, Dietl J. A subset of CD 56 large granular lymphocytes in first-trimester human decidua are proliferating cells. *Fertil.Steril.* 1999;71(1):74-79.
- (153) Lobo SC, Huang ST, Germeyer A, Dosiou C, Vo KC, Tulac S, et al. The immune environment in human endometrium during the window of implantation. *Am.J.Reprod.Immunol.* 2004 Oct;52(4):244-251.

- (154) Peel S, Stewart I. Rat granulated metrial gland cells differentiate in pregnant chimeric mice and may be cytotoxic for mouse trophoblast. *Cell Differ.Dev.* 1989 Oct;28(1):55-64.
- (155) Chantakru S, Miller C, Roach LE, Kuziel WA, Maeda N, Wang WC, et al. Contributions from Self-Renewal and Trafficking to the Uterine NK Cell Population of Early Pregnancy 1. *J.Immunol.* 2002;168(1):22-28.
- (156) Marzusch K, Ruck P, Geiselhart A, Handgretinger R, Dietl JA, Kaiserling E, et al. Distribution of cell adhesion molecules on CD56<sup>++</sup>, CD3<sup>-</sup>, CD16<sup>-</sup> large granular lymphocytes and endothelial cells in first-trimester human decidua. *Hum.Reprod.* 1993 Aug;8(8):1203-1208.
- (157) Ruck P, Marzusch K, Kaiserling E, Horny HP, Dietl J, Geiselhart A, et al. Distribution of cell adhesion molecules in decidua of early human pregnancy. An immunohistochemical study. *Lab.Invest.* 1994 Jul;71(1):94-101.
- (158) Burrows TD, King A, Loke YW. The role of integrins in adhesion of decidual NK cells to extracellular matrix and decidual stromal cells. *Cell.Immunol.* 1995 Nov;166(1):53-61.
- (159) Heuvel MJ, Xie X, Tayade C, Peralta C, Fang Y, Leonard S, et al. A review of trafficking and activation of uterine natural killer cells. *Am J Reprod Immunol* 2005;54(6):322-331.
- (160) Chantakru S, Wang WC, van den Heuvel M, Bashar S, Simpson A, Chen Q, et al. Coordinate Regulation of Lymphocyte-Endothelial Interactions by Pregnancy-Associated Hormones 1. *J.Immunol.* 2003;171(8):4011-4019.
- (161) Cavagna M, Mantese J. Biomarkers of endometrial receptivity—a review. *Placenta* 2003;24:39-47.
- (162) Genbacev OD, Prakobphol A, Foulk RA, Krtolica AR, Ilic D, Singer MS, et al. Trophoblast L-Selectin-Mediated Adhesion at the Maternal-Fetal Interface. *Science* 2003 January 17;299(5605):405-408.
- (163) Fazleabas AT, Kim JJ. Development: What Makes an Embryo Stick? *Science* 2003 January 17;299(5605):355-356.
- (164) Lai T, Shih I, Vlahos N, Ho C, Wallach E, Zhao Y. Differential expression of L-selectin ligand in the endometrium during the menstrual cycle. *Fertil.Steril.* 2005 4;83(4, Supplement 1):1297-1302.



- (165) Zak I, Lewandowska E, Gnyp W. Selectin glycoprotein ligands. *Acta Biochim.Pol.* 2000;47(2):393-412.
- (166) Shimizu Y, Newman W, Tanaka Y, Shaw S. Lymphocyte interactions with endothelial cells. *Immunol.Today* 1992;13(3):106-112.
- (167) Picker L, Butcher E. Physiological and molecular mechanisms of lymphocyte homing. *Annu.Rev.Immunol.* 1992;10(1):561-591.
- (168) Smolen JE, Petersen TK, Koch C, O'Keefe SJ, Hanlon WA, Seo S, et al. L-Selectin Signaling of Neutrophil Adhesion and Degranulation Involves p38 Mitogen-activated Protein Kinase. *J.Biol.Chem.* 2000 May 19;275(21):15876-15884.
- (169) Quenby S, Nik H, Innes B, Lash G, Turner M, Drury J, et al. Uterine natural killer cells and angiogenesis in recurrent reproductive failure. *Hum.Reprod.* 2009 January 1;24(1):45-54.
- (170) IHC World Team. Knowledge Centre, Introduction to Immunohistochemistry. 2007; Available at: <http://www.ihcworld.com/introduction.htm>, 2009.
- (171) Lachapelle MH, Miron P, Hemmings R, Baron C, Roy D. Flow-cytometric characterization of hematopoietic cells in non-pregnant human endometrium. *Am J Reprod Immunol* 1996;35(1):5-13.
- (172) King A, Wellings V, Gardner L, Loke YW. Immunocytochemical characterization of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle. *Hum.Immunol.* 1989 Mar;24(3):195-205.
- (173) Kamat B, Isaacson P. The immunocytochemical distribution of leukocytic subpopulations in human endometrium. *Am.J.Pathol.* 1987;127(1):66.
- (174) Rai R, Sacks G, Trew G. Natural killer cells and reproductive failure--theory, practice and prejudice. *Hum.Reprod.* 2005 May 1;20(5):1123-1126.
- (175) Hamann A, Jablonski-Westrich D, Jonas P, Thiele HG. Homing receptors reexamined: mouse LECAM-1 (MEL-14 antigen) is involved in lymphocyte migration into gut-associated lymphoid tissue. *Eur.J.Immunol.* 1991;21(12).
- (176) Watson SR, Fennie C, Lasky LA. Neutrophil influx into an inflammatory site inhibited by a soluble homing receptor-IgG chimera. *Nature* 1991;349:164-167.

- (177) Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994 Jan 28;76(2):301-314.
- (178) Tedder TF, Steeber DA, Pizcueta P. L-selectin-deficient mice have impaired leukocyte recruitment into inflammatory sites. *J.Exp.Med.* 1995 Jun 1;181(6):2259-2264.
- (179) Spertini O, Luscinskas FW, Kansas GS, Munro JM, Griffin JD, Gimbrone MA, Jr, et al. Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. *J.Immunol.* 1991 Oct 15;147(8):2565-2573.
- (180) Spertini O, Luscinskas FW, Gimbrone MA, Jr, Tedder TF. Monocyte attachment to activated human vascular endothelium in vitro is mediated by leukocyte adhesion molecule-1 (L-selectin) under nonstatic conditions. *J.Exp.Med.* 1992 Jun 1;175(6):1789-1792.
- (181) Brady H, Spertini O, Jimenez W, Brenner B, Marsden P, Tedder T. Neutrophils, monocytes, and lymphocytes bind to cytokine-activated kidney glomerular endothelial cells through L-selectin (LAM-1) in vitro. *J.Immunol.* 1992;149(7):2437-2444.
- (182) Knol E, Tackey F, Tedder T, Klunk D, Bickel C, Sterbinsky S, et al. Comparison of human eosinophil and neutrophil adhesion to endothelial cells under nonstatic conditions. Role of L-selectin. *J.Immunol.* 1994;153(5):2161-2167.
- (183) Munro J, Briscoe D, Tedder T. Differential regulation of leucocyte L-selectin (CD62L) expression in normal lymphoid and inflamed extralymphoid tissues. *Br.Med.J.* 1996;49(9):721-727.
- (184) Pizcueta P, Luscinskas FW. Monoclonal antibody blockade of L-selectin inhibits mononuclear leukocyte recruitment to inflammatory sites in vivo. *Am.J.Pathol.* 1994 Aug;145(2):461-469.
- (185) Picker LJ, Kishimoto TK, Smith CW, Warnock RA, Butcher EC. ELAM-1 is an adhesion molecule for skin-homing T cells. *Nature* 1991 Feb 28;349(6312):796-799.
- (186) Yedwab GA, Paz G, Homonnai TZ, David MP, Kraicer PF. The temperature, pH, and partial pressure of oxygen in the cervix and uterus of women and uterus of rats during the cycle. *Fertil Steril* 1976;27:304-309.
- (187) Rodesch F, Simon P, Donner C, Jauniaux E. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol* 1992;80:283-285.

- (188) Hung TH, Skepper JN, Burton GJ. In Vitro Ischemia-Reperfusion Injury in Term Human Placenta as a Model for Oxidative Stress in Pathological Pregnancies. *Am.J.Pathol.* 2001 September 1;159(3):1031-1043.
- (189) Croy BA, Chantakru S, Esadeg S, Ashkar AA, Wei Q. Decidual natural killer cells: key regulators of placental development (a review). *J.Reprod.Immunol.* 2002;57:151-168.
- (190) Robertson WB. *The endometrium.* : Butterworth-Heinemann; 1981.
- (191) Dallenbach-Hellweg G. *The normal histology of the endometrium. Histopathology of the endometrium.*4th ed.Berlin: Springer-Verlag 1987:36-39.
- (192) Noyes R, Hertig A, Rock J. Dating the endometrial biopsy. *Fertil Steril* 1950;1:3-25.
- (193) Strong DM, Ortaldo JR, Pandolfi F. Cryopreservation of human mononuclear cells for quality control in clinical immunology. I. Correlations in recovery of K- and NK-cell functions, surface markers, and morphology. *J.Clin.Immunol.* 1982;2(3):214-221.
- (194) Porzolt F, Gaus W, Heimpel H. The evaluation of serial measurements of the NK cell activity in man. *Immunobiology* 1983;165(5):475-484.
- (195) Pross HF, Maroun JA. The standardization of NK cell assays for use in studies of biological response modifiers. *J.Immunol.Methods* 1984;68(1-2):235-249.
- (196) Reichert T, DeBruyere M, Deney V, Totterman T, Lydyard P, Yuksel F, et al. Lymphocyte subset reference ranges in adult caucasians. *Clin.Immunol.Immunopathol.* 1991;60(2):190-208.
- (197) Plackett TP, Boehmer ED, Faunce DE, Kovacs EJ. Aging and innate immune cells. *J.Leukoc.Biol.* 2004;76(2):291-299.
- (198) Shaw AJ. *Epithelial Cell Culture A Practical Approach.* Oxford: Oxford University Press; 1996.

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**APPENDIX**

**Abstract submitted and accepted for a poster presentation for British Maternal and Foetal Medicine Society on 18-19/06/09.**

**UNK CELLS IN PATIENTS WITH RECURRENT MISCARRIAGE SHOW EVIDENCE OF PROLIFERATION AND DIFFERENTIATION BUT NOT TRAFFICKING**

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**Introduction:** There is considerable controversy concerning the origin of uterine natural killer cells (uNK). There are two competing hypotheses. uNK cells could be the result of peripheral NK cells trafficking into the uterus or the result of proliferation of uNK cells in the endometrium.

**Method:** Serial sequential sections of endometrial tissue from 20 recurrent miscarriage patients of extreme phenotype at LH+7 days were stained using immunohistochemistry with antibodies to CD56, KI67, NKp30, L-Selectin and CD16, uNK cell markers of proliferation, differentiation and trafficking. Endometrium was examined using a single-blinded method and samples with high and low density of CD56+ cells were compared in areas of epithelium, oedema and vessels.

**Results:** Preliminary semi-quantitative analysis showed more positively stained cells for all markers in samples with high-uNK cell density, except for CD16. The majority of CD56+ cells appeared to be CD16-. The CD56+ was seen more clustered around vessels.

CD56 and NKp30 are frequently found nearer the epithelium in samples with high-uNK cell density. This sub-epithelial location is significant as it is where initial feto-maternal interaction occurs. The localisation of KI67 suggests that uNK cells surrounding vessels are the result of proliferation. The paucity of L-Selectin and CD16+ expression in either group suggests that uNK cells are not trafficking from peripheral blood.

**Conclusion:** Preliminary data from recurrent miscarriage patients provides evidence of proliferation and differentiation of uNK cells but not trafficking from the peripheral circulation.

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A Karam, L Khan, J Drury, A Tang, M Turner, S Quenby. Uterine Natural Killer Cells In Patients With Recurrent Miscarriage Show Evidence Of Proliferation And Differentiation But Not Trafficking. *Arch Dis Child Fetal Neonatal Ed* 2009;94:Fa58-Fa71.

**Abstract submitted and accepted for a poster presentation for Blair Bell Meeting on 05/11/09.**

**THE ROLE AND ORIGINS OF UTERINE NATURAL KILLER (uNK) CELLS IN WOMEN WITH RECURRENT MISCARRIAGE (RM)**

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**Background:** uNK cells are said to cause RM. However, the exact role and function of these unique cells is yet to be determined.

**Method:** Immunohistochemical staining of endometrial sections from RM patients of extreme phenotype between LH +5-9 days. 20 RM patients analysed, 10 patients with high uNK density ( $\geq 5\%$ ) and 10 low uNK ( $\leq 2.5\%$ ). Samples were stained for antibodies to CD56, KI67, NKp30, L-Selectin and CD16, markers of proliferation, differentiation and trafficking. Epithelial edge, areas of low stromal cell density and peri-vascular stroma were studied.

**Results:** High uNK had higher levels for all antibodies ( $P < 0.0001$ ). uNK were more proliferative and differentiated in high group and varied across location sites. Majority of uNK cells positive for CD56, KI67 and NKp30 were clustered around vessels ( $P = 0.021$ ,  $0.001$  and  $P = 0.004$  respectively).

**Conclusion:** The paucity of L-Selectin<sup>+</sup> and CD16<sup>+</sup> in both groups suggests uNK are not trafficking from peripheral blood. The KI67<sup>+</sup> and NKp30<sup>+</sup> uNK assist angiogenesis of uterine arteries which increases blood flow and initiates oxidative stress in developing foetus causing RM.



# uNK CELLS IN PATIENTS WITH RECURRENT MISCARRIAGE SHOW EVIDENCE OF PROLIFERATION AND DIFFERENTIATION BUT NOT TRAFFICKING

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## Introduction

Miscarriage occurs in 15-20% of all clinically diagnosed pregnancies of reproductive aged women, with the most occurring in the first twelve weeks of gestation. Recurrent miscarriage (RM) occurs within 1-2% of these women. There is considerable controversy concerning the origin of uterine natural killer cells (uNK) and their role in pregnancy. There are two competing hypotheses, uNK cells could be the result of peripheral NK cells trafficking into the uterus or the result of proliferation and differentiation of uNK cells resident in the endometrium.

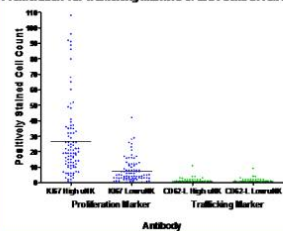
## Methods

Serial sequential paraffin sections of endometrial tissue from 20 recurrent miscarriage patients (RM ≥3 consecutive miscarriages) of extreme phenotype at LH+7 days were stained using immunohistochemistry with antibodies to CD56, KI67, NKp30, L-Selectin and CD16, uNK cell markers of proliferation, differentiation and trafficking. Endometrium was examined using a single-blinded method and samples with high and low density of CD56+ cells were compared in areas of epithelium, oedema and vessels.

## Results

A quantitative analysis showed more positively stained cells for all markers in samples with high uNK cell density in all locations studied. More CD56+ is seen clustered significantly around vessels in both high and low uNK cell density groups.

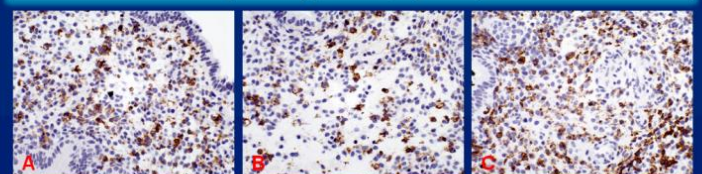
Proliferation vs. Trafficking Markers of uNK Cells in RM Patients



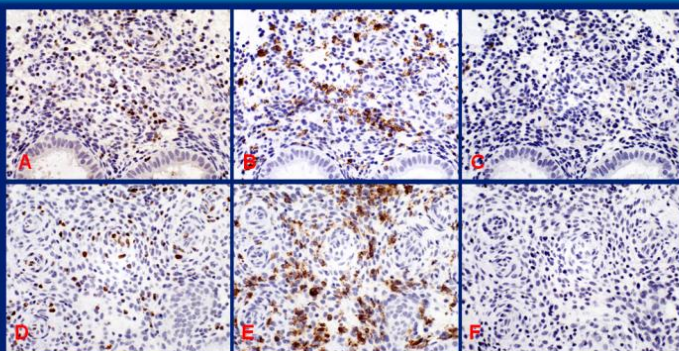
Clinical Demographics of Sample Population

RM uNK Group	Age	% uNK	Number of RM	1st Trim Loss	2nd Trim Loss	3rd Trim Loss	Live birth
10 High Median (Range)	37.5 (32-44)	8.1 (5.2-13.8)	7 (5-7)	6 (5-7)	0 (0-3)	0 (0-1)	0 (0-5)
10 Low Median (Range)	36 (31-44)	1.95 (1-2.4)	7 (5-17)	7 (5-17)	0 (0)	0 (0)	0 (0-1)

Endometrial micrographs demonstrating uNK cells (CD56+) at different locations in a High uNK patient. A- Epithelium Edge, B-Oedema, C-Spiral Artery



Endometrial micrographs of different antibodies staining vessels in High uNK patient. A - NKp30 (Differentiation), B - CD56 (uNK), C - CD16 (Trafficking), D - KI67 (Proliferation), E - CD56, F - L-Selectin (Trafficking)



Antibody	Location	Average % per Stromal Cell		Mann Whitney U-Test Distribution Compared to Vessels (P Values)	
		High	Low	High	Low
CD16	Epithelium	1.32	0.55	NS	NS
	Oedema	1.63	1.04	0.04	0.003
	Vessel	2.40	0.60	-	-
CD56	Epithelium	6.98	1.69	<0.0001	<0.0001
	Oedema	7.37	2.30	0.004	<0.0001
	Vessel	10.27	4.18	-	-
CD62-L	Epithelium	0.03	0.04	NS	NS
	Oedema	0.12	0.10	NS	NS
	Vessel	0.11	0.07	-	-
KI67	Epithelium	2.16	0.59	<0.0001	<0.0001
	Oedema	3.20	0.64	0.011	0.001
	Vessel	4.78	1.49	-	-
NKp30	Epithelium	4.90	1.82	<0.001	0.03
	Oedema	4.49	2.31	<0.001	NS
	Vessel	8.06	3.39	-	-

## Discussion

The peri-vascular localisation of KI67 and NKp30 in both high and low uNK cell density groups suggests that uNK cells surrounding vessels are proliferating and are more differentiated compared to those in oedema and epithelium. This finding is significantly higher for the high uNK cell density group (P<0.0001). The paucity of L-Selectin and CD16+ expression in either group suggests that uNK cells are not trafficking from peripheral blood.

## Conclusion

Low expression of trafficking markers for uNK cells at LH+7-9 challenges the theory that uNK cells migrate from peripheral blood. uNK cells of patients in the high uNK cell density group are more differentiated and proliferating surrounding vessels. These resident uNK cells could be involved in angiogenesis and the induction of a hostile endometrial environment.



# THE ROLE AND ORIGINS OF UTERINE NATURAL KILLER (uNK) CELLS IN WOMEN WITH RECURRENT MISCARRIAGE (RM)

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## Introduction

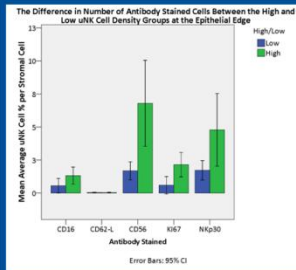
Miscarriage occurs in 15-20% of all clinically diagnosed pregnancies of reproductive aged women, with the most occurring in the first twelve weeks of gestation. Recurrent miscarriage (RM) occurs within 1-2% of these women. There is considerable controversy concerning the origin of uterine natural killer cells (uNK) and their role in pregnancy. There are two competing hypotheses, uNK cells could be the result of peripheral NK cells trafficking into the uterus or the result of proliferation and differentiation of uNK cells resident in the endometrium.

## Methods

Serial sequential paraffin sections of endometrial tissue from 20 recurrent miscarriage patients (RM  $\geq 3$  consecutive miscarriages) of extreme phenotype at LH+7 days were stained using immunohistochemistry with antibodies to CD56, KI67, NKp30, L-Selectin and CD16, uNK cell markers of proliferation, differentiation and trafficking. Endometrium was examined using a single-blinded method and samples with high and low density of CD56+ cells were compared in areas of epithelium, oedema and vessels.

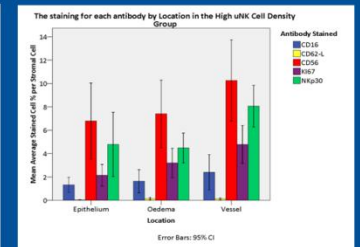
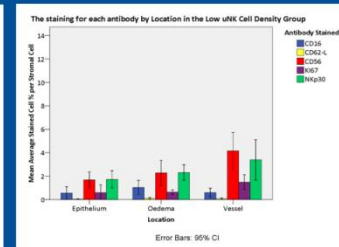
## Results

ANOVA showed more positively stained cells for all markers in samples with high uNK cell density in all locations studied. More CD56+ is seen clustered significantly around vessels in both high and low uNK cell density groups.

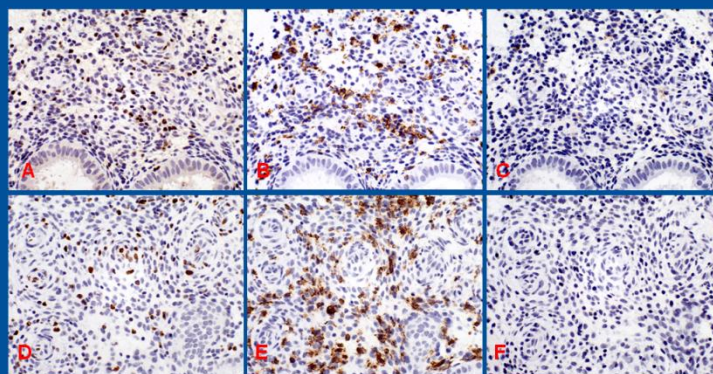


## Clinical Demographics of Sample Population

RM uNK Group	Age	% uNK	Number of RM	1st Trim Loss	2nd Trim Loss	3rd Trim Loss	Live birth
<b>10 High</b> Median (Range)	37.5 (32-44)	8.1 (5.2-13.8)	7 (5-7)	6 (5-7)	0 (0-3)	0 (0-1)	0 (0-5)
<b>10 Low</b> Median (Range)	36 (31-44)	1.95 (1-2.4)	7 (5-17)	7 (5-17)	0 (0-0)	0 (0-0)	0 (0-1)



Endometrial micrographs of different antibodies staining vessels in High uNK patient. A—NKp30 (Differentiation), B—CD56 (uNK), C—CD16 (Trafficking), D—KI67 (Proliferation), E—CD56, F—L-Selectin (Trafficking)



Antibody Stained	High/Low		Location		Contrast to explore % difference (95% CI)	% Significant difference
	Overall % mean difference (95% CI)	% significant difference	Overall significant difference	Location comparison		
CD16	1.556 (0.993-2.179)	P<0.0001	NS	Oedema Vs. Epithelium	0.531 (-0.212-1.275)	NS
				Vessel Vs. Epithelium	0.817 (0.073-1.561)	P=0.032
L-Selectin (CD62-L)	0.035 (-0.034-0.104)	NS	NS	Oedema Vs. Epithelium	0.085 (0.002-0.167)	P=0.044
				Vessel Vs. Epithelium	0.061 (-0.021-0.144)	NS
CD56	4.591 (2.470-6.712)	P<0.0001	P=0.055	Oedema Vs. Epithelium	0.77 (-1.761-3.302)	NS
				Vessel Vs. Epithelium	3.011 (0.490-5.543)	P=0.021
KI67	2.092 (1.358-2.826)	P<0.0001	P=0.003	Oedema Vs. Epithelium	0.643 (-0.233-1.519)	NS
				Vessel Vs. Epithelium	1.567 (0.691-2.443)	P=0.001
NKp30	2.819 (1.369-4.269)	P<0.0001	P=0.008	Oedema Vs. Epithelium	0.335 (-1.397-3.066)	NS
				Vessel Vs. Epithelium	2.586 (0.855-4.318)	P=0.004

## Discussion

The perivascular localisation of KI67 and NKp30 in both high and low uNK cell density groups suggests that uNK cells surrounding vessels are proliferating and are more differentiated compared to those in oedema and epithelium. This finding is significantly higher for the high uNK cell density group (P<0.0001). The paucity of L-Selectin and CD16+ expression in either group suggests that uNK cells are not trafficking from peripheral blood.

## Conclusion

Low expression of trafficking markers for uNK cells at LH+7-9 challenges the theory that uNK cells migrate from peripheral blood. uNK cells of patients in the high uNK cell density group are more differentiated and proliferating surrounding vessels. These resident uNK cells could be involved in premature angiogenesis, which increases uterine blood flow initiating oxidative stress, which is a hostile endometrial environment for the developing foetus and therefore results in recurrent miscarriage.

